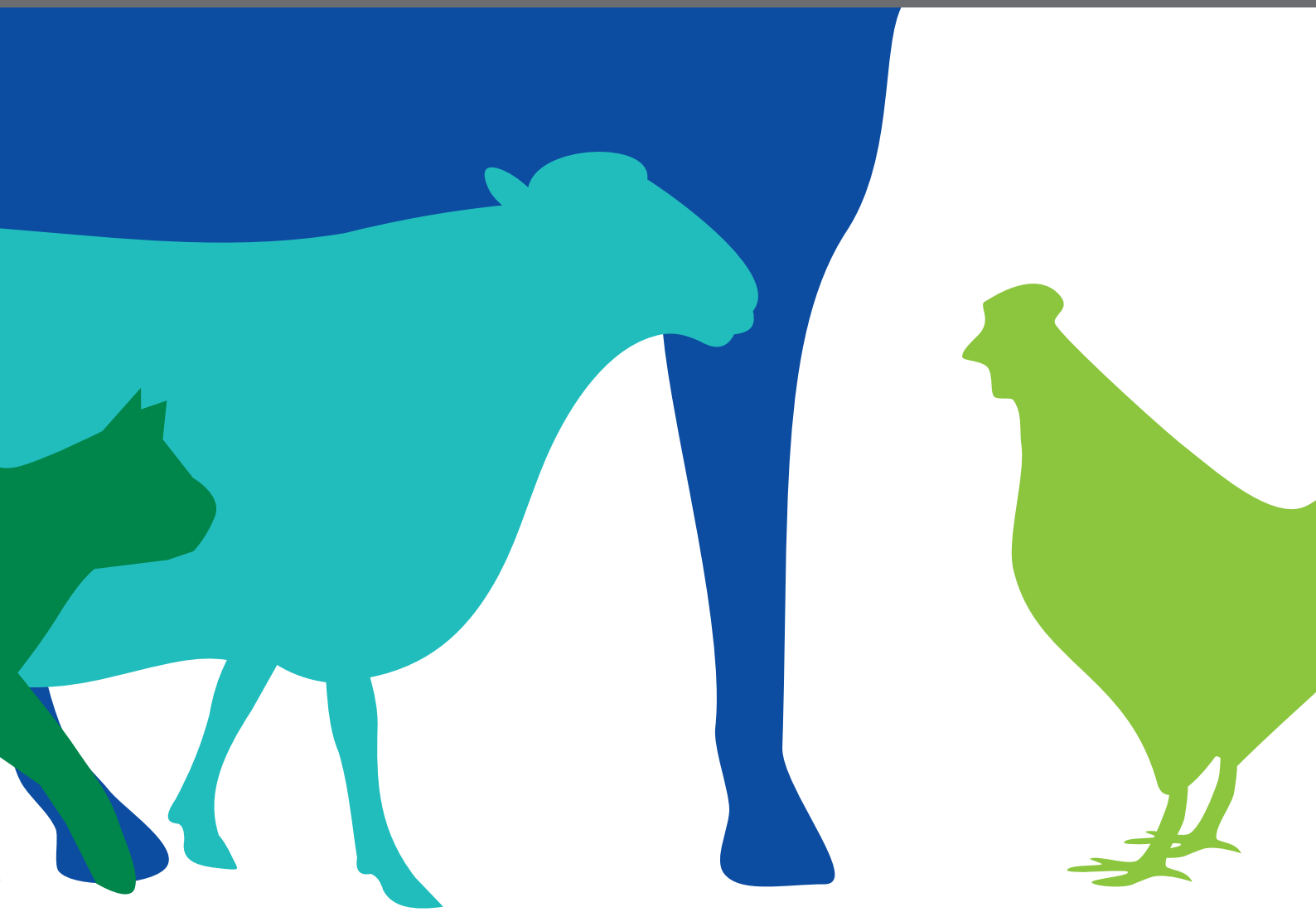
THE ADVANCES IN SEMEN EVALUATION

EDITED BY: Manuel Alvarez Rodriguez, Michael Robert McGowan, Szabolcs Nagy and Heriberto Rodriguez-Martinez

PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-845-7
DOI 10.3389/978-2-88974-845-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

THE ADVANCES IN SEMEN EVALUATION

Topic Editors:
Manuel Alvarez Rodriguez, Universitat Autònoma de Barcelona, Spain
Michael Robert McGowan, The University of Queensland, Australia
Szabolcs Nagy, Hungarian University of Agriculture and Life Sciences, Hungary
Heriberto Rodriguez-Martinez, Linköping University, Sweden

Citation: Rodriguez, M. A., McGowan, M. R., Nagy, S., Rodriguez-Martinez, H., eds. (2022). The Advances in Semen Evaluation. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-845-7

Table of Contents

frontiers in Veterinary Science

EDITORIAL
published: 14 March 2022
doi: 10.3389/fvets.2022.874937

Editorial: The Advances in Semen Evaluation

Heriberto Rodriguez-Martinez[1], *Michael Robert McGowan*[2], *Szabolcs Nagy*[3] *and Manuel Alvarez-Rodríguez*[1,4]*

[1] Department of Biomedical and Clinical Sciences (BKV), BKH/Obstetrics and Gynaecology, Faculty of Medicine and Health Sciences, Linköping University, Linköping, Sweden, [2] School of Veterinary Science, The University of Queensland, Gatton, QLD, Australia, [3] Hungarian University of Agriculture and Life Sciences, Keszthely, Hungary, [4] Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain

Keywords: semen, fertility, cryopreservation, animal production, OMICS

Editorial on the Research Topic

The Advances in Semen Evaluation

Although there have been significant advances in our ability to accurately identify infertile and moderate to severely subfertile breeding males, our ability to accurately identify highly fertile males continues to be quite challenging. Apart from motility and kinetic parameter analysis, a number of studies investigating the impact on fertility outcomes of spermatozoa radical oxidative stress (ROS), Total Antioxidant Capacity (TAC), DNA fragmentation, membrane status (acrosome damage, lipid peroxidation, cholesterol influx, apoptosis-like changes), and capacitation-like status have been conducted. However, there is still a lack of a complete definition of the components of semen, not only the spermatozoa itself but also seminal plasma components that affect fertility outcomes. Thus, the development of new analytical tools to accurately define sperm quality is critical.

In the first paper, Anel-López et al. compared the effects on sperm quality and fertility outcomes of use of three commonly used antibiotics in ram semen extenders. Using computer assisted sperm analysis, sperm motility parameters were significantly lower for semen extended with gentamycin. Further, it was shown that there were no significant differences in fertility outcomes between semen extended without an antibiotic vs. semen treated with penicillin-streptomycin, globally the most commonly used extender antibiotic. This finding was consistent with immediate post-thaw results of simultaneous flow cytometric assessment of sperm viability (Caspase 3 and 7 Activity, ROS generation) and prompts the question, "in the absence of venereal pathogens is the antibiotic treatment of semen necessary". In a larger study examining the relationship between a range of conventional and new post-thaw laboratory measures of ram sperm quality, Mendoza et al. demonstrated that the percentage of intact membrane, non-capacitated (IM-NC) spermatozoa (evaluated using the chlortetracycline assay in combination with ethidium homodimer) in extended chilled semen used for cervical artificial insemination was positively correlated with fertility outcomes.When the analysis was restricted to insemination conducted during the breeding season, an additional three new measures of sperm quality (oxygen consumption, apoptotic-like markers caspase activation) were significantly correlated with fertility outcomes. Modeling indicated that the use of semen with higher percentages of IM-NC and DNA-intact sperm would result in fertility outcomes greater than the population mean.

Three papers examined the impact on sperm quality of direct or indirect administration of plant derived medicinal substances. Sobeh et al. reported the findings of a study investigating the impact on sperm quality of addition of a polyphenol-rich extract, derived from the bark of *Entada abyssinica*, to a standard semen extender. It was concluded that the extracts's potent antioxidant

OPEN ACCESS

Edited and reviewed by:
Ahmed Tibary,
Washington State University,
United States

***Correspondence:**
Manuel Alvarez-Rodríguez
manualvro@gmail.com

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *13 February 2022*
Accepted: *14 February 2022*
Published: *14 March 2022*

Citation:
Rodriguez-Martinez H, McGowan MR, Nagy S and Alvarez-Rodríguez M (2022) Editorial: The Advances in Semen Evaluation. Front. Vet. Sci. 9:874937. doi: 10.3389/fvets.2022.874937

capacity contributed to the observed improvement in sperm progressive motility and plasma membrane integrity. Greco et al. investigated the effect of administration of Tetrahydrocannabinol (THC), maca (a traditional Andean crop), and their combination on testicular tissue and semen parameters in mice. The natural anti-oxidant properties of maca have been shown to protect testicular cell membranes and mitochondria from oxidative stress. Overall, administration of maca, reduced the deleterious effect of THC on testicular parenchyma and semen production. Mao et al. tested the effect of dietary *Echinacea purpurea* (EPE) treatment on the reproductive function of streptozotocin-nicotinamide-induced diabetic rats. EPE treatment significantly increased sperm progressive motility and decreased the percentage of sperm abnormalities. Interestingly, they demonstrated that feeding of EPE increased sperm enzymatic antioxidants (superoxide dismutase, catalase activities, and glutathione), whereas proinflammatory cytokines, such as NO, IL-1b, and TNF-a were decreased.

Three papers examined the impact of chilled and frozen semen storage on sperm quality. Suwimonteerabutr et al. investigated the impact on sperm quality of the addition of butaphosphan and cyanocobalamin to a standard semen extender for chilled boar semen. Overall, supplementation of the semen extender resulted in significant improvements in progressive motility, sperm viability and plasma membrane integrity through to Day 7 of chilled storage. Using a unique approach Wang et al. investigated the molecular mechanisms of sperm cryoinjury and cryoresistance by comparing the piRNA profiles of boar sperm with that of Giant Panda sperm, the latter being known to be quite cryotolerant. They concluded that observed species difference in the profiles involved in the cAMP signaling pathway may be responsible for the difference in cryotolerance of boar and Giant Panda sperm. Continuing in this theme O'Brien et al. investigated whether domestication of ungulates is likely to have altered the sensitivity of their sperm to laboratory manipulation and chilled storage. Using principal components analysis they showed that mitochondrial membrane integrity, oxidative stress level (percentage of low levels of reactive oxidative species) and curvilinear velocity of sperm after chilled storage were the most important biomarkers defining differences between the sperm of domesticated and wild species of pigs, sheep, and goats, respectively.

It is now recognized that instead of semen containing a homogenous population of spermatozoa, it consists of subpopulations of spermatozoa related to period of spermatogenesis and spermatozoal maturation. Gacem et al., using a computer-aided sperm motility analysis system recording sequences at high frequency (250 frames per second), compared the kinematics of sub-populations of spermatozoa from two different horse breeds with that of donkeys. Three distinct sub-populations of motile spermatozoa were defined for both species. The predominant subpopulation consisted of spermatozoa with very fast velocity characteristics and a linear trajectory with a high beat frequency.

In the first of the four reviews, Khan et al. focused on how studies using OMICS technologies (proteomics and transcriptomics) can improve our understanding of the mechanisms of cryo-injury and cryo-tolerance. Systematic application of these technologies could contribute to the optimization of current farm animal cryopreservation protocols. The ultimate goal is the identification of biomarkers that accurately predict spermatozoa freezability. Continuing this focus, Evans et al. examined how lipidomics technologies could be applied to accurately characterize the fatty acid composition and their biological roles in ejaculated, stored, and cryopreserved spermatozoa. Özbek et al. reviewed how OMICS technologies have been and further could be applied to improve identification and selection of more fertile bulls. The final review focused on the critical issue of the level of agreement between laboratories, specifically for the assessment of the percentage of morphologically normal spermatozoa (Perry). A programme to support standardized examination of fixed samples of bull semen by a network of commercial laboratories is described, together with the standardized reporting of specific abnormalities and the maximum percentages of each, based on published reports of their impact on fertility.

AUTHOR CONTRIBUTIONS

HR-M, MM, SN, and MA-R contributed to the conceptualization, writing of the original draft, and review and editing. All authors approved the final version of this editorial.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 01 December 2020
doi: 10.3389/fvets.2020.592162

Butaphosphan and Cyanocobalamin Supplementation in Semen Extender on Chilled Boar Sperm Quality and Life Span

J. Suwimonteerabutr[1,2], *S. Chumsri*[1], *P. Tummaruk*[1,2] *and Morakot Nuntapaitoon*[1,2*]

[1] Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, [2] Swine Reproduction Research Unit, Chulalongkorn University, Bangkok, Thailand

The objective of the present study was to determine the effect of butaphosphan and cyanocobalamin supplementation in semen extender on chilled boar sperm quality and life span. A total of 35 ejaculates of boar semen were included. The semen was diluted with Beltsville thawing solution extender supplemented with different concentrations of butaphosphan and cyanocobalamin [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%] in the diluted semen. The semen samples were evaluated using a computer-assisted sperm analysis system to determine sperm motility and sperm kinetic parameters (i.e., the curvilinear velocity, VCL; straight line velocity, VSL; average path velocity, VAP; linearity, LIN; straightness, STR; amplitude of lateral head, ALH; wobble, WOB; and beat cross frequency, BCF). Additionally, sperm viability, acrosome integrity, mitochondrial activity, and plasma membrane integrity were evaluated after 4 (day 0), 72 (day 3), 120 (day 5), and 168 (day 7) h of storage using SYBR-14–ethidium homodimer-1 (EthD-1), EthD-1, JC-1, and the short hypo-osmotic swelling test, respectively. The analyses were carried out by using the general linear mixed model (MIXED) procedure of SAS. The statistical models for each data set included group, day after storage, and interaction between group and day after storage. The boar was included as a random effect. On day 0 after storage, progressive motility, VCL, VSL, VAP, and plasma membrane integrity of boar sperm in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the 0.4 and 0.5% groups ($P < 0.05$). On day 3 after storage, total motility and progressive motility, VCL, VSL, VAP, LIN, WOB, BCF, and plasma membrane integrity in 0.3% of butaphosphan and cyanocobalamin supplementation were significantly greater than those in the control group ($P < 0.05$). The total motility and progressive motility, VAP, and WOB in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the control group on day 5 after storage ($P < 0.05$). No effects of butaphosphan and cyanocobalamin supplementation on acrosome integrity and mitochondria activity were found on days 3, 5, and 7 after storage. However, the motility and progressive motility and the values for all sperm kinetic parameters except ALH in 0.3% of butaphosphan and cyanocobalamin supplementation

OPEN ACCESS

Edited by:
Michael Robert McGowan, The University of Queensland, Australia

Reviewed by:
Sherrie Gayle Clark-Deener, Virginia Tech, United States
Maria Alejandra Stornelli, National University of La Plata, Argentina

***Correspondence:**
Morakot Nuntapaitoon
morakot.n@chula.ac.th

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *06 August 2020*
Accepted: *27 October 2020*
Published: *01 December 2020*

Citation:
Suwimonteerabutr J, Chumsri S, Tummaruk P and Nuntapaitoon M (2020) Butaphosphan and Cyanocobalamin Supplementation in Semen Extender on Chilled Boar Sperm Quality and Life Span. Front. Vet. Sci. 7:592162. doi: 10.3389/fvets.2020.592162

were greater than those in the control group on day 7 after storage ($P < 0.05$). In conclusion, 0.3% of butaphosphan and cyanocobalamin supplementation in semen extender improved sperm motility, sperm activity, morphology, and life span in chilled boar sperm.

Keywords: butaphosphan, chilled boar semen, cyanocobalamin, life span, sperm quality

INTRODUCTION

Artificial insemination (AI) mostly contributes to improved genetics in the modern commercial swine farm. AI reduces the risk of reproductive disease transmission from direct contact between the boar and dam. Moreover, collected semen from pathogen-free boar in the AI unit mainly reduced the risk for introduction or transmission of boar pathogens in the sow herd (1). Over the last decades, chilled boar semen has considerably increased worldwide swine production due to inexpensive and high ratios of the number of boars per sow, reduced stockmanship, and the high impact of reproductive performance in farms, compared to natural mating (2). The intrinsic quality in chilled boar semen influences swine fertility (3). Moreover, fertility rate and litter size positively correlate with sperm kinetic parameters assessed by the computer-assisted sperm analysis (CASA) system and sperm morphology (3). Sperm activity must use energy from the mitochondria in the form of adenosine triphosphate (ATP) from the mitochondria in the midpiece of the sperm for movement and fertilization in the sow's reproductive tract.

Phosphorus is a crucial part of the energy [i.e., ATP and adenosine monophosphate (AMP), creatinine, nucleotide, and glucose production (4)] and important for the metabolism of sperm energy (5). Cyanocobalamin is a cofactor of the methylmalonyl-CoA mutase which is an enzyme used in the conversion of propionate to succinyl-CoA in the Krebs cycle and related to gluconeogenesis (6, 7). Moreover, cyanocobalamin is an antioxidant which reduces free radicals in the body including in sperm production (8, 9). In general, an intramuscular injection of butaphosphan and cyanocobalamin combination reduces the adverse effects from using dexamethasone in dogs, reduces ketosis in dairy cows, improves the energetic status in postpartum ewes, and increases sperm motility in horses (10–14). Therefore, the combination of butaphosphan and cyanocobalamin may be associated with increased sperm energy supply, enhanced sperm membrane stability, and reduced oxidative agents in boar semen. It is currently unknown whether butaphosphan and cyanocobalamin supplementation in chilled boar semen has an influence on sperm quality and life span. Therefore, the objective of the present study was to determine the effect of butaphosphan and cyanocobalamin supplementation on sperm quality and life span in chilled boar semen.

MATERIALS AND METHODS

The present study was approved by the Chulalongkorn University Animal Care and Use Committee (animal use protocol number 1831110). The protocols followed the guidelines documented in the ethical principles and guidelines for the use of animals for scientific purposes published by the National Research Council of Thailand.

Animal

The present study was performed in an evaporative cooling system of a commercial swine herd located in the western part of Thailand. A total of 35 ejaculates of semen from 16 Duroc, 9 Landrace, and 10 Yorkshire boars aged between 1 and 3 years were included in the experiment. The experiment was conducted between May 2019 and February 2020. Boars were kept in individual pens (2.5 × 2.5 m) on a slatted floor. Boars had access to water *ad libitum* and were fed a commercial lactation diet twice a day. Composition and nutrient followed the nutrient recommendations from the NRC (15).

Semen Collection

The semen was collected by using the gloved-hand method. The boars were allowed at least 7 days of collecting interval. The semen samples were transported to the laboratory immediately after collection. Sperm samples were evaluated for sperm concentration and sperm motility. The semen samples with more than 100 million sperm per milliliter, a volume of more than 100 ml, and sperm motility of more than 70% were selected for the experiment.

Semen Processing

The semen was diluted with Beltsville thawing solution (BTS) (modified BTS®, Kubus Co. Ltd., Madrid, Spain) extender supplemented with different concentrations of butaphosphan and cyanocobalamin combination (Octafos®, Octa Memorial Co., Ltd., Bangkok, Thailand) (1 ml included 100 mg butaphosphan and 0.05 mg cyanocobalamin) [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%]. The diluted semen samples ($3{,}000 \times 10^4$ sperm/ml) were dispersed into 100 ml plastic tubes and equilibrated for 4 h at 16°C (Magapor®, Magapor S.L., Zaragoza, Spain). The sperm motility, sperm kinetic parameters, sperm viability, mitochondrial activity, acrosome integrity, and plasma membrane integrity were evaluated in the diluted semen after 4 (day 0), 72 (day 3), 120 (day 5), and 168 (day 7) h of storage.

Sperm Evaluation

Computer-Assisted Sperm Analysis

The semen samples were evaluated for sperm motility and sperm kinetic parameters using the CASA system (SCA®, Proiser S.L., Valencia, Spain). Sperm kinetic parameters consisted of the curvilinear velocity (VCL), straight line velocity (VSL),

average path velocity (VAP), linearity (LIN), straightness (STR), amplitude of lateral head (ALH), wobble (WOB), and beat cross frequency (BCF).

Sperm Morphology

Sperm viability

Sperm vitality was evaluated using SYBR-14/ethidium homodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, Netherlands). Briefly, 10 μl of aliquot of the sperm sample was thoroughly mixed with 1 μl of 14-μM EthD-1 (Molecular Probes Inc., OR, USA) in 1 ml PBS and 2.7 μl of 0.38-μM SYBR-14 (Dead/Alive Kit; Molecular Probes Inc.) in 1 ml dimethyl sulfoxide (DMSO) at 37°C for 15 min. The sperm was placed on a glass slide and covered with a coverslip. Sperm membrane integrity was assessed using an epifluorescent microscope (CX-31; Olympus, Tokyo, Japan) at ×1,000 magnification. Two hundred sperm were evaluated in five different areas and classified into two categories: live and dead sperm which were stained only green from SYBR-14 (live) and stained both green and red or stained only red from EthD-1 (dead). The percentages of intact sperm membrane were calculated.

Mitochondrial activity

Sperm mitochondrial membrane was determined by using fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazoly-carbocyanine iodide (Molecular Probes, Molecular Probes Inc., Eugene, OR). JC-1 is considered to make it possible to distinguish the mitochondrial membrane potential status (high and low). Briefly, 12.5 μl of aliquot of the sperm sample was mixed with 25 μM final concentration of JC-1 in DMSO and then incubated in the dark at 37°C for 30 min. Counting of the sperm was conducted on individual spermatozoa until 200 sperm had been counted using an epifluorescent microscope (CX-31; Olympus, Tokyo, Japan) at ×1,000 magnification.

Acrosome integrity

Acrosome integrity was evaluated using EthD-1 (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, Netherlands). Briefly, 10 μl of aliquot of the sperm sample was thoroughly mixed with 10 μl of 14-μM EthD-1 (Molecular Probes Inc., OR, USA) at 37°C for 15 min. Five microliters of the mixture was placed on a glass slide and dropped into 95% ethyl alcohol for 30 s and then added to 15 μl FITC-PNA solution [FITC-PNA in PBS (1:10, v/v)] at 4°C for 30 min and removed by PBS. Acrosome integrity was assessed using an epifluorescent microscope (CX-31; Olympus, Tokyo, Japan) at ×1,000 magnification. Two hundred sperm were evaluated in five different areas.

Plasma membrane integrity

Sperm membrane integrity was determined using the short hypo-osmotic swelling test (sHOST). Briefly, 10 μl of aliquot of the sperm sample was thoroughly mixed with 200 μl citrate buffer (75 mOsm), incubated in the dark at 37°C for 30 min, and then, added to 175 μl Hos solution with 5% formaldehyde (75 mOsm). The sperm sample was placed on a glass slide and covered with a coverslip. Counting of the sperm was conducted on individual spermatozoa until 200 sperm had been counted under a light microscope (×400).

Statistical Analysis

Statistical analyses were carried out by using SAS (SAS Institute, Cary, NC, USA). Sperm parameters including sperm motility, sperm kinetic parameters, sperm viability, acrosome integrity, mitochondrial activity, and functional membrane integrity were analyzed by using multiple analysis of variance (ANOVA). The analyses were carried out by using the general linear mixed model (MIXED) procedure of SAS. The statistical models for each data set included group (control, 0.1, 0.2, 0.3, 0.4, and 0.5% of butaphosphan and cyanocobalamin), day after storage (days 0, 3, 5, and 7), and interaction between group and day after collection. The boar was included as a random effect. Least square means were obtained from each class of the factor and were compared by using the least significant test (LSD). For all analyses, $P < 0.05$ was regarded to be statistically significant.

RESULTS

The levels of significance for sperm characteristics, day after collection, and interactions included in the statistical model are presented in **Table 1**. Sperm motility, all sperm kinetic parameters except ALH, sperm viability, and plasma membrane integrity were affected by butaphosphan and cyanocobalamin supplementation over the entire experimental period (**Table 1**). Sperm motility, all sperm kinetic parameters except ALH, sperm viability, and plasma membrane integrity in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the control group (**Table 2**). All sperm characteristics decreased during the day after collection ($P < 0.001$).

Effects of Different Concentrations of Butaphosphan and Cyanocobalamin and Day of Storage on Sperm Motility

The total motility and progressive motility in 0.3% of butaphosphan and cyanocobalamin supplementation were highest in all of the day of collection. On day 0 after storage, no effect of butaphosphan and cyanocobalamin supplementation on total motility was found (**Figure 1A**). Progressive motility of boar sperm in 0.3% of butaphosphan and cyanocobalamin supplementation (66.0%) was greater than that in the 0.4% (59.6%, $P = 0.024$) and 0.5% supplementation (59.3%, $P = 0.017$) (**Figure 1B**). On days 3, 5, and 7 after storage, total motility and progressive motility in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the control ($P < 0.05$).

Effects of Different Concentrations of Butaphosphan and Cyanocobalamin and Day of Storage on Sperm Kinetic Parameters

All sperm kinetic parameters were assessed by the CASA system on each day after collection (**Tables 3**, **4**). The values of all sperm kinetic parameters decreased during storage. On day 0 after storage, the values for VCL, VSL, and VAP in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the 0.4 and 0.5% supplementation ($P < 0.05$) (**Table 3**). On day 3 after storage, the values for VCL, VSL, VAP, LIN, WOB, and BCF in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the control group ($P < 0.05$) (**Table 3**). On day 5 after storage, the values for VAP and WOB in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the control group ($P < 0.05$) (**Table 4**). On day 7 after storage, the values for all parameters except for ALH in 0.3% of butaphosphan and cyanocobalamin supplementation were greater than those in the control group ($P < 0.05$) (**Table 4**).

TABLE 1 | Level of significance for sperm characteristic, day after collection, and interactions included in the statistical model using the MIXED procedure of SAS.

Sperm characteristic	Group	Day	Group × Day
Total motility, %	<0.001	<0.001	0.877
Progressive motility, %	<0.001	<0.001	0.862
VCL, μm/s	0.002	<0.001	0.927
VSL, μm/s	<0.001	<0.001	0.428
VAP, μm/s	<0.001	<0.001	0.892
LIN, %	<0.001	<0.001	0.114
STR, %	0.009	<0.001	0.264
WOB, %	<0.001	<0.001	0.258
ALH, μm	0.327	0.049	0.760
BCF, beats/s	<0.001	<0.001	0.242
Viability, %	0.018	<0.001	0.987
Acrosome, %	0.118	<0.001	0.717
Membrane, %	<0.001	<0.001	0.899
Mitochondria, %	0.058	<0.001	0.999

VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; ALH, amplitude of lateral head; WOB, wobble; BCF, beat cross frequency.

Effects of Different Concentrations of Butaphosphan and Cyanocobalamin and Day of Storage on Sperm Morphology

The effect of butaphosphan and cyanocobalamin supplementation on sperm quality, assessed by fluorescence staining in different extenders, is presented in **Figure 2**. On day 0 after storage, no effect of butaphosphan and cyanocobalamin supplementation on sperm viability and mitochondria activity was found (**Figures 2A,B**). Acrosome activity was higher in 0.1% of butaphosphan and cyanocobalamin supplementation (87.7%) and was greater than that in the control (85.2%, $P = 0.028$) and in the 0.5% supplementation (85.2%, $P = 0.034$) (**Figure 2C**). Moreover, sperm membrane permeability in 0.3% of butaphosphan and cyanocobalamin supplementation (53.2%) was greater than that in the 0.5% (49.6%, $P = 0.005$) and had a tendency to be higher than that in the 0.4% supplementation (50.8%, $P = 0.060$) (**Figure 2C**). On day 3 after storage, no effects of 0.3% of butaphosphan and cyanocobalamin supplementation

TABLE 2 | Effect of 100 mg of butaphosphan and 0.05 mg of cyanocobalamin in different concentrations [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%] and day after storage on semen characteristics from 35 ejaculates analyzed using the MIXED procedure of SAS.

Parameters	Group						SEM*	Day				SEM
	Control	0.1	0.2	0.3	0.4	0.5		0	3	5	7	
Total motility, %	60.7^{c}	66.2^{ab}	64.7^{b}	**67.6**^{a}	64.1^{ab}	61.6^{c}	2.4	73.7^{a}	63.1^{b}	61.7^{b}	58.0^{c}	2.4
PR, %	48.7^{d}	54.0^{ac}	52.5^{bc}	**56.5**^{a}	51.6^{ce}	49.0^{de}	2.5	62.2^{a}	51.2^{b}	49.3^{b}	45.4^{c}	2.5
VCL, μm/s	76.7^{d}	80.1^{abc}	79.2^{bc}	**82.4**^{a}	78.1^{cd}	77.7^{cd}	3.4	83.2^{a}	78.9^{b}	77.9^{bc}	76.2^{c}	3.4
VSL, μm/s	16.4^{c}	17.6^{ab}	17.2^{bc}	**18.4**^{a}	17.0^{bc}	16.6^{bc}	0.9	19.5^{a}	17.4^{b}	16.4^{c}	15.4^{d}	0.8
VAP, μm/s	35.6^{c}	36.8^{b}	36.1^{b}	**38.3**^{a}	36.0^{bc}	35.4^{bc}	1.8	37.7^{a}	36.7^{b}	35.8^{b}	34.6^{c}	1.8
LIN, %	20.6^{d}	21.4^{bc}	21.2^{bcd}	**22.2**^{a}	21.5^{ac}	20.5^{d}	0.6	23.0^{a}	21.8^{b}	20.6^{c}	19.6^{d}	0.6
STR, %	43.4^{b}	44.2 ^{ab}	44.1^{ab}	**45.1**^{a}	44.1^{ab}	43.1^{b}	1.0	48.2^{a}	44.3^{b}	42.3^{c}	41.1^{d}	0.9
WOB, %	43.6^{c}	44.7^{b}	44.3^{bc}	**45.5**^{a}	45.0^{ab}	43.6^{c}	0.6	44.0^{a}	45.6^{b}	44.7^{b}	43.9^{a}	0.6
ALH, μm	1.8	1.9	1.9	1.9	2.0	1.8	0.1	2.0^{a}	1.9^{ab}	1.9^{b}	1.8^{b}	0.1
BCF, beats/s	7.6^{b}	8.1^{ac}	8.0^{b}	**8.4**^{a}	7.8^{bc}	7.7^{b}	0.3	9.1^{a}	8.0^{b}	7.6^{c}	7.0^{d}	0.3
Viability, %	81.3^{bc}	82.6^{ac}	81.0^{bc}	**83.4**^{a}	81.8^{bc}	82.0^{ac}	1.0	85.2^{a}	83.5^{b}	81.1^{c}	78.2^{d}	1.0
Acrosome, %	84.4^{ad}	**85.8**^{c}	84.2^{b}	84.8^{abc}	84.6^{bd}	84.9^{bc}	0.9	86.0^{a}	85.1^{b}	84.3^{bc}	83.8^{c}	0.9
Membrane, %	42.4^{d}	46.2^{ac}	45.3^{bc}	**46.9**^{a}	44.2^{b}	42.6^{d}	1.9	52.0^{a}	46.5^{b}	42.0^{c}	38.0^{d}	1.8
Mitochondria, %	74.0^{ac}	74.9^{ab}	72.8^{bc}	**76.2**^{a}	72.2^{bc}	74.6^{ac}	1.9	77.8^{a}	75.2^{b}	73.3^{b}	70.2^{c}	1.8

^{a,b,c,d,e}*Different superscript letters within rows indicate significant differences ($P < 0.05$).*

**Greatest standard error of the mean (SEM). The bold values provide the maximum values in each parameter.*

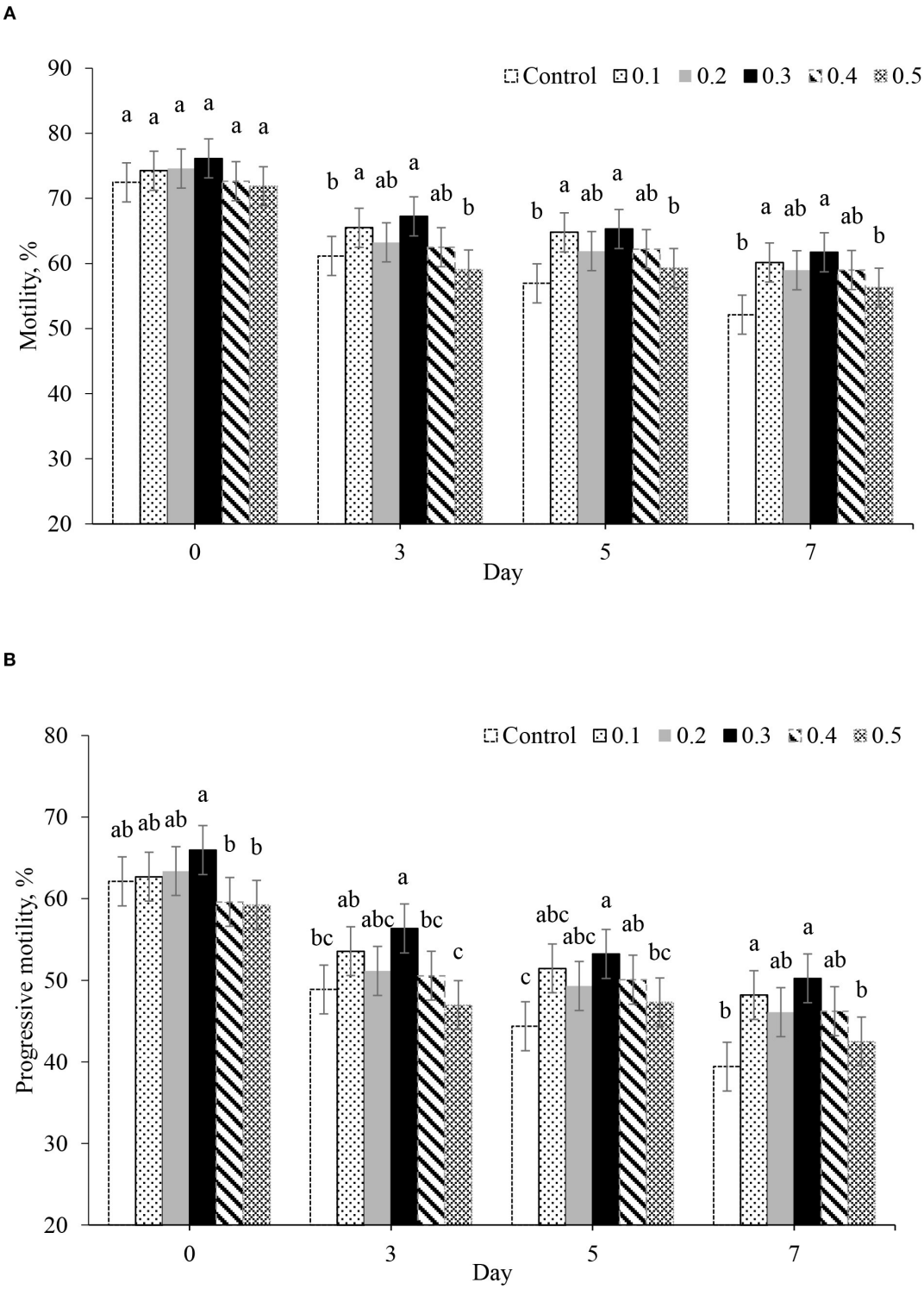


FIGURE 1 | Effect of 100 mg of butaphosphan and 0.05 mg of cyanocobalamin in different concentrations [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%] on total motility **(A)** and progressive motility **(B)** by day after storage ($n = 35$ ejaculations). [a,b,c]Significant differences among groups in each day after storage ($P < 0.05$).

on sperm viability, acrosome integrity, and mitochondria activity were found. Sperm plasma membrane integrity in 0.3% of butaphosphan and cyanocobalamin supplementation was greater than that in the 0.5% and the control group on days 3, 5, and 7 after storage ($P < 0.001$) (**Figure 2D**). On day 5 after storage, sperm viability in 0.1 and 0.3% of butaphosphan

TABLE 3 | Effect of 100 mg of butaphosphan and 0.05 mg of cyanocobalamin in different concentrations [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%] on semen characteristics at days 0 and 3 after storage from 35 ejaculates.

Parameters	Day 0							Day 3						
	Control	0.1	0.2	0.3	0.4	0.5	SEM*	Control	0.1	0.2	0.3	0.4	0.5	SEM
VCL, μm/s	83.2^{ab}	83.0^{ab}	83.8^{ab}	**88.1^{a}**	80.4^{b}	80.7^{b}	3.8	76.9^{b}	80.2^{ab}	79.2^{ab}	**82.8^{a}**	77.4^{ab}	76.8^{b}	3.9
VSL, μm/s	19.9^{ab}	19.2^{b}	20.0^{ab}	**21.1^{a}**	18.2^{b}	18.9^{b}	1.0	16.2^{b}	17.9^{ab}	17.4^{ab}	**18.5^{a}**	17.7^{ab}	16.8^{ab}	1.0
VAP, μm/s	37.5^{ab}	37.5^{ab}	37.9^{ab}	**40.3^{a}**	36.3^{b}	36.6^{b}	2.0	35.0^{b}	37.4^{ab}	36.6^{ab}	**38.9^{a}**	36.5^{ab}	35.8^{b}	2.0
LIN, %	23.3	22.7	23.3	**23.5**	22.2	22.8	0.8	20.7^{c}	21.7^{abc}	21.8^{abc}	22.3^{ab}	**23.0^{a}**	21.0^{b}	0.8
STR, %	48.6	47.7	**48.9**	48.8	47.1	48.1	1.2	43.2^{b}	44.4^{ab}	44.6^{ab}	45.1^{ab}	**45.6^{a}**	43.1^{b}	1.2
WOB, %	43.8	43.8	44.0	**44.5**	43.6	43.9	0.8	44.3^{b}	45.5^{ab}	45.0^{ab}	46.0^{a}	**46.3^{a}**	44.9^{ab}	0.8
ALH, μm	1.9^{b}	1.9^{b}	1.9^{b}	**2.0^{b}**	2.4^{a}	1.9^{b}	0.1	1.8	1.9	1.9	1.9	1.9	1.8	0.1
BCF, beats/s	9.3^{abc}	8.9^{bc}	9.3^{ac}	**9.7^{a}**	8.6^{b}	8.8^{bc}	0.4	7.6^{b}	8.3^{ab}	8.1^{ab}	**8.4^{a}**	8.0^{ab}	7.7^{ab}	0.4

a,b,c Different superscript letters within rows indicate significant differences ($P < 0.05$).

**Greatest standard error of the mean (SEM). The bold values provide the maximum values in each parameter.*

TABLE 4 | Effect of 100 mg of butaphosphan and 0.05 mg of cyanocobalamin in different concentrations [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%] on semen characteristics at days 5 and 7 after storage from 35 ejaculates.

Parameters	Day 5							Day 7						
	Con.	0.1	0.2	0.3	0.4	0.5	SEM*	Con.	0.1	0.2	0.3	0.4	0.5	SEM
VCL, μm/s	75.3	78.2	78.0	**79.3**	78.6	77.8	3.9	71.5^{b}	79.0^{a}	75.8^{ab}	**79.3^{a}**	76.2^{ab}	75.4^{ab}	3.9
VSL, μm/s	15.6	16.4	16.3	**17.1**	17.1	16.1	2.0	13.8^{b}	16.8^{ac}	15.0^{bc}	**16.9^{a}**	15.1^{abc}	14.8^{b}	2.0
VAP, μm/s	34.1^{b}	36.1^{ab}	35.5^{ab}	**37.1^{a}**	36.7^{ab}	35.5^{ab}	2.0	31.8^{b}	36.1^{ac}	34.3^{ab}	**37.0^{a}**	34.6^{ab}	33.9^{bc}	2.0
LIN, %	20.1^{ab}	20.6^{ab}	20.2^{ab}	**21.6^{a}**	21.5^{a}	19.9^{b}	0.8	18.4^{b}	20.6^{ac}	19.6^{bc}	**21.4^{a}**	19.4^{bc}	18.2^{bc}	0.8
STR, %	42.0	42.0	42.2	**43.2**	43.0	41.6	1.2	39.8^{b}	42.5^{ac}	40.9^{bc}	**43.1^{a}**	40.5^{bc}	39.5^{b}	1.2
WOB, %	44.0^{bc}	45.1^{ac}	44.2^{bc}	**46.1^{a}**	45.9^{a}	42.6^{b}	0.8	42.4^{c}	44.4^{ab}	44.2^{ab}	**45.5^{a}**	44.1^{ab}	42.9^{bc}	0.8
ALH, μm	1.8	1.9	1.9	1.9	1.9	1.9	0.1	1.7	1.9	1.8	1.9	1.9	1.8	0.1
BCF, beats/s	7.2	7.7	7.6	**7.8**	7.8	7.5	0.4	6.3^{b}	7.6^{ac}	6.8^{b}	**7.7^{a}**	6.9^{bc}	6.7^{bc}	0.4

a,b,c Different superscript letters within rows indicate significant differences ($P < 0.05$).

**Greatest standard error of the mean (SEM). The bold values provide the maximum values in each parameter.*

and cyanocobalamin supplementation was significantly greater than that in the 0.2% and the control group. No effects of butaphosphan and cyanocobalamin supplementation on sperm viability, acrosome integrity, and mitochondria activity were found on day 7 after storage.

DISCUSSION

The present study is the first report to provide information on the effects of an injectable product including butaphosphan and cyanocobalamin supplementation in chilled boar semen extender on sperm quality and life span. Our results indicated that 0.3% of butaphosphan and cyanocobalamin supplementation in the chilled boar semen extender increased sperm motility, sperm kinetic parameters, and sperm morphology. Therefore, butaphosphan and cyanocobalamin supplementation in semen extender improved chilled boar sperm quality and life span and may be applied in commercial swine herds. This finding will improve our knowledge in improving sperm quality in chilled boar semen and could be used to modify injectable products to be used in chilled boar semen to improved swine fertility.

Effect of Butaphosphan and Cyanocobalamin Supplementation on Sperm Quality

Butaphosphan or phosphorus plays an important role in increasing sperm motility because phosphorus has a crucial role in sperm energy metabolism including ATP and AMP, the production of creatinine and nucleotides, gluconeogenesis, and glycogenesis (4, 5). Moreover, phosphorus stimulates protein function in phosphorylation (16). López Rodríguez et al. (17) found that phosphate concentration in seminal plasma positively correlated with sperm concentration and sperm motility in boars. Furthermore, cyanocobalamin involves energy and glucose metabolism. Cyanocobalamin is a cofactor of methylmalonyl-CoA mutase which is used to convert propionate to succinyl-CoA in the Krebs cycle (6) and is used in gluconeogenesis (7). The results of the present study demonstrated that all concentrations of butaphosphan and cyanocobalamin improved both sperm total motility and progressive motility and the values of VCL, VSL, VAP, LIN, STR, WOB, and BCF. In agreement with Beltrama et al. (18), an intramuscular injection of butaphosphan and cyanocobalamin combination increased sperm motility in

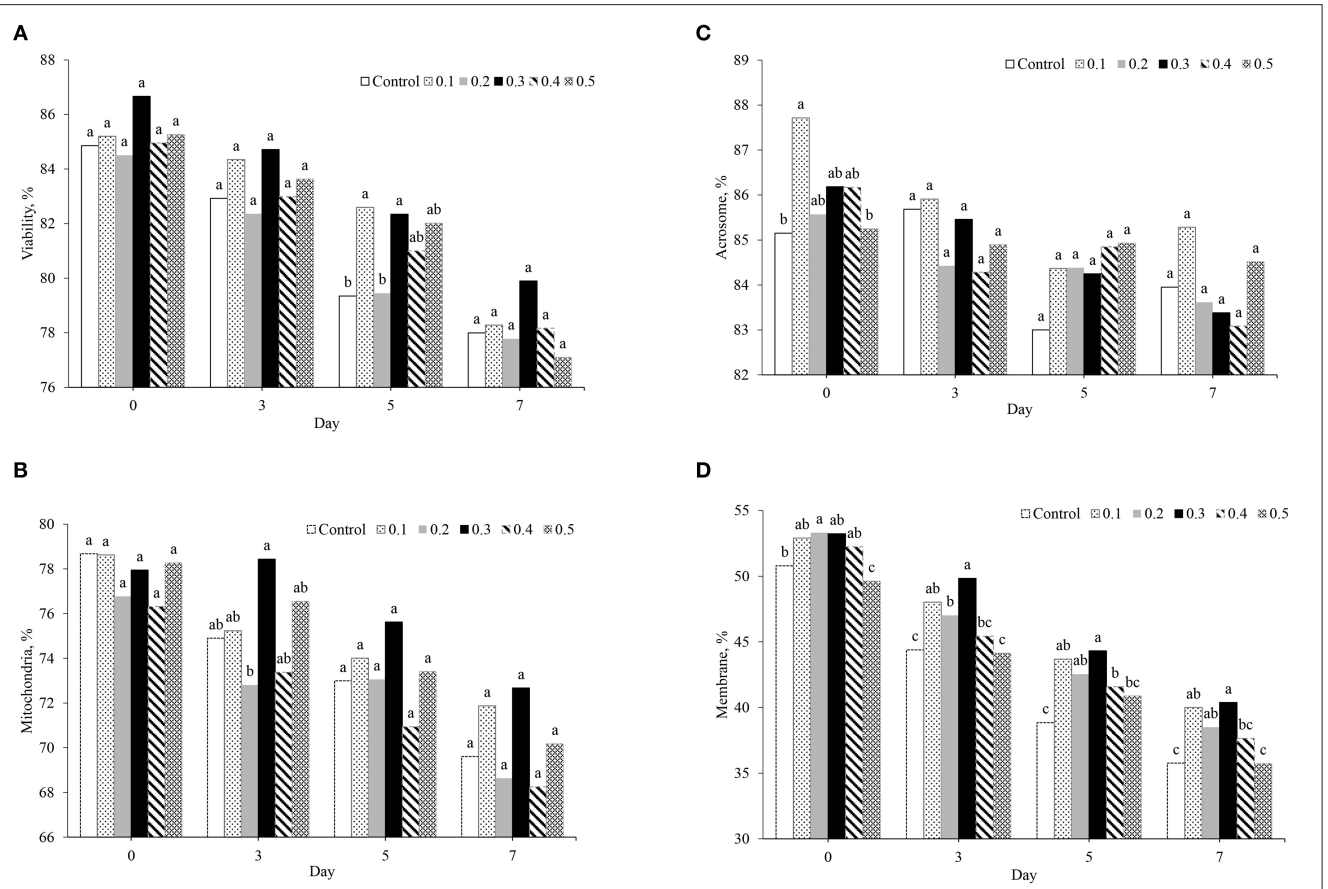


FIGURE 2 | Effect of 100 mg of butaphosphan and 0.05 mg of cyanocobalamin in different concentrations [0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5%] on sperm viability **(A)**, mitochondria activity **(B)**, acrosome integrity **(C)**, and plasma membrane integrity **(D)** by day after storage ($n = 35$ ejaculations). [a,b,c]Significant differences among groups on each day after storage ($P < 0.05$).

mice. Sperm viability and membrane integrity were increased by butaphosphan and cyanocobalamin supplementation in chilled boar semen. From our results, 0.3% of butaphosphan and cyanocobalamin supplementation increased 2.1% of sperm viability and 4.5% of sperm plasma membrane integrity in the chilled boar semen extender and increased semen quality when compared with the control group. Similarly, many previous studies have reported the effect of cyanocobalamin supplementation during the thawing of frozen semen on semen quality and fertilization in many species (8, 9, 19, 20). In boars, supplementation of 0.5 and 1.0 μg cyanocobalamin increased progressive sperm motility and plasma membrane viability (20). In rams, Hamedani et al. (9) supplemented 2.0 mg/ml of cyanocobalamin in the extender preserved at 5°C, and it improved sperm motility, viability, the number of normal sperm, and plasma membrane viability in pre- and post-freezing conditions. Moreover, in Hu et al. (19), the supplementation of 2.5 mg/ml of cyanocobalamin during post-thawing increased the sperm quality. However, Beltrama et al. (18) found that an intramuscular injection of butaphosphan and cyanocobalamin supplementation in mice improved mitochondria activity and acrosome integrity. This contrasts with our results, in which the supplementation of butaphosphan and cyanocobalamin did not result in improvement. The intramuscular injection of the combination of butaphosphan and cyanocobalamin administered exerts a potential role in spermatogenesis and structure, while supplementation in the extender increases sperm activity.

Increasing sperm quality by butaphosphan and cyanocobalamin supplementation improved fertility in swine herds. The values of VSL, VAP, LIN, and STR were positively correlated with litter size in pig (21) and with fertility in humans (22, 23). Additionally, cyanocobalamin impairs reactive oxygen species (ROS) and positively relates with sperm quality, concentration, and fertility rates in humans (24, 25). In accordance with Barranco et al. (26), who measured the total antioxidant capacity in seminal plasma in boars, they found that individual total antioxidant capacity in boars was greatly varied. Moreover, the total antioxidant capacity in seminal plasma in boars positively correlated with sperm concentration,

conception rate, and fertility. Therefore, it can be concluded that the beneficial effects of butaphosphan and cyanocobalamin supplementation in chilled boar semen were increased energy and increased functionality of the plasma membrane leading to improved semen quality in boars.

Effect of Butaphosphan and Cyanocobalamin Supplementation on Semen Life Span

Semen preservation by an extender has been widely used for enhancing semen life span. The extender provides the preserved sperm cells and components, source of energy, proper pH and osmotic pressure depended on the ingredients of the sperm preservation. The short time preservation [i.e., BTS, Illinois variable temperature (IVT), and Kiev] can preserve sperm for about 1–3 days. The BTS is generally used in the swine production industry due to its inexpensiveness, ease of use, and appropriated preservation time. Therefore, BTS was used to preserve semen in the present study. Free radicals gradually increased from oxidative stress conditions such as stresses during cooling and storage time (27), which could damage membrane structure and mitochondria function (28). Sperm membranes are rich in polyunsaturated fatty acids (PUFAs), which are highly sensitive to lipid peroxidation. Sperm membranes were destroyed by lipid peroxidation, leading to leaking of sperm intracellular organisms and inhibiting the respiratory systems of the sperm cell (29). Moreover, free radicals in sperm cells declined ATP utilization at the contractile apparatus of the flagellum (30). Sperm cells rapidly decreased motility and death from lipid peroxidation (31). Butaphosphan may provide energy reserves and cyanocobalamin protects sperm cells from ROS during storage at low temperatures (24), thereby increasing the sperm motility and life span. The present study demonstrated that sperm in 0.3% of butaphosphan and cyanocobalamin supplementation has a higher percentage of viability than that in the control group at day 5 of storage. Similarly, a previous study found that supplementation with vitamin B_{12} in bull cow semen increases semen quality and increases semen lifetime (32). In agreement with our results, sperm motility, sperm viability, and plasma membrane integrity increased after day 3 of storage. In general, above 60% of sperm total motility after dilution was used in AI in swine herds. At day 5 of storage, semen with 0.1–0.4% butaphosphan and cyanocobalamin supplementation in chilled boar semen had total motility above 60%, whereas the control group and 0.5% supplementation had total motility below 60% in the present study. Moreover, semen with 0.1 and 0.3% butaphosphan and cyanocobalamin supplementation in chilled boar semen still had total motility above 60% at day 7 of storage. Additionally, concerning sperm morphology, semen with 0.1 and 0.3% butaphosphan and cyanocobalamin supplementation has significantly higher viability than in the control group at day 5 after storage and has higher plasma membrane integrity at day 7 after storage. Therefore, it can be concluded that the supplementation with 0.3% butaphosphan and cyanocobalamin in the extender could significantly enhance semen quality and prolong the life span period of the sperm.

High concentrations of butaphosphan and cyanocobalamin supplementation have an adverse effect on semen. Hu et al. (8) reported that 3.75 mg/ml of cyanocobalamin supplementation reduced cow semen quality. The present study found that semen with 0.4 and 0.5% of butaphosphan and cyanocobalamin supplementation (equivalent to 0.020 and 0.025 mg/ml, respectively) did not improve semen quality and life span when compared with the control group. The mechanism of butaphosphan and cyanocobalamin supplementation having an adverse effect on semen quality is still not clear. Inappropriate antioxidative supplementation may cause increased cell death because antioxidants cannot distinguish between advantageous and disadvantageous radicals. High antioxidant supplementation acts as prooxidants by increasing oxidative stress and disturbs the ROS formation and neutralization balance (33). It can be concluded that supplementation with butaphosphan and cyanocobalamin in an extender should not be used in excess of 0.02 mg/ml.

CONCLUSIONS

In conclusion, the beneficial effects of 0.3% butaphosphan and cyanocobalamin supplementation on chilled boar semen were increased energy and increased functionality of the plasma membrane leading to improved semen quality, sperm activity, morphology, and life span of chilled boar sperm.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The present study was approved by the Chulalongkorn University Animal Care and Use Committee (animal use protocol number 1831110). The protocols followed the guidelines documented in the ethical principles and guidelines for the use of animals for scientific purposes published by the National Research Council of Thailand. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Financial support for the present study was provided by the National Science Technology and Innovation Policy Office and the Office of the Higher Education Commission (Talent Mobility program) [Grant Number 0862015, 2019].

REFERENCES

1. Maes D, Van SA, Appeltant R, Arsenakis I, Nauwynck H. Porcine semen as a vector for transmission of viral pathogens. *Theriogenology*. (2016) 85:27–38. doi: 10.1016/j.theriogenology.2015.09.046
2. Juonala T, Lintukanges S, Nurttila T, Andersson M. Relationship between semen quality and fertility in 106 AI-Boars. *Reprod Dom Anim*. (1998) 33:155–8. doi: 10.1111/j.1439-0531.1998.tb01334.x
3. Broekhuijse MLWJ, Šoštarić E, Feitsma H, Gadella BM. Application of computer-assisted semen analysis to explain variations in pig fertility. *J Anim Sci*. (2012) 90:779–89. doi: 10.2527/jas.2011-4311
4. Klingenberg M. The ADP and ATP transport in mitochondria and its carrier. *Biochim Biophys Acta*. (2008) 1778:1978–2021. doi: 10.1016/j.bbamem.2008.04.011
5. Cunningham JG. *Textbook of Veterinary Physiology*. 3rd ed. Philadelphia, PA: W.B. Saunders (2002).
6. Kennedy DG, Cannavan A, Molloy A, O'Harte F, Taylor SM, Kennedy S, et al. Methylmalonyl-CoA mutase (EC 5.4.99.2) and methionine synthetase (EC 2.1.1.13) in the tissues of cobalt-vitamin B12 deficient sheep. *Brit J Nut*. (1990) 64:721–32. doi: 10.1079/BJN19900074
7. Mcdowell LR. *Vitamins in Animal and Human Nutrition*. 2nd ed. Ames, IA: Iowa State University Press (2000).
8. Hu JH, Tian WQ, Zhao XL, Zan LS, Xin YP, Li QW. The cryoprotective effects of vitamin B12 supplementation on bovine semen quality. *Reprod Dom Anim*. (2011) 46:66–73. doi: 10.1111/j.1439-0531.2009.01575.x
9. Hamedani MA, Tahmasbi AM, Ahangari YJ. Effects of vitamin B12 supplementation on the quality of Ovine spermatozoa. *Open Vet J*. (2013) 3:140–4.
10. Deniz A, Spiecker-Hauser U, Rehagen M. Efficacy of a butafosfan and vitamin B12 combination (Catosal®) on biochemical and hematological blood parameters in dogs treated with dexamethasone. *Int J App Res Vet Med*. (2009) 7:116–29.
11. Rollin E, Berghaus RD, Rapnicki P, Godden SM, Overton MW. The effect of injectable butaphosphan and cyanocobalamin on postpartum serum β-hydroxybutyrate, calcium, and phosphorus concentrations in dairy cattle. *J Dairy Sci*. (2010) 93:978–87. doi: 10.3168/jds.2009-2508
12. Pereira RA, Fensterseifer S, Barcelos VB, Martins CF, Schneider A, Schmitt E, et al. Metabolic parameters and dry matter intake of ewes treated with butaphosphan and cyanocobalamin in the early postpartum period. *Small Ru Res*. (2013) 114:140–5. doi: 10.1016/j.smallrumres.2013.05.016
13. Pereira RA, Silveira PA, Montagner P, Schneider A, Schmitt E, Rabassa VR, et al. Effect of butaphosphan and cyanocobalamin on postpartum metabolism and milk production in dairy cows. *Animal*. (2013) 7:1143–7. doi: 10.1017/S1751731113000013
14. Cazales NP, de Oliveira GS, Farias MR, de Araujo HBB, Henrique GZW, Cunha IBF, et al. Effect of intramuscular injection of butafosfan and cobalamin on the quality of Fresh and Cooled Stallion Semen. *Semina Ciências Agrárias Londrina*. (2015) 36:2603–10. doi: 10.5433/1679-0359.2015v36n4p2603
15. National Research Council (NRC). *Nutrient Requirements of Swine*. 11th Rev ed. Washington, DC: National Academy Press (2012).
16. Tash JS, Bracho GE. Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. *J Andro*. (1994) 15:505–9.
17. López Rodríguez A, Rijsselaere T, Beek J, Vyt P, Van SA, Maes D. Boar seminal plasma components and their relation with semen quality. *Sys Biol Reprod Med*. (2013) 59:5–12. doi: 10.3109/19396368.2012.725120
18. Beltrame FL, de Santi F, Vendramini V, Cabral REL, Miraglia SM, Cerri PS, et al. Vitamin B12 prevents cimetidine-induced androgenic failure and damage to sperm quality in rats. *Front Endocrinol*. (2019) 10:309. doi: 10.3389/fendo.2019.00309
19. Hu JH, Li QW, Chen YL, Jiang ZL, Jia YH, Wang LQ, et al. Effects of addition of vitamin B_{12} to the extender on post-thaw motility, acrosome morphology, and plasma membrane integrity in bull semen. *Turkey J Vet Anim Sci*. (2009) 33:379–84. doi: 10.3906/vet-0712-19
20. Mello AR, Hyde AM, Elsea LE, Whitaker BD. The effect of cyanocobalamin supplementation during the thawing of frozen boar semen on spermatozoa, *in vitro* fertilization and embryonic development. *Anim Reprod*. (2013) 10:119–23.
21. Holt C, Holt WV, Moore HD, Reed HC, Curnock RM. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. *J Andro*. (1997) 18:312–23.
22. Krause W. Computer-assisted semen analysis systems: comparison with routine evaluation and prognosis values in male infertility and assisted reproduction. *Hum Reprod*. (1995) 10:60–6. doi: 10.1093/humrep/10.suppl_1.60
23. Larsen L, Scheike T, Jensen TK, Bonde JP, Ernst E, Hjollund NH, et al. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. The Danish First Pregnancy Planner Study Team. *Hum Reprod*. (2000) 15:1562–7. doi: 10.1093/humrep/15.7.1562
24. Chen QX, Mei J, Ng V, Chia SE, Ling WH, Ong CN. Semen folate, vitamin B12 and reactive oxygen species and their relationships with sperm parameters. *Acta Nutrimenta Sinica*. (2001) 23:160–3.
25. Boxmeer JC, Smit M, Utomo E, Romijn JC, Eijkemans MJ, Lindemans J, et al. Low folate in seminal plasma is associated with increased sperm DNA damage. *Fert Ster*. (2009) 92:548–56. doi: 10.1016/j.fertnstert.2008.06.010
26. Barranco I, Tvarijonaviciute A, Perez-Patiño C, Parrilla I, Ceron JJ, Martinez EA, et al. High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Sci Rep*. (2015) 5:18538. doi: 10.1038/srep18538
27. Sikka SC, Rajasekaran M, Hellstrom WJ. Role of oxidative stress and antioxidants in male infertility. *J Andro*. (1995) 16:464–8.
28. Radomil L, Pettitt MJ, Merkies KM, Hickey KD, Buhr MM. Stress and dietary factors modify boar sperm for processing. *Reprod Dom Anim*. (2011) 46:39–44. doi: 10.1111/j.1439-0531.2011.01865.x
29. White IG. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod Ferti Dev*. (1993) 5:639–58. doi: 10.1071/RD9930639
30. Verma A, Kanwar KC. Human sperm motility and lipid peroxidation in different ascorbic acid concentrations: an *in vitro* analysis. *Andrologia*. (1998) 30:325–9. doi: 10.1111/j.1439-0272.1998.tb01178.x
31. Guthrie HD, Welch GR. Effects of reactive oxygen species on sperm function. *Theriogenology*. (2012) 78:1700–8. doi: 10.1016/j.theriogenology.2012.05.002
32. Yufan L. The effects of vitamin B12 on the quality of freezing bull semen sperm. *J Hebei Nor Uni Sci Tech*. (1998). Available online at: http://en.cnki.com.cn/Article_en/CJFDTOTAL-HBNS801.009.htm
33. Poljsak B, Šuput D, Milisav I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. *Oxid Med Cell Longev*. (2013) 2013:1–11. doi: 10.1155/2013/956792

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 December 2020
doi: 10.3389/fvets.2020.604477

A Polyphenol-Rich Extract From *Entada abyssinica* Reduces Oxidative Damage in Cryopreserved Ram Semen

Mansour Sobeh[1,2]*, *Soha A. Hassan*[3], *Mahmoud A. E. Hassan*[4], *Wael A. Khalil*[5]*, *Mohamed A. O. Abdelfattah*[6], *Michael Wink*[2] and *Abdelaziz Yasri*[1]

[1] AgroBioSciences Research Division, Mohammed VI Polytechnic University, Ben-Guerir, Morocco, [2] Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Heidelberg, Germany, [3] Basic Science Department, Faculty of Dentistry, October 6 University, Cairo, Egypt, [4] Animal Production Research Institute, Giza, Egypt, [5] Department of Animal Production, Faculty of Agriculture, Mansoura University, Mansoura, Egypt, [6] College of Engineering and Technology, American University of the Middle East, Kuwait

The Splinter bean, *Entada abyssinica*, is widely used in folk medicine. In the current work, we profiled the secondary metabolites from *E. abyssinica* bark extract using LC-MS and investigated its effect on cryopreserved ram semen. Twenty-eight compounds, including tannins and gallic acid derivatives that prevailed in the extract, were tentatively identified. Results showed that the quality of the post-thawed semen showed a significant improvement when the extract was added to the extender at a concentration of 375 μg/mL. The progressive motility and plasma membrane integrity of sperm cells were significantly increased in the post-thawed semen; however, the total antioxidant capacity (TAC) was insignificantly increased. A significant decrease in the concentration of hydrogen peroxide was detected as well. No significant changes were observed in activities of lactate dehydrogenase (LDH), alanine aminotransaminase (ALT), and aspartate transaminase (AST) within the treated samples. Intact sperm percentage was significantly increased, while apoptotic and necrotic sperm percentages were reduced significantly. Molecular docking of some individual components from the extract revealed their potential to interfere with the apoptosis cascade in which Bcl-2 is involved. In conclusion, *Entada abyssinica* appears to be useful for cryopreservation presumably owing to its polyphenol content that has potent antioxidant capacity scavenging reactive oxygen species (ROS), enhancing the endogenous antioxidant system and inhibiting lipid peroxidation.

Keywords: ***Entada abyssinica*, polyphenolics, semen cryopreservation, sperm ultrastructure, Antioxidant biomarker**

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Marcia De Almeida Monteiro Melo Ferraz, Ludwig Maximilian University of Munich, Germany
Abouzar Najafi, University of Tehran, Iran

***Correspondence:**
Mansour Sobeh
mansour.sobeh@um6p.ma
Wael A. Khalil
w-khalil@mans.edu.eg

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *09 September 2020*
Accepted: *23 October 2020*
Published: *02 December 2020*

Citation:
Sobeh M, Hassan SA, Hassan MAE, Khalil WA, Abdelfattah MAO, Wink M and Yasri A (2020) A Polyphenol-Rich Extract From Entada abyssinica Reduces Oxidative Damage in Cryopreserved Ram Semen. Front. Vet. Sci. 7:604477. doi: 10.3389/fvets.2020.604477

INTRODUCTION

Artificial insemination (AI) is a widely applied technique that uses fresh semen and frozen–thawed sperms. Thus, sperm preservation has many applications in different areas including AI, species conservation, and clinical medicine (1, 2). One of the most growing interests in many countries is ram semen cryopreservation, aiming to increase the quality of productive parameters of the selected flocks. The use of ram frozen–thawed semen eliminates the geographical barriers, helps in preserving endangered breeds, and conserves the biodiversity (3).

The viability of the preserved sperms may be affected by many factors including storage temperature, cryoprotectant concentration, cooling rate, extender composition, free radical contents, seminal plasma contents, and antiseptic factors (4, 5). Scientists are still facing undesirable results of the process plausibly owing to intracellular ice crystal formation, osmotic and chilling damage, which cause sperm cell injury, DNA damage, and cytoplasmic injury (6). Ice crystallization and recrystallization during freezing and thawing techniques induce biochemical and cellular changes and alter the sperm efficiency (7). Sperm motility and morphology may be affected as well by increased membrane permeability after cryopreservation (8, 9). In addition, these post-thawing-induced changes could impair sperm transport and survival inside the female reproductive tract, affecting fertilizing capacity and embryogenesis (8). Therefore, many efforts to maintain and improve sperm viability in these techniques have been developing recently.

The primary antioxidant system in seminal plasma acts as defensive machine by the aid of some enzymes such as glutathione peroxidase, superoxide dismutase, and catalase, which scavenge reactive oxygen species (ROS) resulting from lipid peroxidation (10). This system alone is insufficient to face the oxidative stress during cryopreservation and thawing processes. The dilution of semen during extending causes a decrease in the concentration of the system's antioxidant enzymes leading to strong oxidative stress (11). Moreover, the high content of saturated and polyunsaturated fatty acids in the plasma membrane of ram sperm makes it more susceptible to oxidative stress (12). This emphasizes the importance of supplementing the freezing media with added antioxidants that would minimize the negative effect of ROS and maintain the quality of post-thawed sperm (13). In this regard, different types of supplements are added to the freezing media as protective agents; however, antioxidants of natural origin are of special interest in this concern (14–16).

The Splinter bean, *Entada abyssinica* (Fabaceae), is a widely spread tree in central and eastern tropical Africa. Extracts from *E. abyssinica* bark and leaves have been traditionally used in folk medicine to manage a large number of ailments, such as sleeping sickness, coughs, rheumatic fever, abdominal pain, and diarrhea in west and east Africa (17, 18). A plethora of biological activities were documented for different plant parts. For instance, a complex extract from the bark containing alkaloids, diterpenoids, saponins, and flavonoids exhibited antimicrobial, antifungal, and antiviral activities as well as cytotoxic properties against drug-resistant cancer cells (19, 20). For the leaves, anti-inflammatory and antioxidant activities among other various beneficial effects were also described (21).

In this work, we characterized the chemical constituents of a methanol extract from *E. abyssinica* bark utilizing HPLC-MS/MS. The potential antioxidant activities of the extract were evaluated *in vitro*. We investigated the cryopreservative and antioxidative effects of adding the extract to semen extender on the quality of Ossimi ram (*Ovis aries*) semen. Sperm vitality and morphology were investigated in detail. Sperm ultrastructure was also evaluated after the thawing process. Oxidative biomarkers and enzymatic activities in the post-thawed extender were studied. Moreover, the major identified compounds in the extract were docked to the Bcl-2:BH3 interface to evaluate their individual antiapoptotic potential.

MATERIALS AND METHODS

Plant Material and Extraction

Entada abyssinica bark material was collected from trees growing in Lupaga Site in Shinyanga, Tanzania, and kept under accession number P7301, at IPMB, Heidelberg University. The bark sample was ground and extracted with 100% methanol at ambient temperature for 3 days (6 × 500 mL). The filtered extracts were evaporated under reduced pressure at 40°C. The frozen residue was lyophilized yielding 25% extraction yield based on the initial dry weight.

In vitro Antioxidant Activities

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric-reducing ability (FRAP), and total phenolic content assays were done according to Sobeh et al. (22). All assays were done in triplicates.

HPLC-PDA-ESI-MS/MS

The chemical constituents of the bark extract were annotated utilizing a ThermoFinnigan LCQ-Duo ion trap mass spectrometer (ThermoElectron Corporation, Waltham, MA, USA) with an ESI source (ThermoQuest Corporation, Austin, TX, USA) as detailed in Sobeh et al. (22).

Ethical Approval

The semen samples were collected from a sheep flock belonging to the Animal Production Research Station, El-Karada, Kafrelsheikh, Animal Production Research Institute (APRI), Agricultural Research Center, Ministry of Agriculture, Egypt, in cooperation with the Physiology and Biotechnology Laboratory, Animal Production Department, Faculty of Agriculture, Mansoura University, Egypt. This study was approved by the Ethical Committee of Mansoura University.

Animal Management

Five sexually mature and clinically healthy Ossimi rams (60–80 kg LBW and 2–4 years old) were trained to serve an artificial vagina for collection of semen ejaculates. The animals were kept under natural photoperiod in open shaded stockyard, raised under the same environmental conditions, and fed on concentrate feed mixture with free access to trace mineralized salt lick blocks and drinking water all time.

Collection of Semen

Ejaculates were collected as per conventional artificial vaginal method once weekly from each ram for 5 weeks before feeding at 7–8 a.m. A total of 25 ejaculates were transferred immediately to a water bath at 37°C. Only ejaculates with overall motility ≥70% and minimum sperm concentration ≥2.2×10^9 sperm cells/mL were selected for the experiment, pooled, and then divided into five aliquots to be subjected to the different experimental treatments.

Preparation of Semen Extenders

Tris-citric-soybean lecithin extender (TSBL) was used in this study as a control. It is composed of 250 mM Tris (AppliChem, Germany), 87.5 mM citric acid monohydrate (AppliChem, Germany), 69 mM glucose (Sigma Aldrich, USA), 1% (w/v) soybean lecithin (L-a-phosphatidyl choline, LAB: product number MC041), 5% (v/v) glycerol, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. The extender was shaken gently and warmed in a water bath up to 37°C before use. Osmolarity level and pH value were adjusted to 300 mOsmol and pH 7.3, respectively, before the addition of cryoprotectants.

Biocompatibility of the Extract

The compatibility of the extract with the ram semen was investigated by evaluating the sperm characteristics (progressive motility, vitality, abnormality, membrane integrity, and acrosome integrity) after diluting the fresh semen with different concentrations of the extract and equilibrating for 4 h at 5°C before cryopreservation.

Semen Freezing and Thawing

Dilution of the collected semen was carried out at 37°C with a ratio of 1:10 (semen:extender). Final sperm concentrations were adjusted to 220×10^6 sperm/mL. Extended semen was cooled gradually to 5°C for 4 h, and then the equilibrated semen was aspirated into 0.25 mL French straws and sealed (IMV technologies, France). The straws were exposed to liquid nitrogen vapors for 10 min and finally placed into liquid nitrogen at −196°C. The straws remained in liquid nitrogen until thawing at 37°C in a water bath for 30 s.

Experimental Design

Using supplemented TSBL extender, the semen was extended by adding different concentrations of the bark extract (0, 125, 250, 375, and 500 μg/mL extender) before cryopreservation in liquid nitrogen.

Semen Evaluation

Sperm Progressive Motility

The percentage of progressive sperm motility, which was defined as the ability of a spermatozoa to move forward in a long semi-arc pattern, was determined to analyze the sperm motility. An aliquot (10 μL) of diluted semen was mounted on a previously warmed slide, then covered and investigated by phase-contrast microscope (DM 500, Leica, Switzerland) supplied with a hot stage at 37°C at 100x magnification. A total number of 200 spermatozoa per slide were counted, and the analysis was conducted in three replicates.

Sperm Vitality

Sperm vitality was investigated in a smear of semen stained with 5% eosin (vital stain) and 10% nigrosin (background stain) to estimate alive and dead sperm cells according to Moskovtsev and Librach (23). Percentage of alive sperm cells (unstained ones) was calculated for 300 sperm cells per sample and examined under light microscope at magnification (400x).

Morphological Sperm Abnormalities

Abnormalities were assessed in 300 sperm cells during vitality test using a light microscope. The following criteria were considered: (i) abnormal tails (coiled tail, broken tail, terminally coiled tail, double tail), (ii) abnormal heads (microcephalic head, pear shaped head, round short head, loose head, double head), and (iii) cytoplasmic droplets proximal and distal droplets according to Aamdal et al. (24).

Plasma Membrane Integrity

Plasma membrane integrity of spermatozoa was assessed using hypo-osmotic swelling test (HOS-t) according to the protocol described by Neild et al. (25). Briefly, 50 μL of semen was incubated for 30 min at 37°C in a hypo-osmotic solution (500 μL at osmolarity level of 75 mOsm/kg H_2O), containing fructose (6.75 g/L) and sodium citrate (3.67 g/L). A sample of the mixture was placed on a slide and covered with a cover slip. Sperm cells showing coiled or swollen tails (with functional intact membranes) were counted in all samples using phase-contrast microscope (400x) within total count of 300 sperm cells per slide.

Acrosome Integrity

A drop of frozen–thawed semen was placed on a pre-warmed glass slide and allowed to air-dry. The slide was then fixed in 5% formaldehyde for 30 min, washed afterward under running water, dried, and then immersed in a Giemsa solution for 3 h at 37°C. Finally, the slides were washed under running tap water before dried. All slides were investigated under phase-contrast microscope using oil immersion lens with 200 sperm cells counted.

Biochemicals Assay in the Extender After Thawing

The following parameters in seminal extender were evaluated using the available commercial kits (Biodiagnostic, Egypt) according to the manufacturers' instructions and the mentioned biochemical methods. Total antioxidant concentration (26), hydrogen peroxide, H_2O_2 (27), enzymatic activity of lactic dehydrogenase (LDH) (28), alanine transaminase (ALT), and aspartate transaminase (AST) (29) were noted. The tested parameters were measured using a spectrophotometer (Spectro UV-VIS Auto, UV-2602, Labomed, USA).

Ultrastructure Changes by Transmission Electron Microscope

Semen samples were prepared for transmission electron microscope (TEM) as per the method described by Oliveira et al. (30) with some modifications. In brief, semen extender samples (500 μl) were centrifuged and suspended in 2.5% glutaraldehyde in phosphate-buffered saline for 2 h at 4°C to allow for first fixation. Washing the post-fixed samples was carried out by 1% osmium tetroxide for 90 min at room temperature and followed by dehydration through ascending grades of ethanol. The dehydrated samples were treated with propylene oxide, infiltrated in an equal mixture of epon: propylene oxide, and finally embedded in Epon resin (Epon 812; FlukaChemie, Switzerland). Specimens were transferred into polyethylene capsules using toothpick then placed in an

oven for polymerization at 60°C for 24 h. Ultrathin-sections (60–70 nm) were cut using an ultramicrotome. Observation of the obtained sections was done using a JEOL-JEM 2100 TEM operated at 80 KV. The ultrastructure of sperm was examined in 300 sperms per sample. The observed results were categorized into three patterns and defined according to sperm criteria as described by Baccetti et al. (31): (i) Intact spermatozoa: The ultra-structure of all sperm components (plasma membrane, acrosome, nucleus, and cytoplasm) is normal with no defects. (ii) Apoptotic sperm: altered nuclear structure with irregular marginated chromatin, cytoplasmic residue and binucleate and multinucleate sperm. Discontinuous or deformed plasma membrane or deformed acrosomal structure. (iii) Necrotic spermatozoa: distorted nuclear structure with necrotic chromatin and cytoplasmic residue. Broken or discontinuous plasma membrane deformity or acrosomal absence.

Molecular Docking

The *in silico* molecular docking computational tool was applied to evaluate, on a molecular level, the antiapoptotic potential of the major compounds identified in *Entada abyssinica* bark extract. The X-ray crystallographic structure of the Bcl-2:BH3 interface complex (PDB code: 4B4S) was downloaded from the Protein Data Bank (www.rcsb.org). The docking protocol was applied using Molecular Operating Environment (2010.10; Chemical Computing Group Inc., Montreal, Canada) software. Downloaded protein was protonated to add the hydrogen atoms that were not detected during the crystallization process. Chemical structure of the compounds selected for docking was downloaded directly from PubChem database or drawn using the MOE builder tool. Compounds were then washed to set their ionization state. The MMFF94x force field was used to do the energy minimization for the compounds. Docking was done applying the default settings of placement and scoring.

Statistical Analysis

The general linear model analysis of variance (ANOVA) was applied for data statistical analysis using SAS software (32). Different concentrations of the bark extract were statically tested for their effect. Tukey's test was applied to examine the significant differences among treatments for all considered parameters. Arcsine transformation was performed before the analysis of variance for all percentage values.

RESULTS

Chemical Profiling of the Bark Extract

Altogether, 28 secondary metabolites were tentatively identified in the methanol extract from *E. abyssinica* bark based on their molecular weight, mass fragmentation pattern, available authentic compounds, in-house library, and online literature. Tannins and gallic acid derivatives dominated the extract (**Table 1** and **Supplementary Figure 1**). Among the annotated compounds, one showed a signal at 37.86 min and demonstrated a molecular ion peak at $[M - H]^-$ *m/z* 521 with three daughter ions at 331, 271, 169; it was tentatively characterized as dimethyl caffeoyl galloylglucose (**Supplementary Figure 2**).

TABLE 1 | Phytochemical profiling of *Entada abyssinica* bark extract.

No.	RT	M-H$^-$	MS/MS	Tentatively identified compounds
1	1.53	133	115	Malic acid[a]
2	2.37	417	153, 241, 285	Gentisic acid dipentoside
3	3.18	609	179, 305, 441	(epi)Gallocatechin-(epi)gallocatechin
4	5.25	593	289, 407, 425	(epi)Catechin-(epi)gallocatechin[a]
5	6.26	305	179, 221, 287	(epi)Gallocatechin[a]
6	10.97	761	305, 423, 609	(epi)Gallocatechin-(epi)gallocatechin gallate
7	11.50	183	125, 169, 183	Methylgallate
8	12.19	483	169, 271, 331	Digalloyl glucose
9	13.05	289	179, 205, 245	(epi)Catechin[a]
10	17.47	457	179, 305	Gallocatechin gallate[a]
11	18.17	745	289, 407, 593	(epi)Catechin-(epi)gallocatechin gallate[a]
12	18.98	457	169, 305, 331	(epi)Gallocatechin gallate[a]
13	20.17	729	289, 559, 577	(epi)Catechin-(epi)catechin gallate[a]
14	20.65	457	179, 305	Gallocatechin gallate
15	21.78	617	285, 493, 599	Kaempferol syringyl gallate
16	22.60	729	289, 559, 577	(epi)Catechin-(epi)catechin gallate
17	25.19	541	169, 211, 271, 541	Hydroxybenzoylbenzyl-*O*-galloyl-glucoside
18	26.35	615	271, 301, 463	Quercetin galloylglucose
19	27.73	441	179, 245, 289	Catechin gallate
20	28.73	441	179, 245, 289	(epi)Catechin gallate[a]
21	30.34	477	169, 315, 331, 417	Coumaroyl-*O*-galloylglucose
22	32.19	507	169, 323, 445, 447	Methyl gallate caffeoylglucose
23	34.12	601	169, 313, 439	Caffeoyl pyrogallol galloylglucose
24	36.38	435	273	Phlorizin
25	37.86	521	169, 271, 331	Dimethyl caffeoyl galloylglucose
26	39.10	585	169, 313, 439	*p*-Coumaroyl pyrogalloylgalloylglucose
27	41.88	477	169, 313, 327	Coumaroyl-*O*-galloylglucose
28	43.45	461	169, 313, 401	Cinnamoyl-*O*-galloylglucose

[a] *Identified according to Sobeh et al. (22).*

Another compound exhibiting $[M - H]^-$ *m/z* at 585 and three main ions at 331, 271, 169, was tentatively annotated as *p*-coumaroyl pyrogalloylgalloylglucose (**Supplementary Figure 3**). Additionally, a signal with $[M - H]^-$ *m/z* 461 was identified as cinnamoyl-*O*-galloylglucose (**Supplementary Figure 4**).

Antioxidant Activity of the Bark Extract and Its Effect on Post-thawed Semen Extender of *Ovis aries*

To initially investigate the antioxidant potential of the extract, DPPH and FRAP assays were performed, and TPC was determined. The extract demonstrated substantial activity in

both assays and showed an appreciable TPC of 240 mg GAE/gm extract (**Table 2**).

The compatibility of the extract with the fresh ram semen was investigated. The extract was found to be safe in concentrations up to 500 μg/mL. Progressive motility, vitality, abnormality, and acrosome integrity were not affected (**Table 3**). Then after, the potential value of the extract as a supplement during cryopreservation was investigated through examining the characteristics, morphological abnormalities, the oxidative stress biomarkers, and the ultrastructural changes in the post-thawed ram sperms. The extract significantly enhanced the progressive motility and membrane integrity (**Table 4**). However, other parameters including sperm vitality, abnormal morphology, and acrosome integrity were not significantly affected when compared to the control.

In sperm-based cell assays, the total antioxidant capacity (TAC) was increased insignificantly at a concentration of 375 μg/mL, while the concentration of H_2O_2 was significantly reduced when compared to the control group without significant change in the concentration of LDH, AST, and ALT activities (**Table 5**).

TABLE 2 | Antioxidant activity of the methanol bark extract of *Entada abyssinica*.

Sample	DPPH (EC_{50} μg/mL)	FRAP (mM $FeSO_4$ equivalent/mg sample)	TPC mg GAE/g extract
Bark extract	35.8	13.21	240
Ascorbic acid	2.92 ± 0.29	–	–
Quercetin	–	24.04 ± 1.23	–

Ascorbic acid and quercetin are positive controls. DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; FRAP, ferric-reducing antioxidant power assay; TPC, total phenolic content.

Effect of the Bark Extract on Sperm Ultrastructure Post-thawing

The sperm characterization in different groups was assessed based on the criteria mentioned in the Materials and Methods section. The normal sperms appear intact with continuous plasma membrane (PM) along the acrosomal cap (AC), intermediate, and tail regions as seen in **Figure 1A**. Normal structure of dense elongated nucleus was also noticed. The abnormal sperms in different groups show dented plasma membrane (PM) with gaps observed between membrane and nucleus (**Figure 1B**). **Figures 1C,D** show notable abnormalities in sperm membrane system exhibited as cytoplasmic residues (CPR), destruction in acrosomal region (DAC), and complete lysis of plasma membrane (LPM). At the level of 375 μg/mL, the extract significantly increased the percentages of intact sperm cells and significantly decreased apoptotic spermatozoa without significant change in necrotic spermatozoa when compared to the control (**Table 6**).

Molecular Docking Study

The major compounds identified in the extract were docked to the surface interface of the Bcl-2:BH3 complex. As shown in **Table 7**, the docked compounds showed appreciable free binding energy manifested by the low value of the docking scores. This reveals the potential of the extract components to

TABLE 3 | Sperm characteristics in extender of post equilibrated (at 5°C for 4 h) fresh ram semen supplemented with different concentrations of the bark extract (means ± SE, $n = 5$).

Sample	Progressive motility	Vitality	Abnormality	Membrane integrity	Acrosome integrity
			%		
Control	81.0 ±1.87	77.6 ±3.50	7.6 ±1.03	75.4 ±2.42^{b}	92.4 ±0.51
Extract 125 μg/mL	84.0 ±1.87	82.6 ±1.60	8.4 ±0.51	81.2 ±2.01^{a}	93.4 ±1.33
Extract 250 μg/mL	84.0 ±1.87	79.6 ±1.63	10.2 ±1.46	82.8 ±1.59^{a}	94.0 ±1.30
Extract 375 μg/mL	86.0 ±1.87	82.8 ±1.85	7.8 ±0.49	84.0 ±1.70^{a}	93.0 ±0.45
Extract 500 μg/mL	80.0 ±2.24	77.6 ±1.44	10.4 ±1.44	78.2 ±1.53^{b}	94.0 ±0.71

^{a,b}*Means denoted within the same column with different superscripts are significantly different at $p < 0.05$ compared with the control group.*

TABLE 4 | Sperm characteristics in extender of post-thawed ram semen supplemented with different concentrations of the bark extract (means ± SE, $n = 5$).

Sample	Progressive motility	Vitality	Membrane integrity	Acrosome integrity	Abnormal morphology
			%		
Control	48.0 ±2.55^{b}	44.6 ±3.63	43.4 ±2.80^{b}	88.2 ±0.86	12.4 ±0.51
Extract 125 μg/mL	56.0 ±1.87^{a}	48.6 ±2.34	45.6 ±2.16^{b}	88.8 ±0.58	12.8 ±0.97
Extract 250 μg/mL	56.0 ±2.92^{a}	48.8 ±1.91	45.4 ±2.36^{b}	88.0 ±1.73	14.4 ±2.01
Extract 375 μg/mL	59.0 ±1.87^{a}	51.0 ±1.90	53.6 ±2.87^{a}	86.8 ±0.80	15.6 ±1.29
Extract 500 μg/mL	52.0 ±2.00^{ab}	45.8 ±2.08	41.2 ±2.03^{b}	88.0 ±0.84	14.8 ±0.97

^{a,b}*Means denoted within the same column with different superscripts are significantly different at $p < 0.05$.*

TABLE 5 | Antioxidant capacity, oxidative stress, and enzymatic activity in extender of post-thawed ram semen supplemented with different concentrations of the bark extract (Means ± SE, $n = 3$).

Sample	TAC	H_2O_2	LDH	AST	ALT
	Mm/L	nm/L	U/mL	U/L	
Control	0.20 ±0.07	0.36 ±0.05^{ab}	147.5 ±24.84	67.3 ±3.33	18.7 ±0.67
Extract 125 μg/mL	0.31 ±0.05	0.27 ±0.01^{bc}	139.4 ±38.17	52.7 ±6.36	15.3 ±2.40
Extract 250 μg/mL	0.36 ±0.03	0.27 ±0.03^{bc}	143.9 ±19.48	48.7 ±7.69	16.0 ±1.15
Extract 375 μg/mL	0.45 ±0.03	0.2 ±0.06^{c}	134.0 ±10.94	49.3 ±9.33	16.7 ±1.76
Extract 500 μg/mL	0.30 ±0.09	0.45 ±0.05^{a}	118.7 ±6.79	68.0 ±4.00	16.0 ±2.31

^{a,b,c}*Means denoted within the same column with different superscripts are significantly different at* $p < 0.05$.

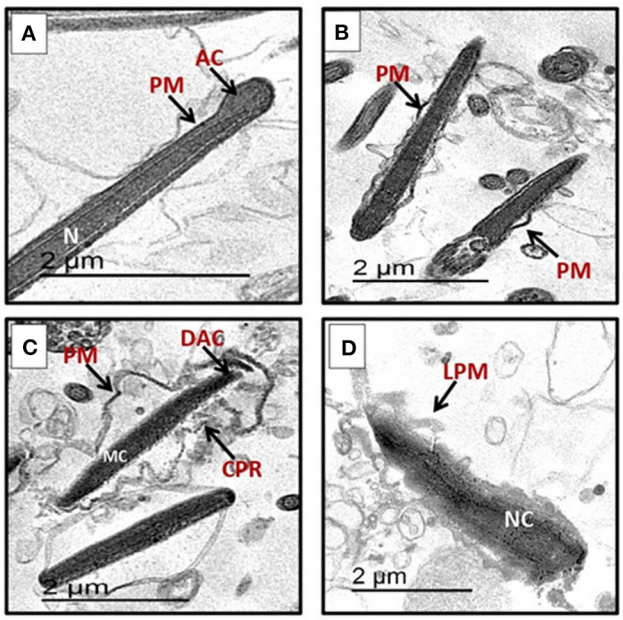


FIGURE 1 | Transmission electron microscope (TEM) micrographs of ram spermatozoa in post-thawed semen showing **(A)** normal sperm with complete nuclei (N), homogenous condensed chromatin, intact acrosomal cap (AC), normal plasma membrane (PM). **(B)** Early apoptotic sperm cells with mildly swollen plasma membrane (PM). **(C)** Sperm with apoptotic nucleus characterized by marginated chromatin (MC), cytoplasmic residue (CPR), broken plasma membrane (PM), detached with apical ridge formed near the tip of acrosomal cap (DAC), degenerated acrosome. **(D)** Sperm with necrotic chromatin (NC), with cytoplasmic residue (CPR), lost plasma membrane (LPM), and degenerated acrosome (DAC).

inhibit the dimerization of the Bcl-2 with the BH3 domain of the proapoptotic Bim protein, thus hindering the apoptosis cascade.

DISCUSSION

Previous studies reported that sperm viability and motility, the integrity of both plasma membrane and acrosome in post-thawed semen are negatively affected during the cryopreservation process (33, 34). The mechanism by which the cryopreservation affects motility has not been fully explained; however, a solid correlation links the percentage of immotile spermatozoa and mitochondrial defects in post-thawing sperm (33). Sperm cells have a large surface with small size; thus, they are sensitive to the damage caused by cryopreservation and the consequences of ROS production (35). In this study, the H_2O_2 concentration was significantly decreased in the sperm extender upon adding the plant extract in increasing concentrations (125, 250, 375, and 500 μg/mL). It was noticeable that the higher the extract concentration used, the stronger the antioxidant effect until a concentration of 375 μg/mL.

Reactive oxygen species (ROS) may cause apoptosis and DNA damage plus other cellular alterations such as lipid peroxidation, disruption of plasma membrane, and mitochondria (36). The observed significant improvement of the sperms' membrane integrity and progressive motility in the group supplemented with the extract (375 μg/mL) could be explained by the extract's substantial antioxidant potential that counters the effect of oxidative stress produced by cryopreservation. By increasing the concentration of the extract to 500 μg/mL, the post-thawed sperm quality started to decline significantly compared to the other concentrations. This may be due to the high concentration of tannins that potentially inhibit the activity associated with apoptosis regulation (37). Our findings are in agreement with previous reports that recommend the addition of different antioxidants in semen extenders during cryopreservation (13, 14, 38, 39).

Cryopreservation induces negative changes in plasma membrane and acrosomal structure (14). In the current study, the percentage of the intact sperms was significantly increased upon adding the extract to the extender in a dose-dependent manner until a concentration of 500 μg/mL. On the contrary, the percentage of the apoptotic sperms was significantly decreased in the groups where the extract was added in the concentrations 125, 250, and 375 μg/mL. The last group (500 μg/mL) recorded the highest value of apoptotic sperms.

Plasma membrane defects may impair the sperm vitality and motility (40). The decreased motility of the preserved spermatozoa was reported to be as a result of the ultrastructural changes taking place during the process (41). The physical and chemical factors to which a sperm is exposed are the main causes of such alterations. Ice crystals formation around the cell membranes and increasing of the permeability are the most probable reasons. Formation of ROS, on the other side, affects

TABLE 6 | Percentage of sperm groups characterized using TEM in extender of post-thawed ram semen supplemented with different concentrations of the bark extract (Means ± SE, $n = 3$).

Sample	Intact sperm	Apoptotic sperm	Necrotic sperm
	%		
Control	38.3 ± 2.03^{b}	40.3 ± 0.88^{b}	21.3 ± 1.86^{c}
Extract 125 μg/mL	48.0 ± 2.08^{a}	31.7 ± 1.86^{c}	21.7 ± 2.03^{c}
Extract 250 μg/mL	41.7 ± 0.88^{b}	30.3 ± 0.88^{c}	28.0 ± 0.58^{ab}
Extract 375 μg/mL	53.3 ± 1.45^{a}	21.7 ± 1.45^{d}	25.0 ± 1.73^{bc}
Extract 500 μg/mL	16.3 ± 2.03^{c}	53.3 ± 2.19^{a}	30.3 ± 0.67^{a}

^{a,b,c,d}*Means denoted within the same column with different superscripts are significantly different at $p < 0.05$.*

TABLE 7 | Docking scores of the docking poses obtained upon docking major compounds identified in the bark extract to Bcl-2:BH3 complex interface.

Compound number	Compound name	Docking score (kcal/mol)
5	Epigallocatechin	−13.16
6	Epigallocatechin-epigallocatechin gallate	−16.49
8	Digalloyl glucose	−12.72
12	Epigallocatechin gallate	−15.69
13	Epicatechin-epicatechin gallate	−16.66
18	Quercetin galloylglucose	−13.62
20	Epicatechin gallate	−13.89
24	Phlorizin	−9.44
25	Dimethyl caffeoyl galloylglucose	−13.57
26	*p*-Coumaroyl pyrogalloyl galloylglucose	−13.23
27	*p*-Coumaroyl galloylglucose	−13.12
28	Cinnamoyl galloylglucose	−10.32

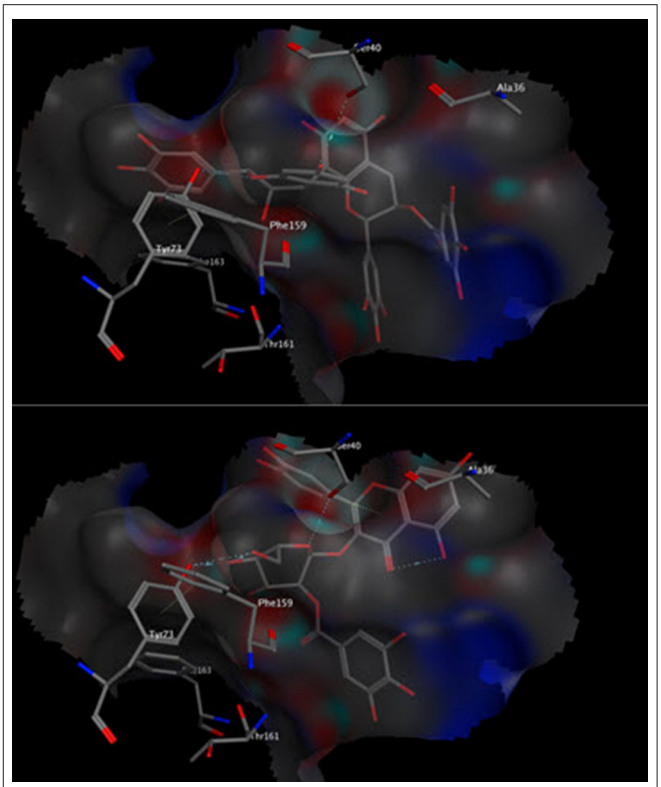


FIGURE 2 | Three-dimensional poses of compounds 6 (top) and 18 (bottom) docked into the Bcl-2:BH3 surface interface.

plasma membrane integrity, nuclear structure, and leads to apoptosis (42). Previous reports described the antioxidant effect of caffeic acid and its derivatives on normal cells. Epigallocatechin 3-gallate (EGCG) has been studied *in vitro* and *in vivo*. It was reported that EGCG scavenges hydroxyl radicals that react with plasma membrane phospholipids and proteins, which, in turn, improves DNA fragmentation (43). Epicatechin, gallocatechin gallate, and quercetin galloylglucose, secondary metabolites identified in the extract, exhibited similar activities (14, 44).

Molecular modeling was conducted to gain more insights about the antiapoptotic potential of the extract. The major identified compounds were docked into the surface interface of Bcl-2:BH3 complex. It is reported that the programmed cell death (apoptosis) is regulated by the family of B-cell lymphoma-2 proteins (Bcl-2), which comprises antiapoptotic proteins such as Bcl-2 and proapoptotic proteins such as Bim (45). It is well accepted that apoptotic pathways are activated through heterodimerization between pro- and antiapoptotic members into a protein complex such as that of Bcl-2 and Bim. The BH3 domain of the proapoptotic Bim protein binds to a hydrophobic cleft on the Bcl-2 surface initiating the apoptotic pathways (46). Interfering with such interaction by small organic molecules could hinder such interaction and prohibit cell death. The docked compounds were able to bind successfully to the Bcl-2:BH3 interface with appreciable binding energies affording several polar and non-polar interactions with the amino acid residues in the binding site. Compounds 6, 18, 24, 25, and 27 were even able to interact with the Tyr73 residue, which was reported to be among the residues contributing favorably to the binding energy of the Bcl-2:BH3 complex (47). Out of these compounds, epigallocatechin–epigallocatechin gallate (6) and quercetin galloylglucose (18) showed the minimal binding energy of 16.49 and 13.62 kcal/mol, respectively (**Figure 2**). Because polyphenols are partially ionized in physiological pH, we considered the docking of the ionized form of the compounds. They showed comparable docking scores with some additional ionic interactions with the basic amino acid residues in the binding site. Our results are in agreement with other studies that have reported antiapoptotic activity for tannin-rich extracts such as *Lannea stuhlmannii*, *Lannea humilis*, and *Senna sengueana* (22, 48).

CONCLUSION

The present study profiled the chemical composition of *Entada abyssinica* bark extract. In addition, it highlights the antioxidant activities of the extract *in vitro* and in a semen-based model.

Taken together, the obtained results suggest that *Entada abyssinica* extract could be useful as a natural antioxidant that have a potential activity to protect cryopreserved sperm cells against oxidative stress. Nevertheless, the ability of the extract to attain higher fertilization rates in reproductive technologies is recommended to be studied in more detail.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of the Mansoura University.

AUTHOR CONTRIBUTIONS

MS took part in the conceptualization, methodology, software, data curation, writing, review, and editing, and visualization. SH took part in the conceptualization, methodology, data curation, writing the original draft, and visualization. MH took part in the methodology, software, writing the original draft, and writing, review, and editing. WK took part in the conceptualization, methodology, formal analysis, data curation, and writing the original draft, reviewing, and Editing. MA was in charge of the methodology, formal analysis, data curation, and writing the original draft. MW was involved in the conceptualization, writing, reviewing, and editing, and project administration. AY took part by writing, reviewing, and editing and by project administration. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.604477/full#supplementary-material

Supplementary Figure 1 | LC-MS profile of *Entada abyssinica* bark extract.

Supplementary Figure 2 | (A) MS/MS profile of dimethyl caffeoyl galloylglucose. **(B)** Proposed fragmentation pattern of compound 25.

Supplementary Figure 3 | (A) MS/MS profile of *p*-coumaroyl pyrogalloylgalloylglucose. **(B)** Proposed fragmentation pattern of compound 26.

Supplementary Figure 4 | MS/MS profile of cinnamoyl-*O*-galloylglucose (compound 28).

REFERENCES

1. Flores E, Ramió-Lluch L, Bucci D, Fernández-Novell J, Peña A, Rodríguez-Gil J. Freezing-thawing induces alterations in histMeth Enzymolne H1-DNA binding and the breaking of protein-DNA disulfide bonds in boar sperm. *Theriogenology*. (2011) 76:1450–64. doi: 10.1016/j.theriogenology.2011.05.039
2. Masoudi R, Sharafi M, Shahneh AZ, Towhidi A, Kohram H, Esmaeili V, et al. Fertility and flow cytometry study of frozen-thawed sperm in cryopreservation medium supplemented with soybean lecithin. *Cryobiology*. (2016) 73:69–72. doi: 10.1016/j.cryobiol.2016.05.010
3. Andrabi S, Maxwell W. A review on reproductive biotechnologies for conservation of endangered mammalian species. *Anim Reprod Sci.* (2007) 99:223–43. doi: 10.1016/j.anireprosci.2006.07.002
4. Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. *Reprod Biomed Online.* (2018) 37:327–39. doi: 10.1016/j.rbmo.2018.05.012
5. Lv C, Wu G, Hong Q, Quan G. Spermatozoa cryopreservation: state of art and future in small ruminants. *Biopreserv Biobank.* (2019) 17:171–82. doi: 10.1089/bio.2018.0113
6. Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reprod Biomed Online.* (2003) 6:191–200. doi: 10.1016/S1472-6483(10)61710-5
7. Salamon S, Maxwell W. Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Anim Reprod Sci.* (1995) 38:1–36. doi: 10.1016/0378-4320(94)01328-J
8. Salamon S, Maxwell W. Storage of ram semen. *Anim Reprod Sci.* (2000) 62:77–111. doi: 10.1016/S0378-4320(00)00155-X
9. Gandini L, Lombardo F, Lenzi A, Spano M, Dondero F. Cryopreservation and sperm DNA integrity. *Cell Tissue Bank.* (2006) 7:91–8. doi: 10.1007/s10561-005-0275-8
10. López-Fernández C, Crespo F, Arroyo F, Fernández J, Arana P, Johnston S, et al. Dynamics of sperm DNA fragmentation in domestic animals: II. The stallion. *Theriogenology*. (2007) 68:1240–50. doi: 10.1016/j.theriogenology.2007.08.029
11. Tavilani H, Goodarzi MT, Vaisi-Raygani A, Salimi S, Hassanzadeh T. Activity of antioxidant enzymes in seminal plasma and their relationship with lipid peroxidation of spermatozoa. *Int Braz J Urol.* (2008) 34:485–91. doi: 10.1590/S1677-55382008000400011
12. Evans G, Maxwell WC. *Salamons' Artificial Insemination of Sheep and Goats.* Sydney, NSW: Butterworths (1987).
13. Kankofer M, Kolm G, Aurich J, Aurich C. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. *Theriogenology*. (2005) 63:1354–65. doi: 10.1016/j.theriogenology.2004.07.005
14. Sobeh M, Hassan SA, El Raey MA, Khalil WA, Hassan MA, Wink M. Polyphenolics from Albizia harveyi exhibit antioxidant activities and counteract oxidative damage and ultra-structural changes of cryopreserved bull semen. *Molecules.* (2017) 22:1993. doi: 10.3390/molecules22111993
15. Abdelnour SA, Hassan MA, Mohammed AK, Alhimaidi AR, Al-Gabri N, Al-Khaldi KO, et al. The effect of adding different levels of curcumin and its nanoparticles to extender on post-thaw quality of cryopreserved rabbit sperm. *Animals.* (2020) 10:1–13. doi: 10.3390/ani10091508
16. Ismail AA, Abdel-Khalek AE, Khalil, WA, Yousif AI, Saadeldin IM, Abomughaid MM, et al. Effects of mint, thyme, and curcumin extract nanoformulations on the sperm quality, apoptosis, chromatin decondensation, enzyme activity, and oxidative status of cryopreserved goat semen. *Cryobiology*. (2020). doi: 10.1016/j.cryobiol.2020.09.002. [Epub ahead of print].
17. Olajide OA, Alada A. Studies on the anti-inflammatory properties of *Entada abyssinica*. *Fitoterapia.* (2001) 72:492–6. doi: 10.1016/S0367-326X(01)00273-8
18. Teke GN, Lunga PK, Wabo HK, Kuiate JR, Vilarem G, Giacinti G, et al. Antimicrobial and antioxidant properties of methanol extract, fractions and compounds from the stem bark of *Entada abyssinica* Stend ex A. Satabie. *BMC Complement Altern Med.* (2011) 11:57. doi: 10.1186/1472-6882-11-57
19. Kuete V, Voukeng IK, Tsobou R, Mbaveng AT, Wiench B, Beng VP, et al. Cytotoxicity of Elaoephorbia drupifera and other Cameroonian medicinal plants against drug sensitive and multidrug resistant cancer cells. *BMC Complement Altern Med.* (2013) 13:1. doi: 10.1186/1472-6882-13-250

20. Dzoyem JP, Melong R, Tsamo AT, Tchinda AT, Kapche DG, Ngadjui BT, et al. Cytotoxicity, antimicrobial and antioxidant activity of eight compounds isolated from *Entada abyssinica* (Fabaceae). *BMC Res Notes.* (2017) 10:118. doi: 10.1186/s13104-017-2441-z
21. Olajide OA, Akinola Alada AR, Kolawole OT. Anti-inflammatory properties of *Entada abyssinica*. *Leaves Pharmaceutic Biol.* (2005) 43:583–5. doi: 10.1080/13880200500301654
22. Sobeh M, Mahmoud MF, Hasan RA, Abdelfattah MA, Sabry OM, Ghareeb MA, et al. Tannin-rich extracts from Lannea stuhlmannii and Lannea humilis (Anacardiaceae) exhibit hepatoprotective activities *in vivo* via enhancement of the anti-apoptotic protein Bcl-2. *Sci Rep.* (2018) 8:1–16. doi: 10.1038/s41598-018-27452-8
23. Moskovtsev S, Librach C. Methods of sperm vitality assessment. *Spermatogenesis*. (2013) 927:13–9. doi: 10.1007/978-1-62703-038-0_2
24. Aamdal J, Andersen K, Fougner J. Insemination with frozen semen in the blue fox. In: *Proceedings of the 7th International Congress on Animal Reproduction and Artificial Insemination.* Munich (1972). p. 1713–6.
25. Neild D, Chaves G, Flores M, Mora N, Beconi M, Agüero A. Hypoosmotic test in equine spermatozoa. *Theriogenology.* (1999) 51:721–7. doi: 10.1016/S0093-691X(99)00021-7
26. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Colorimetric method for determination of total antioxidant capacity. *J Clin Pathol.* (2001) 54:356–61. doi: 10.1136/jcp.54.5.356
27. Aebi H. Catalase *in vitro*. *Meth Enzymol.* (1984) 105:121–6. doi: 10.1016/S0076-6879(84)05016-3
28. Bais R, Philcox M. IFCC methods for the measurement of catalytic concentration of enzymes. Part 8. IFCC method for lactate dehydrogenase (L-lactate: NAD. *J. Anal. Methods Chem*. (1994) 16:167–182. doi: 10.1155/S1463924694000210
29. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol.* (1957) 28:56–63. doi: 10.1093/ajcp/28.1.56
30. Oliveira LZ, Hossepian De Lima VF, Levenhagen MA, Dos Santos RM, Assumpção TI, Jacomini JO, et al. Transmission electron microscopy for characterization of acrosomal damage after Percoll gradient centrifugation of cryopreserved bovine spermatozoa. *J Vet Sci.* (2011) 12:267–72. doi: 10.4142/jvs.2011.12.3.267
31. Baccetti B, Collodel G, Piomboni P. Apoptosis in human ejaculated sperm cells (notulae seminologicae 9). *J Submicrosc Cytol Pathol.* (1996) 28:587–96.
32. SAS (2007). *Statistical Analysis System. Stat-User's Guid*. Release 9.1.3. Cary, NC: SAS Institute.
33. Ozkavukcu S, Erdemli E, Isik A, Oztuna D, Karahuseyinoglu S. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. *J Assist Reprod Genet.* (2008) 25:403–11. doi: 10.1007/s10815-008-9232-3
34. Khalil WA, El-Harairy MA, Zeidan AE, Hassan MA, Mohey-Elsaeed O. Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights. *Int J Vet Sci Med*. (2018) 6:S49–56. doi: 10.1016/j.ijvsm.2017.11.001
35. Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol Reprod Dev.* (2001) 59:451–8. doi: 10.1002/mrd.1052
36. Bollwein H, Fuchs I, Koess C. Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. *Reprod Domest Anim.* (2008) 43:189–95. doi: 10.1111/j.1439-0531.2007.00876.x
37. Nam S, Smith DM, Dou QP. Tannic acid potently inhibits tumor cell proteasome activity, increases p27 and Bax expression, and induces G1 arrest and apoptosis. *Cancer Epidemiol Prevent Biomark.* (2001) 10:1083–8.
38. Khalil WA, El-Harairy MA, Zeidan AE, Hassan MA. Impact of selenium nano-particles in semen extender on bull sperm quality after cryopreservation. *Theriogenology.* (2019) 126:121–7. doi: 10.1016/j.theriogenology.2018.12.017
39. Shahin MA, Khalil WA, Saadeldin IM, Swelum AA-Z, El-Harairy MA. Comparison between the effects of adding vitamins, trace elements, and nanoparticles to shotor extender on the cryopreservation of dromedary camel epididymal spermatozoa. *Animals.* (2020) 10:1–16. doi: 10.3390/ani10010078
40. Ezzati M, Shanehbandi D, Hamdi K, Rahbar S, Pashaiasl M. Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. *Cell Tissue Bank.* (2020) 21:1–15. doi: 10.1007/s10561-019-09797-0
41. Holt W, Penfold L, Johnston S, Temple-Smith P, Shaw J, Mccallum C, et al. Cryopreservation of macropodid spermatozoa: new insights from the cryomicroscope. *Reprod Fertil Dev.* (1999) 11:345–53. doi: 10.1071/RD99076
42. Peris-Frau P, Soler AJ, Iniesta-Cuerda M, Martín-Maestro A, Sánchez-Ajofrín I, Medina-Chávez DA, et al. Sperm Cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *Int J Mol Sci.* (2020) 21:1–22. doi: 10.3390/ijms21082781
43. Kim H-S, Quon MJ, Kim J-A. New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. *Redox Biol.* (2014) 2:187–95. doi: 10.1016/j.redox.2013.12.022
44. Tvrda E, Straka P, Galbavy D, Ivanic P. Epicatechin provides antioxidant protection to bovine spermatozoa subjected to induced oxidative stress. *Molecules.* (2019) 24:3226. doi: 10.3390/molecules24183226
45. Reed JC. Mechanisms of apoptosis. *Am J Pathol.* (2000) 157:1415–30. doi: 10.1016/S0002-9440(10)64779-7
46. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat RevMol Cell Biol.* (2008) 9:47–59. doi: 10.1038/nrm2308
47. Rautureau G, Yabal M, Yang H, Huang D, Kvansakul M, Hinds M. The restricted binding repertoire of Bcl-B leaves Bim as the universal BH3-only prosurvival Bcl-2 protein antagonist. *Cell Death Dis.* (2012) 3:e443. doi: 10.1038/cddis.2012.178
48. Sobeh M, Mahmoud MF, Hasan RA, Cheng H, El-Shazly AM, Wink M. Senna singueana: Antioxidant, hepatoprotective, antiapoptotic properties and phytochemical profiling of a methanol bark extract. *Molecules.* (2017) 22:1–16. doi: 10.3390/molecules22091502

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 25 February 2021
doi: 10.3389/fvets.2021.609180

Impact of Cryopreservation on Spermatozoa Freeze-Thawed Traits and Relevance OMICS to Assess Sperm Cryo-Tolerance in Farm Animals

*Ibrar Muhammad Khan[1], Zubing Cao[1], Hongyu Liu[1], Adnan Khan[2], Sajid Ur Rahman[3], Muhammad Zahoor Khan[4], Anucha Sathanawongs[5] and Yunhai Zhang[1]**

[1] Anhui Provincial Laboratory of Local Livestock and Poultry Genetical Resource Conservation and Breeding, College of Animal Science and Technology, Anhui Agricultural University, Hefei, China, [2] Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agriculture Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China, [3] Key Laboratory of Animal Parasitology of Ministry of Agriculture, Laboratory of Quality and Safety Risk Assessment for Animal Products on Biohazards (Shanghai) of Ministry of Agricultural Sciences, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China, [4] State Key Laboratory of Animal Nutrition, Beijing Engineering Technology Research Center of Raw Milk Quality and Safety Control, College of Animal Science and Technology, China Agriculture University, Beijing, China, [5] Department of Veterinary Biosciences and Veterinary Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Ana Josefa Soler, University of Castilla-La Mancha, Spain
Abouzar Najafi, University of Tehran, Iran

***Correspondence:**
Yunhai Zhang
yunhaizhang@ahau.edu.cn

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *22 September 2020*
Accepted: *01 February 2021*
Published: *25 February 2021*

Citation:
Khan IM, Cao Z, Liu H, Khan A, Rahman SU, Khan MZ, Sathanawongs A and Zhang Y (2021) Impact of Cryopreservation on Spermatozoa Freeze-Thawed Traits and Relevance OMICS to Assess Sperm Cryo-Tolerance in Farm Animals. Front. Vet. Sci. 8:609180. doi: 10.3389/fvets.2021.609180

Sperm cryopreservation is a powerful tool for the livestock breeding program. Several technical attempts have been made to enhance the efficiency of spermatozoa cryopreservation in different farm animal species. However, it is well-recognized that mammalian spermatozoa are susceptible to cryo-injury caused by cryopreservation processes. Moreover, the factors leading to cryo-injuries are complicated, and the cryo-damage mechanism has not been methodically explained until now, which directly influences the quality of frozen–thawed spermatozoa. Currently, the various OMICS technologies in sperm cryo-biology have been conducted, particularly proteomics and transcriptomics studies. It has contributed while exploring the molecular alterations caused by cryopreservation, identification of various freezability markers and specific proteins that could be added to semen diluents before cryopreservation to improve sperm cryo-survival. Therefore, understanding the cryo-injury mechanism of spermatozoa is essential for the optimization of current cryopreservation processes. Recently, the application of newly-emerged proteomics and transcriptomics technologies to study the effects of cryopreservation on sperm is becoming a hotspot. This review detailed an updated overview of OMICS elements involved in sperm cryo-tolerance and freeze-thawed quality. While also detailed a mechanism of sperm cryo-injury and utilizing OMICS technology that assesses the sperm freezability potential biomarkers as well as the accurate classification between the excellent and poor freezer breeding candidate.

Keywords: spermatozoa cryo-biology, functional traits, cryo-injuries, cryo-tolerance fingerprints, molecular tools

INTRODUCTION

Sperm cryopreservation has become a popular technique for the long-lasting semen preservation of genetically superior animals, related transgenic lines, and mammalian endangered species (1, 2). Besides, cryopreservation assists the wide spread of genetic diversity, and contributed greatly into the extension of reproductive technologies worldwide, such as artificial insemination and *in-vitro* fertilization (3).

However, cryopreservation can have a detrimental effect on the normal physiology of sperm, causing damage and modifications that eventually lead to the death of the sperm, thereby reducing freeze-thawed quality parameters (2). Furthermore, the conflicts in sperm size, shape, and lipid-protein content among the species demonstrate that cryopreservation methods are not fairly efficient in all species (4). It has been recorded by Grötter et al. (5) that farm animals like bull, ram, and boar produce more cryo-sensitive spermatozoa than human, rabbit, cat, and dog. In addition to the interspecies variability, many other variables such as freezing-thawing rates, type of semen extenders or cryo-protectants, the origin of spermatozoa (epididymal or ejaculate sperm), seasonal fluctuations, and even inter-or intra-individual variations also influence the success of the cryopreservation method (6, 7).

In 1937, glycerol was used as freezing medium for semen of bull, ram, stallion, boar, and rabbit at cooling (−21°C) phase. The good cryo-protective effects were obtained when the glycerol concentrations ranged from 0.5 to 2 M (8). Then, about 10 years later, the Polge et al. (9) further confirmed the positive effects of glycerol on frozen poultry semen. However, the glycerol causes toxicity in sperm by denaturation of protein, alteration *via* actin interactions, and induction of plasma membrane fragility during cryopreservation (9–11). Another significant breakthrough was achieved during the 1950s, when dry ice was replaced by liquid nitrogen as a freezing medium; since sperm can be preserved viable at −196°C unlimitedly. On the contrary, dry ice cannot completely stop the metabolic activity of mammalian cells (12). However, it should be noted that some drawbacks still exist about the concept whether storage in liquid nitrogen is completely harmless to the viability of frozen sperm (13, 14).

Impact of cryopreservation on sperm biology produced novel consequences; and has led to the development of modern cryopreservation techniques where particular proteins, antioxidants, and cryo-protective agents are integrated into the freezing medium to enhance the cryo-survival of spermatozoa (15). There has been no genetic selection of the breeding stocks for semen cryopreservation in animal breeding programs, even though improvement has been found in outlining the major genes involved in spermatozoa cryo-biological function (16). Although it has been proved that some sperm protein markers are correlated with high cryo-tolerance, their function is reliant on the presence of mRNA (7). It has been recommended that spermatozoa RNAs evaluation provides valuable information on their biological function (16, 17).

However, to date, there is a limited collection of literature about the associations of OMICS with spermatozoa freeze-thawed quality of farm animals. The spermatozoa freeze-thawing resilience varies based on their physical characteristics, such as size, shape, and lipid content. Therefore, it is difficult to establish a standardized freezing technique for the breeding management in various species of animal. The review explored how cryopreservation alters the structural and molecular integrity of freeze-thawed spermatozoa. Additionally, the review also details the current understanding of the OMICS element present in the farm animal spermatozoa and their potential use in predicting sperm cryo-tolerance.

CRYOPRESERVATION DETERIORATES SPERMATOZA FREEZE-THAWED QUALITY

Cryopreservation damages the sperm in a variety of ways such as ultra-structural damage and sub-lethal damages that encourage oxidative and osmotic stresses, which amend lipid and protein configuration, decrease motility and viability, cause injury to mitochondria and spermatozoa tail, and intensify sperm DNA fragmentation, leading to a decline in freeze-thawed sperm quality as shown in **Figure 1** (2, 18). A spermatozoon consists of several membranes, such as plasma membrane, mitochondrial membrane, and the acrosomal membrane. These membranes act as physiological barriers that must remain intact to ensure sperm viability, particularly after cryopreservation (13, 19). Cryopreservation induces structural damages of mitochondria, altering the biochemical processes involved in ATP production and ultimately reducing spermatozoa freeze-thawed viability and motility (20).

Structural and Molecular Integrity of Freeze-Thawed Spermatozoa

The spermatozoa plasma membrane is the midline between the inner and outer environments. The plasma membrane plays a vital role for male and female gametes, displaying receptors responsible for sperm–oocyte interactions (21). Integrity of membrane-intact spermatozoa is required for survival in the female genital tract. Alterations in membrane structures may be associated with dysregulation of the lipids, resulting in oxidative stress (22). The higher ratio of unsaturated to saturated fatty acid in the plasma membrane makes more susceptible to cryopreservation-related damage and peroxidation (23). More damage has been detected in the plasma membrane and acrosome membranes during freezing-thawing cycle because these parts are more exposed to cryo-environment and thus suffering from ultra-structural biochemical and functional changes. These changes inhibit spermatozoa movement in the female reproductive tract, reducing fertility in animal species (24).

The structural and functional integrity of the spermatozoa acrosome is considered necessary to attain high fertility; however, cryopreservation can damage the acrosomal layer, diminishing the ability of spermatozoa to penetrate the zona pellucida (25). Cryopreservation can affect the acrosomal membrane and induce a pre-acrosomal reaction, thus influencing the viability and quality of the spermatozoa. Sperm freeze-thawing induces

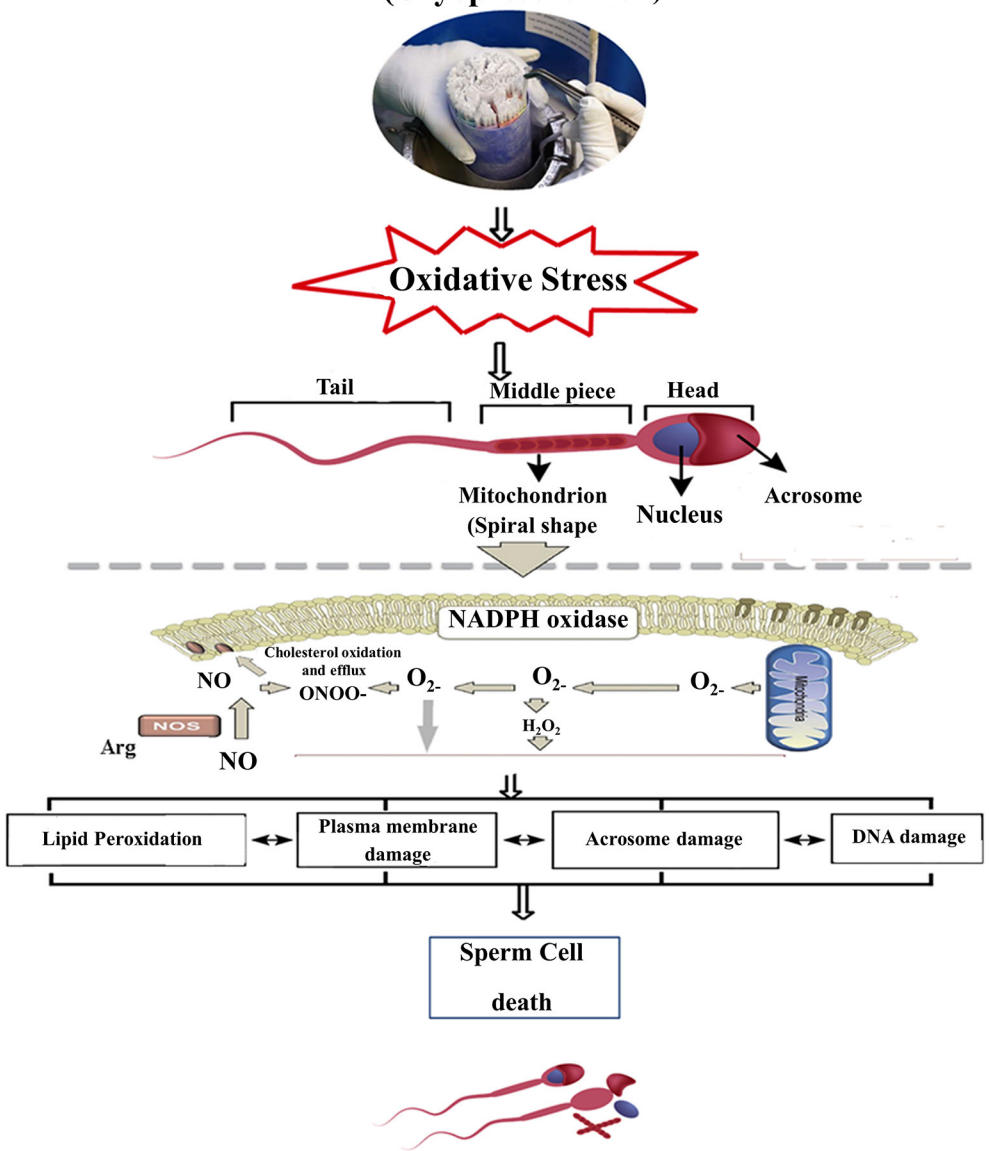


FIGURE 1 | Scheme represents cryopreservation damages in the sperm cell, whereas an excess induction of oxidative stress in resulting ROS production can deteriorate the sperm plasma membrane and acrosomal membrane and eventually alter the molecular structure (DNA). ROS, Reactive oxygen species; H_2O_2, Hydrogen peroxide; $^{\bullet}O_2^-$, Superoxide radical; NADPH, Nicotinamide adenine dinucleotide phosphate; $ONOO^-$, Peroxy nitrate, NO; Nitric oxide.

capacitation, and sudden occurrence of acrosome reaction-like changes in mammalian spermatozoa (1, 2). The acrosomal reaction further assists the sperm to achieve fertilization, hence sperm cell quality is evaluated based on proper capacitation, acrosomal reaction, regular fertilization, and early embryonic development (26).

Spermatozoa DNA integrity is considered very important because it protects the genetic material and transfers the paternal characteristics into offspring, It has been found that damaged DNA may harm fertilization, embryogenesis, and the healthy live birth rate in mammals (27). Spermatozoa DNA disintegration is characterized by single and double-stranded DNA breaks, which occur during or after DNA wrapping; some of these breaks might escape the DNA repairing mechanism and be transferred into mature spermatozoa. Aberrant spermatozoa chromatin packaging, oxidative stress, and abortive apoptosis are the etiological factors that lead to DNA strand breaks (28, 29). For successful fertilization after sperm penetrates the oocyte, the spermatozoa chromatin material must undergo de-condensation (30). Cryopreservation can damage spermatozoa DNA integrity, influencing the sperm functional potential and the successful fetal development (31).

The Mechanism of Spermatozoa Cryo-Injury in the Cryopreservation Methods

During the cryopreservation process, the mammalian spermatozoa have to endure various types of stresses caused by ice formation, chemical toxicity, and oxidative stress, which mainly damage cytoplasm membrane, consequently leading to a lower post-thawed quality and fertility (10, 32, 33). According to the traditional theory, the cryo-damages of mammalian cells are mainly derived from ice crystal formation and chemical toxicity. However, different from other somatic cells, spermatozoa cells contain lower water content and higher protein concentration. In general, the water content in spermatozoa is ∼60% and lower than that of typical somatic cells (>80%) (34). Therefore, it is presumed that the effects of ice formation on spermatozoa may be less as compared to other somatic cells. However, despite the above hypothesis, some researchers still think that it is necessary to prevent ice formation in spermatozoa. Some specific protectants, such as antifreeze proteins (35, 36) or synthetic ice blockers (37) were used to modify ice crystal shape during sperm cryopreservation. But, it should be noted that disputes related to ice formation still exist. Additionally, the sperm plasma membrane is extremely sensitive to osmotic stress. However, ice formation can aggravate the effects of osmotic stress on sperm during freezing (38). In addition to cited factors, the oxidative stress caused by cryopreservation should not be neglected, because long-time exposure to oxygen cannot be completely avoided during cooling or freezing (39–41).

When reactive oxygen species (ROS) exceeds the defense mechanisms of sperm, consequent damage occurs in the cell membrane structure and molecular modification as well. This damage can reduce post-thawed fertility of spermatozoa, and the zygotes or embryo often fail to be carried through to full-term pregnancy (42, 43). The equine spermatozoa have the potential to produce ROS, and the average level of ROS plays a vital role in the signaling events that control sperm capacitation (31, 44), spermatozoa acrosome reaction, hyper-activation, and sperm–oocyte fusion (45). High levels of ROS production can cause polypeptide chains in the spermatozoa to become fractured that may reduce ATP production, which leads to inadequate axonemal phosphorylation, increased lipid peroxidation, and loss of motility. When the equilibrium between ROS and antioxidants is disturbed, leading to malformed spermatozoa and eventually male infertility (46, 47), and it is considered the main causative factor for spermatozoa DNA damage (39). The only reactions that can occur in frozen aqueous systems at −196°C are photophysical events such as the formation of free radicals and the production of breaks in macromolecules, and these events support the damaging of sperm DNA material (48). However, the expected increase occurs in ROS production during freeze-thawing; thus cells become under rescue and facing oxidative stress. ROS manufactured as byproducts of redox reactions, it is essential for cellular function and acts as signaling agents, the stimulation of specific transcription factor-like "NF-kB and AP-1" to sustain energy metabolism and hence to rescue the cell (49). The manufacturing of ROS during spermatozoa freezing is well-reputable, although the freezing and thawing cycle altered the electron transport chain in mitochondria and oxidase NADPH in the plasma membrane (50).

RELEVANCE OMICS EXPLORATION AND SPERMATOZOA CRYO-TOLERANCE

Semen from bulls, boars, and rams were tested for motility parameters using the computer-assisted sperm analyzer (CASA) and found to be statistically significant, although there are still major variations in their ability to develop viable embryos, both *in vitro* and *in vivo* (51, 52). The transcriptome and proteome monitor the genome expression, and along with phenotypic traits and environmental knowledge provide an opportunity for a systematic OMICS approaches to understanding normal and abnormal cell biology (53). Identification and validation of OMICS biomarkers, such as genes, transcripts, proteins, and metabolites, primarily associated with seminal plasma and spermatozoa of livestock species, have a great potential to improve the reproductive efficiency of farm animals. Single nucleotide polymorphisms (SNPs) are the most frequent type of mutation in the genome, and these single base substitutions are correlated with perceived genetic features in the DNA code (54, 55). For example, nucleotide substitutions in the coding region of FSHβ, the beta-subunit follicle-stimulating hormone (FSH) gene, were associated with reduced semen quality, sperm cryosurvival, and conception rates in beef cattle (56). Metabolites such as 2-oxoglutaric acid and fructose are potential biomarkers of the quality and fertility of the frozen sperm of the bull (57). The proteome (PEBP4) also appears to be a reservoir of potential biomarkers related to bull spermatozoa—freezing and fertility (58). Increasing evidence suggests that transcriptoms such as mRNA, microRNA (miRNA), small non-coding RNAs, and piwi-interacting RNA (piRNA) may have a functional role in early embryogensis and serve as biomarkers of male reproductive performance. To that purpose, RNA sequencing (RNA-Seq) and other approaches have been used to assess the occurrence and quantity of RNA in animal freeze-thawed spermatozoa (59–61). The use of current omics technology in cellular biology is the need of the day and an excellent tool for exploring spermatozoa molecular occupation. Cryo-biology plays a crucial role in the preservation of genetics, but it can degrade the consistency of spermatozoa. The wide range of genetic variations in freezing-thawing spermatozoa has encouraged the selection of breeding animals whose semen can tolerate cryopreservation stresses (**Figure 2**).

Proteomics May Provide an Opportunity for the Elucidation of Spermatozoa Cry-Tolerance

Currently, seminal plasma proteins are considered the basic units in advanced reproductive technology, and it is evident that proteins are involved in different spermatozoa biological mechanisms such as energy production, the glycolysis cycle, the citric acid cycle, and oxidative phosphorylation, which maintain the sperm in a physically active state (62). Some studies illustrate common issues regarding frozen stimulus damages of

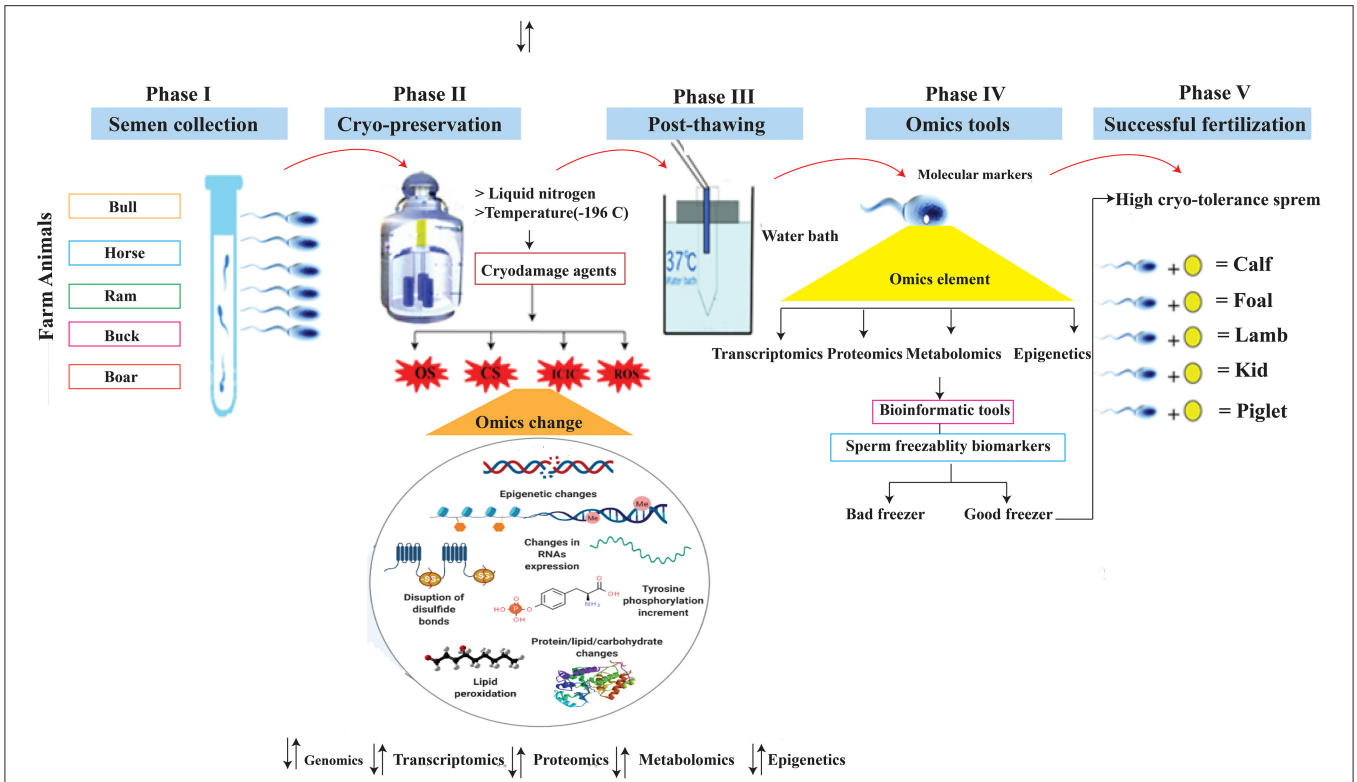


FIGURE 2 | Schemes showed five important phases of sperm biotechnology: Phase I highlighted the semen collection from farm animals, phase II showed semen cryo-storage at liquid nitrogen where temperature is −196°C and produces various stresses which changed the omics elements, phase III highlighted the sperm thawing process, phase IV suggested utilization of OMICS tools for development of cryo-markers, and the last phase supposed the sperm cryo-tolerance efficacy and led to successful fertilization. OS, Osmotic stress; CS, Cold shock; ICIC, Intracellular ice crystal Formation; ROS, Reactive oxygen species.

bovine spermatozoa. A former study matched protein levels in pre- and post-thawed sperm using isobaric tags for comparative and complete quantitation (iTRAQ) technology and found that variations in the identified proteins affected the quality of freeze-thawed sperm, probably decreasing the fertilizing capacity in swine (63). There are some definite spermatozoa proteomic markers of the good freezer and bad freezer animals that have been identified in domestic animals (64); a higher level of voltage-dependent anion channel 2, heat shock protein 90, and low level of triosephosphate isomerase is associated with good freezability in boar sperm (65, 66).

There is considerable variability in spermatozoa ability to withstand cryopreservation procedures between and within ejaculates. Some sperm-specific proteins have been identified as associated with the post-thawing phenomena, and their expression patterns are involved with cell resistance against freeze-thaw damage. Furthermore, the differential expression patterns of seminal plasma and sperm proteins could be developed as freezability biomarkers (63, 64, 67). Vilagran et al. (68) recognized VDAC2 as a possible positive biomarker of spermatozoa cryopreservation in swine, whereas the occurrence of VDAC2 in higher quantities in good cryo-tolerance spermatozoa suggests its contribution in the protection of spermatozoa from changes in membrane fluidity through improved regulation of ion transportation across the membrane during cold shock trials in the cryopreservation process. The higher level of fertility-linked 28-30-kDa heparin-binding proteins (HPBs) in seminal plasma enhances the conception rate by 13% while comparing to lack of these proteins, and also provides better cryo-protective support during the cryopreservation (69). It has been reported that the higher levels of fertility-linked 28–30-kDa heparin-binding proteins (*HPBs*) in semen could provide better cryo-protective support to sperm morphology and membrane integrity, achieving a 13% higher conception rate compared with that induced by semen lacking these proteins (22).

We acknowledged some enzymes in the good freezability semen that guarded sperm against oxidative stress, and it found in two forms (Rho and Pi) of glutathione S-transferase (GST) group. Hence, an enrichment of defensive intracellular proteins and membrane enzymes in spermatozoa of good freezability would be a great advantage, as these sperm cells are wide-open to ROS production during cryo-stimulus and their function could be related to enhanced protection of sperm membrane (70). Boar spermatozoa genomics analysis indicated that the protein level of outer dense fiber 2 (ODF2), heat shock protein (HSP90AA1), A-kinase-anchoring proteins 3 and 4 (AKAP3 and AKAP4), voltage-dependent anion channel 2 (VDAC2), triosephosphate isomerase 1 (TP1), and acrosin-binding protein (ACRBP), were

associated with good freezability semen (63, 64). AKAP4 and AKAP3 were found in the fibrous sheath of spermatozoa flagellum and are involved in sperm motility and morphology. High expression of AKAP4 or AKAP3 in freeze-thawed spermatozoa was linked with premature capacitation (71).

Adenylate kinase isoenzyme 1 (AK1) and phosphatidylethanolamine-binding protein 1(PEBP1) were found abundantly in bull, horse, and boar spermatozoa with higher cryo-associated rates. In contrast, the T-complex protein 1 subunits (CCT5 and CCT8), epididymal sperm-binding protein E12 (ELSPBP1), proteasome subunit α type-6, and binder of sperm 1 (BSP1) were predominately found in bull spermatozoa with lower fertility and freeze-thawing rates (72). In cattle bull, many studies have attempted to identify protein markers of sperm cryo-tolerance or freeze-thawed semen fertility by the quantifying seminal plasma proteins (73–75). These studies identified BSPs as negatively related to the freezing ability or fertility in sperm cell either in seminal plasma (76, 77). The sperm-enriched proteomes identified based on access code, regulation, location, and function in different mammals are shown in **Table 1**.

Could the Spermatozoa Transcriptomics Profiling Provide Some Inspirations?

The underline mechanisms behind the effect of cryopreservation on sperm characteristics are not entirely understood. Genes and mRNA stability, protein expression, and epigenetic content of spermatozoa are thought to be modulated during the freezing-thawing process. Though, Ostermeier et al. (89), trusted that transcripts were expressed during spermatogenesis and that resistant transcript are assisted the sperm in struggling against the injury persuaded by the freezing-thawing cycle, the other residents of sperm transcripts were promptly degraded in response to cryo-stimulus. Some constraints of their study were that the authors could not elucidate why some novel transcripts were present. Some transcripts were upregulated after the freezing and thawing cycle (89).

Cryopreservation can affect the expression of critical genes such as genes encoding α, and β inhibin are potential candidates as fertility markers because both are significantly associated with sperm acrosomal integrity and motility (90). The embryogenesis-linked BCL2 like 11 (*BCL2L11*), BRCA1, and DNA that repair linked full-length transcripts in fresh bull semen were found abundantly in spermatozoa and are associated with structural components of ribosomes, while the transcripts detected in the lowest amounts are connected with ion transporter activity (91). Xue-Bing (92) described the ribosomal protein L31 (*RPL31*), which belongs to the ribosome multipart and is situated in the 60S subunit of the ribosome, as being differentially expressed between fresh and frozen-thawed sperm. The authors concluded that the RPL31 gene could be among other growth regulation genes in early embryonic growth. Nonetheless, the high expression of *RPL31* in cryopreserved sperm may be a result of cold stress and demands further exploration. Sperms are susceptible to oxidative damage due to their high polyunsaturated fatty acid content. Hence, glutamate-cysteine ligase catalytic subunit (*GCLC*) gene regulation in freeze-thawed sperm could be a protective comeback of the sperm to cold shock and oxidation stress. Besides, we found in a preceding work that the protein glutathione transferase mu5 (*GSTM5*), a fellow of the glutathione metabolic pathways, was upregulated in freeze–thawed sperm (93).

The role of transcriptomes such as sperm motility cation channel sperm associated 1 (CATSPER1) and sperm associated antigen 1 (SPAG1) in fertility and development of sperm hyperactivated motility has been clearly demonstrated in infertile male candidates; the knockout studies indicated that these transcripts are indispensable for the structural integrity of sperm (94, 95). Chen et al. (96) discovered four novel genes (e.g., *R1A10, R1C4, R4A1,* and *R4D2*), in fresh and cryo-preserved bull spermatozoa, were differentially expressed, and sequence results declared all four genes are regulated by ncRNAs transcripts, which may play a significant role during the freezing-thawing cycle and require further study (96). Cytochrome c oxidase polypeptide 5 (*COX5 A*) and (*COXI1*) are essential for mitochondrial function (24, 97). During mammalian sperm and oocyte fusion, phospholipase C zeta1 (*PLCZ1*) and phospholipase C beta1 (*PLCB1*) monitor the calcium signaling and aid sperm activation. High levels of PLCZ1 were found in spermatozoa, which are associated with phosphatidylinositol-linked enhancement of oocyte maturation *via* Ca^{2+} oscillations (98). The freeze-thawed sperm enriched transcripts related to fertility and cryo-sensitivity identified with a gene symbol, gene name, and functions are shown in **Table 2**.

The Potential Metabolomics Profiling and Sperm Cryo-Tolerance

A wide range of metabolomics biomarkers have been identified in sperm cells from boars (99), bulls (100), and goats (101), and these studies indicated that seminal fluid and spermatozoa metabolites might suggestively be connected to male breeding capability. The metabolites are assessed through developmental biological studies and thereby serve as metabolomics markers. In mouse sperm, the role and interaction of glycolytic metabolites with tyrosine phosphorylation were analyzed, whereas the outcome of this interaction is energy production which is vital for sperm freeze-thawed viability and motility (102). Amino acids play important roles in cellular physiology while also participating in the crucial phase of sperm cryobiology (103). In ram sperm, amino acid provides protection and regulation of metabolic activity and protects spermatozoa during cryopreservation, thereby decreasing lipid peroxidation and injury caused by free radicals (104).

In the meanwhile, carbohydrates are also present in the seminal plasma of animals and solely utilized in spermatozoa energy metabolism pathway (105). Spermatozoa consumed the surrounding seminal nutrients available in semen plasma and that nutrient metabolites, one way or another, control the signaling pathways and elaborate in spermatozoa hyperactivation, motility, capacitation, acrosomal reaction, freeze-thawing cycle, and spermatozoa–oocyte combination (106). Spermatozoa can be genetically (e.g., transcription and

TABLE 1 | The freeze-thawed sperm enriched proteomes identified based on access code, regulation, location, and function in different farm animals and could be evaluated as a cryo-tolerance biomarkers.

Protein name	Protein symbol	Organism	Access code	Protein regulation	Location	Function during cryopreservation	References
Dihydrolipo amidedehydrogenase precursor	DLD	*Sus scrofa*	P09622	Up	Mitochondria	Hyperactivation of spermatozoa during capacitation and acrosome reaction	(78)
Inositol-1(or 4)- Monophosphatase	IMPA1	*Bos Taurus/Sus scrofa*	P29218	Up	Cytosol	Key enzyme of the phosphatidylinositol signaling pathway	(78)
S100 calcium binding protein A9	S100A9	*Bos taurus/Sus scrofa*	P06702	Down	Cytosol	Ca2+ binding protein	(78)
Soluble adenylyl cyclase (sAC)	ADCY10	*Oryctolagus cuniculus*	Q8C0T9		Fibrous sheet	cAMP production	(79)
β1,4galactosyltransferase 1 (GalT)	B4GALT1	*Bos taurus*	P15535		Apical Region	ZP3 (N-acetyl glucosamine)	(80)
Cysteine rich secretory protein 1	CRISP1	*Bos taurus/Equus caballus/Sus scrofa*	Q03401		Equatorial segment in capacitated sperm	Sperm-Oolemma Penetration	(81)
Cysteine rich secretory protein 1	CRISP2	*Capra hircus/Bos taurus/Sus scrofa*	P16563		Inner acrosome membrane	Sperm-Oolemma Penetration	(81)
ADAM metallopeptidase domain 2	ADAM2	*Bos taurus/Oryctolagus cuniculus*	Q99965		Integral membrane protein	Sperm-Oolemma Penetration	(82)
ADAM metallopeptidase domain 3A	ADAM3	*Bos taurus/Sus scrofa*	Q62287		Integral membrane protein	Sperm-Oolemma Penetration	(83)
Tektin 1	TEKT1	*Bos taurus/Sus scrofa*	Q969V4	Down	Flagella	Flagella- related	(84)
Septin 4	SEPT4	*Bos taurus*	O43236	Down	Annulus	Flagella- related	(85)
Isocitrate dehydrogenase subunit α	IDH3A	*Bos taurus*	P50213	Down	Mitochondria	Energy- Related	(139)
Izumo sperm-egg fusion 1	IZUMO1	*Bos taurus/Capra hircus/Sus scrofa*	Q9D9J7		Sperm cell-surface protein	Fertilization	(64)
Prostaglandin D2 synthase	PTGDS	*Ovis aries/Sus scrofa/Bos Taurus*	O02853		Testis, epididymis and prostate	Male reproductive system	(71)
Outer dense fiber protein 2	ODF2	*Sus scrofa/Bos taurus*	Q6AYX5		Sperm tail outer dense fibers	Association- with semen freezability	(64)
Voltage-dependent anion channel 2	VDAC2	*Sus scrofa Bos taurus*	CAB94711		Testis	Semen freezability	(63)
Phosphatidylethanolamine-binding protein 1	PEBP1	*Bos taurus*	NP001028795		Spermatozoa	Related to conception	(86)
Seminal plasma protein PDC-109 precursor	BSP1	*Bos taurus*	NP001001145		Plasma membrane	Sperm capacitation	(86)
Sperm acrosome associated 1	SPACA1	*Sus scrofa/Bos taurus*	Q9HBV2		Sperm acrosomal membrane-associated protein	Association with sperm freezability	(87)
Epididymal sperm-binding protein 1	ELSPBP1	*Bubalus bubalis/Sus scrofa*	Q96BH3		Epididymal origin	Sperm fertility	(88)

translation events) switched off, but metabolically is always switched on (107). The metabolic biomarker like "2-oxoglutarate aminotransferase" was mainly spotted in the boar spermatozoa (108), and was significantly found in low viable freeze-thawed sperm (109). The bioinformatics tools showed that metabolic pathways are playing an essential role in sperm cryopreservation, and hereby include the following pathways–citrate cycle "TCA cycle," gluconeogenesis, dicarboxylate metabolism, glyoxylate, pyruvate metabolism, and galactose metabolism (110).

Single Nucleotide Polymorphisms Markers Can Be Used for the Study of Sperm Cryo-Tolerance

The genome-wide association studies (GWAS) observed a sequence variation in the genome so-called SNPs, together with the pedigree and phenotypic evidence, thereby performing an association analysis and identifying genes or regulatory omics element that are significant for the trait of interest. GWAS approaches are much needed and practical in humans while

TABLE 2 | The freeze-thawed spermatozoa enriched transcripts identified based on their functions, location, transcripts per million (TPM), and unique gene reads (UGR) and can be evaluated as freezability biomarkers in farm animals.

Gene Symbol	Gene Name	Access Code	Function during cro-preservation	Organism	Location	TPM	UGR
PRM1	Protamine 1	NM_174156	Sperm progressive motility	*Bos taurus/Sus scrofa*	Chromosome25/ Chromosome 03/	8,659	120
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	NM_174814	Association with Y chromosome	*Bos taurus/Sus scrofa/Equus caballus*	Chromosome 14/Chromosome 04/Chromosome 09	3,050	84
FABP1	Fatty acid binding protein 1	NM_001443	Sperm metabolism	*Bos taurus/Sus scrofa*	Chromosome 11/Chromosome 03/	2,923	1,074
SCP2D1	Sterol-binding domain containing 1	NM_001040507		*Bos taurus/Equus caballus*	Chromosome 13/Chromosome 22	2,726	182
THSD4	Thrombo spondin type 1 domain containing 4	NM_001078030	Hydrolase, peptidase activity	*Bos taurus/Equus caballus*	Chromosome 10/Chromosome 01	1,961	2,506
CHMP5	Charged multi vesicular body protein 5	NM_001034682	Inhibit apoptosis	*Bos taurus/Sus scrofa/Equus caballus*	Chromosome 08/Chromosome 10/Chromosome 23	1,693	260
NR2E3	Nuclear receptor subfamily2 group E member 3	NM_001167900	Maintenance of proper cell function	*Bos taurus*	Chromosome 10/	1,610	1,241
SV2C	Synaptic vesicle glycoprotein 2C	NM_001192019	Positively regulates the releasable pool of secretory vesicles	*Bos taurus/Equus caballus*	Chromosome 10/Chromosome 14	1,518	2,592
MGC137055	Det1and ddb1 associated	NM_001077080	Oxygen binding and carrier activity	*Bos taurus*	Chromosome 19	1,434	74
GTSF1L	Gametocyte specific factor 1-like	NM_001079601	Spermatogenesis	*Bos taurus/Equus caballus*	Chromosome 13/Chromosome 22	1,416	155
TOE1	Target of EGR1, member1 (nuclear)	NM_001075594	Cellular signaling, growth and proliferation	*Bos taurus/Gallus gallus/Equus caballus*	Chromosome 03/Chromosome 08/Chromosome 02	1,359	1,743
SLC16A7	Solute carrier family 16 member 7	NM_001076336	Monocarbooxylic acid trans-membrane transporter activity	*Bos taurus*	Chromosome 05/	1,284	2,831
MCOLN2	Mucolipin 2	NM_001192734	Carbonate dehydratase activity and zinc ion binding	*Bos taurus/Equus caballus*	Chromosome 03/Chromosome 05	1,231	1,756
UNC119	Unc-119 lipid binding chaperone	NM_001034645	Role in the mechanism of photoreceptor neurotransmitter release through the synaptic vesicle cycle	*Bos taurus/Equus caballus*	Chromosome 19/Chromosome 11	1,136	790
CXCR4	C-X-C motif chemokine receptor 4	NM_174301	Chemokine activity and heparin binding	*Bos taurus*	Chromosome 2/	1,095	975
PAG5	Pregnancy-associated glycoprotein 5	NM_176616	Aspartic-type endopeptidas activity	*Bos taurus/Ovis aries/Capra hircus*	Chromosome 29/Chromosome 21/Chromosome 13/	971	962
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase)	NM_174745	Stimulating Ca_2+ ATPase activity	*Bos taurus/Sus scrofa*	Chromosome 18/Chromosome 06	933	1,417
ITPA	Inosine triphosphatase (nucleoside triphosphate pyrophosphatas)	NM_001076282	Chromosome organization	*Bos taurus/Equus caballus*	Chromosome 13/Chromosome 22/	919	458
CCDC181	Coiled-coil domain containing 181	NM_001205801	Coiled-coil proteins are important for the function of the centrosome, and help cell division	*Bos taurus/Capra hircus/Sus scrofa*	Chromosome 16/Chromosome 16/Chromosome 04	919	144
DNAJB12	DNAJ heat shock protein family (Hsp40) member B12	NM_001017946	Regulate molecular chaperone activity by stimulating ATPase activity	*Bos taurus/Bos indicus*	Chromosome 28/Chromosome 28/	914	2315

also required in farm animals to develop SNPs biomarkers for phenotypic traits (111). Hering et al. (112) conducted the GWAS study upon high and low semen motility of Holstein bulls groups and identified the candidate gene *INCENP*, which is closely located to SNPs markers (rs109416157), associated with semen freezing quality. The progressive advancement in OMICS techniques made possible to measure the link between gene polymorphism and sperm freeze-thawed activity. RNA-Seq datasets were used to identify SNPs, and a total of 40 SNPs were genotyped, while several polymorphisms in *MS4A2*, *MAP3K20*, and *ROBO1* genes were significantly associated with sperm motility, membrane integrity, reduced cryo-induced lipid per-oxidase, and DNA damage in the boar spermatozoa (17). The genotyping frequencies are different among the genotype groups, while the Gene Ontology terminology (e.g., stress response) is relevant to polymorphisms, such as *MAP3K20* (rs340643892), *APPL1* (rs339379734), and *MS4A2* (rs339836492) and play an important role in the cryopreservation stresses (113). Different reports and evidence highlighted that polymorphisms in boar spermatozoa could be used as SNPs markers for semen quality (114). Nikbina et al. (137) performed a molecular experiment and analyzed the four SNPs related to caprine LHβ genes in exon3; these markers regulate the fresh and freeze-thawed semen quality characteristics of the boar. The most powerful SNPs such as FSHβ3 SNPs, FSHβ3-c, and FSHβ loci polymorphisms have been tested and investigated by Dai et al. (56) in semen freeze-thawed consistency characteristics and libido in goats. The results were consistent with previously available reports showing the impact of (FSHβ3) SNPs on semen quality in cattle bulls (56). Five SNP markers have been identified and are closely correlated with sperm freeze-thawed consistency and possible GnRH gene polymorphism in Chinese water buffalo. An association study found that g.3424T > C and g.3462C > A were used as high ejaculate volume markers, while g.991T > C, g.1041T > C, g.3424T > C, and g.3462C > A were used for decreasing sperm abnormality markers (115). Although evidence is present among the 3-UTR variants of the targeted mRNA, an association with semen quality has been shown (116).

BIOINFORMATICS TOOLS FOR CRYO-MARKERS DISCOVERY

Transcriptomic Tools

Advances in bioinformatics techniques have made it possible to isolate high-quality RNA from sperm and to develop novel non-invasive approaches to evaluate cryo-tolerance and post-thawed quality biomarkers in animals (86, 117).

Spermatozoa contain a subset of RNAs, including mRNAs, non-coding RNAs [ncRNA including microRNAs (miRNAs)], mitochondrial (mtRNA), and ribosomal RNAs (rRNAs) that can be routinely isolated from the sperm of several species including bulls, horses, and pigs (16, 72, 118). This novel approach is based on sperm RNA-sequencing (RNAseq) data analysis, by comparing the mRNA profile between higher and lower post-thawing semen to identify marker genes for mammalian semen post-thawing (16, 17).

The bovine spermatozoa transcript profile remains incomplete because previous studies have relied on hybridization-based techniques and did not provide information about full-length transcripts. In contrast, RNA-Seq studies based on high-throughput sequencing technology can assemble complete transcript sequences, including full-length mRNAs, and identify novel splicing junctions (119–121). RNA-seq (e.g., Illumina RNA-seq), using high-throughput next-generation sequencing (HT-NGS) technology that provides more excellent resolution for transcriptome profiling compared with other microarray technologies (122) and can identify candidate genes associated with more or less cryo-tolerant sperm. Gene annotation and gene analysis enable the researchers to investigate the genes relevant to multiple spermatozoa functions. Furthermore, the multiple candidate genes need to be validated for their link with high semen cryopreservation potential (61).

Differentially expressed genes are validated by quantitative real-time PCR (qRT-PCR), whereas the KASP™ assay analyzes SNP biomarkers. Combined studies of the transcriptome and proteome provide a clear picture of the genome, which could differentiate individuals likely to have high and low sperm cryo-tolerance (64). Microarray technology has been used to study the molecular mechanisms of spermatogenesis and the genomic etiology of male infertility. High-throughput technology has been effectively used for global gene profiling for mouse and bovine spermatozoa. A bovine oligonucleotide microarray (Affymetrix Bovine Gene-Chip) has been used to profile the transcript "fingerprints" of spermatozoa collected from high low-fertility bulls (117). Bioinformatics tools were used to select the differentially express genes and putative SNP markers potentially associated with good post-thawing and low post-thawing spermatozoa quality (113).

Next-generation sequencing (NGS) is the most reliable method to determine the small RNA profile in bull and pig spermatozoa. The sequencing of miRNAs and piRNAs in the semen of the bull was performed concerning different traits such as fertility, cryo-tolerance, and normal embryonic development (123).

Proteomics Tools

High-throughput proteomic technology is especially useful to discover the biomarkers. Once the clinical value of proteomics markers are confirmed that it should be possible to develop the other cheaper tools, such as protein microarrays, mass spectrometry selective reaction monitoring (SRM), or multiplexed ELISA for routine biomarkers testing in the reproductive clinics (124). Proteins adenylate kinase isoenzyme1 (*AK1*), phosphatidyl ethanolamine-binding protein 1 (*PEBP1*), epididymal sperm-binding protein E12 (*ELSPBP1*), and binder sperm1 (*BSP1*) were noticed abundantly in the spermatozoa from the bulls with higher artificial insemination (AI) fertility rates and confirmed their differential expression by Western blotting analyses. Moreover, a linear regression model was also used to determine the link between the fertility rate and protein abundance. This model investigated proteins like such as CCT5 and AK1, both of which influence spermatozoa cryo-tolerance and fertility rates in higher AI rates (88). Mass

spectrometry-based targeted proteomics approaches such as selected reaction monitoring are developed as a gifted tool for the verification of candidate proteins in biological and biomedical submissions. The unbiased "discovery" proteomics examination, e.g., "shotgun' proteomics," can now deliver genome-scale coverage and quantification of both proteomics and post-translational modifications using extensive fractionation and stable isotope labeling (125).

Differential labeling followed by the LC-MS/MS technique was used to carry out proteomic analysis, and high numbers of differentially expressed proteins were identified in asthenozoospermia patients. The other non-proteomic techniques such as *ELISA*, immunofluorescence, enzymatic activity, flow cytometry, immunochemistry, and Western blotting were also used to detect differentially expressed sperm protein (126–128). More significantly, proteins are the primary driving force in almost all cellular developments. Hence, protein microarrays were established as a high-throughput apparatus to overcome the constraint of DNA microarrays and provide a direct platform for protein function analyses. At about the same time, an additional protein microarray was settled through the immobilization of purified proteins on glass slides. To discriminate this type of array from the antibody arrays, they are separated into analytical and functional (138).

Lipidomics and Metabolomics Tools

Like all tiny molecules, lipids are produced and metabolized by enzymes that are influenced by the environmental factors of a given biological system, for instance, by the nutrition and temperature. Initial reports of mass spectrometric analysis using soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization of multifaceted lipid mixtures were published by Wenk (129). The foremost objective of lipidomics is the complete classification of different lipid species and their natural roles concerning the expression of proteins involved in lipid metabolism and function, including gene regulation (130). Lipidomics is relatively a new area of research that has seen rapid progress in analytical technologies such as mass spectrometry (MS), fluorescence spectroscopy, dual-polarization interferometry, spectroscopy of NMR, and computational methods that help the identification of the position of molecular species of lipids (131). The phospholipids and fatty acid configurations of boar spermatozoa for cryo-resistance are compared by using matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) in combination with thin-layer chromatography and 31P NMR spectroscopy. Metabolomics techniques like NMR and GC-MS have been widely used to identify possible biomarkers for freeze-thawed sperm fertility in cattle bulls (132) and men (133–135). Two well-known techniques are used to study metabolomics biomarkers on a wide range, MS-based methods and NMR spectroscopy-based methods. Organic acids, carbohydrates, amino acids, and lipid anti-oxidants are the major metabolites in seminal fluids, and these classes were measured by spectrophotometric, colorimetric, and thin-layer chromatography methods such as "TLC" and NMR. High-resolution proton NMR spectroscopy has proved to be one of the most potent bio-fluid and intact tissue analysis technologies, providing a wide-ranging profile of metabolite signals without isolation, derivatization, and pre-selected parameters of measurement (136).

CONCLUSIONS AND FUTURE PERSPECTIVE

The OMICS profiling data from various spermatozoa freezability groups, in combination with advanced bioinformatics technology consisting of Illumina RNA-seq, high-throughput next-generation sequencing (HT-NGS) technology, multiplexed ELISA, should be used to identify the routine biomarkers for good and poor cryo-tolerance farm animals. Combining these powerful technologies would provide a deeper insight into the molecular and cellular changes induced by the freezing-thawing process, and would allow data analysis in different cryopreserved samples to determine the spermatozoa freezing capacity of farm animal species. Besides, a validation technique is required to approve whether candidate genes and putative SNP markers may contribute to high cryo-tolerance of sperm. This useful knowledge, which has been extensively presented in this report, is important for the identification of potential biomarkers to predict spermatozoa freezability more accurately and for the development of new policies to improve the results of cryo-preserved spermatozoa. Nevertheless, systematic analysis of the specific genetic markers that may facilitate the post-thawing cycle would be a feasible approach to distinguish a male breeding stock, which has the excellent genetic potential for cryopreservation. A long-lasting follow-up study on the subsequent offspring obtained from good cryo-resistance freeze-thawed spermatozoa is suggested for future works.

AUTHOR CONTRIBUTIONS

IK and YZ: conceptualization. SR: formal analysis and software. YZ, ZC, and HL: funding acquisition. IK, AK, and MK: investigation. IK: writing original draft. YZ: project administration. HL: resources. ZC: visualization. IK, YZ, and AS: writing review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by National Research and Development Program of China (2018YFD0501700), Anhui Provincial Livestock Genebank.

ACKNOWLEDGMENTS

We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

REFERENCES

1. Bailey J, Morrier A, Cormier N. Semen cryopreservation: successes and persistent problems in farm species. *Can J Anim Sci*. (2003) 83:393–401. doi: 10.4141/A03-024
2. Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl*. (2000) 21:1–7. doi: 10.1002/j.1939-4640.2000.tb03268.x
3. Kumar A, Prasad JK, Srivastava N, Ghosh SK. Strategies to minimize various stress-related freeze–thaw damages during conventional cryopreservation of mammalian spermatozoa. *Biopreserv Biobank*. (2019) 17:603–12. doi: 10.1089/bio.2019.0037
4. Lv C, Wu G, Hong Q, Quan G. Spermatozoa cryopreservation: state of art and future in small ruminants. *Biopreserv Biobank*. (2018) 17:171–82. doi: 10.1089/bio.2018.0113
5. Grötter LG, Cattaneo L, Estela P, Kjelland ME, Ferré LB. Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post—thaw quality optimization. *Reprod Domest Anim*. (2019) 54:655–65. doi: 10.1111/rda.13409
6. Lemma A. Effect of Cryopreservation on Sperm Quality and Fertility, Artificial Insemination in Farm Animals. Milad Manafi: IntechOpen (2011). doi: 10.5772/16563
7. Yeste M. Sperm cryopreservation update: cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology*. (2016) 85:47–64. doi: 10.1016/j.theriogenology.2015.09.047
8. Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reprod Biomed Online*. (2003) 6:191–200. doi: 10.1016/S1472-6483(10)61710-5
9. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*. (1949) 164:666. doi: 10.1038/164666a0
10. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*. (1995) 7:871–91. doi: 10.1071/RD9950871
11. Curry MR, Redding BJ, Watson PF. Determination of water permeability coefficient and its activation energy for rabbit spermatozoa. *Cryobiology*. (1995) 32:175–81. doi: 10.1006/cryo.1995.1016
12. Foote RH. The history of artificial insemination: selected notes and notables. *J Anim Sci*. (2002) 80:1–10. doi: 10.2527/animalsci2002.80E-Suppl_21a
13. Benson JD, Woods EJ, Walters EM, Critser JK. The cryobiology of spermatozoa. *Theriogenology*. (2012) 78:1682–99. doi: 10.1016/j.theriogenology.2012.06.007
14. Li J, Parrilla I, Ortega MD, Martinez EA, Rodriguez-Martinez H, Roca J. Post-thaw boar sperm motility is affected by prolonged storage of sperm in liquid nitrogen. A retrospective study. *Cryobiology*. (2018) 80:119–25. doi: 10.1016/j.cryobiol.2017.11.004
15. Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. *Reprod Biomed Online*. (2018) 37:327–39. doi: 10.1016/j.rbmo.2018.05.012
16. Card C, Anderson EJ, Zamberlan S, Krieger KBE, Sartini BL. Cryopreserved bovine spermatozoal transcript profile as revealed by high-throughput ribonucleic acid sequencing. *Biol Reprod*. (2013) 88:49. doi: 10.1095/biolreprod.112.103788
17. Mańkowska A, Brym P, Paukszto Ł, Jastrzebski J.P., and Fraser, L. (2020). Gene Polymorphisms in Boar Spermatozoa and Their Associations with Post-Thaw Semen Quality. *Int J Mol Sci*. 21:1902. doi: 10.3390/ijms21051902
18. Pons-Rejraji H, Bailey JL, Leclerc P. Cryopreservation affects bovine sperm intracellular parameters associated with capacitation and acrosome exocytosis. *Reprod Fertil Dev*. (2009) 21:525–37. doi: 10.1071/RD07170
19. Prien S, Iacovides S. Cryoprotectants and cryopreservation of equine semen: a review of industry cryoprotectants and the effects of cryopreservation on equine semen membranes. *J Dairy Vet Anim Res*. (2016) 3:1–7. doi: 10.15406/jdvar.2016.03.00063
20. Farias JG. Effects of cryopreservation on mitochondria of fish spermatozoa. *Rev Aquacult*. (2017) 9:76–87. doi: 10.1111/raq.12105
21. Graham JK, Moce E. Fertility evaluation of frozen/thawed semen. *Theriogenology*. (2005) 64:492–504. doi: 10.1016/j.theriogenology.2005.05.006
22. Karunakaran M, Devanathan TG. Evaluation of bull semen for fertility-associated protein, *in vitro* characters and fertility. *J Appl Anim Res*. (2017) 45:136–44. doi: 10.1080/09712119.2015.1129343
23. Halliwell B, Gutteridge JM. Lipid peroxidation, oxygen radicals, cell damage, and antioxidant therapy. *Lancet*. (1984) 1:1396–7. doi: 10.1016/S0140-6736(84)91886-5
24. Maxwell SSW. Storage of ram semen. *Anim Reprod Sci*. (2000) 62:77–111. doi: 10.1016/S0378-4320(00)00155-X
25. Wassarman PM. Mammalian fertilization. *Physiol Reprod*. (1999) 96:175–83. doi: 10.1016/S0092-8674(00)80558-9
26. Maduro MR, Lo KC, Chuang WW, Lamb DJ. Genes and male infertility: what can go wrong? *J Androl*. (2003) 24:485–93. doi: 10.1002/j.1939-4640.2003.tb02697.x
27. Ron-El R, Nachum H, Herman A, Golan A, Caspi E, Soffer Y. Delayed fertilization and poor embryonic development associated with impaired semen quality. *Fertil Steril*. (1991) 55:338–44. doi: 10.1016/S0015-0282(16)54127-2
28. Tarlatzis BC, Goulis DG. Sperm DNA fragmentation assessment: is it really helpful? *Gynecol Endocrinol*. (2010) 26:315–6. doi: 10.3109/09513590903507370
29. Shamsi MB, Imam SN, Dada R. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. *J Assist Reprod Genet*. (2011) 28:1073–85. doi: 10.1007/s10815-011-9631-8
30. Flaherty SP, Payne D, Swann NJ, Matthews C. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod*. (1995) 10:2623–9. doi: 10.1093/oxfordjournals.humrep.a135757
31. Silva PF, Gadella BM. Detection of damage in mammalian sperm cells. *Theriogenology*. (2006) 65:958–78. doi: 10.1016/j.theriogenology.2005.09.010
32. Holt WV. Basic aspects of frozen storage of semen. *Anim Reprod Sci*. (2000) 62:3–22. doi: 10.1016/S0378-4320(00)00152-4
33. Salamon S, Maxwell WM. Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Anim Reprod Sci*. (1995) 38:1–36. doi: 10.1016/0378-4320(94)01328-J
34. Kleinhans FW, Travis VS, Du J, Villines PM, Colvin KE, Critser JK. Measurement of human sperm intracellular water volume by electron spin resonance. *J Androl*. (1992) 13:498–506.
35. Payne SR, Oliver JE, Upreti GC. Effect of antifreeze proteins on the motility of ram spermatozoa. *Cryobiology*. (1994) 31:180–4. doi: 10.1006/cryo.1994.1021
36. Qadeer S, Khan MA, Ansari MS, Rakha BA, Ejaz R, Husna AU. Evaluation of antifreeze protein III for cryopreservation of Nili-Ravi (*Bubalus bubalis*) buffalo bull sperm. *Anim Reprod Sci*. (2014) 148:26–31. doi: 10.1016/j.anireprosci.2014.04.013
37. Quan GB, Wu SS, Lan ZG, Yang HY, Shao QY, Hong QH. The effects of 1,4-cyclohexanediol on frozen ram spermatozoa. *Cryo Lett*. (2013) 34:217–27.
38. Watson, P. F. (2000).The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*. (2016) 60–61:481–92. doi: 10.1016/S0378-4320(00)00099-3
39. Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol*. (2008) 59:2–11. doi: 10.1111/j.1600-0897.2007.00559.x
40. Desai N, Sharma R, Makker K, Sabanegh E, Agarwal A. Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. *Fertil Steril*. (2009) 92:1626–31. doi: 10.1016/j.fertnstert.2008.08.109
41. Gonçalves FS, Barretto LSS, Arruda RP, Perri SHV, Mingoti GZ. Effect of antioxidants during bovine *in vitro* fertilization procedures on spermatozoa and embryo development. *Reprod Domest Anim*. (2010) 45:129–35. doi: 10.1111/j.1439-0531.2008.01272.x
42. Aitken RJ, Iuliis GND, Mclachlan RI. Biological and clinical significance of DNA damage in the male germ lineandnbsp. *Int J Androl*. (2008) 32:46–56. doi: 10.1111/j.1365-2605.2008.00943.x
43. Gurler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, et al. Effects of cryopreservation on sperm viability, synthesis of reactive

oxygen species, and DNA damage of bovine sperm. *Theriogenology*. (2016) 86:562–71. doi: 10.1016/j.theriogenology.2016.02.007
44. Guthrie HD, Welch GR. Effects of reactive oxygen species on sperm function. *Theriogenology*. (2012) 78:1700–8. doi: 10.1016/j.theriogenology.2012.05.002
45. Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, et al. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev*. (1997) 47:468–82.
46. Wang X, Sharma RK, Sikka SC, Thomas AJJr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril*. (2003) 80:531–5. doi: 10.1016/S0015-0282(03)00756-8
47. Smith R, Kaune H, Parodi D, Madariaga M, Rios R, Morales I, et al. Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress. *Hum Reprod*. (2006) 21:986–93. doi: 10.1093/humrep/dei429
48. Özkavukçu S. Cryopreservation: basic knowledge and biophysical effects. *J Ankara Med Sch*. (2002) 24:187–96. doi: 10.1501/Jms_0000000030
49. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol*. (1998) 39:67–101. doi: 10.1146/annurev.pharmtox.39.1.67
50. Agarwal A. Prevention of oxidative stress injury to sperm. *J Androl*. (2005) 26:654–60. doi: 10.2164/jandrol.05016
51. Larsson B, Rodríguez-Martínez H. Can we use *in vitro* fertilization tests to predict semen fertility? *Anim Reprod Sci*. (2000) 60–1:327–36. doi: 10.1016/S0378-4320(00)00089-0
52. Sellés E, Gadea J, Romar R, Matás C, Ruiz S. Analysis of *in vitro* fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. *Reprod Domest Anim*. (2003) 38:66–72. doi: 10.1046/j.1439-0531.2003.00406.x
53. Carrell DT, Aston KI, Oliva R, Emery BR, De Jonge CJ. The "omics" of human male infertility: integrating big data in a systems biology approach. *Cell Tissue Res*. (2016) 363:295–312. doi: 10.1007/s00441-015-2320-7
54. Silvestri E, Lombardi A, De Lange P, Glinni D, Senese R, Cioffi F, et al. Studies of complex biological systems with applications to molecular medicine: the need to integrate transcriptomic and proteomic approaches. *J Biomed Biotechnol*. (2011) 2011:810242. doi: 10.1155/2011/810242
55. Long JA. The 'omics' revolution: use of genomic, transcriptomic, proteomic and metabolomic tools to predict male reproductive traits that impact fertility in livestock and poultry. *Anim Reprod Sci*. (2020) 220:106354. doi: 10.1016/j.anireprosci.2020.106354
56. Dai L, Zhao Z, Zhao R, Xiao S, Jiang H, Yue X, et al. Effects of novel single nucleotide polymorphisms of the FSH beta-subunit gene on semen quality and fertility in bulls. *Anim Reprod Sci*. (2009) 114:14–22. doi: 10.1016/j.anireprosci.2008.08.021
57. Menezes EB, Velho ALC, Santos F, Dinh T, Kaya A, Topper E, et al. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genomics*. (2019) 18:714. doi: 10.1186/s12864-019-6074-6
58. Somashekar L, Selvaraju S, Parthipan S, Patil SK, Binsila BK, Venkataswamy MM, et al. Comparative sperm protein profiling in bulls differing in fertility and identification of phosphatidylethanolamine-binding protein 4, a potential fertility marker. *Andrology*. (2017) 5:1032–51. doi: 10.1111/andr.12404
59. Gòdia M, Estill M, Castelló A, Balasch S, Rodríguez-Gil JE, Krawetz SA, et al. RNA-Seq analysis to describe the boar sperm transcriptome and its seasonal changes. *Front Genet*. (2019) 16:299. doi: 10.3389/fgene.2019.00299
60. Card CJ, Krieger KE, Kaproth M, Sartini BL. Oligo-dT selected spermatozoal transcript profiles differ among higher and lower fertility dairy sires. *Anim Reprod Sci*. (2017) 177:105–23. doi: 10.1016/j.anireprosci.2016.12.011
61. Fraser L. A novel approach to assess semen freezability. *Vet Med*. (2016) 1:e5–6. doi: 10.17140/VMOJ-1-e003
62. Holland A, Ohlendieck K. Comparative profiling of the sperm proteome. *Proteomics*. (2015) 15:632–48. doi: 10.1002/pmic.201400032
63. Chen X, Zhu H, Hu C, Hao H, Zhang J, Li K, et al. Identification of differentially expressed proteins in fresh and frozen-thawed boar spermatozoa by iTRAQ-coupled 2D LC-MS/MS. *Reproduction*. (2014) 147:321–30. doi: 10.1530/REP-13-0313
64. Yeste M. Recent advances in boar sperm cryopreservation: state of the art and current perspectives. *Reprod Domest Anim*. (2015) 50(Suppl. 2):71–9. doi: 10.1111/rda.12569
65. Vilagran I, Castillo J, Bonet S, Sancho S, Yeste M, Estanyol JM, et al. Acrosin-binding protein. (ACRBP) and triosephosphate isomerase. (TPI) are good markers to predict boar sperm freezing capacity. *Theriogenology*. (2013) 80:443–50. doi: 10.1016/j.theriogenology.2013.05.006
66. Casas E, Vavouri T. Sperm epigenomics: challenges and opportunities. *Front Genet*. (2014) 5:330. doi: 10.3389/fgene.2014.00330
67. Wang P, Wang YF, Wang H, Wang CW, Zan LS, Hu JH, et al. HSP90 expression correlation with the freezing resistance of bull sperm. *Zygote*. (2014) 22:239–45. doi: 10.1017/S096719941300004X
68. Vilagran I, Yeste M, Sancho S, Casas I, Rivera Del Alamo MM, Bonet S. Relationship of sperm small heat-shock protein 10 and voltage-dependent anion channel 2 with semen freezability in boars. *Theriogenology*. (2014) 82:418–26. doi: 10.1016/j.theriogenology.2014.04.023
69. Kumar P, Kumar D, Singh I, Yadav PS. Seminal plasma proteome: promising biomarkers for bull fertility. *Agric Res*. (2012) 1:78–86. doi: 10.1007/s40003-011-0006-2
70. Li P, Hulak M, Koubek P, Sulc M, Dzyuba B, Boryshpolets S, et al. Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, *Cyprinus carpio* L. *Theriogenology*. (2010) 74:413–23. doi: 10.1016/j.theriogenology.2010.02.024
71. Intasqui P, Camargo M, Del Giudice PT, Spaine DM, Carvalho VM, Cardozo KH, et al. Unraveling the sperm proteome and post-genomic pathways associated with sperm nuclear DNA fragmentation. *J Assist Reprod Genet*. (2013) 30:1187–202. doi: 10.1007/s10815-013-0054-6
72. Das PJ, Paria N, Gustafson-Seabury A, Vishnoi M, Chaki SP, Love CC, et al. Total RNA isolation from stallion sperm and testis biopsies. *Theriogenology*. (2010) 74:1099–106. doi: 10.1016/j.theriogenology.2010.04.023
73. Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod*. (1993) 49:1202–7. doi: 10.1095/biolreprod49.6.1202
74. Gerena RL, Irikura D, Urade Y, Eguchi N, Chapman DA, Killian GJ. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biol Reprod*. (1998) 58:826–33. doi: 10.1095/biolreprod58.3.826
75. Moura AA, Memili E. Functional aspects of seminal plasma and sperm proteins and their potential as molecular markers of fertility. *Anim Reprod Sci*. (2016) 13:191–9. doi: 10.21451/1984-3143-AR884
76. Roncoletta M, Morani Eda S, Esper CR, Barnabe VH, Franceschini PH. Fertility-associated proteins in Nelore bull sperm membranes. *Anim Reprod Sci*. (2006) 91:77–87. doi: 10.1016/j.anireprosci.2005.03.014
77. Sarsaifi K, Vejayan J, Wahid Haron A, Yusoff R, Hani H, Rasoli M, et al. Protein profile and functionality of spermatozoa from two semen collection methods in Bali bulls. *Livest Sci*. (2015) 172:96–105. doi: 10.1016/j.livsci.2014.12.004
78. Martinez-Heredia J, Estanyol JM, Ballesca JL, Oliva R. Proteomic identification of human sperm proteins. *Proteomics*. (2006) 6:4356–69. doi: 10.1002/pmic.200600094
79. Muratori M, Luconi M, Marchiani S, Forti G, Baldi E. Molecular markers of human sperm functions. *Int J Androl*. (2009) 32:25–45. doi: 10.1111/j.1365-2605.2008.00875.x
80. Yoshida M, Kawano N, Yoshida K. Control of sperm motility and fertility: diverse factors and common mechanisms. *Cell Mol Life Sci*. (2008) 65:3446–57. doi: 10.1007/s00018-008-8230-z
81. Bronson RA, Fusi FM,Calzi F, Doldi N, Ferrari A. Evidence that a functional fertilin-like ADAM plays a role in human sperm–oolemmal interactions. *Mol Hum Reprod*. (1999) 5:433–40. doi: 10.1093/molehr/5.5.433
82. Yamaguchi R, Muro Y, Isotani A, Tokuhiro K, Takumi K, Adham I, et al. Disruption of ADAM3 impairs the migration of sperm into oviduct in mouse. *Biol Reprod*. (2009) 81:142–6. doi: 10.1095/biolreprod.108.074021
83. Siva AB, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, et al. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. *Mol Hum Reprod*. (2010) 16:452–62. doi: 10.1093/molehr/gaq009

84. Lhuillier P, Rode B, Escalier D, Lores P, Dirami T, Bienvenu T, et al. Absence of annulus in human asthenozoospermia: case report. *Hum Reprod.* (2009) 24:1296–303. doi: 10.1093/humrep/dep020
85. Jana A, Fatima E, Petr N. Increased expression of secretory actin-binding protein on human spermatozoa is associated with poor semen quality. *Hum Reprod.* (2007) 22:1396–404. doi: 10.1093/humrep/del511
86. D'amours O, Frenette G, Fortier M, Leclerc P, Sullivan R. Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes. *Reproduction.* (2010) 139:545–56. doi: 10.1530/REP-09-0375
87. Arai MM, Minami K, Ogura Y, Otsuka N, Hama S, Harayama H, et al. Variation among individual bulls in the distribution of acrosomal tyrosine-phosphorylated proteins in epididymal and ejaculated spermatozoa. *Reprod Fertil Dev.* (2017) 29:1297–305. doi: 10.1071/RD15483
88. Harayama H, Minami K, Kishida K, Noda T. Protein biomarkers for male artificial insemination subfertility in bovine spermatozoa. *Reprod Med Biol.* (2017) 16:89–98. doi: 10.1002/rmb2.12021
89. Ostermeier GC, Goodrich RJ, Diamond MP, Dix DJ, Krawetz SA. Toward using stable spermatozoal RNAs for prognostic assessment of male factor fertility. *Fertil Steril.* (2005) 83:1687–94. doi: 10.1016/j.fertnstert.2004.12.046
90. Maharana BR. Candidate gene markers for sperm quality and fertility in bulls. *Vet World.* (2013) 6:905–10. doi: 10.14202/vetworld.2013.905-910
91. Selvaraju S, Parthipan S, Somashekar L, Kolte AP, Krishnan Binsila B, Arangasamy A, et al. Occurrence and functional significance of the transcriptome in bovine (*Bos taurus*) spermatozoa. *Sci Rep.* (2017) 7:42392. doi: 10.1038/srep42392
92. Xue-Bing LI. Study on the gene expression of preimplantation IVF bovine embryos. *J Anhui Agric Sci.* (2010) 11:95–111.
93. Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. *Mol Cell Endocrinol.* (2004) 15:31–9. doi: 10.1016/j.mce.2003.10.069
94. Sapiro R, Kostetskii I, Olds-Clarke P, Gerton GL, Radice GL, Strauss IJ. Male infertility, impaired sperm motility, and hydrocephalus in mice deficient in sperm-associated antigen 6. *Mol Cell Biol.* (2002) 22:6298–305. doi: 10.1128/MCB.22.17.6298-6305.2002
95. Tamburrino L, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. *Hum Reprod.* (2014) 29:418–28. doi: 10.1093/humrep/det454
96. Chen X, Wang Y, Zhu H, Hao H, Zhao X, Qin T, et al. Comparative transcript profiling of gene expression of fresh and frozen-thawed bull sperm. *Theriogenology.* (2015) 83:504–11. doi: 10.1016/j.theriogenology.2014.10.015
97. Selvaraju S, Ravindra JP, Ghosh J, Gupta PS, Suresh KP. Evaluation of sperm functional attributes in relation to *in vitro* sperm-zona pellucida binding ability and cleavage rate in assessing frozen thawed buffalo. (Bubalus bubalis) semen quality. *Anim Reprod Sci.* (2008) 106:311–21. doi: 10.1016/j.anireprosci.2007.05.005
98. Nomikos M, Elgmati K, Theodoridou M, Calver BL, Nounesis G, Swann K, et al. Phospholipase Czeta binding to PtdIns(4,5)P2 requires the XY-linker region. *J Cell Sci.* (2011) 124:2582–90. doi: 10.1242/jcs.083485
99. Holden SA, Fernandez-Fuertes B, Murphy C, Whelan H, O'gorman A, Brennan L, et al. Relationship between *in vitro* sperm functional assessments, seminal plasma composition, and field fertility after AI with either non-sorted or sex-sorted bull semen. *Theriogenology.* (2017) 87:221–8. doi: 10.1016/j.theriogenology.2016.08.024
100. Marin S, Chiang K, Bassilian S, Lee WN, Boros LG, Fernandez-Novell JM, et al. Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. *FEBS Lett.* (2003) 554:342–6. doi: 10.1016/S0014-5793(03)01185-2
101. Patel AB, Srivastava S, Phadke RS, Govil G. Arginine activates glycolysis of goat epididymal spermatozoa: an NMR study. *Biophys J.* (1998) 75:1522–8. doi: 10.1016/S0006-3495(98)74071-8
102. Goodson SG, Qiu Y, Sutton KA, Xie G, Jia W, O'brien DA. Metabolic substrates exhibit differential effects on functional parameters of mouse sperm capacitation. *Biol Reprod.* (2012) 87:75. doi: 10.1095/biolreprod.112.102673
103. Patel AB, Srivastava S, Phadke RS, Govil G. Identification of low-molecular-weight compounds in goat epididymis using multinuclear nuclear magnetic resonance. *Anal Biochem.* (1999) 266:205–15. doi: 10.1006/abio.1998.2888
104. Sangeeta S, Arangasamy A, Kulkarni S, Selvaraju S. Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Anim Reprod Sci.* (2015) 161:82–8. doi: 10.1016/j.anireprosci.2015.08.008
105. Juyena NS, Stelletta C. Seminal plasma: an essential attribute to spermatozoa. *J Androl.* (2012) 33:536–51. doi: 10.2164/jandrol.110.012583
106. Odet F, Gabel S, London RE, Goldberg E, Eddy EM. Glycolysis and mitochondrial respiration in mouse LDHC-null sperm. *Biol Reprod.* (2013) 88:95. doi: 10.1095/biolreprod.113.108530
107. Binsila BK, Selvaraju S, Somashekar L, Archana SS, Arangasamy A, Ravindra JP, et al. Molecular advances in semen quality assessment and improving fertility in bulls. *Indian J Anim Reprod.* (2018) 39:1–10.
108. Van Der Horst CJ. The occurrence of phenylalanine- and alpha-aminocaprylic acid-alpha-ketoglutarate transaminase in boar spermatozoa. *Experientia.* (1970) 26:249. doi: 10.1007/BF01900073
109. Moore HD, Hibbitt KG. Fertility of boar spermatozoa after freezing in the absence of seminal vesicular proteins. *J Reprod Fertil.* (1977) 50:349–52. doi: 10.1530/jrf.0.0500349
110. Fu L, Liu Y, An Q, Zhang J, Tong Y, Zhou F, et al. Glycolysis metabolic changes in sperm cryopreservation based on a targeted metabolomic strategy. *Int J Clin Exp Pathol.* (2019) 12:1775–81.
111. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet.* (2005) 6:95–108. doi: 10.1038/nrg1521
112. Hering DM, Olenski K, Kaminski S. Genome-wide association study for poor sperm motility in Holstein-Friesian bulls. *Anim Reprod Sci.* (2014) 146:89–97. doi: 10.1016/j.anireprosci.2014.01.012
113. Fraser L, Dziekonska A, Strzezek R, Strzezek J. Dialysis of boar semen prior to freezing-thawing: its effects on post-thaw sperm characteristics. *Theriogenology.* (2007) 67:994–1003. doi: 10.1016/j.theriogenology.2006.12.002
114. Xing Y, Ren J, Ren D, Guo Y, Wu Y, Yang G, et al. A whole genome scanning for quantitative trait loci on traits related to sperm quality and ejaculation in pigs. *Anim Reprod Sci.* (2009) 114:210–8. doi: 10.1016/j.anireprosci.2008.08.008
115. Wang S, Zhang Y, Cheng Y, Lu G, Yang R, Geng H, et al. Association of SNPs in GnRH gene with sperm quality traits of Chinese water buffalo. *Reprod Domest Anim.* (2020) 55:384–92. doi: 10.1111/rda.13634
116. Liu J, Sun Y, Yang C, Zhang Y, Jiang Q, Huang J, et al. Functional SNPs of INCENP affect semen quality by alternative splicing mode and binding affinity with the target Bta-miR-378 in Chinese Holstein bulls. *PLoS ONE.* (2016) 11:e0162730. doi: 10.1371/journal.pone.0162730
117. Gilbert I, Bissonnette N, Boissonneault G, Vallee M, Robert C. A molecular analysis of the population of mRNA in bovine spermatozoa. *Reproduction.* (2007) 133:1073–86. doi: 10.1530/REP-06-0292
118. Kempisty B, Antosik P, Bukowska D, Jackowska M, Lianeri M, Jaskowski JM, et al. Analysis of selected transcript levels in porcine spermatozoa, oocytes, zygotes and two-cell stage embryos. *Reprod Fertil Dev.* (2008) 20:513–8. doi: 10.1071/RD07211
119. Bissonnette N. Transcriptome analysis of bull semen with extreme nonreturn rate: use of suppression-subtractive hybridization to identify functional markers for fertility. *Biol Reprod.* (2008) 78:618–35. doi: 10.1095/biolreprod.106.059030
120. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet.* (2009) 10:57–63. doi: 10.1038/nrg2484
121. Werner T. Next generation sequencing allows deeper analysis and understanding of genomes and transcriptomes including aspects to fertility. *Reprod Fertil Dev.* (2011) 23:75–80. doi: 10.1071/RD 10247
122. Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. *J Appl Genet.* (2011) 52:413–35. doi: 10.1007/s13353-011-0057-x
123. Capra E, Turri F, Lazzari B, Cremonesi P, Gliozzi TM, Fojadelli I, et al. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between High- and Low-motile sperm populations. *BMC Genomics.* (2017) 18:14. doi: 10.1186/s12864-016-3394-7

124. Jodar M, Soler-Ventura A, Oliva R. Molecular biology of reproduction and development research group. Semen proteomics and male infertility. *J Proteomics*. (2017) 6:125–34. doi: 10.1016/j.jprot.2016.08.018
125. Silva JV, Yoon S, De Bock PJ, Goltsev AV, Gevaert K, Mendes JF, et al. Construction and analysis of a human testis/sperm-enriched interaction network: unraveling the PPP1CC2 interactome. *Biochim Biophys Acta Gen Subj*. (2017) 1861:375–85. doi: 10.1016/j.bbagen.2016.11.041
126. Tomar R, Mishra AK, Mohanty NK, Jain AK. Altered expression of succinic dehydrogenase in asthenozoospermia infertile male. *Am J Reprod Immunol*. (2012) 68:486–90. doi: 10.1111/aji.12023
127. Bhilawadikar R, Zaveri K, Mukadam L, Naik S, Kamble K, Modi D, et al. Levels of Tektin 2 and CatSper 2 in normozoospermic and oligoasthenozoospermic men and its association with motility, fertilization rate, embryo quality and pregnancy rate. *J Assist Reprod Genet*. (2013) 30:513–23. doi: 10.1007/s10815-013-9972-6
128. Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. *Hum Reprod Update*. (2014) 20:40–62. doi: 10.1093/humupd/dmt046
129. Wenk MR. Lipidomics: new tools and applications. *Cell*. (2010) 143:888–95. doi: 10.1016/j.cell.2010.11.033
130. Tyagi MG. Analysis of phospholipids using the high performance liquid chromatography technique. *Int J Curr Res Biosci Plant Biol*. (2016) 3:28–31. doi: 10.20546/ijcrbp.2016.304.005
131. Vijayakumar A, Vijayaraj P, Vijayakumar AK, Rajasekharan R. The arabidopsis ABHD11 mutant accumulates polar lipids in leaves as a consequence of absent acylhydrolase activity. *Plant Physiol*. (2016) 170:180–93. doi: 10.1104/pp.15.01615
132. Kumar A, Kroetsch T, Blondin P, Anzar M. Fertility-associated metabolites in bull seminal plasma and blood serum: 1H nuclear magnetic resonance analysis. *Mol Reprod Dev*. (2015) 82:123–31. doi: 10.1002/mrd.22450
133. Hamamah S, Seguin F, Bujan L, Barthelemy C, Mieusset R, Lansac J. Quantification by magnetic resonance spectroscopy of metabolites in seminal plasma able to differentiate different forms of azoospermia. *Hum Reprod*. (1998) 13:132–5. doi: 10.1093/humrep/13.1.132
134. Gilany K, Moazeni-Pourasil RS, Jafarzadeh N, Savadi-Shiraz E. Metabolomics fingerprinting of the human seminal plasma of asthenozoospermic patients. *Mol Reprod Dev*. (2014) 81:84–6. doi: 10.1002/mrd.22284
135. Qiao S, Wu W, Chen M, Tang Q, Xia Y, Jia W, et al. Seminal plasma metabolomics approach for the diagnosis of unexplained male infertility. *PLoS ONE*. (2017) 12:e0181115. doi: 10.1371/journal.pone.0181115
136. Everett JR. Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. *Concepts Magn Reson*. (2000) 12:289–320. doi: 10.1002/1099-0534(2000)12:5<289::AID-CMR3>3.0.CO;2-W
137. Nikbina S, Panandamb JM, Yaakubb H, Murugaiyahc M. Association of novel SNPs in gonadotropin genes with sperm quality traits of Boer goats and Boer crosses. *J Appl Anim Res*. (2018) 46:459–66. doi: 10.1080/09712119.2017.1336441
138. Zhou SM, Cheng L, Guo, SJ, Zhu H, Tao S. Functional protein microarray: an ideal platform for investigating protein binding property. *Front Biol*. (2012) 7:336–49. doi: 10.1007/s11515-012-1236-9
139. Zhao D, Wu W, Xu B, Niu X, Cui H, Zhang Y, et al. Variants in the SRD5A2 gene are associated with quality of semen. *Mol Med Rep*. (2012) 6: 639–44. doi: 10.3892/mmr.2012.965

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 16 April 2021
doi: 10.3389/fvets.2021.601794

Advancing Semen Evaluation Using Lipidomics

*Holly C. Evans[1], Thu T. N. Dinh[1], Madison L. Hardcastle[1], Alicia A. Gilmore[1], Muhammet R. Ugur[1], Mustafa Hitit[1,2], Frank Dean Jousan[1], Molly C. Nicodemus[1] and Erdogan Memili[1]**

[1] Department of Animal and Dairy Sciences, Mississippi State University, Starkville, MS, United States, [2] Department of Animal Genetics, Kastamonu University, Kastamonu, Turkey

Developing a deeper understanding of biological components of sperm is essential to improving cryopreservation techniques and reproductive technologies. To fully ascertain the functional determinants of fertility, lipidomic methods have come to the forefront. Lipidomics is the study of the lipid profile (lipidome) within a cell, tissue, or organism and provides a quantitative analysis of the lipid content in that sample. Sperm cells are composed of various lipids, each with their unique contribution to the overall function of the cell. Lipidomics has already been used to find new and exciting information regarding the fatty acid content of sperm cells from different species. While the applications of lipidomics are rapidly evolving, gaps in the knowledge base remain unresolved. Current limitations of lipidomics studies include the number of available samples to analyze and the total amount of cells within those samples needed to detect changes in the lipid profiles across different subjects. The information obtained through lipidomics research is essential to systems and cellular biology. This review provides a concise analysis of the most recent developments in lipidomic research. This scientific resource is important because these developments can be used to not only combat the reproductive challenges faced when using cryopreserved semen and artificial reproductive technologies in livestock such as cattle, but also other mammals, such as humans or endangered species.

Keywords: fatty acids, lipids, biomarkers, livestock, cryotolerance

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Daniel Marcelo Lombardo, University of Buenos Aires, Argentina
Jamie L. Stewart, Virginia-Maryland College of Veterinary Medicine, United States

***Correspondence:**
Erdogan Memili
em149@msstate.edu

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *01 September 2020*
Accepted: *11 March 2021*
Published: *16 April 2021*

Citation:
Evans HC, Dinh TTN, Hardcastle ML, Gilmore AA, Ugur MR, Hitit M, Jousan FD, Nicodemus MC and Memili E (2021) Advancing Semen Evaluation Using Lipidomics. Front. Vet. Sci. 8:601794. doi: 10.3389/fvets.2021.601794

INTRODUCTION

Fertility—An Economically Vital Trait

Male fertility may be defined as the ability of a sperm cell to fertilize an oocyte and support development to produce viable offspring. There are numerous factors that contribute to the overall fertility of a given male. The variation within these factors makes it challenging to determine what makes one male more fertile than another. Previous research in humans has demonstrated that aspects of the ejaculate, such as sperm volume, presence of abnormal components (i.e., urine or blood), and seminal plasma volume, along with more specific spermatozoan characteristics including morphology, motility, DNA integrity, acrosome integrity, and membrane integrity can further illustrate differences of fertility and performance (1, 2). However, these characteristics alone may not be enough to ascertain the true fertility of an individual male. With technological advancements in animal management toward highly efficient and productive livestock, this industry has shifted toward the utilization of sperm cryopreservation techniques to allow for the broader distribution of top-quality genetics, while preserving their impact for future generations.

Cryopreservation is the freezing of biological samples with the intent of preserving the integrity of the sample for later use (3). In the case of spermatozoa, the frozen sample would later be used for artificial insemination (AI) of an open female. Freezing and thawing semen samples can be extremely detrimental to sperm due to cellular damage, membrane breakage, acrosome damage, and cell death that is caused by ice crystal formation, reactive oxygen species (ROS), lipid peroxidation, and other factors (4). However, an individual male that maintains and produces high-quality, fresh sperm could perform at critically lower levels after cryopreservation due to damages incurred during the process of cryopreservation. Cryopreserved sperm still suffer from structural damage that affect sperm physiology including low motility and viability that results in the considerable extent of fertility loss (5, 6), and yet, advances in knowledge concerning semen cryopreservation protocols have led to the commercialization of this process to market genetically superior livestock on a worldwide basis with tremendous positive economic impact.

Spermatogenesis

Spermatogenesis is the continuous generation of sperm cells in the male, which ensures the replenishment of spermatozoa. In bulls for instance, the process of spermatogenesis takes ∼61 days to complete with new cells being added to the process every 13.5 days (7)). Spermatozoa, along with various hormones, proteins, and seminal fluids, are produced in the testicles, which contribute to normal reproductive physiological processes in males. Testicles are the site of testosterone production. Inhibin is produced by Sertoli cells within the testicles and elicits negative feedback on sperm production and estrogen release, aiding in spermatogenesis, the modulation of male libido, and the male erection (8).

Spermatogenesis is compartmentalized in the seminiferous tubules of the testicles. In the basal compartment, mitosis takes place to allow for the proliferation of spermatogonia to obtain the primary spermatocytes. Primary spermatocytes undergo two cycles of meiosis to further mature to become secondary spermatocytes, and then, spermatids. Spermiogenesis, the final stage of spermatogenesis, completes the cellular transformation of spermatids into mature, motile spermatozoa. Finally, spermiation allows for mature spermatozoa to be released from the Sertoli, or sustentacular, cells and into the lumen of the seminiferous tubules. Spermatozoa are stored in the caput (head) of the epididymis. While in the caput of the epididymis, sperm are immotile and have a low membrane fluidity due to the high cholesterol to phospholipid ratio (9). When sperm reach maturity, they are pushed into the corpus (body) of the epididymis. On a molecular basis, mature sperm will have a higher degree of membrane fluidity, contain disulfide bonds, and have lower cholesterol to phospholipid ratio than immature sperm cells (9). From the corpus of the epididymis, mature spermatozoa are moved into the caudal (tail) of the epididymis for storage and transport to the vas deferens to await ejaculation. Sperm are altered as they move through the epididymis, including the modification of lipids and sugars found on the sperm cell's plasma membrane (10). This allows for the development of cellular motility and changes in membrane fluidity in preparation for fertilization.

Sperm Structure

Sperm cells have several structures that allow for the fertilization of the female oocyte. The head piece of the bovine sperm cell is shaped like a round kernel of corn. In other models such as the rooster, the sperm head is slender and elongated (11). If the head of the sperm cell, regardless of species, is not properly shaped, its ability to maneuver with forwarding, progressive motility to fertilize an oocyte can be compromised. Abnormalities have been associated with immaturity of sperm and reduced fertility (12).

The sperm head is encapsulated by several membrane layers which include theplasma membrane, the outer acrosomal membrane, the acrosome, and the inner acrosomal membrane. The acrosome, which deteriorates once the acrosome reaction occurs, allows for penetration of the zona pellucida (13). Through capacitation, the acrosomal matrix proteins are exposed and allow for interaction with the zona pellucida of the oocyte (14). Proteins such as sp56 and zonadhesin have been identified as key sperm-zona binding agents (15, 16). The nucleus of sperm cells is held within these layers to prevent damage to the genetic material needed to fertilize an oocyte for successful fetal development. On the front portion of the sperm cell, the acrosome bends posteriorly to create the apical ridge, which is responsible for the recognition and binding of the cell to the zona pellucida of the oocyte (17). Toward the center of the sperm head, the acrosome ends and forms the equatorial segment. The structures of the head that lie between the apical segment and the equatorial segment are grouped into the principal segment of the sperm head.

The midpiece (neck) of the spermatozoa connects to the implantation socket at the caudal end of the sperm head via the capitulum. Mitochondrial adenosine triphosphate (ATP) production occurs in the midpiece and fuels the movement of sperm cells. The midpiece is composed of mitochondrial helices and inner tubules that are responsible for the bending of the sperm tail. There are coarse outer fibers that form a fibrous sheath surrounding the sperm tail. The endpoint of the midpiece is the annulus. The axial filament complex originating from the distal centriole is a matrix of the microtubules. The sliding motion of these tubules allows for the lateral movement of the sperm tail and is driven by ATPases (18). This is of great importance because ATP production is dependent upon environmental temperature, which allows for greater mobility. Mitochondria are biomarkers of sperm cell fertility and are necessary for motility (19). They have two sets of membranes, the inner and outer mitochondrial membranes. This creates an environment for energy-transduction and allows for oxidative phosphorylation to occur (20).

LIPIDS OF THE SPERM MEMBRANE

Lipid Classes

Lipids are biological components that are broadly defined as naturally occurring substances that are not soluble in water. From storing energy to providing structure or flexibility to

cellular components, lipids have variable functions in a biological system. There are many ways to classify lipids which range from simple to complex categorization. For this discussion, the Lipid Metabolites and Pathways Strategy (LIPID MAPS®) classification system will be utilized (LIPID Metabolites and Pathways Strategy; http://www.lipidmaps.org). LIPID MAPS classify lipids into eight different categories: fatty acyls (FAc), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK) (21). Of the listed lipid classes, they can be further separated based on their basic subunits. Ketoacyl subunits are found in polyketides, saccharolipids, fatty acyls, glycerolipids, glycerophospholipids, and sphingolipids (22). Ketoacyl subunits are acetyl compounds and propionyl compounds (21). The remaining lipid classes of sterols and prenol lipids contain organic isoprene subunits, which are unsaturated hydrocarbon compounds.

Lipids classified within the fatty acyl (FAc) group are synthesized via chain elongation by acetyl-CoA with malyonyl-CoA. The FAc group is composed of diverse lipids that are further classified into subgroups. The FAc group can be thought of as one of the most fundamental building blocks in biological lipids because they are the foothold, if you will, for the formation of larger, more complex lipids. Glycerolipids typically function as an energy storage system in mammalian tissue. Mono-, di-, or tri-substituted glycerols comprise glycerolipids (GL). However, GLs are also play a major role in both cell signaling and act as structural components of cell membranes. These molecules are composed of neutral lipids and have a glycerol backbone (23). One of the most widely recognized GLs are triglycerides, which store energy in the body in the form of glycerol unit and three long-chain fatty acid units. These units are broken down via β-oxidation to help fuel the body with energy when sugars are not available or when exercise is prolonged.

Glycerophospholipids (GP), or phospholipids, are long-chain fatty acids that perform as both structural and functional components of cells. In the most basic of forms, GPs are composed of a negatively charged phosphate head, two fatty acids, which can vary, and a glycerol molecule. They are readily observed in the membranes of cells, including lipid bilayers, in addition to serving as secondary messengers and binding sites. This is because the fatty acid chains, or tails, are uncharged and hydrophobic, whereas the phosphate group is hydrophilic. These lipids assist with cellular signaling and metabolism in both animal and plant cells (24, 25). Lipids with a backbone of sphingoid bases are grouped into the SP category. These lipids also contain aliphatic amino alcohols like sphingosine, which is an important phospholipid. These lipids are pivotal to the vitality and protection of cellular membrane protection. This is due in part to the formation of outer leaflets of the plasma membrane lipid that are not only stable structurally, but also resistant to chemical changes (26).

Cholesterol and its derivatives are components of the sterol lipids (ST) category. Cholesterol is commonly discussed in human medicine due to its role in cardiovascular disease development and control. This wax-like lipid is necessary for normal bodily functions, but it can become harmful in copious amounts. On a cellular level, cholesterol and other ST lipids give structure to membranes. Cholesterol efflux in the plasma membrane of sperm can alter acrosomal responsiveness, and thus, negatively impact fertilization success due to changes in stability (27, 28). In rodent models, high-fat diets decreased sperm motility, increased serum lipid concentrations, and altered hormone levels to include heightened estradiol levels and decreased testosterone levels (29). In addition to structural functions, sterol lipids also act as signaling molecules and hormones. General groups of these include progesterone, estrogen, and androgen.

Prenol lipids include classes of vital compounds such as vitamins K and E, which act as antioxidant agents, preventing cell membrane oxidation and controlling the production of free radicals. They are synthesized from 5-carbon precursors, which include compounds like isopentenyl diphosphate and dimethylallyl diphosphate (30). These products are a result of the mevalonic acid pathway (30). Additional functions include cell signaling and anabolism.

Lipids that have a sugar backbone where fatty acids attach are grouped into the saccharolipids (SL) category. Saccharolipids have a similar structure to that of glycerolipids because the glycerol is replaced with monosaccharides. The structure of SLs is compatible with lipid bilayers. Polyketides are considered to be secondary metabolites. They are synthesized by the polymerization of acetyl and propionyl subunits. This is made possible by classic, iterative, and multimodular enzymes, which share features from a mechanical standpoint with fatty acid synthases. This group of molecules may contain methylene groups or varying carbonyl groups. Polyketides are important from a pharmaceutical standpoint in that PKs are often components of anticancer, antifungal, anticholesteremic agents, antibiotics, immunomodulators, and parasiticides due to their antimicrobial and immunosuppressive qualities (21). Within the simple category are STs, such as cholesterol and FAc (including all derivatives), compared to the complex category, which would include SPs, GLs, and GPs (31). While new technology is being applied to the study of lipidomics, commonly used tools include, but are not limited to, GC, MS, LC-MS, thin layer chromatography, and NMR.

As FAs are structural compounds of cell membranes, the composition of the fatty acids may play a critical role in sperm function through regulation of membrane structure (32). Dietz et al. (33) suggested lipid concentration of bovine semen to be 4.10 mg/ml and were able to identify a total of nine fatty acids: SFA 12:0, 14:0, 15:0, 16:0, 16:1, 17:0, 18:0, MUFA 18:1, and PUFA 18:2. Of those, 16:0 (palmitic acid) was the most abundant lipid group with a relative percentage of 40.9% followed by palmitic acid 14:0, 18:0, and 18:1 as the most predominant FA with relative concentrations of 26.4, 12.9, and 10.5%, respectively. Komarek et al. (34) analyzed the lipid composition of bull sperm and seminal plasma samples separately using thin-layer chromatography and reported that total lipid content of bovine spermatozoa and seminal plasma accounts for 12.0 and 1.35% of the total dry weight, respectively. Fractions of lipids were detected, including phospholipids, cholesterol, diglycerides, triglycerides,

and wax esters (34). The most abundant lipid groups were phospholipids and cholesterol with 73 and 14.5% of the total lipid composition, respectively.

Cholesterol and Precursors of Steroid Hormones

Cholesterol is a steroid hormone found in all mammals. Cholesterol serves as the precursor molecule for all other steroid hormones, as well as, to vitamin D and bile acids/salts. There are five major classes of sterol hormones: androgens, estrogens, progestogens, glucocorticoids, and mineralocorticoids. Androgens are especially important in males due to their role in fertility and reproduction. Testosterone is a cholesterol derivative responsible for the development of the male's sexual behavior, maintenance of the testes, the onset of puberty, and development of muscle mass (35). While cholesterol serves as a building block for sterol hormones, cholesterol also performs an important structural function in cellular membranes and contributes to the fluidity of plasma membranes and their functionality (36). Cholesterol is key for the process of capacitation of sperm cells. Amounts of the cholesterol in sperm membranes may determine cryotolerance of the cell because higher levels of cholesterol result in more rigid and cohesive sperm membranes. Bull sperm (0.89 $\mu M/10^9$ sperm) and ram sperm (0.722 $\mu M/10^9$ sperm) contain lower levels of cholesterol compared to human sperm (1.438 $\mu M/10^9$ sperm) (37). In addition, the ratio of the polyunsaturated FAs to saturated FAs in bull sperm (3.5) are greater than human (1.0) and ram sperm (2.5) (38). Researchers have also studied comparative cholesterol content in neutral lipids of sperm and seminal plasma from bulls and water buffalos. While the cholesterol content of the sperm cells and seminal plasma from the bull were 23.3 and 18.8%, respectively, these values in water buffalo were 22.2 and 24.7%, respectively (39).

The loss of cholesterol from the sperm membrane leads to an imbalance that affects its permeability (40). This membrane alteration allows for calcium, bicarbonate, and potassium ions to cross freely through the membrane, thus, increasing the internal ion concentration. As the intracellular ion concentration increases, the acrosome reaction is induced. A method has been developed for total lipid extraction and purification that is still widely used with modifications for cholesterol analysis (41). The method developed employs methanol and chloroform as analytical reagents. When using the Bligh and Dyer method, the volumes of cholorform:methanol: water, both before and after dilution, should be kept in the proportions of 3:2:0.8 and 2:2:1.8, respectively. The ratios presented account for water present within a given sample. For samples with higher water volume, methanol, and chloroform volumes should be adjusted. Samples lacking water volumes can de diluted with water. Samples are prepared using a vortex and centrifugation to establish distinct layers, a chloroform layer and an organic layer which contains the lipids. The organic layer containing the lipids is then separated and evaporated under liquid nitrogen. Samples can then be analyzed using the preferred method of the researcher, such as microscopy or LC-MS (42, 43). Previous studies have focused on manipulating cholesterol levels to determine the effect on post-thaw viability (44). In a rodent based study, it was determined that rabbits that were fed high-fat diets had significantly lower semen quality, motility, capacitation, and acrosome reaction (45). This could be a result of increased cholesterol incorporation to the plasma membrane, which increases membrane rigidity and resistant to alteration by reducing the fluidity. The ability to quantify cholesterol within the sperm membrane allows for the ratio comparison of cholesterol to other lipids as well as proteins in both high and low freezability and fertility sperm, allowing for a clearer picture of the dynamics.

Fat-Soluble Vitamins

Lipids are also transporters of vitamins A, D, E, and K, which contribute to functions and metabolism in the body. Vitamin E, which is found in the cell membrane, has been demonstrated to have important antioxidant properties. It destroys free hydroxyl radicals and superoxide anion, reducing lipid peroxidation of the plasma membrane (46). In study completed by Hu et al. (47) vitamin E was used as a supplementation at various concentrations to bull sperm subjected to cryopreservation. When samples were supplemented with 1.5 mg/ml concentrations of vitamin E, there was a significantly improved level in sperm motility, straight-line velocity, and straightness ($P < 0.05$). In addition, the percentage of acrosome-intact and membrane-intact sperm was significantly improved ($P < 0.05$). While vitamin E supplementation has demonstrated the ability to reduce the potential of lipid peroxidation, allowing for improved semen quality post-thaw, this is still an area of research interest to further evaluate the role of vitamin E in reproduction.

Vitamin A is required for normal mammalian spermatogenesis and has antioxidant properties. This vitamin breaks chains by attaching to peroxyl radicals, thus preventing lipid peroxidation (48). Zervos et al. (49) examined the effects of vitamin A on acrosin activity. Fifteen rams were split into three groups and received different concentrations of vitamin A, given as retinyl acetate. The three groups included a control group, the 12,500 IU/animal per day group, and the 50,000 IU/animal per day. Acrosin activity was measured using spectrophotometry. There was no statistical difference found between the control group and 12,500 IU group, but a significant decrease in acrosin activity was found in the 50,000 IU groups in comparison to the control group ($P < 0.05$). It was concluded that excessive vitamin A intake does not affect acrosin activity, but deprivation of vitamin A can reduce acrosin activity.

Vitamin D is thought to function in regulating intracellular Ca and Ca-binding proteins in the testis. Jueraitetibaike et al. (50) investigated the associations between seminal plasma vitamin D levels and semen quality. Vitamin D levels were detected using electrochemiluminescence in 220 fertile men. Seminal plasma 25(OH)D levels were positively correlated with semen volume and kinetic values of the sperm cells. Research suggests that vitamin D in seminal plasma could be linked to the regulation of sperm motility by promoting ATP synthesis via the cAMP/PKA pathway.

Vitamin K is a key modulator of extracellular calcium homeostasis within sperm cells and the epididymis, facilitates energy production within the mitochondria, and contains antioxidant properties. The intracellular compartmentalization of the vitamin K cycle may contain a more localized defense system against ROS attack (51). In addition, the reduced form of vitamin K, KH2, has been demonstrated to protect plasma membranes from peroxidation by ROS uptake in humans (52).

Oxidation of Membrane Lipids—Primary Oxidation, Secondary Oxidation

Sperm cells are highly susceptible to oxidative stress (OS) due to the concentration of PUFAs found within the plasma membranes. Antioxidant concentrations are low in the cytoplasm of sperm cells as compared to that of somatic cells, which have larger quantities that contribute to defending against oxidative damage. Oxidative stress is the imbalance between reactive oxidative species (ROS) and antioxidants (4). Several types of ROS exist, including oxygen free radicals, non-radical species, and reactive nitrogen species. Oxygen free radicals are highly reactive compounds that can affect any cellular component (53). Examples of oxygen free radicals include compounds such as hydroxyl radicals and superoxide anions. Non-radical species are moderately reactive and are formed after both protonation and univalent reduction occur. Some examples of these would include hydrogen peroxide and hypochlorous radicals (54). These compounds react with proteins and form other ROS-like hydroxyl radicals.

Both the oxygen free radicals and the non-radical species are created by the partial reduction of oxygen within a given compound (55). Reactive nitrogen species are a little different from the other two categories because they are produced by enzymatic activity of nitric oxide synthase 2 and NADPH oxidase. Additionally, these compounds are derived from nitric oxide compounds (56). Two examples of these antimicrobial molecules include superoxide and nitric oxide. There are several potential consequences to an overabundance of ROS. One major consequence of OS is lipid peroxidation, which compromises the integrity of cell membranes (57). However, many laboratory techniques have been developed to measure lipid peroxidation in spermatozoa and to combat ROS from harming spermatozoa (57, 58).

Sperm lipids are abundant in the membranes and they are largely in the form of PUFAs, which contain unconjugated double bonds between methylene bridges (59). The double bond adjacent to methylene group weakens the methyl carbon–hydrogen bond, thus, making hydrogen excessively vulnerable to oxidative damage. Because the intracellular levels of ROS elevate excessively, ROS establishes a cascade of reactions, which eventually culminate in lipid peroxidation (LPO) (60–62). Then, a great amount of membrane fatty acids is demolished, and fluidity decreases with the loss of function of sperm cell (63). The functions of membrane receptors and enzymes are suppressed (64). Therefore, LPO initiates an autocatalytic self-propagating chemical reaction, which causes unsuccessful fertilization due to impairment of sperm function (59, 60, 65).

The machinery of lipid peroxidation can occur in three main stages: initiation, propagation, and termination. Initiation mainly comprises abstraction of hydrogen from the carbon–carbon double bonds, therefore, leading to free radicals, which then, produces lipid radicals, and subsequently, interacts with oxygen, generating the peroxyl radicals (60, 66). The chain of autocatalytic reactions is preceded with abstraction of hydrogen atoms from the PUFA by peroxyl radicals, leading to formation of organic hydroperoxides, one of the possible limiting factors of the lifespan of mammalian sperm (67). With interaction of the formed radicals with successive lipids, the propagation stage progresses with the formed radicals that then produce cytotoxic aldehydes due to decay of hydroperoxide (68, 69). Subsequently, the development of alkyl and peroxyl radicals maintained in a repeated cycle until the end product is produced as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), and the chain reaction ceases. The physiological levels of lipid peroxidation indicate the functional effects of ROS on sperm metabolism improving the ability of sperm to contact with oocyte (70). Nevertheless, the lipid peroxidation is regarded as the primary molecular mechanism (71) implicated in the oxidative damage to the cell that induces death. The two major consequences of this are structural damage to cell membrane and production of secondary products (72).

PUFAs with the presence of double bonds are susceptible to free radical attack and induction of LPO, which results in morphological and membrane abnormalities, in addition to impaired motility (57, 73). In this regard, due to free radical attack on PUFA in sperm, the lipid peroxidation cascade through mitochondrial generation of ROS propels cytotoxic lipid aldehydes such as 4-hydroxynonenal (4HNE) (74). Hence, mammalian sperm has been reported to be susceptible to loss of motility (75, 76) and acrosome integrity (77) due to the exogenous oxidant as a result of LPO. This may arise from the set of complexes of acrosome reaction which causes changes in membrane phospholipid/cholesterol ratio, membrane fluidity, and net charge of sperm cellular surface because the lipid composition and metabolism play a significant role in mammalian acrosome reaction (78).

Moreover, excessive production of ROS in cryopreservation causes alterations in the levels of carbohydrate, protein, and lipid in the sperm membrane, owing to the reduction of disulfide bonds between membrane proteins (79) and the increase in the peroxidation of membrane phospholipids, along with changes of sperm glycocalyx. As a result of peroxidative damage, phosphatidylcholine, phosphatidylethanolamine, and cholesterol molecules are released along with loss of phosphatidylcholine and phosphatidylethanolamine (67, 80). This leads to ultrastructural alterations of sperm plasma membrane in which cryopreservation influences membrane integrity severely (6, 81).

Although fresh sperm had slight lipid peroxidation, cryopreserved sperm suffer from higher lipid peroxidation (82, 83). This may result from the reason that cryopreserved sperm cells can be more susceptible to peroxidases than fresh sperm cells (84) and endogenous phosphatidylcholine is subject to excessive peroxidation, which is detected particularly in the

mitochondrial midpiece and tail (85). Ram sperm, due to its high sensitivity to lipid peroxidation, demonstrated greater vulnerability to chromatin damage (86), owing to changes in expression of genes regulating the protamination process, and in bulls, it is sperm positively correlated with DNA integrity (87, 88). Also, this is consistent with results that cryopreserved bovine sperm suffered from low chromatin damage when low levels of lipid peroxidation were experienced (89).

Roles of Lipid Components in Cryopreservation

Cryopreservation and the shipment of frozen semen are necessary for the advancement of the livestock industry as it allows for customized breeding of females to genetically superior sires, thereby, increasing the progeny from these males. Nevertheless, sperm from certain sires are more resilient to cryopreservation than others due to differences among lipid compositions of the sperm cell membranes (72, 90). There are differences in the composition of spermatozoa within an ejaculate, in addition to the quantity and quality of components among sperm cells, but the ability of the sperm cell to migrate through the reproductive tract of the female to fertilize an oocyte is dependent upon the form and function of the anatomical piece being evaluated.

The protein to phospholipid components, as well as the ratios of proteins to phospholipids and cholesterol to phospholipids, vary greatly when comparing the component constituents of the plasma membrane to the outer acrosomal membrane (91). The protein to phospholipid ratio is the greatest in whole sperm, followed by the outer acrosomal membrane and then the plasma membrane, due in part to their form and function (91). The cholesterol to phospholipid ratio is lower in the whole sperm and the outer acrosomal membrane, but the plasma membrane has a greater ratio of those components (91). While much is already understood with regards to their responsibilities and functions as energy sources and structural components to cells, the role of fatty acids in fertility and cryopreservation has not been well-elucidated (92). Docosahexaenoic acid (DHA, 22:6), has been positively correlated with sperm motility and improved semen parameters under heat-stress conditions, but the mechanism of how DHA affects motility is not well-understood (92, 93). Additionally, DHA and stearic acid (18:0) are involved in motility parameters before and after freezing sperm and having high quantities of these fatty acids generally means that sperm will have better post-cryopreservation motility than those with lower quantities (94). In a study performed by Maldjian et al. (95), the introduction of 3% fish oil to the diet of boars increased DHA content in sperm from 33 to 45% and increased ejaculate concentrations but did not improve or preserve sperm parameters upon post-thaw.

Fertility and functionality of sperm cells are impacted by the structural characteristics of the spermatozoon itself. Membrane layers surrounding the nucleus and cytoplasm, as well as the tail, all contain critical lipids and fatty acids that are vital to cellular integrity and overall functionality (96). For example, the head and tail of bull sperm contain large quantities of very long-chain fatty acids followed by saturated fatty acids with choline being a predominant portion in both the head and the tail (97). Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) make up the composition of lipid membranes in addition to other materials such as sugars and proteins. These components are vital to successful fertilization. This membrane matrix varies from male to male and from cell to cell within an ejaculate. Compositional characteristics of the plasma membrane give way to fluidity and freezability of sperm cells, and sperm cells with more fluid membranes display improved responses after cryopreservation procedures (98). Destabilization of the membrane is caused by temperature-induced stress in addition to osmotic stressors like water or cryoprotectants, causing damage or swelling of the membrane (99). However, detailed mechanisms behind the functionality of fatty acids in these fluid membrane roles are not well understood. Sperm with greater proportions of PUFA compared to SFA tend to demonstrate higher fertility due to the degree of fluidity and strength of the cell membrane that is provided by PUFA having multiple double bonds (94). Saturated fatty acids do not contain double bonds and are less structurally stable when encountered by stressors or challenges such as freezing temperatures from cryopreservation.

The plasma membrane of the sperm cell can be destroyed by osmotic stressors, ice crystal formation, and dehydration of the membrane from cooling rates (100, 101). These factors disrupt the integrity of the cell and hinder the ability of the membrane to be selectively permeable to important molecules, leaving them incapable of delivering genetic material to the oocyte and prevents pregnancy. In addition to this damage, sperm cells with smaller acrosomes could be at greater risk for damage or attack by these factors (102). Specie differences exist in lipid compositions of the sperm plasma membrane, as well as variations among sires within a given species, thereby, making lipid profiling a vital component to sperm evaluation.

Lipidomics—Study of Lipid Composition and Functions

Lipidomics is the study of the lipid profile (lipidome) within a cell, tissue, or organism and provides a quantitative analysis of the lipid content in the sample being studied. This can also be thought of as a branch of metabolomics, which is the characterization and quantification of the major classes of metabolites in a given sample. Lipidomics has already been used to find new and exciting information regarding the fatty acid content of sperm cells from different species. In the stallion, mass spectrometry revealed the presence of (O-acyl)-ω-hydroxy-fatty acids, specifically in the sperm head and tail, which had not been previously detected (103). While the exact functions of these compounds are unclear, complex fatty acids, such as (O-acyl)-ω-hydroxy-fatty acids, which contain carbon chains of up to 52 carbons, are important to sperm cell membrane functionality (103). In canine species, changes in the fatty acid composition of sperm cells throughout the process of sperm maturation have been documented. The concentrations of SFA, MUFA, and PUFA were high in those sperm cells that were collected from the cauda

epididymis. In addition, sperm collected from the cauda portion of the epididymis had significantly greater amounts of 8:0, 18:0, and 15:0 as compared to that found in sperm from the caput and corpus of the epididymis. Differences were also present in the epididymal fluids of samples, with the caput having significantly less 18:0, 15:0, and 18:2 than that of the cauda fluid (104). In boars, the supplementation of both n-3 and n-6 fatty acids to the diet was shown to alter the composition of sperm cell fatty acids and had a positive correlation of DHA content with viability and progressive motility of sperm cells (105).

Mendeluk et al. (106) reported that several fatty acid concentrations, including 16:1 cis9, 18:2 (ω-6, LA), 20:5 (ω-3, EPA), and 22:6 (ω-3, DHA), increased significantly in erythrocytes after dietary supplementation was provided. Recently, research efforts have explored the relationship between season and lipid profiles of bull semen (107) identified and quantified the lipid profile of semen samples from five Holstein–Friesian bulls during the summer (August to September) and winter (December to January) months. While the average volume of ejaculates and the total sperm numbers per ejaculate did not differ between seasons, sperm concentration was lower in winter than in summer. Despite lower sperm concentration in the winter months, the proportion of spermatozoa defined as morphologically normal was higher in addition to the motility, progressive motility, and velocity of spermatozoa collected in the summer months (107). Further studies could use these initial results to develop predictors of sperm fertilization competence.

Lipidomic Techniques and Applications

The fatty acid composition of sperm cells has been a topic of investigation for several years. Previous research has elucidated groups or classes of fatty acids in spermatozoa from bulls, boars, roosters, stallions, and human males. However, quantifying the levels or amounts of these fatty acids has proven to be more difficult than qualifying the fatty acids and detecting their presence. This is a rather difficult task because of the number of cells may be limited and the calibration of the technologies used to identify and quantify the fatty acids could be set to higher threshold levels than what is present. Recent efforts have been made to design a streamlined method to fractionate then quantify the fatty acids in sperm cells via GC-MS methods (108). Lipidomics has also been utilized to identify lipid profile differences between healthy and diseased human patients. For example, blood plasma from patients with diseases, such as acute lung infections, pulmonary embolism, or acute exacerbation of the chronic pulmonary disease, had a more than 2-fold increase in various lipids compared to healthy patients (109). Lipidomics and liquid chromatography-mass spectrometry may be used to diagnose subclinical coronary artery disease (110) determined that patients with severe coronary calcification tended to have greater levels of monounsaturated triacylglycerols and saturated triacylglycerols. This led to the suggestion that calcification could be associated with cellular autophagy dysfunction.

Researchers have started to explore the possibility of using sperm as an indicator of health and risk of cancer in male subjects. For example, post-thaw semen quality of cancer patients is of lower quality as compared to samples before being frozen (111). Furthermore, men with testicular cancer have significantly lower sperm cell concentrations, but patients with other cancer types have been shown to have no differences in normal sperm (112). It has also been noted that diet affects the quality of fatty acids and stability of the sperm plasma membrane. In a study performed by Marchiani et al. (2015), rabbits were fed high-fat diets to determine if sperm quality changed due to metabolic status. The sperm cells from these rabbits showed marked decreases in motility measurement of both progressive and total motility, in addition to reduced normal morphology. The authors noted that hypertension could be a potential indicator of sperm quality in humans. These structures and their composition help determine the fertility of a given sire, but there are still many unknowns that need exploring.

A variety of microscopy tools are readily available to ascertain and evaluate the sperm membrane structure and integrity. Advances in electron microscopy has allowed for the development of a clearer, more accurate depiction of the landscape of the sperm cell. Using staining techniques in conjunction with microscopy, the composition of membrane regions has become more apparent (113). Scanning electron microscopy is commonly used to evaluate semen samples, such as in the study completed by Khalil et al. (6), which assessed the structural damage of cryopreservation by examining sperm cells for detached and cracked heads as well as damaged tails. The researchers also used transmission electron microcopy to assess the plasma membrane, acrosome, and nucleus by recording the appearance of swelling in the membrane, the typicalness of the acrosome, and the damage to the mitochondria and chromatin. In the study by Dobranić et al. (114), functional membrane integrity of canine spermatozoa was evaluated using hyper-osmotic swelling test (HOST). With HOST, sperm cells are incubated in a hypoosmotic solution such as fructose solution with Na-citrate to determine intactness of membranes in the sperm cells. Sperm with curled or more flaccid tail appearance indicate intact or damaged tails, respectively (115).

Lipidomics involve characterization of lipid content and their biological roles in each biological sample using analytical methods. Currently, there are two strategies for the lipid analyses: targeted and non-targeted lipid analysis. Targeted lipidomics is applied when researchers focus on known and specific lipids. Since the selected reaction monitoring (SRM) method is utilized in targeted lipidomics, it provides high sensitivity for quantitative lipid analyses 44, 80 [(116); 101]. Lipid classes that show unique fragmentation patterns and low abundant lipids are suitable for targeted lipid analyses. Non-targeted lipid analysis helps detect all lipids simultaneously in a single run. Although this method provides an overall profile of lipids that are detectable, it is not a sensitive analysis. Combining targeted and non-targeted lipid strategies may help to produce more powerful data.

Since lipidomic techniques are relatively new, several challenges exist. The use of gas chromatography-mass spectrometry (GC-MS) to elucidate lipid profiles has proven to be a promising avenue for determination of bull fertility, but this machinery and use of the technology are not widely available and it requires trained personnel to produce reliable data. One of the major limitations often encountered is the lack

of subjects or samples utilized for analysis. Having a greater number of cells to analyze could provide more comprehensive results or lead to the discovery of other compounds. In our recent study, a GC-MS method was used to evaluate the differences in Holstein bull sperm freezability and to compare the quality and quantity of fatty acids (108). When compared to similar studies, we noted that the calibration and detection techniques can vary which will yield different results, thereby making the use of GC-MS beneficial because you can collect a breadth of spectral data while also challenging due to the number of variables and settings that can alter specificity of the analysis.

With gas chromatography, modifications and adjustments can be made to the gas flow rate, column specifications, and temperature which can prove beneficial when quantifying lipids. Gas chromatography is an analytical tool that allows for the separation of compounds via vaporization. The carrier gas transports the injected liquid sample. Carrier gases are typically inert gases, such as helium. The sample is carried from the injector to the column that is located within the oven (117). Columns vary in length, ranging from a couple of meters to 100 meters, and type, such as polarized vs. non-polarized. Common detectors used with GC are MS or flame ionization (FID). Compounds assessed using GC should be compared against a standard for validation (118). Internal standards can be obtained for the various lipids, but not with the same ease of access. Approximately 80 analytical standards are commercially available for GP of the complex category, limiting the ability to perform absolute quantification (118). Sample preparation is another drawback of GC in that it typically requires using large sample volumes in addition to samples requiring derivatization (119).

Much like GC, a standard is needed for absolute quantification, and for simple lipids these are available commercially. Mass spectrometry (MS) is another analytical technique. By using the masses of atoms and molecules, the identities of the various components that make up a sample is revealed. The data gathered can also be used to quantify the components of the sample as well. The MS works by converting molecules to ions, sorting the ions based on their mass and charge, and then, detection. The electron ionizer is an electron beam that molecules pass through that strips the electrons, thus, producing a positive ion that travels to the mass analyzer component, which is an electric field that accelerates the ions into a magnetic field where they are then deflected based on the mass of the ions. Lastly, the ions impact a charged plate that generates a signal that can be used for analysis (120). The MS is useful in quantifying a substance when it is known and determining the composition of an unknown sample, in addition to, allowing researchers to conclude the structure and properties of various molecules (118). The MS determines the abundance of ions according to their mass to charge ration or m/z (119). When compared to nuclear magnetic resonance (NMR), MS often offers heightened sensitivity and selectivity between various lipids (121).

Recent advances in analytical technologies, such as MS, NMR, and high-performance liquid chromatography (HPLC), have helped researchers to improve lipidomics (121). Among these technologies, MS-based methods are commonly used in lipid analyses due to the higher sensitivity, throughput, and specificity (122). In addition, a great number of ionization technologies, such as electron ionization (EI), Matrix-Assisted Laser Desorption Ionization (MALDI), Electrospray Ionization (ESI), and Fast Atom Bombardment (FAB) in MS, have been developed as well. Each of these ionization methods can be used for the analyses of different lipid groups, such as FAB commonly being applied to identify fatty acids, monoacylglycerols, and glycerophospholipids (123, 124).

Although the NMR is not as sensitive as MS, NMR is the only method of analysis that allows for lipid analysis of cells and tissues when they are intact (119). Nuclear magnetic resonance spectroscopy is composed of a coiled wire surrounded by a magnet. One of the coils generates electromagnetic radiation at a constant frequency, whereas the relative strength of the magnetic field increases. The growing magnetic field strength splits the nuclei in the samples until the nuclei reach a point of resonance, after which, the nuclei fall back to a lower energy level remitting a radiation signal that the second coil records. The signals recorded by the various nuclei in the sample are then analyzed and processed producing the NMR spectrum (125). Typically, 1H and 31P NMR spectroscopy are used for analysis due to their sensitivity. Proton NMR is commonly used to investigate diseases, poisons, and disorders that induce changes in the lipid composition; 31P NMR is commonly used to quantify GPs. In the past, one-dimensional NMR has been the most prevalent tool, however, two-dimensional NMR is becoming a useful tool. The rise in popularity for two-dimensional NMR is centered around the ability of better resolution (118).

The thiobarbituric acid (TBARS) assay is used to assess changes in Malonaldehyde (MDA), a reactive compound formed when lipids undergo oxidation (126). In conjunction with Thiobarbituric acid (TBA), MDA reacts to form the MDA-TBA adduct and can be measured colorimetrically or fluorometrically to determine the levels of lipid peroxidation in each sample (126). The TBARS assay needs to be carried out under high temperatures and in an acidic environment. To run this assay, semen samples are thawed and diluted in PBS (127). Then, 100 μL of spermatozoa are mixed with 200 μL of 5% trichloroacetic acid, 0.375% TBA and 0.25 N HCl reagent. The mixture is then heated to 90°C for 15 min to stimulate the reaction. Following the incubation period, samples are transferred to an ice-water bath for 5 min. After cooling, the samples are centrifuged at 1,500 × g for 15 min. The supernatant is then collected and transferred into a well-plate so the absorbance can be measured by a microplate reader to calculate MDA concentration. This method has the benefit of being well-recognized and can utilize a variety of sample types such as tissue homogenates, urine samples, cell lysates, serum, and plasma. However, it is necessary to standardize TBARS by using multiple fatty acid concentrations rather than selecting an arbitrary fatty acid to use as a standard or reference (128). This method lacks specificity, but it can help determine the amount of lipid peroxidation present if the sample is uncomplicated (127, 128).

The BODIPY C_{11} probe colorimetric assay measures lipid peroxidation of cell membranes via flow cytometry. BODIPY (581/591) C_{11} easily incorporates into sperm cells and undergoes

a spectral emission shift when attacked by ROS that can be measured to determine change (129). To conduct this assay, semen samples are collected, and then, allowed to sit for 30 min to liquify from its gel-like stage post-ejaculation (129). Following the waiting period, sperm cells are separated via a Percoll gradient, and then, the BODIPY (581/591) C_{11} probe is added to 5μM of cells for 30 min. Sperm cells are washed twice by centrifuging at 650 g for 5 min. An 80 μM ferrous sulphate promoter is incubated for 15 min. The sample is then evaluated using a flow cytometer. The BODIPY probe colorimetric assay has been demonstrated to have good repeatability and sensitivity when evaluating deer sperm (127).

The TBA-TCA Reagent Colorimetric Method Assay is used to measure lipid peroxidation by determining MDA levels through the TBA assay, which produces a red absorbance. This assay is run by thawing and centrifuging sperm cells in Tris buffer (130). The sperm pellet is then resuspended in PBS. A 2 mL of TBA-TCA reagent is added to 1 mL of sperm cell suspension and incubated in boiling water for 40 min. The sample is cooled and centrifuged at 500 × g for 10 min. The supernatant is aspirated, and absorbance is read at 535 nm under a UV spectrophotometer. Final MDA levels are determined by the absorbance coefficient of $1.56 \times 105/mol/cm^3$. The TBA can react with a wide assortment of oxidized lipids, both saturated and unsaturated varieties, but it does lack sensitivity and specificity (131). To combat these weaknesses, researchers have incorporated high-performance liquid chromatography to increase specificity and sensitivity of the assay (132).

The 4-Hydroxynonenal (HNE)-His Adduct ELISA/HNE Adduct Competitive ELISA is an immunoassay that helps detect HNE-His protein adducts, which are formed when 4-HNE reacts with lysine, histidine, or cysteine residues in sperm cells (133). This assay is run with a 96-well titer ELISA plate where sperm cell samples and bovine serum albumin (BSA) standards are added to wells (134). The HNE-protein adducts present in the samples are probed with an anti-HNE-His antibody, followed by an HRP secondary antibody. Using a microplate reader, the absorbance of each well is read at 450 nm to quantify the HNE-protein adducts. This method has proven to be accurate and repeatable; however, care must be taken when selecting antibodies for the sample specimen (133, 135).

In the *Glutathione peroxidase test*, glutathione peroxidase (GSH) reacts with hydrogen peroxide to form glutathione disulfide (GSSG). Adding glutathione reductase and NADPH reduces GSSG to GSH and results in consumption of NADPH, which is related to the peroxide content of the sample (136). Sperm cell samples are centrifuged at 12,000 g for 5 min (137). Fifty μL of sperm cells are added to a 930 μL solution of EDTA 1 mM, sodium azide, and potassium phosphate buffer (137). Then, a 10 μL secondary solution, composed of 0.02 g of 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, is placed into the cuvette of the spectrophotometer with the aliquots of the first solution. Finally, 20 μL of 500 IU/mL of GSH-S transferase in phosphate buffer is added to initiate reaction (137). The absorbance is monitored at 340 nm until it reached the plateau. Calculations are then performed using the volume of the sample, light path length, corresponding dilution factors, absorbance decrease, and molar extinction coefficient. This test has been applied to human seminal plasma samples to quantify the presence of glutathione peroxidase (138). It was found that glutathione peroxidase activity was significantly lower in those samples with oligozoospermia, asthenozoospermia, or teratozoospermia conditions in which Crisol et al. (138) speculate is related to overall sperm quality. When utilizing this test, it is vital to consider other avenues of assessment because this test only evaluates one fraction of the antioxidant system that is in place to protect the spermatozoa.

CONCLUSIONS

The knowledge base of lipids and their composition in livestock sperm and the difficulty of data accuracy and interpretation of results have been documented. There is a need for more detailed lipidomics studies utilizing sperm from livestock with distinct phenotypes of economically important traits such as sperm freezability and male fertility. Growing interests and platforms with various techniques such as GC-MS, MS-MS, and LC-MS enable researchers to profile comprehensive metabolomic signatures of diverse tissues in livestock, including sperm. This is important because lipids play critical roles in molecular morphology and function in the cells. Combined with other methods in cell and molecular biology, such as bioinformatics, lipidomics can be applied to harness the power of integrated studies to decipher sperm markers for freezability and male fertility. Potential markers uncovered through discovery research can be further studied through mechanistic experiments to determine the molecular and cellular underpinnings of male fertility. However, there is a need for more comprehensive studies involving different stages of animal development, nutrition, environment, and season using single cell analyses. Because of the significant similarities between livestock and other organisms, including human and endangered species, results generated using various livestock models can be applied to advance basic and applied reproduction of other mammals.

AUTHOR CONTRIBUTIONS

HE, TD, MLH, AG, MU, MH, FJ, MN, and EM assisted in the conception of the study and contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2017-67016-26507 from the USDA National Institute of Food and Agriculture. Partial funding was provided by Mississippi Agricultural Forestry Experiment Station. MU was funded through a competitive graduate fellowship from the Turkish Ministry of National Education. MH was funded through a competitive international postdoctoral fellowship by the Scientific and Technological Research Council of Turkey (TUBITAK-1059B191800016).

REFERENCES

1. Aziz N, Said T, Paasch U, Agarwal A. The relationship between human sperm apoptosis, morphology and the sperm deformity index. *Hum Reprod.* (2007) 22:1413–9. doi: 10.1093/humrep/dem016
2. Zorn B, Golob B, Ihan A, Kopitar A, Kolbezen M. Apoptotic sperm biomarkers and their correlation with conventional sperm parameters and male fertility potential. *Off Publ ALPHA Sci Reprod Med.* (2012) 29:357–64. doi: 10.1007/s10815-012-9718-x
3. Lederman L. Cryopreservation. *Biotechniques*. (2008) 45:619–21. doi: 10.2144/000112986
4. Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, et al. Advances in cryopreservation of bull sperm. *Front Vet Sci.* (2019) 6:268. doi: 10.3389/fvets.2019.00268
5. Bailey JL, Bilodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl.* (2000) 21:1–7. doi: 10.1002/j.1939-4640.2000.tb03268.x
6. Khalil WA, El-Harairy MA, Zeidan AEB, Hassan MAE, Mohey-Elsaeed O. Evaluation of bull spermatozoa during and after cryopreservation: structural and ultrastructural insights. *Int J Vet Sci Med.* (2018) 6(Suppl):S49–56. doi: 10.1016/j.ijvsm.2017.11.001
7. Staub C, Johnson L. Review: Spermatogenesis in the bull. *Animal*. (2018) 12:S27–35. doi: 10.1017/S1751731118000435
8. Senger PL. *Pathways to Pregnancy and Parturition.* Redmond, OR: Current Conceptions (2012). Available online at: https://search.ebscohost.com/login.aspx?direct=trueanddb=cat00043aandAN=mstate.2640080andauthtype=ssoandcustid=magn1307andsite=eds-liveandscope=siteandcustid=magn1307.
9. Gervasi MG, Visconti PE. Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology*. (2017) 5:204–18. doi: 10.1111/andr.12320
10. Tecle E, Gagneux P. Sugar-coated sperm: unraveling the functions of the mammalian sperm glycocalyx. *Mol Reprod Dev*. (2015) 82:635–50. doi: 10.1002/mrd.22500
11. Villaverde-Morcillo S, Esteso MC, Castaño C, Toledano Díaz A, López-Sebastián A, Campo JL, et al. Influence of staining method on the values of avian sperm head morphometric variables. *Reprod Domest Anim*. (2015) 50:750–5. doi: 10.1111/rda.12574
12. Boe-Hansen GB, Fortes MRS, Satake N. Morphological defects, sperm DNA integrity, and protamination of bovine spermatozoa. *Andrology*. (2018) 6:627–33. doi: 10.1111/andr.12486
13. Primakoff P, Myles DG. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science.*(2002) 296:2183–5. doi: 10.1126/science.1072029
14. Anifandis G, Messini C, Dafopoulos K, Sotiriou S, Messinis I. Molecular and cellular mechanisms of sperm-oocyte interactions opinions relative to in vitro fertilization (IVF). *Int J Mol Sci.* (2014) 15:12972–97. doi: 10.3390/ijms150712972
15. Cohen N, Wassarman PM. Association of egg zona pellucida glycoprotein mZP3 with sperm protein sp56 during fertilization in mice. *Int J Dev Biol.* (2001) 45:569–76.
16. Bi M, Hickox JR, Winfrey VP, Olson GE, Hardy DM. Processing, localization and binding activity of zonadhesin suggest a function in sperm adhesion to the zona pellucida during exocytosis of the acrosome. *Biochem J.* (2003) 375:477–88. doi: 10.1042/bj20030753
17. van Gestel RA, Brewis IA, Ashton PR, Brouwers JF, Gadella BM. Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte. *Mol Hum Reprod.* (2007) 13:445–54. doi: 10.1093/molehr/gam030
18. Purves W, Sadava D, Orians G, Heller C. *Life: The Science of Biology*. 7th ed. Sinauer Associates and W. H. Freeman (2003).
19. Losano JDA, Fernando Padin J, Mendez-Lopez I, Angrimani DSR, Garcia AG, Barnabe VH, et al. The stimulated glycolytic pathway is able to maintain atp levels and kinetic patterns of bovine epididymal sperm subjected to mitochondrial uncoupling. *Oxid Med Cell Longev.* (2017) 2017:1682393. doi: 10.1155/2017/1682393
20. Mannella CA. Structure and dynamics of the mitochondrial inner membrane cristae. *Mol Cell Res.* (2006) 1763:542–8. doi: 10.1016/j.bbamcr.2006.04.006
21. Fahy E, Cotter D, Sud M, Subramaniam S. Lipid classification, structures, and tools. *Biochim Biophys Acta*. (2011) 1811:637–47. doi: 10.1016/j.bbalip.2011.06.009
22. Yamada T, Taguchi K, Bamba T. Separation of Lipids. In: *Supercritical Fluid Chromatography*, ed. C F B T-S F C Poole. St. Louis, MO:Elsevier (2017). p. 419–38. doi: 10.1016/B978-0-12-809207-1.00014-8
23. Christie WW, Han X. *Lipid Analysis: Isolation, Separation, Identification and Lipidomic Analysis.* 4th ed. (2012).
24. Munnik T, Testerink C. Plant phospholipid signaling: "in a nutshell." *J Lipid Res.* (2008) 50:S260–5. doi: 10.1194/jlr.R800098-JLR200
25. Nozawa Y. Phospholipid signaling and cell function. In: Lajtha A, Mikoshiba K, editors. *Handbook of Neurochemistry and Molecular Neurobiology*. 3rd ed. Springer (2009). p. 297–311. doi: 10.1007/978-0-387-30370-3_15
26. Van Meer G, Voelker DR, Feigenson GW. Membrane lipids: Where they are and how they behave. *Nat Rev Mol Cell Biol.* (2008) 9:112–24. doi: 10.1038/nrm2330
27. Cross NL. Role of cholesterol in sperm capacitation1. *Biol Reprod.* (1998) 59:7–11. doi: 10.1095/biolreprod59.1.7
28. Sheriff DS, Ali EF. Perspective on plasma membrane cholesterol efflux and spermatozoal function. *J Hum Reprod Sci.* (2010) 3:68–75. doi: 10.4103/0974-1208.69337
29. Fan Y, Liu Y, Xue K, Gu G, Fan W, Xu Y, et al. Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS ONE*. (2015) 10:e0120775. doi: 10.1371/journal.pone.0120775
30. Han X. *Lipidomics: Comprehensive Mass Spectrometry of Lipids*. Hoboken, NJ:John Wiley and Sons, Ltd (2016). doi: 10.1002/9781119085263
31. Miniewska K, Godzien J, Mojsak P, Maliszewska K, Kretowski A, Ciborowski M. Mass spectrometry-based determination of lipids and small molecules composing adipose tissue with a focus on brown adipose tissue. *J Pharm Biomed Anal.* (2020) 191:113623. doi: 10.1016/j.jpba.2020.113623
32. Aksoy Y, Aksoy H, Altinkaynak K, Aydin HR, Özkan A. Sperm fatty acid composition in subfertile men. *Prostagl Leukot Essent Fat Acids*. (2006) 75:75–9. doi: 10.1016/j.plefa.2006.06.002
33. Dietz RW, Pickett BW, Komarek RJ, Jensen RG. Fatty acid composition of bovine semen. *J Dairy Sci.* (1963) 46:468–72. doi: 10.3168/jds.S0022-0302(63)89075-X
34. Komarek RJ, Pickett BW, Lanz RN, Jensen RG. Lipid Composition of Bovine Spermatozoa and Seminal Plasma. *J Dairy Sci.* (1964) 47:531–4. doi: 10.3168/jds.S0022-0302(64)88704-X
35. Sèdes L, Thirouard L, Maqdasy S, Garcia M, Caira F, Lobaccaro JMA, et al. Cholesterol: A gatekeeper of male fertility? *Front Endocrinol.* (2018) 9:369. doi: 10.3389/fendo.2018.00369
36. Leahy T, Gadella BM. Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction*. (2011) 142:759–78. doi: 10.1530/REP-11-0310
37. Darin-Bennett A, White IG. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology*. (1977) 14:466–470. doi: 10.1016/0011-2240(77)90008-6
38. Poulos A, Darin-Bennett A, White IG. The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. *Comp Biochem Physiol Part B Comp Biochem.* (1973) 46:541–9. doi: 10.1016/0305-0491(73)90094-1
39. Jain YC, Anand SR. Fatty acids and fatty aldehydes of buffalo seminal plasma and sperm lipid. *J Reprod Fertil.* (1976) 47:261–7. doi: 10.1530/jrf.0.0470261
40. Gadella BM. Sperm membrane physiology and relevance for fertilization. *Anim Reprod Sci.* (2008) 107:229–36. doi: 10.1016/j.anireprosci.2008.05.006
41. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* (1959) 37:911–7. doi: 10.1139/o59-099
42. Müller K, Müller P, Pincemy G, Kurz A, Labbe C. Characterization of sperm plasma membrane properties after cholesterol modification: consequences for cryopreservation of rainbow trout spermatozoa1. *Biol Reprod.* (2008) 78:390–9. doi: 10.1095/biolreprod.107.064253
43. Brouwers JF, Boerke A, Silva PFN, Garcia-Gil N, van Gestel RA, Helms JB, et al. Mass spectrometric detection of cholesterol oxidation in bovine sperm. *Biol Reprod.* (2011) 85:128–36. doi: 10.1095/biolreprod.111.091207

44. Purdy PH, Graham JK. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility1. *Biol Reprod.* (2004) 71:522–7. doi: 10.1095/biolreprod.103.025577
45. Saez Lancellotti TE, Boarelli PV, Monclus MA, Cabrillana ME, Clementi MA, Espínola LS, et al. Hypercholesterolemia Impaired Sperm Functionality in Rabbits. *PLoS ONE.* (2010) 5:e13457. doi: 10.1371/journal.pone.0013457
46. Majzoub A, Agarwal A. Systematic review of antioxidant types and doses in male infertility: Benefits on semen parameters, advanced sperm function, assisted reproduction and live-birth rate. *Arab J Urol.* (2018) 16:113–24. doi: 10.1016/j.aju.2017.11.013
47. Hu JH, Zhao XL, Tian WQ, Zan LS, Li QW. Effects of vitamin E supplementation in the extender on frozen-thawed bovine semen preservation. *Animal.* (2011) 5:107–12. doi: 10.1017/S1751731110001679
48. Palace VP, Khaper N, Qin Q, Singal PK. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. *Free Radic Biol Med.* (1999) 26:746–61. doi: 10.1016/S0891-5849(98)00266-4
49. Zervos IA, Tsantarliotou MP, Vatzias G, Goulas P, Kokolis NA, Taitzoglou IA. Effects of dietary vitamin A intake on acrosin- and plasminogen-activator activity of ram spermatozoa. *Reproduction*. (2005) 129:707–15. doi: 10.1530/rep.1.00182
50. Jueraitetibaike K, Ding Z, Wang DD, Peng LP, Jing J, Chen L, et al. The effect of Vitamin D on sperm motility and the underlying mechanism. *Asian J Androl.* (2019) 21:400–7. doi: 10.4103/aja.aja_105_18
51. Vervoort LMT, Ronden JE, Thijssen HHW. The potent antioxidant activity of the vitamin K cycle in microsomal lipid peroxidation. *Biochem Pharmacol.* (1997) 54:871–6. doi: 10.1016/S0006-2952(97)00254-2
52. Simes DC, Viegas CSB, Araújo N, Marreiros C. Vitamin K as a diet supplement with impact in human health: current evidence in age-related diseases. *Nutrients*. (2020) 12:138. doi: 10.3390/nu12010138
53. Martínez-Cayuela M. Oxygen free radicals and human disease. *Biochimie*. (1995) 77:147–61. doi: 10.1016/0300-9084(96)88119-3
54. Hitit M, Ugur MR, Dinh T, Sajeev D, Kaya A, Topper E, et al. Cellular and functional physiopathology of bull sperm with altered sperm freezability. *Front Vet Sci.* (2020) 7:581137. doi: 10.3389/fvets.2020.581137
55. Ray PD, Huang B-W, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal.* (2012) 24:981–90. doi: 10.1016/j.cellsig.2012.01.008
56. Martínez MC, Andriantsitohaina R. Reactive nitrogen species: molecular mechanisms and potential significance in health and disease. *Antioxid Redox Signal.* (2009) 11:669–702. doi: 10.1089/ars.2007.1993
57. Bucak MN, Sariözkan S, Tuncer PB, Sakin F, Ateşşahin A, Kulaksiz R, et al. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. *Small Rumin Res.* (2010) 89:24–30. doi: 10.1016/j.smallrumres.2009.11.015
58. Barranco I, Tvarijonaviciute A, Perez-Patinõ C, Parrilla I, Ceron JJ, Martinez EA, et al. High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Sci Rep.* (2015) 5:18583. doi: 10.1038/srep18538
59. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. *World J Mens Health.* (2014) 12:138. doi: 10.5534/wjmh.2014.32.1.1
60. Aitken RJ. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev.* (1995) 7:659–68. doi: 10.1071/RD9950659
61. Makker K, Agarwal A, Sharma R. Oxidative stress and male infertility. *Indian J Med Res.* (2009) 129:357–67.
62. Kothari S, Thompson A, Agarwal A, du Plessis SS. Free radicals: Their beneficial and detrimental effects on sperm function. *Indian J. Exp. Biol.* (2010) 48:425–35.
63. Aitken RJ, Sawyer D. The human spermatozoon - not waving but drowning. *Adv Exp Med Biol.* (2003) 518:85–98. doi: 10.1007/978-1-4419-9190-4_8
64. Bonanno O, Romeo G, Asero P, Pezzino FM, Castiglione R, Burrello N, et al. Sperm of patients with severe asthenozoospermia show biochemical, molecular and genomic alterations. *Reproduction*. (2016). doi: 10.1530/REP-16-0342
65. Opuwari CS, Henkel RR. An update on oxidative damage to spermatozoa and oocytes. *Biomed Res Int.* (2016) 2016:9540142. doi: 10.1155/2016/9540142
66. Dutta S, Majzoub A, Agarwal A. Oxidative stress and sperm function: a systematic review on evaluation and management. *Arab J Urol.* (2019) 17:87–97. doi: 10.1080/2090598X.2019.1599624
67. Alvarez JG, Storey BT. Spontaneous lipid peroxidation in rabbit epididymal spermatozoa: its effect on sperm motility. *Biol Reprod.* (1982) 27:1102–8. doi: 10.1095/biolreprod27.5.1102
68. Baker MA, Krutskikh A, Aitken RJ. Biochemical entities involved in reactive oxygen species generation by human spermatozoa. *Protoplasma*. (2003) 221:145–51. doi: 10.1007/s00709-002-0057-0
69. Aitken RJ. Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Mol Reprod Dev.* (2017) 84:1039–52. doi: 10.1002/mrd.22871
70. John Aitken R, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod.* (1989) 41:183–97. doi: 10.1095/biolreprod41.1.183
71. Mann T, Jones R, Sherins R. Oxygen damage, lipid peroxidation and motility of spermatozoa. In: *Testicular Development, Structure and Function*. New York, NY: Raven Press (1980). p. 497–501.
72. Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. *Theriogenology*. (1992) 38:209–22. doi: 10.1016/0093-691X(92)90231-F
73. Garcia-Oliveros LN, de Arruda RP, Batissaco L, Gonzaga VHG, Nogueira VJM, Florez-Rodriguez SA, et al. Heat stress effects on bovine sperm cells: a chronological approach to early findings. *Int J Biometeorol.* (2020) 64:1367–78. doi: 10.1007/s00484-020-01917-w
74. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem.* (2012) 287:P33048–60. doi: 10.1074/jbc.M112.366690
75. Rao B, Soufir JC, Martin M, David G. Lipid peroxidation in human spermatozoa as relatd to midpiece abnormalities and motility. *Gamete Res.* (1989) 24:127–34. doi: 10.1002/mrd.1120240202
76. Lone SA, Prasad JK, Ghosh SK, Das GK, Balamurugan B, Verma MR. Study on correlation of sperm quality parameters with antioxidant and oxidant status of buffalo bull semen during various stages of cryopreservation. *Andrologia*. (2018) 9:648684. doi: 10.1111/and.12970
77. Singh RK, Kumaresan A, Chhillar S, Rajak SK, Tripathi UK, Nayak S, et al. Identification of suitable combinations of *in vitro* sperm-function test for the prediction of fertility in buffalo bull. *Theriogenology*. (2016) 86:2263–2271. doi: 10.1016/j.theriogenology.2016.07.022
78. Kant R, Atreja SK, Hasan SS. Evaluation of lipid changes in buffalo (*Bubalus bubalis*) spermatozoa during *in-vitro* capacitation and acrosome reaction. *Indian J Anim Res.* (2017) 51:40–3. doi: 10.18805/ijar.7085
79. Chatterjee S, De Lamirande E, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. *Mol Reprod Dev.* (2001) 60:498–506. doi: 10.1002/mrd.1115
80. Pini T, Leahy T, de Graaf SP. Sublethal sperm freezing damage: manifestations and solutions. *Theriogenology.* (2018) 118:172–81. doi: 10.1016/j.theriogenology.2018.06.006
81. Pons-Rejraji H, Bailey JL, Leclerc P. Cryopreservation affects bovine sperm intracellular parameters associated with capacitation and acrosome exocytosis. *Reprod Fertil Dev.* (2009) 21:525–37. doi: 10.1071/RD07170
82. Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions. *Vet Med Int.* (2011) 2011:686137. doi: 10.4061/2011/686137
83. Lone SA, Prasad JK, Ghosh SK, Das GK, Kumar N, Balamurugan B, et al. Effect of cholesterol loaded cyclodextrin (CLC) on lipid peroxidation and reactive oxygen species levels during cryopreservation of buffalo (*Bubalus bubalis*) spermatozoa. *Asian Pacific J Reprod.* (2016) 5:445–536. doi: 10.1016/j.apjr.2016.10.003
84. Beconi MT, Francia CR, Mora NG, Affranchino MA. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology.* (1993) 40:841–51. doi: 10.1016/0093-691X(93)90219-U
85. Brouwers JFHM, Gadella BM. *In situ* detection and localization of lipid peroxidation in individual bovine sperm cells. *Free Radic BiolMed.* (2003) 35:1382–91. doi: 10.1016/j.freeradbiomed.2003.08.010

86. Dos Santos Hamilton TR, De Castro LS, De Carvalho Delgado J, De Assis PM, Siqueira AFP, Mendes CM, et al. Induced lipid peroxidation in ram sperm: semen profile, DNA fragmentation and antioxidant status. *Reproduction*. (2016) 151:379–90. doi: 10.1530/REP-15-0403
87. Kasimanickam R, Kasimanickam V, Thatcher CD, Nebel RL, Cassell BG. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology*. (2007) 67:1004–12. doi: 10.1016/j.theriogenology.2006.11.013
88. Ahmed S, Khan MI, Ur R, Ahmad M, Iqbal S. Effect of age on lipid peroxidation of fresh and frozen-thawed semen of Nili-Ravi buffalo bulls. *Ital J Anim Sci.* (2018) 17:730–5. doi: 10.1080/1828051X.2018.1424569
89. Simões R, Feitosa WB, Siqueira AFP, Nichi M, Paula-Lopes FF, Marques MG, et al. Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo *in vitro* development outcome. *Reproduction*. (2013) 146:433–41. doi: 10.1530/REP-13-0123
90. Poulos A, Voglmayr JK, White IG. Phospholipid changes in spermatozoa during passage through the genital tract of the bull. *Biochim Biophys Acta*. (1973) 306:194–202. doi: 10.1016/0005-2760(73)90225-7
91. Parks E, Arion W, Foote RH. Lipids of plasma membrane. *Biol Reprod*. (1987) 1249–58. doi: 10.1095/biolreprod37.5.1249
92. Amaral A, Castillo J, Maria Estanyol J, Luis Ballesca J, Ramalho-Santos J, Oliva R. Human sperm tail proteome suggests new endogenous metabolic pathways. *Mol Cell Proteomics*. (2013) 12:330–342. doi: 10.1074/mcp.M112.020552
93. Gholami H, Chamani M, Towhidi A, Fazeli MH. Improvement of semen quality in holstein bulls during heat stress by dietary supplementation of omega-3 fatty acids. *Int J Fertil Steril.* (2011) 4:160–7.
94. Martínez-Soto JC, Landeras J, Gadea J. Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success. *Andrology*. (2013) 1:365–75. doi: 10.1111/j.2047-2927.2012.00040.x
95. Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, Noble R. Changes in sperm quality and lipid composition during cryopreservation of boar semen. *Theriogenol.* (2005) 411–21. doi: 10.1016/j.theriogenology.2004.09.021
96. Peddinti D, Nanduri B, Kaya A, Feugang JM, Burgess SC, Memili E. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. *BMC Syst Biol.* (2008) 2:19. doi: 10.1186/1752-0509-2-19
97. Furland NE, Oresti GM, Antollini SS, Venturino A, Maldonado EN, Aveldaño MI. Very long-chain polyunsaturated fatty acids are the major acyl groups of sphingomyelins and ceramides in the head of mammalian spermatozoa. *J Biol Chem*. (2007) 282:18151–61. doi: 10.1074/jbc.M700709200
98. Giraud MN, Motta C, Boucher D, Grizard G. Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. *Hum Reprod.* (2000) 15:2160–4. doi: 10.1093/humrep/15.10.2160
99. Barthelemy C, Royere D, Hammahah S, Lebos C, Tharanne MJ, Lansac J. Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. *Arch Androl.* (1990) 25:29–40. doi: 10.3109/01485019008987591
100. Morris GJ, Faszer K, Green JE, Draper D, Grout BWW, Fonseca F. Rapidly cooled horse spermatozoa: Loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. *Theriogenology*. (2007) 68:804–12. doi: 10.1016/j.theriogenology.2007.06.009
101. Sieme H, Oldenhof H, Wolkers WF. Sperm membrane behaviour during cooling and cryopreservation. *Reprod Domest Anim.* (2015) 50:20–6. doi: 10.1111/rda.12594
102. Menkveld R, El-Garem Y, Schill W-B, Henkel R. Relationship between human sperm morphology and acrosomal function. *J Assist Reprod Genet.* (2003) 20:432–8. doi: 10.1023/A:1026288710638
103. Wood PL, Scoggin K, Ball BA, Troedsson MH, Squires EL. Lipidomics of equine sperm and seminal plasma: Identification of amphiphilic (O-acyl)-ω-hydroxy-fatty acids. *Theriogenology*. (2016) 86:1212–21. doi: 10.1016/j.theriogenology.2016.04.012
104. Ramos Angrimani DS, Nichi M, Losano JDA, Lucio CF, Lima Veiga GA, Franco MVMJ, et al. Fatty acid content in epididymal fluid and spermatozoa during sperm maturation in dogs. *J Anim Sci Biotechnol.* (2017) 8:18. doi: 10.1186/s40104-017-0148-6
105. Liu Q, Zhou Y-F, Duan R-J, Wei H-K, Peng J, Jiang S-W. Dietary n-6:n-3 ratio and Vitamin E improve motility characteristics in association with membrane properties of boar spermatozoa. *Asian J Androl.* (2017) 19:223–9. doi: 10.4103/1008-682X.170446
106. Mendeluk GR, Cohen MI, Ferreri C, Chatgilialoglu C. Nutrition and reproductive health: sperm versus erythrocyte lipidomic profile and ω-3 intake. *J Nutr Metab VO.* (2015) 2015:670526. doi: 10.1155/2015/670526
107. Argov-Argaman N, Mahgrefthe K, Zeron Y, Roth Z. Variation in lipid profiles within semen compartments-the bovine model of aging. *Theriogenology*. (2013) 80:712–21. doi: 10.1016/j.theriogenology.2013.05.024
108. Evans HC, Dinh TTN, Ugur MR, Hitit M, Sajeev D, Kaya A, et al. Lipidomic markers of sperm cryotolerance in cattle. *Sci Rep*. (2020) 10:20192. doi: 10.1038/s41598-020-77089-9
109. Gao D, Zhang L, Song D, Lv J, Wang L, Zhou S, et al. Values of integration between lipidomics and clinical phenomes in patients with acute lung infection, pulmonary embolism, or acute exacerbation of chronic pulmonary diseases: a preliminary study. *J Transl Med.* (2019) 17:162. doi: 10.1186/s12967-019-1898-z
110. Djekic D, Pinto R, Repsilber D, Hyotylainen T, Henein M. Serum untargeted lipidomic profiling reveals dysfunction of phospholipid metabolism in subclinical coronary artery disease. *Vasc Health Risk Manag.* (2019) 15:123–35. doi: 10.2147/VHRM.S202344
111. Dearing CG, Jayasena CN, Lindsay KS. Human sperm cryopreservation in cancer patients: links with deprivation and mortality. *Cryobiology*. (2017) 79:9–13. doi: 10.1016/j.cryobiol.2017.10.003
112. Bonetti TC, Pasqualotto FF, Queiroz P, Iaconelli A Jr, Borges E Jr. Sperm banking for male cancer patients: social and semen profiles. *Int Braz J Urol.* (2009) 35:190–7; discussion 197–8. doi: 10.1590/s1677-55382009000200009
113. Bearer EL, Friend DS. Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *J Electron Microsc Tech.* (1990) 16:281–97. doi: 10.1002/jemt.1060160403
114. Dobranić T, Cergolj M, Samardžija M. Determination of membrane integrity of canine spermatozoa. *Veterinarski Arhiv.* (2005) 75.
115. Jeyendran RS, Van der Ven HH, Zaneveld LJ. The hypoosmotic swelling test: an update. *Arch Androl.* (1992) 29:105–16. doi: 10.3109/01485019208987714
116. Tsugawa H, Arita M, Kanazawa M, Ogiwara A, Bamba T, Fukusaki E. MRMPROBS: a data assessment and metabolite identification tool for large-scale multiple reaction monitoring based widely targeted metabolomics. *Anal Chem*. (2013) 85:5191–9. doi: 10.1021/ac400515s
117. Zuo HL, Yang FQ, Huang WH, Xia ZN. Preparative gas chromatography and its applications. *J Chromatogr Sci.* (2013) 51:704–15. doi: 10.1093/chromsci/bmt040
118. Khoury S, Canlet C, Lacroix M, Berdeaux O, Jouhet J, Bertrand-Michel J. Quantification of lipids: model, reality, and compromise. *Biomolecules*. (2018) 8:174. doi: 10.3390/biom8040174
119. Barbosa LFSP, Oliveira WVC, Pereira MHC, Moreira MB, Vasconcelos CGC, Silper BF, et al. Somatic cell count and type of intramammary infection impacts fertility from *in vitro* produced embryo transfer. *Theriogenology*. (2018) 108:291–6. doi: 10.1016/j.theriogenology.2017.12.025
120. Urban PL. Quantitative mass spectrometry: an overview. *Philos Trans R Soc A Math Phys Eng Sci.* (2016) 374:20150382. doi: 10.1098/rsta.2015.0382
121. Lee HC, Yokomizo T. Applications of mass spectrometry-based targeted and non-targeted lipidomics. *Biochem Biophys Res Commun.* (2018) 504:576–81. doi: 10.1016/j.bbrc.2018.03.081
122. Li L, Han J, Wang Z, Liu J, Wei J, Xiong S, et al. Mass spectrometry methodology in lipid analysis. *Int J Mol Sci.* (2014) 15:10492–507. doi: 10.3390/ijms150610492
123. Korachi M, Blinkhorn AS, Drucker DB. Analysis of phospholipid molecular species distributions by fast atom bombardment mass spectrometry (FAB-MS). *Eur J Lipid Sci Technol.* (2002) 104:50–6. doi: 10.1002/1438-9312(200201)104:1<50::AID-EJLT50>3.0.CO;2-Z

124. Ji H, Voinov VG, Deinzer ML, Barofsky DF. Distinguishing between cis/trans isomers of monounsaturated fatty acids by FAB MS. *Anal Chem.* (2007) 79:1519–22. doi: 10.1021/ac061155d
125. Zia K, Siddiqui T, Ali S, Farooq I, Zafar MS, Khurshid Z. Nuclear magnetic resonance spectroscopy for medical and dental applications: a comprehensive review. *Eur J Dent.* (2019) 13:124–8. doi: 10.1055/s-0039-1688654
126. Agarwal A, Sharma RK, Gupta S, Harlev A, Ahmad G, Plessis SS, et al. Oxidative stress in human reproduction shedding light on a complicated phenomenon. *Springer.* (2017) 17–46. doi: 10.1007/978-3-319-48427-3
127. Domínguez-Rebolledo Á, Martínez-Pastor F, Fernández-Santos MR, Del Olmo E, Bisbal A, Ros-Santaella JL, et al. Comparison of the TBARS assay and BODIPY C11 probes for assessing lipid peroxidation in red deer spermatozoa. *Reprod Domest Anim.* (2010) 45:360–8. doi: 10.1111/j.1439-0531.2009.01578.x
128. Rael LT, Thomas GW, Craun ML, Curtis CG, Bar-Or R, Bar-Or D. Lipid peroxidation and the thiobarbituric acid assay: standardization of the assay when using saturated and unsaturated fatty acids. *J Biochem Mol Biol.* (2004) 37:749–52. doi: 10.5483/BMBRep.2004.37.6.749
129. Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod.* (2007) 13:203–11. doi: 10.1093/molehr/gal119
130. Srivastava N, Pande M. Mitochondrion: Features, functions and comparative analysis of specific probes in detecting sperm cell damages. *Asian Pacific J Reprod.* (2016) 5:445–52. doi: 10.1016/j.apjr.2016.10.008
131. Moselhy HF, Reid RG, Yousef S, Boyle SP. A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. *J Lipid Res.* (2013) 54:852–8. doi: 10.1194/jlr.D032698
132. Boyle SP, Dobson VL, Duthie SJ, Hinselwood DC, Kyle JA, Collins AR. Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study. *Eur. J Clin Nutr.* (2000) 54:774–82. doi: 10.1038/sj.ejcn.1601090
133. Borovic S, Rabuzin F, Waeg G, Zarkovic N. Enzyme-linked immunosorbent assay for 4-hydroxynonenal–histidine conjugates. *Free Radic Res.* (2006) 40:809–20. doi: 10.1080/10715760600693422
134. Samanta L, Parida R, Dias TR, Agarwal A. The enigmatic seminal plasma: a proteomics insight from ejaculation to fertilization. *Reprod Biol Endocrinol.* (2018) 16:1–11. doi: 10.1186/s12958-018-0358-6
135. Weber D, Milkovic L, Bennett SJ, Griffiths HR, Zarkovic N, Grune T. Measurement of HNE-protein adducts in human plasma and serum by ELISA—Comparison of two primary antibodies. *Redox Biol.* (2013) 1:226–33. doi: 10.1016/j.redox.2013.01.012
136. Cai Z. Lipid Peroxidation. *Encyclopedia of Toxicology.* 2nd ed. (2005). p. 730–4. doi: 10.1016/B0-12-369400-0/00564-0
137. Garrido N, Meseguer M, Alvarez J, Simón C, Pellicer A, Remohí J, et al. Relationship among standard semen parameters, glutathione peroxidase / glutathione reductase activity, and mRNA expression and reduced glutathione content in ejaculated. *Fertil Steril.* (2004) 82(Suppl 3):1059–66. doi: 10.1016/j.fertnstert.2004.04.033
138. Crisol L, Matorras R, Aspichueta F, Expósito A, Hernández ML, Ruiz-Larrea MB, et al. Glutathione peroxidase activity in seminal plasma and its relationship to classical sperm parameters and in vitro fertilization-intracytoplasmic sperm injection outcome. *Fertil Steril.* (2012) 97:852–7. doi: 10.1016/j.fertnstert.2012.01.097

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 April 2021
doi: 10.3389/fvets.2021.635013

Comparative Analysis of piRNA Profiles Helps to Elucidate Cryoinjury Between Giant Panda and Boar Sperm During Cryopreservation

Yihan Wang[1†], Yingmin Zhou[2†], Malik Ahsan Ali[1,3†], Jiaman Zhang[1], Wencan Wang[1], Yan Huang[2], Bo Luo[2], Heming Zhang[2], Ziyue Qin[1], Yan Zhang[1], Ming Zhang[1], Guangbin Zhou[1] and Changjun Zeng[1]*

[1] College of Animal Sciences and Technology and Farm Animal Genetic Resources Exploration and Innovation Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu, China, [2] China Conservation and Research Center for the Giant Panda, Chengdu, China, [3] Department of Theriogenology, Riphah College of Veterinary Sciences, Lahore, Pakistan

Cryopreservation induces sperm cryoinjuries, including physiological and functional changes. However, the molecular mechanisms of sperm cryoinjury and cryoresistance are still unknown. Cryoresistance or the freeze tolerance of sperm varies across species, and boar sperm is more susceptible to cold stress. Contrary to boar sperm, giant panda sperm appears to be strongly freeze-tolerant and is capable of surviving repeated cycles of freeze-thawing. In this study, differentially expressed (DE) PIWI-interacting RNAs (piRNAs) of fresh and frozen-thawed sperm with different freeze tolerance capacity from giant panda and boar were evaluated. The results showed that 1,160 (22 downregulated and 1,138 upregulated) and 384 (110 upregulated and 274 downregulated) DE piRNAs were identified in giant panda and boar sperm, respectively. Gene ontology (GO) enrichment analysis revealed that the target DE messenger RNAs (mRNAs) of DE piRNAs were mainly enriched in biological regulation, cellular, and metabolic processes in giant panda and boar sperm. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that the target DE mRNAs of DE piRNAs were only distributed in DNA replication and the cyclic adenosine monophosphate (cAMP) signaling pathway in giant panda, but the cAMP, cyclic guanosine monophosphate (cGMP), and mitogen-activated protein kinase (MAPK) signaling pathways in boar sperm were considered as part of the olfactory transduction pathway. In conclusion, we speculated that the difference in the piRNA profiles and the DE piRNAs involved in the cAMP signaling pathway in boar and giant panda may have contributed to the different freeze tolerance capacities between giant panda and boar sperm, which helps to elucidate the molecular mechanism behind sperm cryoinjury and cryoresistance.

Keywords: piRNAs, sperm cryopreservation, freezability, boar, giant panda

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Gregorio Miguel Ferreira De Camargo, Federal University of Bahia, Brazil
Julián Santiago-Moreno, Instituto Nacional de Investigación y Tecnología Agroalimentaria (INIA), Spain

***Correspondence:**
Changjun Zeng
zengchj@sicau.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *29 November 2020*
Accepted: *17 February 2021*
Published: *22 April 2021*

Citation:
Wang Y, Zhou Y, Ali MA, Zhang J, Wang W, Huang Y, Luo B, Zhang H, Qin Z, Zhang Y, Zhang M, Zhou G and Zeng C (2021) Comparative Analysis of piRNA Profiles Helps to Elucidate Cryoinjury Between Giant Panda and Boar Sperm During Cryopreservation. Front. Vet. Sci. 8:635013. doi: 10.3389/fvets.2021.635013

INTRODUCTION

Sperm cryopreservation is widely used to manage and preserve male fertility in human and domestic animals (1). Then, artificial insemination (AI) is extensively employed with frozen-thawed sperm to enhance the rate of genetic improvement, especially in cattle (2). However, sperm cryoresistance or freeze tolerance and the post-thawed sperm quality vary across species. Less than 1% AI with frozen-thawed boar sperm was carried out due to the low conception rate and litter size (3, 4). It is well-known that various factors during cryopreservation, including rapid temperature transitions, osmotic stress, and ice crystal formation, affect the post-thaw quality of semen (5). Furthermore, the transcriptomics, epigenetics, and proteomics of sperm were also modified during cryopreservation (6–8). Despite the extensive progress that has been achieved in optimizing the cryopreservation process through the selection of friendly cryoprotectants and the design of better freezing and thawing procedures to ameliorate cryodamage, the underlying mechanisms of freeze tolerance or freezability involved in cryopreservation have not been completely elucidated yet.

Compared with other mammals' sperm, the higher level of phospholipids and the lower level of cholesterol in the plasma membrane of boar sperm contribute to the susceptibility to cold shock or cold stress (9). Cold shock causes the rearrangement of phospholipids, destruction of acrosomal integrity, and functional damage to ion transporters and channels in sperm (9, 10). However, compared with boar sperm, giant panda sperm shows a higher freeze tolerance capacity and can sustain repeated freeze–thaw cycles (11). Cryopreservation has no significant impact on sperm viability and motility, and the acrosome integrity and functional capacitation of giant panda sperm were also not affected after repeated freeze–thaw cycles (12). Our previous studies have shown that the transcriptomic profiles were significantly different between boar and giant panda sperm during cryopreservation (13). Furthermore, comparative analysis of the transcriptomic modifications between boar and giant panda sperm during cryopreservation indicated that differentially expressed (DE) messenger RNAs (mRNAs) were mainly distributed in inflammatory-related pathways, the cytokine–cytokine receptor interaction pathway, and membrane signal transduction-related pathways (14). These previous studies demonstrated that cryopreservation induces different transcriptomic modifications and may explain why sperm with different freeze tolerance or cryoresistance capacities are susceptible to cold stress.

PIWI-interacting RNAs (piRNAs) are small non-coding RNAs which are germline-specific and are required to protect genomic integrity from deleterious effects and to preserve RNA homeostasis during male gametogenesis; they are also associated with sperm morphology, motility, and fertility (15). The expression of piRNAs in human sperm was correlated with the sperm concentration and fertilization rate (16). Moreover, a panel of piRNAs discovered in seminal plasma can serve as fertility or infertility markers in males (17). Recently, 79 putative piRNAs were found to be differentially expressed between low and high motile bovine sperm after cryopreservation (18). Therefore, we speculated that piRNAs may be involved in post-thawed sperm cryoinjury or cryoresistance, motility, and fertility during cryopreservation. Thus, in this study, we first evaluated the differences in the piRNA profiles of fresh and frozen-thawed boar and giant panda sperm, which will help to uncover the underlying molecular mechanisms of sperm cryoresistance and freeze tolerance and improve post-thawed sperm quality and fertility.

MATERIALS AND METHODS

Ethical Statement, Semen Collection, and Treatment

Fresh ejaculates from five sexually mature giant pandas with normal physiological parameters were obtained by electrical stimulation from the Bifengxia Base of China Conservation and Research Center for the Giant Panda (Yaán, Sichuan, China) according to a previous protocol (11). Briefly, giant pandas were anesthetized by an intramuscular injection of 10 mg/kg ketamine HCl and maintained with 0–5% isofluorane gas. Electroejaculation was conducted by using an electroejaculator (Boring, OR, USA); the period of electrical stimuli (2–8 V, repeated three times) was 2 s following an intermittent break of 2 s. When penile erection occurs during stimulation, semen was collected into a sterile glass container. Fresh ejaculates from 11 boars were collected with the glove-handed technique. All procedures were carried out while strictly following the Regulations of the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004) and were accredited by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China (under permit no. 2019202012).

All ejaculates from giant panda and boar were pooled separately and two equal groups were generated (fresh sperm and cryopreserved sperm). Direct RNA extraction was performed with fresh sperms, and the other group was cryopreserved according to a previously procedure (19). Briefly, TES–Tris (TEST) egg yolk buffer was used to dilute the giant panda sperm (Irvine Scientific, Santa Ana, CA) to obtain 5% concentration of glycerol. This diluted material was filled into 0.25-ml semen straws and gradually cooled to 4°C in 4 h, then kept at 7.5 cm for 1 min over liquid nitrogen (LN) to obtain the cooling rate of −40°C/min and then at 2.5 cm for 1 min above LN (approximate cooling rate was −100°C/min), before plunging in LN until further processing. Thawing was performed by immersing the semen straws for 30 s in a water bath with constant temperature of 37°C. Semen was diluted with an equal volume of Ham's F10 (HF10) containing 5% fetal calf serum and 25 mM HEPES. Boar sperm was cryopreserved according to the following procedure; firstly, the sperm was centrifuged (for 5 min at 1,800 rpm and 17°C) and then diluted with a lactose–egg yolk (LEY) extender containing 10 ml hen's egg yolk and 40 ml 11% β-lactose. Secondly, the sperm and the extender mixture were cooled to 4°C (at 0.2°C/min), and further dilution with LEY was performed

to obtain a final 3% concentration of glycerol. Lastly, the 0.25-ml semen straws (FHK, Tokyo, Japan) were loaded with this mixture, sealed, and kept 3 cm above LN for 10 min before being submerged into it until future use.

RNA Extraction, Library Preparation, and Sequencing

Before RNA extraction, seminal plasma was removed from all the samples by washing with RNase-free water three times. Then, 0.5% Triton (X-100) was employed in accordance with a previous study (16) to minimize the somatic cell count as they hinder the spermatic RNA extraction process. Then, the TRIzol LS Reagent kit (Invitrogen, Carlsbad, CA, USA) was utilized to extract total RNA from all sperm samples according to the manufacturer's instructions. The RNA samples were pooled together equally in their respective groups before constructing RNA libraries. Furthermore, a Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA) equipment was used to determine the purity and concentration of the RNA and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was employed to check its integrity. Then, a NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490, Ipswich, MA, USA) was utilized to isolate mRNA. The small RNA libraries were built by using the NEB Next Ultra RNA Library Prep Kit for Illumina (NEB, E7530, Ipswich, MA, USA) and the NEBNext Multiplex Oligos for Illumina (NEB, E7500, Ipswich, MA, USA) according to the manufacturer's guidelines. After confirming the quality using Qubit 2.0 and the Agilent Bioanalyzer 2100 system (Agilent Technologies), all libraries were sequenced with the Illumina Hiseq 2500 platform (Illumina, San Diego, CA, USA).

piRNA Identification and Expression Analysis

After removal of low-quality, poly-N, and adapter-containing reads and sequences with <18 or >34 nt, clean reads were acquired. The sequence alignments of giant panda and pig were carried out with their reference genomes (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/004/335/GCF_000004335.2_AilMel_1.0 and ftp://ftp.ensembl.org/pub/release-75/fasta/sus_scrofa/, respectively). Furthermore, to compare the clean reads with the Silva database, Rfam database, Repbase, and the GtRNAdb database and filtering out non-coding RNAs (ncRNAs) such as ribosomal RNA (rRNA), transport RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and repetitive sequences, Bowtie analysis was performed (20). Novel and known piRNAs were sorted out by comparing the obtained piRNA sequences with miRbase RNA sequences using proTRAC (21). Differential expression of piRNAs in the fresh and frozen-thawed groups was determined with the DESeq R package (v. 1.10.1) based on the reads per kilobase million (TPM) and fragments per kilobase million (FPKM) algorithms (22). The piRNAs between both sperm groups were analyzed by iDEG (23), and those with adjusted $p < 0.01$ and absolute value of log2 fold change (FC) >1 were classified as DE piRNAs. Then, hierarchical clustering analysis was performed by R heatmap.2 on the selected DE piRNAs; piRNAs with similar expressions were clustered based on the log10(TPM + 1) value.

piRNA Target Prediction, GO, and KEGG Enrichment Analyses

The prediction of potential piRNA targets was performed by BLAST with non-redundant (NR) (20), Gene Ontology (GO) (20), Kyoto Encyclopedia of Genes and Genomes (KEGG) (24), and EuKaryotic Orthologous Group (KOG) (25) databases to obtain annotation information of the target genes. KEGG pathways and GO enriched in predicted DE piRNA target genes were elucidated using KOBAS software (26) and the GOseqR package (27), respectively.

Comparison of DE piRNAs in Fresh and Post-thawed Boar and Giant Panda Sperm During Cryopreservation

piRBase (http://www.regulatoryrna.org/database/piRNA/) was used to browse the common piRNAs and annotations. The homology of piRNAs was predicted between various species by considering the similarity and conserved sequences of the piRNAs to determine the piRNAs. The software Python 2.7 was used for comparing the sequence similarities of the DE piRNAs in giant panda sperm and boar sperm. During sequence alignment, 1–18 bases were perfectly matched; one mismatch base was allowed after the 19th base to select the best pairing sequence (28).

Statistical Analysis

All data were shown as the means ± SEM. SPSS (v. 20.0) with independent samples t test was used to determine statistical differences, and $p < 0.05$ were considered as statistically significant.

RESULTS

piRNA Profiles of Fresh and Cryopreserved Boar and Giant Panda Sperm

A total of 16,980,071 and 19,571,331 raw reads were obtained from fresh and cryopreserved sperm groups of giant pandas, respectively. Similarly, respective boar sperm groups generated 18,956,444 and 16,507,275 raw reads. After removal of low-quality reads, ploy-N, adapter, and sequences with <24 or >32 nt, 519,311 and 4,488,163 clean reads were generated in respective fresh and frozen-thawed giant panda sperm. Similarly, 9,031,512 and 7,188,244 clean reads were generated in fresh and frozen-thawed boar sperm, respectively (**Table 1**). The 24-nt (21.76%) and 31-nt (34.21%) piRNAs were the most abundant in fresh and frozen-thawed giant panda sperms, respectively. Similarly, the 30-nt (25%) and 32-nt (1.96%) piRNAs showed the highest and the lowest respective abundances, respectively, in boar sperm.

A total of 88 (containing 116,706 piRNAs) and 133 (containing 21,5835 piRNAs) piRNA clusters were identified after mapping to the designated reference genomes of giant panda and boar sperm, respectively. Compared to the 125,435 and 112,708 piRNAs expressed in fresh and frozen-thawed boar

TABLE 1 | Overview of piRNA sequencing of fresh and frozen-thawed sperm in giant panda and boar.

Species	Group	Raw reads	Clean reads	Mapped reads	Mapped ratio (%)
Giant panda	Fresh sperm	16,980,071	519,311	85,586	16.48
	Post-thawed sperm	19,571,331	4,488,163	325,288	7.2
Boar	Fresh sperm	18,956,444	9,031,512	2,988,336	15.76
	Post-thawed sperm	16,507,275	7,188,244	2,087,711	13.0

sperm, respectively, 49,393 and 87,670 piRNAs were expressed in fresh and frozen-thawed giant panda sperm, respectively. Differential analysis depicted the differential expression of 1,160 piRNAs (1,138 upregulated and 22 downregulated) between fresh and frozen-thawed giant panda sperm (**Figure 1A**). In contrast to the giant panda sperm, 384 DE piRNAs (110 upregulated and 274 downregulated) were identified in boar sperm (**Figure 1A**). Moreover, hierarchical clustering analysis was performed for the clustering of all DE piRNAs (**Figure 1B**).

Combined Analysis of piRNAs and Target mRNAs in Boar and Giant Panda Sperm

Two hundred fifty-three (seven upregulated and 246 downregulated) and 453 target DE mRNAs (366 upregulated and 87 downregulated) of the DE piRNAs were obtained between fresh and post-thawed sperm in giant panda and boar, respectively (**Figure 2A**). Twenty-eight DE piRNAs were identified to be the common piRNAs by joint analysis of the DE piRNAs of giant panda and boar sperm (**Figure 2B**). Therefore, 1,132 and 356 DE piRNAs were selected and regarded as the unique piRNAs in giant panda and boar sperm, respectively (**Data Sheet 1**, **2**). Based on the similarity and conservation of the piRNA sequences, 28 DE piRNAs were considered as the homologous piRNAs between giant panda and boar sperm according to the piRBase database (**Data Sheet 3**). However, no target DE mRNAs were found for these common DE piRNAs.

Comparative GO and KEGG Analysis of DE piRNAs in Giant Panda and Boar Sperm

GO enrichment analysis showed that 106 and 3,251 target DE mRNAs of the DE piRNAs were annotated with 41 and 59 GO terms in giant panda and boar sperm, respectively. Most of the target mRNAs of the DE piRNAs were seen to be distributed in cell, cell part, binding and biological regulation, and metabolic terms in giant panda and boar sperm, which are strictly associated with the structural and functional modifications of sperm. The GO term distributions of the target DE mRNAs of DE piRNAs were significantly different in fresh and frozen-thawed giant panda and boar sperm (**Figure 3A**).

Notably, most of the target DE mRNAs of DE piRNAs were distributed in the cyclic adenosine monophosphate (cAMP) signaling pathway in giant panda sperm, except for DNA replication (**Figure 3B**). However, the target mRNAs of the DE piRNAs in boar sperm were mainly distributed in the peroxisome and spliceosome, followed by the membrane-related pathway, such as the cAMP, cyclic guanosine monophosphate (cGMP), mitogen-activated protein kinase (MAPK), and PI3K–Akt signaling pathways. Moreover, the cAMP pathway was found in both giant panda and boar sperm, but was extremely enriched in giant panda sperm. Further analysis indicated that DE piRNAs involved in the cAMP signaling pathway may regulate the post-thawed sperm function by targeting cyclic nucleotide-gated (CNG) ion channel-related genes.

DISCUSSION

It is well-known that differences in the size, shape, and the lipid–protein composition of sperm across various species result in different sensitivities to freezing (29, 30). Esmaeili and colleagues have demonstrated that cryotolerance shows a relation to the ratio of polyunsaturated fatty acids (PUFAs) (omega-3/omega-6) (30). The plasma membrane of boar sperm contains a higher concentration of phospholipids and a lower concentration of cholesterol (9). In addition, the head size of boar sperm is larger than that of the giant panda and is more sensitive to freezing (31). Sperm with smaller heads are usually less cryopreservation-sensitive; thus, the freeze tolerance capacity of giant panda sperm is higher than that of boar sperm after cryopreservation (14).

Nowadays, electroejaculation is the preferred method to collect semen from giant panda. Previous studies have reported that electroejaculation may have an impact on semen parameters, increasing semen osmolarity, disrupting plasma membrane integrity, acrosomal damage, and acrosomal exocytosis (32, 33). However, the sperm morphology remained within acceptable standards (34). Compared with the quality parameters of fresh feline ejaculates collected using three different techniques—urethral catheterization after medetomidine administration, electroejaculation, and epididymal slicing after orchiectomy—the highest quality semen parameters were achieved using electroejaculation (35). Spindler et al. reported that most sperm of giant panda were morphologically normal using electroejaculation, and the sperm parameters (seminal volume, concentration, initial motility, acrosomal integrity, etc.) were consistent with previous reports (11). Therefore, the fertility of frozen-thawed giant panda sperm will be similar to that following the use of fresh sperm (11).

The process of freeze–thawing induces apoptotic-like changes in sperm, and these changes may affect the plasma membrane and acrosomal activity (36) and the mitochondrial activity (37) and also cause abnormal expressions of genes and proteins associated with cryoinjury (38). Moreover, sperm genomic epigenetic elements may be altered during cryopreservation. Previous studies have demonstrated that some genes play

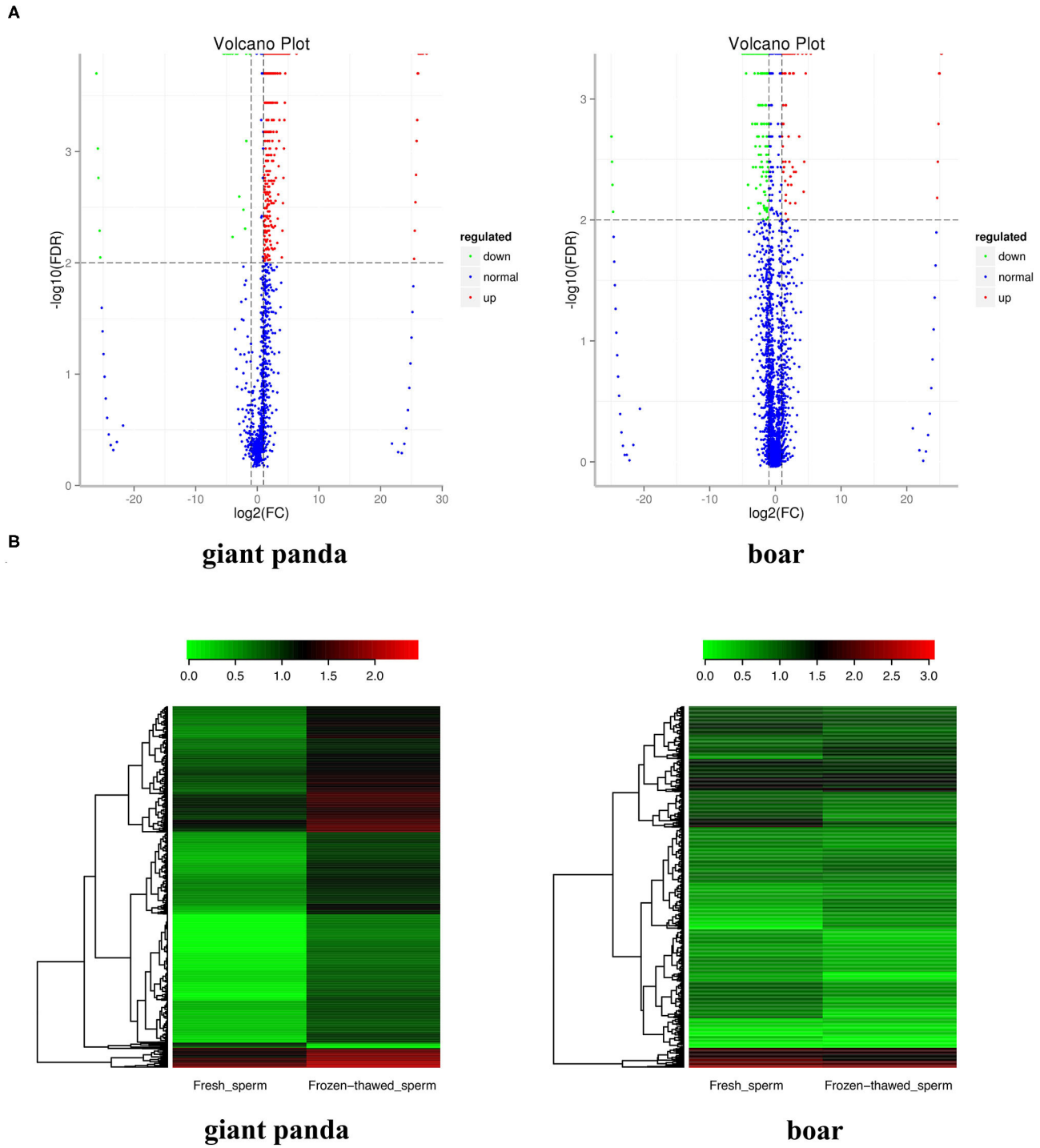


FIGURE 1 | Volcano plot and clustering analysis of differentially expressed PIWI-interacting RNAs (DE piRNAs) in fresh and frozen-thawed giant panda and boar sperm. **(A)** Volcano plot of DE piRNAs in fresh and frozen-thawed giant panda sperm. *Blue dots* represent normal expressed, *green dots* represent the downregulated, and *red dots* represent the upregulated piRNAs. **(B)** Heat maps of the cluster analysis of piRNAs. *Red* indicates high expression while *green* means low expression of piRNAs.

critical roles in freezing, such as PRM1, FSHB, ADD1, ARNT, and SNORD116/PWSAS (39, 40). Some proteins, such as TPI1, ACRBP, HSP90AA1, and PHGPx, were proven to be markers of sperm cryoresistance (38, 41, 42). Furthermore, certain mRNA transcripts encoding related proteins were affected during cryopreservation; for instance, PRM1 mRNA

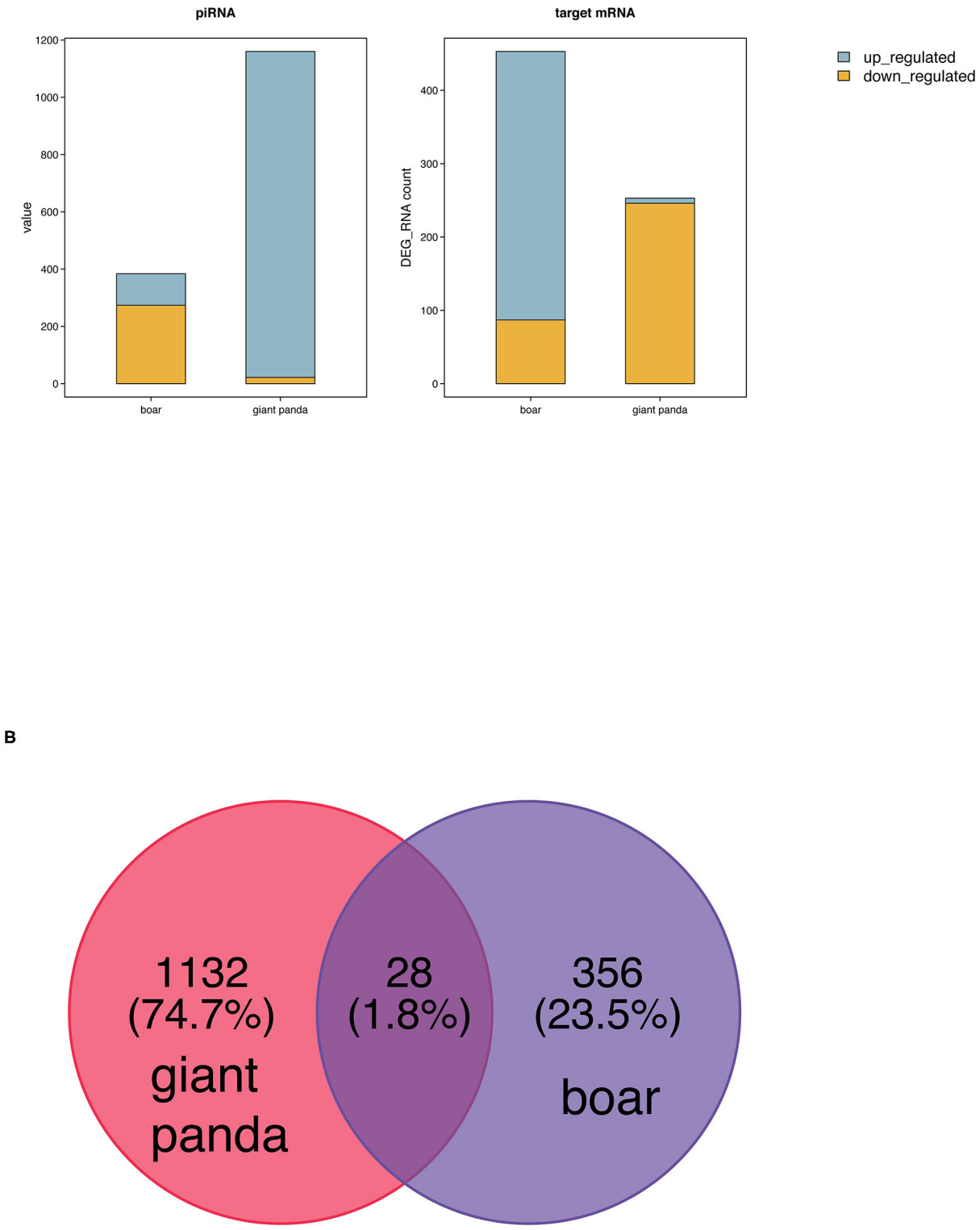


FIGURE 2 | Comparative analysis of differentially expressed PIWI-interacting RNAs (DE piRNAs) in fresh and frozen-thawed giant panda and boar sperm. **(A)** Comparison of DE piRNAs and target DE mRNAs. **(B)** Unique and common DE piRNAs.

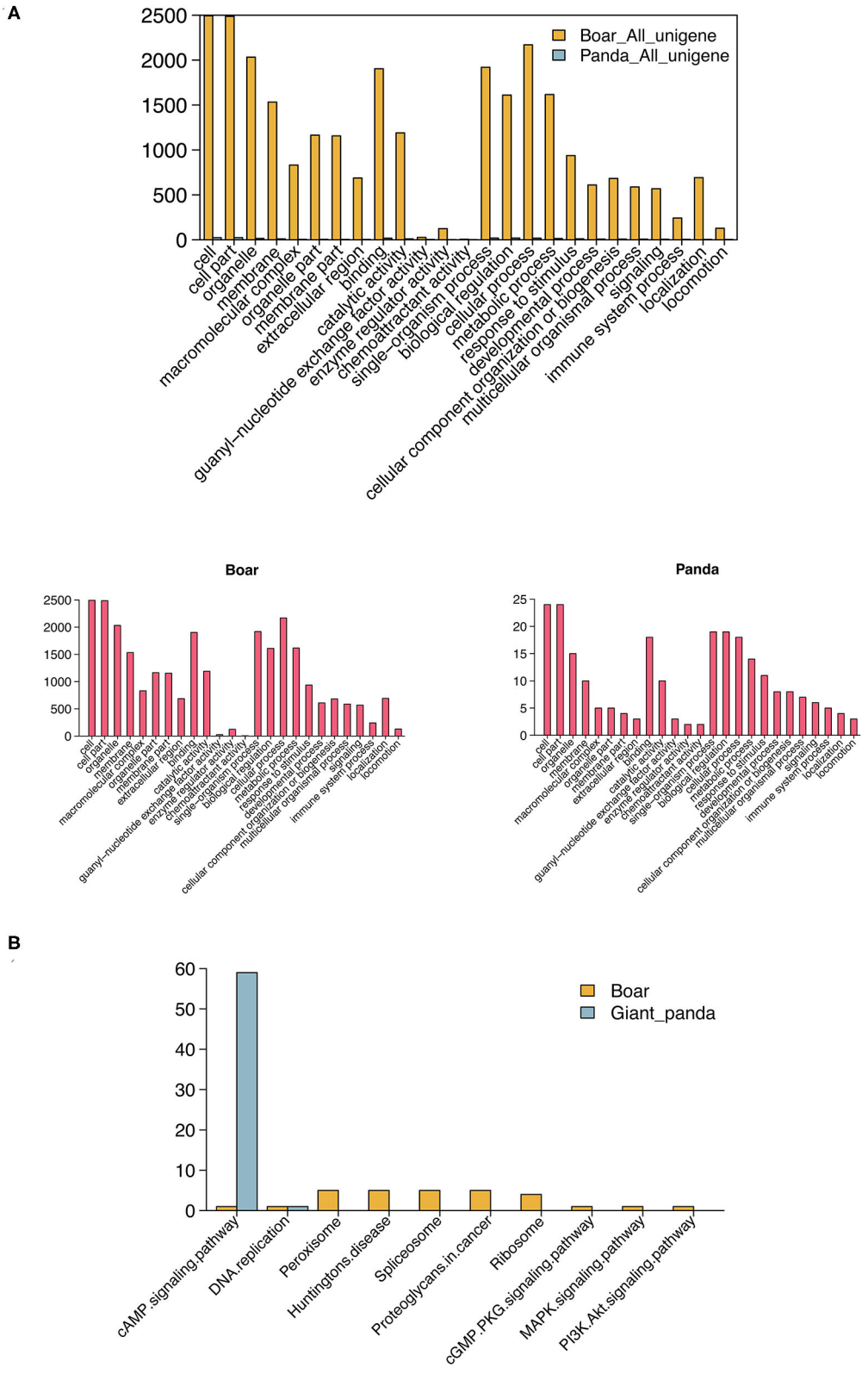


FIGURE 3 | Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed PIWI-interacting RNAs (DE piRNAs) in giant panda and boar sperm. **(A)** GO analysis of the target DE messenger RNAs (mRNAs) of DE piRNAs. **(B)** Top 10 KEGG pathways of the target DE mRNAs of DE piRNAs.

transcripts were reduced in boar, cattle, and human sperm (43–45). Beyond that, some sperm mRNA transcripts associated with early embryo development were downregulated in embryos fertilized with frozen horse sperm compared to those with fresh sperm (46). In fact, some microRNAs (miRNAs) associated with cryopreservation, or named CryomiRs, may affect the expressions of the mRNA transcripts during cryopreservation, which ultimately affects the expressions of genes and proteins associated with sperm metabolism and apoptosis (14, 47).

According to our previous study, the DE miRNAs and target mRNAs of giant panda sperm were mainly enriched in olfactory transduction pathways, including the cAMP and cGMP signaling pathways (14). In the present study, we found that the target DE mRNAs of DE piRNAs in giant panda sperm were mainly distributed in the cAMP signaling pathway and partially involved in DNA replication. Similarly, few targets of the DE piRNAs in boar sperm were also enriched in the cAMP signaling pathway, but the ratio was much lower than that of giant panda sperm. Therefore, we speculated that the 1,132 specific piRNAs involved in the cAMP signaling pathway in giant panda sperm may be closely related to the freeze tolerance of sperm. Therefore, we speculated that cryopreservation can affect the expression levels of olfactory transduction pathway-related genes and is probably involved in the regulation of capacitation, motility, fertility, and even the freeze tolerance of post-thawed sperm. However, the regulatory mechanism of the olfactory transduction signaling pathway on post-thawed sperm is still unknown.

It is well-known that olfactory receptors or odorant receptors are associated with sperm motility and chemotaxis. In the olfactory transduction pathway, after the attachment of odorant molecules with the G protein-coupled receptor (GPCR) in sperm, the concentration of cAMP increases, leading to the opening of CNG ion channels (48). Notably, CNG channels play an important role in the regulation of the intracellular Ca^{2+} level, which causes influx of Ca^{2+}, and then induce sperm hyperactivity (49). In mature sperm, cAMP binding with a target protein is essential for those events during sperm capacitation, including sperm plasma membrane hyperactivation (50, 51), tyrosine phosphorylation (52), and increasing intracellular Ca^{2+} and pH (53–55). It was demonstrated that the intracellular concentrations of cAMP and Ca^{2+} play a primary role in sperm capacitation, motility, acrosomal reaction, lipid remodeling, and hyperpolarization of the plasma membrane (55–58). Furthermore, cAMP is known to be an important second messenger for steroid (hormones) biosynthesis, and the specific role of its downstream protein kinase A (PKA) pathway is regulating steroid biosynthesis (59). Steroid hormones induce sperm capacitation and acrosomal response (60). cAMP–PKA signaling pathways induce steroid biosynthesis in stromal cells by activating certain transcription factors, such as CREB, CREM, and GATA4, and regulating the expressions of downstream target proteins (58, 61). In addition, the synthesis of cAMP also activates a Ca^{2+} signal regulated by PKA or protein kinase C (PKC), which upregulates Nur77 expression, and causes StAR transcription, promoting steroid hormone biosynthesis (62). Previous studies indicated that the intracellular Ca^{2+}, 1,2-diacylglycerol (DAG), and cAMP levels in buffalo sperm were increased significantly after cryopreservation as compared to fresh ejaculates, and the addition of taurine or trehalose reduced the extent of capacitation-like changes in buffalo sperm (56). Likewise, cryopreservation negatively affected the PKA and AMP-activated protein kinase (AMPK) activity in Atlantic salmon sperm (63), and when AMPK was inhibited, the sperm motility decreased accordingly. In this study, the target mRNAs of the DE piRNAs in giant panda sperm are mainly enriched in the cAMP pathway, which indicates that cAMP and calcium may be associated with frozen-thawed sperm quality of giant panda. Differences in the cAMP pathway-related piRNAs and mRNAs between the giant panda and boar sperm may have contributed to sperm cryotolerance. Therefore, our study first revealed that piRNAs might be regulating the cAMP signaling pathway to regulate post-thawed sperm quality, which provides new insights into the cryoinjury, cryoresistance, or the freeze tolerance mechanisms of sperm varying across species. Future exploration should focus on the biological roles of these DE piRNAs in sperm freeze tolerance or cryoresistance and their association with post-thawed sperm quality, which may provide some insights regarding the molecular mechanisms of cryoinjury.

CONCLUSION

In this study, we first conducted a comparative analysis of the piRNAs and target mRNAs between giant panda sperm and boar sperm during cryopreservation. The differentially expressed piRNAs and their target DE mRNAs are mainly involved in the cAMP signaling pathway and DNA replication, which indicated that these piRNAs play a critical role in sperm cryoresistance and cryoinjury during cryopreservation. Our study provides new insights into the cryoinjury, cryoresistance, or freeze tolerance mechanisms of sperm varying across species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Gene Expression Omnibus, GSE163128.

ETHICS STATEMENT

The animal study was reviewed and approved by the Regulations of the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004) the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China, under permit No: 2019202012. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YW, YZho, MA, JZ, WW, and ZQ collected samples, performed the experiments, analyzed the data, and drafted the manuscript. BL, YH, and HZ contributed to samples collection, data analysis, and revised the manuscript. MZ, GZ, and YZha revised the manuscript critically and given final approval to be published. CZ granted, concept, designed the experiment and revised, given final approval version of the manuscript to be published. All authors reviewed and approved the final manuscript.

FUNDING

This research was supported by National Natural Science Foundation of China (No: 31872356 and No: 31570533).

ACKNOWLEDGMENTS

We offer special thanks to the Sichuan TIEQILISHI group for semen collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.635013/full#supplementary-material

Data Sheet 1 | All DE piRNAs in giant panda and boar sperm.

Data Sheet 2 | DE piRNAs and their target DE mRNAs in giant panda and boar sperm.

Data Sheet 3 | The common DE piRNAs in giant panda and boar sperm.

REFERENCES

1. Yeste M, Rodríguez-Gil JE, Bonet S. Artificial insemination with frozen-thawed boar sperm. *Mol Reprod Dev*. (2017) 84:802–13. doi: 10.1002/mrd.22840
2. Flores E, Ramió-Lluch L, Bucci D, Fernández-Novell JM, Peña A, Rodríguez-Gil JE. Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA disulfide bonds in boar sperm. *Theriogenology*. (2011) 76:1450–64. doi: 10.1016/j.theriogenology.2011.05.039
3. Yeste M. Recent advances in boar sperm cryopreservation: State of the art and current perspectives. *Reprod Domest Anim*. (2015) 50(Suppl 2):71–9. doi: 10.1111/rda.12569
4. Kim S, Lee YJ, Kim YJ. Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15°c. *Anim Reprod Sci*. (2011) 124:118–24. doi: 10.1016/j.anireprosci.2011.01.014
5. Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches. *Reprod Biomed Online*. (2018) 37:327–39. doi: 10.1016/j.rbmo.2018.05.012
6. Yeste M, Estrada E, Casas I, Bonet S, Rodríguez-Gil JE. Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. *Theriogenology*. (2013) 79:929–39. doi: 10.1016/j.theriogenology.2013.01.008
7. Flores E, Cifuentes D, Fernández-Novell JM, Medrano A, Bonet S, Briz MD, et al. Freeze-thawing induces alterations in the protamine-1/DNA overall structure in boar sperm. *Theriogenology*. (2008) 69:1083–94. doi: 10.1016/j.theriogenology.2008.01.022
8. Mostek A, Westfalewicz B, Słowińska M, Dietrich MA, Judycka S, Ciereszko A. Differences in sperm protein abundance and carbonylation level in bull ejaculates of low and high quality. *PLoS ONE*. (2018) 13:e0206150. doi: 10.1371/journal.pone.0206150
9. Cerolini S, Maldjian A, Surai P, Noble R. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim Reprod Sci*. (2000) 58:99–111. doi: 10.1016/S0378-4320(99)00035-4
10. Bailey JL, Lessard C, Jacques J, Brèque C, Dobrinski I, Zeng WX, et al. Cryopreservation of boar semen and its future importance to the industry. *Theriogenology*. (2008) 70:1251–9. doi: 10.1016/j.theriogenology.2008.06.014
11. Spindler RE, Huang Y, Howard JG, Wang P, Zhang H, Zhang G, et al. Acrosomal integrity and capacitation are not influenced by sperm cryopreservation in the giant panda. *Reproduction*. (2004) 127:547–56. doi: 10.1530/rep.1.00034
12. Santiago-Moreno J, Esteso MC, Pradiee J, Castaño C, Toledano-Díaz A, O'brien E, et al. Giant panda (*Ailuropoda melanoleuca*) sperm morphometry and function after repeated freezing and thawing. *Andrologia*. (2016) 48:470–4. doi: 10.1111/and.12468
13. Ran MX, Li Y, Zhang Y, Liang K, Ren YN, Zhang M, et al. Transcriptome sequencing reveals the differentially expressed lncRNAs and mRNAs involved in cryoinjuries in frozen-thawed giant panda (*Ailuropoda melanoleuca*) sperm. *Int J Mol Sci*. (2018) 19:3066. 19. doi: 10.3390/ijms19103066
14. Ran MX, Zhou YM, Liang K, Wang WC, Zhang Y, Zhang M, et al. Comparative analysis of microRNA and mRNA profiles of sperm with different freeze tolerance capacities in boar (Sus scrofa) and giant panda (*Ailuropoda melanoleuca*). *Biomolecules*. (2019) 9:432. doi: 10.3390/biom9090432
15. Heejin C, Wang Z, Dean J. *Sperm Acrosome Overgrowth and Infertility in Mice Lacking Chromosome 18 Pachytene piRNA*. BioRxiv (2020).
16. Cui L, Fang L, Shi B, Qiu S, Ye Y. Spermatozoa expression of piR-31704, piR-39888, and piR-40349 and their correlation to sperm concentration and fertilization rate after ICSI. *Reprod Sci*. (2018) 25:733–9. doi: 10.1177/1933719117725822
17. Hong YT, Wang C, Fu Z, Liang HW, Zhang SY, Lu ML, et al. Systematic characterization of seminal plasma piRNAs as molecular biomarkers for male infertility. *Sci Rep*. (2016) 6:24229. doi: 10.1038/srep24229
18. Capra E, Turri F, Lazzari B, Cremonesi P, Gliozzi TM, Fojadelli I, et al. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between High- and Low-motile sperm populations. *BMC Genomics*. (2017) 18:14. doi: 10.1186/s12864-016-3394-7
19. Huang Y, Li D, Zhou Y, Zhou Q, Li R, Wang C, et al. Factors affecting the outcome of artificial insemination using cryopreserved spermatozoa in the giant panda (*Ailuropoda melanoleuca*). *Zoo Biol*. (2012) 31:561–73. doi: 10.1002/zoo.20421
20. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene ontology consortium. *Nat Genet*. (2000) 25:25–9. doi: 10.1038/75556
21. Rosenkranz D, Zischler H. proTRAC–a software for probabilistic piRNA cluster detection, visualization and analysis. *BMC Bioinformat*. (2012) 13:5. doi: 10.1186/1471-2105-13-5
22. Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, Cumbie JS, et al. High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE*. (2007) 2:e219. doi: 10.1371/journal.pone.0000219
23. Romualdi C, Bortoluzzi S, D'alessi F, Danieli GA. IDEG6: a web tool for detection of differentially expressed genes in multiple tag sampling experiments. *Physiol Genomics*. (2003) 12:159–62. doi: 10.1152/physiolgenomics.00096.2002
24. Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M. The KEGG resource for deciphering the genome. *Nucleic Acids Res*. (2004) 32:D277–80. doi: 10.1093/nar/gkh063
25. Koonin EV, Fedorova ND, Jackson JD, Jacobs AR, Krylov DM, Makarova KS, et al. A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. *Genome Biol*. (2004) 5:R7. doi: 10.1186/gb-2004-5-2-r7

26. Mao X, Cai T, Olyarchuk JG, Wei L. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics*. (2005) 21:3787–93. doi: 10.1093/bioinformatics/bti430
27. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol*. (2009) 10:R25. doi: 10.1186/gb-2009-10-3-r25
28. Rosenkranz D. piRNA cluster database: a web resource for pirna producing loci. *Nucleic Acids Res*. (2016) 44:D223–30. doi: 10.1093/nar/gkv1265
29. Lv CR, Wu GQ, Hong QH, Quan GB. Spermatozoa cryopreservation: state of art and future in small ruminants. *Biopreserv Biobank*. (2019) 17:171–82. doi: 10.1089/bio.2018.0113
30. Esmaeili V, Shahverdi AH, Moghadasian MH, Alizadeh AR. Dietary fatty acids affect semen quality: a review. *Andrology*. (2015) 3:450–61. doi: 10.1111/andr.12024
31. Garde JJ, Soler AJ, Cassinello J, Crespo C, Malo AF, Espeso G, et al. Sperm cryopreservation in three species of endangered gazelles (Gazella cuvieri, G. *dama mhorr, and G. dorcas neglecta). Biol Reprod*. (2003) 69:602–11. doi: 10.1095/biolreprod.102.012914
32. Dooley MP, Pineda MH. Effect of method of collection on seminal characteristics of the domestic cat. *Am J Vet Res*. (1986) 47:286–92.
33. Aitken RJ, Wang YF, Liu J, Best F, Richardson DW. The influence of medium composition, osmolarity and albumin content on the acrosome reaction and fertilizing capacity of human spermatozoa: development of an improved zona-free hamster egg penetration test. *Int J Androl*. (1983) 6:180–93. doi: 10.1111/j.1365-2605.1983.tb00337.x
34. Benavides F, Sutovsky P, López V, Kennedy C, Echevarría L. Semen parameters of fertile guinea pigs (cavia porcellus) collected by transrectal electroejaculation. *Animals*. (2020) 10:767. doi: 10.3390/ani10050767
35. Jelinkova K, Vitasek R, Novotny R, Bartoskova A. A comparison of quality parameters of fresh feline ejaculates collected by three different collection techniques. *Reprod Domest Anim*. (2018) 53:1068–74. doi: 10.1111/rda.13205
36. Raad G, Lteif L, Lahoud R, Azoury J, Azoury J, Tanios J, et al. Cryopreservation media differentially affect sperm motility, morphology and DNA integrity. *Andrology*. (2018) 6:836–45. doi: 10.1111/andr.12531
37. Meyers S, Bulkeley E, and Foutouhi A. Sperm mitochondrial regulation in motility and fertility in horses. *Reprod Domest Anim*. (2019) 54(Suppl 3):22–8. doi: 10.1111/rda.13461
38. Chen XL, Zhu HB, Hu CH, Hao HS, Zhang JF, Li KP, et al. Identification of differentially expressed proteins in fresh and frozen-thawed boar spermatozoa by iTRAQ-coupled 2D LC-MS/MS. *Reproduction*. (2014) 147:321–30. doi: 10.1530/REP-13-0313
39. Valcarce DG, Cartón-García F, Riesco MF, Herráez MP, Robles V. Analysis of DNA damage after human sperm cryopreservation in genes crucial for fertilization and early embryo development. *Andrology*. (2013) 1:723–30. doi: 10.1111/j.2047-2927.2013.00116.x
40. Dai LS, Zhao ZH, Zhao RF, Xiao SQ, Jiang H, Yue XP, et al. Effects of novel single nucleotide polymorphisms of the FSH beta-subunit gene on semen quality and fertility in bulls. *Anim Reprod Sci*. (2009) 114:14–22. doi: 10.1016/j.anireprosci.2008.08.021
41. Casas I, Sancho S, Ballester J, Briz M, Pinart E, Bussalleu E, et al. The HSP90AA1 sperm content and the prediction of the boar ejaculate freezability. *Theriogenology*. (2010) 74:940–50. doi: 10.1016/j.theriogenology.2010.04.021
42. Vilagran I, Castillo J, Bonet S, Sancho S, Yeste M, Estanyol JM, et al. Acrosin-binding protein (ACRBP) and triosephosphate isomerase (TPI) are good markers to predict boar sperm freezing capacity. *Theriogenology*. (2013) 80:443–50. doi: 10.1016/j.theriogenology.2013.05.006
43. Seshagiri PB, Mariappa D, Aladakatti RH. Tyrosine phosphorylated proteins in mammalian spermatozoa: molecular and functional aspects. *Soc Reprod Fertil Suppl*. (2007) 63:313–25.
44. Ganguly I, Gaur GK, Kumar S, Mandal DK, Kumar M, Singh U, et al. Differential expression of protamine 1 and 2 genes in mature spermatozoa of normal and motility impaired semen producing crossbred frieswal (HF×Sahiwal) bulls. *Res Vet Sci*. (2013) 94:256–62. doi: 10.1016/j.rvsc.2012.09.001
45. Zeng CJ, Peng WP, Ding L, He L, Zhang Y, Fang DH, et al. A preliminary study on epigenetic changes during boar spermatozoa cryopreservation. *Cryobiology*. (2014) 69:119–27. doi: 10.1016/j.cryobiol.2014.06.003
46. Ortiz-Rodriguez JM, Ortega-Ferrusola C, Gil MC, Martín-Cano FE, Gaitskell-Phillips G, Rodríguez-Martínez H, et al. Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse. *PLoS ONE*. (2019) 14:e0213420. doi: 10.1371/journal.pone.0213420
47. Zhang Y, Dai DH, Chang Y, Li Y, Zhang M, Zhou GB, et al. Cryopreservation of boar sperm induces differential microRNAs expression. *Cryobiology*. (2017) 76:24–33. doi: 10.1016/j.cryobiol.2017.04.013
48. Gibson AD, Garbers DL. Guanylyl cyclases as a family of putative odorant receptors. *Annu Rev Neurosci*. (2000) 23:417–39. doi: 10.1146/annurev.neuro.23.1.417
49. Luddi A, Governini L, Wilmskötter D, Gudermann T, Boekhoff I, Piomboni P. Taste receptors: new players in sperm biology. *Int J Mol Sci*. (2019) 20:967. doi: 10.3390/ijms20040967
50. Alonso CAI, Osycka-Salut CE, Castellano L, Cesari A, Di Siervi N, Mutto A, et al. Extracellular cAMP activates molecular signalling pathways associated with sperm capacitation in bovines. *Mol Hum Reprod*. (2017) 23:521–34. doi: 10.1093/molehr/gax030
51. Zapata-Carmona H, Barón L, Zuñiga LM, Díaz ES, Kong M, Drobnis EZ, et al. The activation of the chymotrypsin-like activity of the proteasome is regulated by soluble adenyl cyclase/cAMP/protein kinase A pathway and required for human sperm capacitation. *Mol Hum Reprod*. (2019) 25:587–600. doi: 10.1093/molehr/gaz037
52. Matamoros-Volante A, Moreno-Irusta A, Torres-Rodriguez P, Giojalas L, Gervasi MG, Visconti PE, et al. Semi-automatized segmentation method using image-based flow cytometry to study sperm physiology: the case of capacitation-induced tyrosine phosphorylation. *Mol Hum Reprod*. (2018) 24:64–73. doi: 10.1093/molehr/gax062
53. Brukman NG, Nuñez SY, Puga Molina LDC, Buffone MG, Darszon A, Cuasnicu PS, et al. Tyrosine phosphorylation signaling regulates Ca^{2+} entry by affecting intracellular pH during human sperm capacitation. *J Cell Physiol*. (2019) 234:5276–88. doi: 10.1002/jcp.27337
54. Stival C, Puga Molina Ldel C, Paudel B, Buffone MG, Visconti PE, Krapf D. Sperm capacitation and acrosome reaction in mammalian sperm. *Adv Anat Embryol Cell Biol*. (2016) 220:93–106. doi: 10.1007/978-3-319-30567-7_5
55. Buffone MG, Wertheimer EV, Visconti PE, Krapf D. Central role of soluble adenylyl cyclase and cAMP in sperm physiology. *Biochim Biophys Acta*. (2014) 1842:2610–20. doi: 10.1016/j.bbadis.2014.07.013
56. Singh VK, Atreja SK, Kumar R, Chhillar S, Singh AK. Assessment of intracellular Ca^{2+}, cAMP and 1,2-diacylglycerol in cryopreserved buffalo (*Bubalus bubalis*) spermatozoa on supplementation of taurine and trehalose in the extender. *Reprod Domest Anim*. (2012) 47:584–90. doi: 10.1111/j.1439-0531.2011.01922.x
57. Orta G, De La Vega-Beltran JL, Martín-Hidalgo D, Santi CM, Visconti PE, Darszon A. Catsper channels are regulated by protein kinase A. *J Biol Chem*. (2018) 293:16830–41. doi: 10.1074/jbc.RA117.001566
58. Balbach M, Beckert V, Hansen JN, Wachten D. Shedding light on the role of cAMP in mammalian sperm physiology. *Mol Cell Endocrinol*. (2018) 468:111–20. doi: 10.1016/j.mce.2017.11.008
59. Selvaraj V, Stocco DM, Clark BJ. Current knowledge on the acute regulation of steroidogenesis. *Biol Reprod*. (2018) 99:13–26. doi: 10.1093/biolre/ioy102
60. Gimeno-Martos S, González-Arto M, Casao A, Gallego M, Cebrián-Pérez JA, Muiño-Blanco T, et al. Steroid hormone receptors and direct effects of steroid hormones on ram spermatozoa. *Reproduction*. (2017) 154:469–81. doi: 10.1530/REP-17-0177
61. Ravnskjaer K, Madiraju A, Montminy M. Role of the cAMP pathway in glucose and lipid metabolism. *Handb Exp Pharmacol*. (2016) 233:29–49. doi: 10.1007/164_2015_32
62. Costa RR, Reis RI, Aguiar JF, Varanda WA. Luteinizing hormone (LH) acts through PKA and PKC to modulate T-type calcium currents and intracellular calcium transients in mice leydig cells. *Cell Calcium*. (2011) 49:191–9. doi: 10.1016/j.ceca.2011.02.003
63. Lee-Estevez M, Herrera L, Díaz R, Beltrán J, Figueroa E, Dumorné K, et al. Effects of cryopreservation on cAMP-dependent protein kinase

and AMP-activated protein kinase in Atlantic salmon (salmo salar) spermatozoa: relation with post-thaw motility. *Anim Reprod Sci*. (2019) 209:106133. doi: 10.1016/j.anireprosci.2019.106133

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 28 April 2021
doi: 10.3389/fvets.2021.651286

Echinacea purpurea Ethanol Extract Improves Male Reproductive Dysfunction With Streptozotocin–Nicotinamide-Induced Diabetic Rats

Chien-Feng Mao[1], *Sabri Sudirman*[2], *Chi-Chih Lee*[1], *David Tsou*[1] *and Zwe-Ling Kong*[1]*

[1] *Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan,* [2] *Fisheries Product Technology, Faculty of Agriculture, Universitas Sriwijaya, Palembang, Indonesia*

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Alessandra Pelagalli, University of Naples Federico II, Italy
Pablo Daniel Cetica, Universidad de Buenos Aires, Argentina
Victor Udo Nna, University of Calabar, Nigeria

***Correspondence:**
Zwe-Ling Kong
kongzl@mail.ntou.edu.tw

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *09 January 2021*
Accepted: *08 March 2021*
Published: *28 April 2021*

Citation:
Mao C-F, Sudirman S, Lee C-C, Tsou D and Kong Z-L (2021) Echinacea purpurea Ethanol Extract Improves Male Reproductive Dysfunction With Streptozotocin–Nicotinamide-Induced Diabetic Rats. Front. Vet. Sci. 8:651286. doi: 10.3389/fvets.2021.651286

As lifestyle changes, the prevalence of diabetes increases every year. Diabetes-induced male reproductive dysfunction is predominantly due to increased oxidative stress and then results in sperm damage and infertility. *Echinacea purpurea* is a traditional medicinal herb and is well-known for its immune-modulatory, antioxidative, anti-inflammatory, anticancer, and antiviral activities. The Toll-like receptor 4 (TLR4) plays a critical role in innate immune responses leading to nuclear factor (NF)-κB phosphorylation and release of proinflammatory cytokines including nitric oxide (NO), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α. However, the relation between *Echinacea purpurea* extract and TLR4 remains unclear. This study aimed to investigate the protective effects on male reproduction of *Echinacea purpurea* ethanol extract (EPE) against diabetic rats and whether the anti-inflammatory effects were through the TLR4 pathway. Diabetic male Sprague–Dawley (SD) rats were induced by streptozotocin (65 mg/kg) and nicotinamide (230 mg/kg). EPE was tested in three doses (93, 279, and 465 mg/kg p.o. daily) for 4 weeks. Besides, metformin administration (100 mg/kg/day) was treated as a positive control. Results indicated that EPE administration for about 4 weeks improved hyperglycemia and insulin resistance. Additionally, EPE increased sperm motility, protected sperm morphology and mitochondrial membrane potential, as well as protein for testosterone synthesis enzyme. In sperm superoxide dismutase, catalase, and glutathione antioxidants were increased, whereas proinflammatory cytokines, such as NO, IL-1β, and TNF-α were decreased. The testis protein content of TLR4 and downstream phospho-NF-κB p65 also were reduced. The EPE might reduce the production of proinflammatory cytokines *via* TLR4 pathways and improve diabetes-induced male infertility.

Keywords: diabetes, *Echinacea purpurea*, inflammation, male reproduction, oxidative stress, Toll-like receptor

INTRODUCTION

Diabetes mellitus (DM) has been identified as a metabolic disorder disease. This disease can occur due to insufficient insulin secretion, abnormal insulin action, or both. Type-1 and type-2 DM are the common types of diabetes disease. Type-1 DM is characterized by autoimmune-mediated pancreatic β-cell results in the deficiency of insulin, whereas type-2 DM is peripheral insulin resistance (1). Hyperglycemia was observed in diabetes disease. This condition causes elevated oxidative stress and some proinflammatory cytokine levels, such as interleukin-1β and tumor necrosis factor-α (2, 3). Diabetes disease also causes an adverse effect on organs, such as the liver, pancreas, kidneys, and testis (4). A previous study reported that DM also decreases some steroidogenesis-related genes, such as steroidogenic acute regulatory (StAR) protein, cytochrome P450 enzyme (CYP11A1), and 17β-hydroxysteroid dehydrogenase (HSD) and resulting in impairment of the spermatogenesis and sperm properties (5).

Oral antidiabetic agents have been used for diabetic management. However, some of these agents reported that it increased the prevalence of cardiovascular and gastrointestinal diseases (6). Therefore, the investigation of an alternative antidiabetic agents with less adverse effects is a major topic for future research. Functional foods or natural products are the potential sources for novel antidiabetic agents, such as fucoxanthin from seaweed and antroquinonol-rich extract from *Antrodia cinnamomea* (7, 8).

Echinacea purpurea (EP, Asteraceae) is a medicinal plant with an important immunostimulatory effect (9). Extracts of EP have been used in North America for wound and infection treatments (10). This extract also shows antimicrobial and antiviral activities (11). A previous study reported that the bioactive compounds of EP ethanol extract are composed of phenolic acid and isobutylamides. The micro-nanoencapsulated *Echinacea purpurea* ethanol extract has been reported for its ameliorative effects on the diabetic model (12). However, the effect of this ethanol extract alone has been not reported. We hypothesized that EP ethanol extracts alone also have a potential to improve reproductive dysfunction in male diabetic rats. Additionally, the EP ethanol extract shows antioxidant and anti-inflammatory activities (13, 14). Therefore, this study aimed to investigate the ameliorative effects of *Echinacea purpurea* ethanol extract on reproductive dysfunction of streptozotocin–nicotinamide-induced diabetic male rats.

MATERIALS AND METHODS

Echinacea purpurea Extraction

Echinacea purpurea ethanol extract (EPE) was supplied by the Taiwan Direct Biotechnology Corporation (Taipei, Taiwan). The EPE contains alkylamides (dodecatetraenoic acid isobutylamide) and phenolic compounds (caffeic acid, chlorogenic acid, cichloric acid, and echinacoside) as analyzed by the Taiwan Direct Biotechnology Corporation by using high-performance liquid chromatography (HPLC) assay (12).

Animals and Treatments

This study used 36 healthy adult male Sprague–Dawley (SD) rats ($N = 36$, 5 weeks old). The animals were obtained from BioLASCO (Yilan City, Taiwan). They were kept under standard laboratory conditions (12-h light/12-h dark cycle and 23 ± 1°C) and fed a standard rodent diet (LabDiet 5001). Feed and water were provided *ad libitum*. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC Approval No. 103033) of the National Taiwan Ocean University. Briefly, the rats were acclimatized for a week and then randomly divided into two main groups (control and diabetes group). The diabetic rat model was intraperitoneally induced by streptozotocin (STZ, 65 mg/kg) and nicotinamide (NA, 230 mg/kg) according to a previous method. The diabetes condition was confirmed by oral glucose tolerance test (OGTT) after a week of STZ–NA injection (15, 16). The rats were confirmed as diabetes if the glucose concentration ≥200 mg/dl at 2-h post-load glucose (17). The diabetes group was divided into five subgroups ($n = 6$) as shown in **Figure 1**. The first subgroup of diabetic rats without any treatment (DM) and other diabetic rats were daily orally administrated by three different doses of EPE (93, 279, and 465 mg/kg of body weight) for 4 weeks. The EPE doses were chosen according to the previous study (12). Besides, a group of diabetic rats was treated with metformin (100 mg/kg) as a positive control (18), whereas the Control and untreated diabetes (DM) groups were oral gavage administered by distillated water (dH_2O). The EPE and metformin were dissolved in dH_2O to make the concentration.

Oral Glucose Tolerance Test

The oral glucose tolerance test (OGTT) was performed based on the previous methods (15, 19). The OGTT was measured at a week after STZ–NA injection (before the treatment) and the last week of the treatment. Briefly, the rats were fasted for 10 h before the study. Glucose was orally administered (2 g/kg of BW). Blood samples were collected sequentially from the tail vein before and 30, 60, 90, and 120 min after the glucose injection. In OGTT, the glucose level and area under the curve (AUC) were calculated.

Sample Collection

The rats were sacrificed after treatment for 4 weeks. Whole blood was collected into tubes and centrifuged at 3,000 rpm at 4°C for 15 min to collect the plasma according to the previous method (20). The plasma, testis, and hypothalamus were stored at −80°C until biochemical analysis. Another testis, epididymis, and fat were removed, cleared of adhering connective tissue, and assayed immediately, whereas, sperms in the epididymis were collected by the swim-up technique (21). Briefly, the semen was diluted with two volumes of Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Life Technologies, Grand Island, New York, USA) and then centrifuged at 200 × *g* for 5 min. The supernatant was transferred to another tube. The tube was slanted and incubated for 30 min at 37°C in a 5% CO_2 incubator for further analysis.

Plasma Biochemical Assays

The plasma glucose concentration was determined by glucose enzymatic kit (Randox, Colorato, USA). Plasma insulin

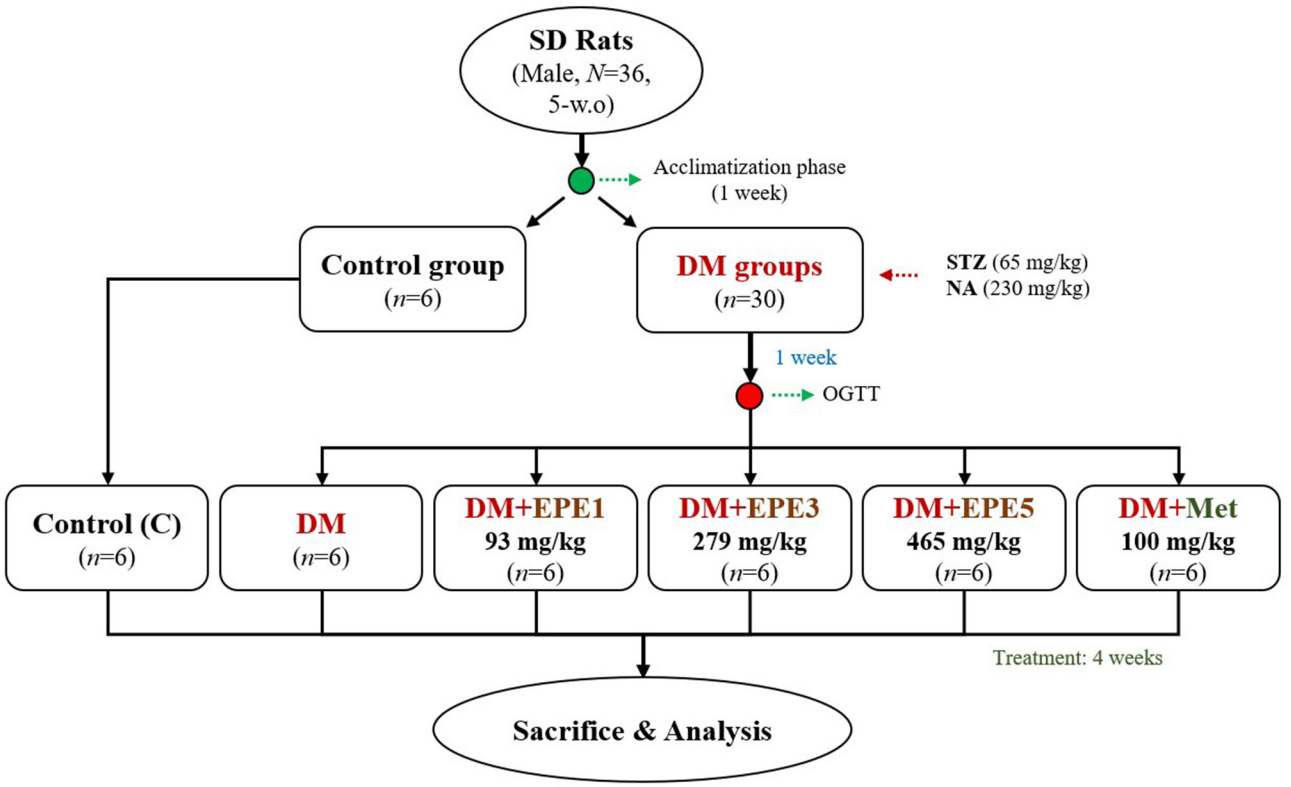


FIGURE 1 | The flowchart of *Echinacea purpurea* ethanol extract treatment against streptozotocin–nicotinamide-induced diabetes male rat model. DM, diabetes mellitus; EPE, *Echinacea purpurea* ethanol extract; Met, metformin.

concentration was measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB Inc., Sylveniusgatan 8A, Uppsala, Sweden). A homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting plasma insulin concentration (mU/ml) times fasting blood glucose (mmol/L) divided by 22.5 according to a previous study (22). Plasma and testis homogenate IL-1β and TNF-α concentrations were measured using ELISA kits (Peprotech, New Jersey, USA) and rat TNF-α ELISA kits (eBioscience, California, USA) according to the manufacturer's instructions, respectively. The protein concentration in the tissue lysate was determined by the Bradford protein assay (23).

Antioxidative Analysis and Reactive Oxygen Species Production

The superoxide dismutase (SOD) and catalase activities, as well as reduced glutathione (GSH) level, were observed in sperms of diabetic rats after 4 weeks of treatments. The SOD was determined by the Ransod kit (Randox, Colorato, USA). Catalase activity was measured according to the previously described method (24). The underlying principle of this approach is that the oxygen bubbles generated from the decomposition of hydrogen peroxide (H_2O_2) by catalase are trapped by the surfactant Triton X-100. The trapped oxygen bubbles are then visualized as foam, the test-tube height of which is measured to quantify the catalase activity. Briefly, each sample (100 μl) was added in a tube. Subsequently, 100 μl of 1% Triton X-100 and 100 μl of undiluted 30% H_2O_2 were added to the solutions and mixed thoroughly and were then incubated at room temperature. Following completion of the reaction, the height of O_2-forming foam that remained constant for 15 min in the test tube was finally measured using a ruler.

The reduced glutathione (GSH) was estimated by using Ellman's reagent (25). The principle of this approach is that Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) reacts with GSH resulting in a product that can be measured at 412 nm. Briefly, plasma or testis homogenate (500 μl) was mixed with 500 μl of 10% trichloroacetic acid. The contents were mixed well for complete precipitation of proteins and centrifuged at 20,000 × *g* for 5 min. An aliquot of clear supernatant (10 μl) was taken and mixed with 85 μl of PBS. Ellman's reagent (5 μl) was added. After 5 min, the optical density was measured at 412 nm against blank.

The plasma and sperm lipid peroxidation levels were measured according to the concentration of thiobarbituric acid reactive species (TBARs), and the amount of produced malondialdehyde (MDA) was used as an index of lipid peroxidation. The testes were homogenized with buffer containing 1.5% potassium chloride to obtain 1:10 (w/v) whole homogenate. Briefly, one volume of the test sample and two volumes of stock reagent (15%, w/v trichloroacetic acid in 0.25 N HCl and 0.375%, w/v thiobarbituric acid in 0.25 N HCl) were mixed in a centrifuge tube. The solution was heated in boiling

water for 15 min. After cooling, the precipitate was removed by centrifugation at 1,500 × *g* for 10 min, and then fluorescence of the supernatant was read at 532 nm against a blank containing all reagents except test sample on a fluorescence spectrophotometer (HITACHI F2000, Tokyo, Japan) (26).

A modified colorimetric nitro blue tetrazolium (NBT) test was used to evaluate superoxide anion production of sperms (27). Briefly, sperm samples, then duplicate samples of 100 μl of washed sperms, were incubated with an equal volume of NBT working reagent (1:10 diluted by RPMI 1640 from 0.01% NBT stock, Sigma-Aldrich, Missouri, USA) at 37°C for 45 min. Following incubation, the samples were washed and centrifuged at 500 × *g* for 10 min in PBS twice to remove all residual NBT solution, leaving only a cell pellet containing formazan. To quantify the formazan product, the intracellular formazan was solubilized in 60 μl of 2 M KOH and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA), and the resulting color reaction was measured spectrophotometrically on a microplate reader (Dynatech MR5000, Switzerland) at 570 nm.

The nitric oxide in the plasma and testis homogenate of the rats was measured by Griess reagent according to the previous method (28). Briefly, the plasma or sperm homogenate was added to a 96-well plate. Then, 100 μl of Griess solution [sulfanilamide in 5% phosphoric acid and N-(1-naphthyl) ethylenediamine in water mixed in the volume ratio 1:1 immediately before use] was added to the wells and incubated for 10 min. After the incubation time, the absorbance was measured at 570 nm by using a spectrophotometer.

Epididymal Sperm Concentration, Motility, and Morphology

After the sperm collection, ∼10 μl of the diluted sperm suspension was transferred to each counting chamber for counting under a light microscope at 200× magnification. Sperm progressive motility was evaluated by an earlier method (29). Briefly, the fluid obtained from the cauda epididymis with a pipette was diluted to 2 ml with buffer solution. A slide was placed on a phase-contrast microscope, and an aliquot of this solution was placed on the slide, and percent motility was evaluated visually at 200× magnification. The method was used for determination of the percentage of morphologically abnormal spermatozoa after adapting the method. A total of 300 sperm cells was examined on each slide, and the head, tail, and total abnormality rates of spermatozoa were expressed as a percentage (30).

Assessment of Sperm Mitochondrial Membrane Potential

The sperm mitochondrial membrane potential (MMP) was measured by using the fluorescent cationic dye Rhodamine 123 (Sigma-Aldrich, Missouri, USA) according to the previous method (31). Rhodamine 123 dissolved in 0.01 M PBS was added to sperm samples at a final concentration of 10 μM and incubated at 37°C for 30 min. After incubation, the tubes were centrifuged at 800 × *g* for 10 min, and sperms were washed twice and resuspended in PBS. The dye fluorescence to reflect the MMP of sperms was measured using FACS Calibur (Becton Dickinson, San Jose, California, USA).

Western Blot

Testis and hypothalamus were lysed by using a radioimmunoprecipitation (RIPA) buffer. Hypothalamus tissue was used to measure the G-protein-coupled receptor 54 (GPR54) expression. The protein concentration in the lysates was determined by Bradford protein assay (23). Equal amounts of protein (50 μg) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and, subsequently, electro-transferred onto polyvinyl difluoride (PVDF) membranes. After blocking with 5% skim milk in TBST for 1 h, the membranes were incubated with the primary antibodies at room temperature for 2–3 h or overnight for 4°C. After washing four times with TBST, the membranes were incubated with the appropriated peroxidase-conjugated secondary antibodies at room temperature for 1 h. The antibody dilution is shown in **Table 1**.

Statistical Analysis

All values were given as mean ± standard error of the mean (SEM). All statistical calculations were done by the SPSS statistics v22.0 (SPSS for Windows Inc., version 22; Chicago, IL, USA) system. One-way ANOVA was used to examine the overall differences between groups, and a Duncan's multiple range test was used to identify significant differences ($p < 0.05$) between the groups.

RESULTS

Glucose, Insulin, and Homeostasis Model Assessment of Insulin Resistance Levels

Figure 2A showed that the glucose level of high dose of EPE (EPE5) and Met groups significantly decreased after 4 weeks of treatment when compared with the untreated diabetes (DM) group. The glucose of medium dose (EPE3) also significantly decreased after 4 weeks of treatment as shown in **Figure 2B** and **Table 2** when compared with the DM group, whereas there are no significant effects on the insulin level. However, the homeostasis model assessment of insulin resistance (HOMA-IR) level increased in the DM group, and its level significantly reduced in the Met, EPE3, and EPE5 groups after treatment for 4 weeks when compared with the DM group.

Antioxidative Properties and Reactive Oxygen Species Levels

The activity of enzymatic antioxidants of the sperm were evaluated after 4 weeks of treatments, such as superoxide dismutase (SOD), catalase, and reduced-type glutathione (GSH) (**Figure 3**). Low SOD activity was shown in the DM group (**Figure 3A**). However, after treatment with the medium and high doses of EPE, the SOD activities were significantly enhanced. The catalase activity was observed to increase in the EPE5 group; however, there was no significant effect (**Figure 3B**), whereas the reduced type of GSH significantly increased in the EPE5 group when compared with the DM group as shown in **Figure 3C**.

The high levels of superoxide anion (O_2^-), nitric oxide (NO), and malondialdehyde (MDA) in sperm and plasma were observed in the DM group (**Figure 4**). The levels of superoxide anion, NO, and MDA significantly reduced in sperms after treatment with medium and high doses of EPE (EPE3 and EPE5) as shown in **Figures 4A,C,E**. The NO and MDA productions in plasma also significantly decreased after treatment with EPE3 and EPE5 (**Figures 4B,D**). As a positive control, metformin administration also showed significant effects on productions of superoxide anion in the sperm and NO and MDA in the plasma.

TABLE 1 | The antibody dilution.

Antibody dilution	Primary antibody	Secondary antibody
Kiss receptor/GPR54	1:1,000 (rabbit)	1:5,000 (goat anti-rabbit)
StAR	1:1,000 (mouse)	1:5,000 (goat anti-mouse)
CYP11A1	1:1,000 (rabbit)	1:5,000 (goat anti-rabbit)
17β-HSD	1:500 (rabbit)	1:5,000 (goat anti-rabbit)
TLR4	1:1,000 (mouse)	1:5,000 (goat anti-mouse)
phosphor-NF-κB p65	1:500 (rabbit)	1:5,000 (goat anti-rabbit)
α-tubulin	1:5,000 (rabbit)	1:5,000 (goat anti-rabbit)
β-actin	1:5,000 (rabbit)	1:5,000 (goat anti-rabbit)
GAPDH	1:5,000 (rabbit)	1:5,000 (goat anti-rabbit)

Toll-Like Receptor, Phosphorylated p65 Subunit of NF-κB, and Proinflammatory Cytokines Expressions

Figure 5 shows that the relative expressions of Toll-like receptor 4 (TLR4) and phosphorylated p65 subunit of NF-κB (phosphor-NF-κB p65) increased in the DM group. These levels significantly reduced after treatment with a high dose of EPE (EPE5) for 4 weeks when compared with the DM group. Metformin also

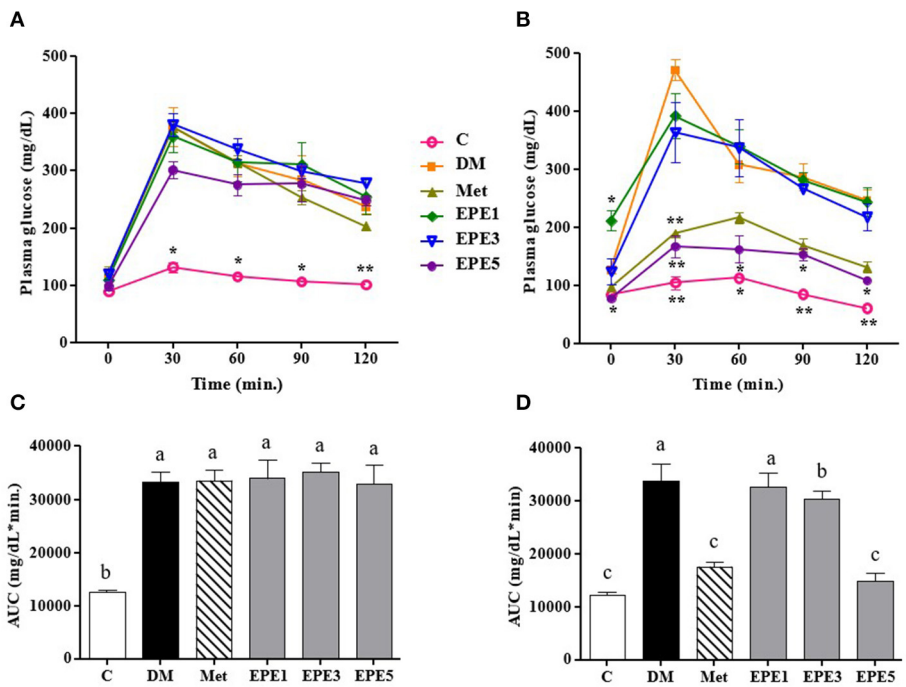


FIGURE 2 | The oral glucose tolerance test (OGTT): a week after streptozotocin (STZ)–nicotinamide (NA) injection or before treatment **(A)** and after treatment **(B)** and the area under the curve (AUC): before treatment **(C)** and after treatment **(D)** in diabetic rats. Data are shown as the mean ± SEM ($n = 6$). Significant difference at $^*p < 0.05$ and $^{**}p < 0.01$ vs. DM, respectively. The values with different superscript letters (a–c) represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

TABLE 2 | Plasma fasting blood glucose level, plasma insulin level, and homeostasis model assessment of insulin resistance (HOMA-IR) in diabetic rats after 4 weeks of treatments.

Properties	C	DM	Met	EPE1	EPE3	EPE5
Glucose (mg/dl)	92.83 ± 16.62^b	162.96 ± 17.17^a	104.63 ± 14.86^{ab}	119.41 ± 20.09^{ab}	80.33 ± 19.61^b	92.59 ± 20.68^b
Insulin (μU/ml)	4.67 ± 1.47^a	5.74 ± 1.60^a	3.62 ± 0.40^a	5.36 ± 1.90^a	5.13 ± 1.93^a	4.70 ± 1.40^a
HOMA-IR	1.35 ± 0.13^b	4.59 ± 0.45^a	1.32 ± 0.09^b	4.33 ± 1.28^a	2.23 ± 0.41^b	1.43 ± 0.33^b

Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a, b) in the same row represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. HOMA-IR = fasting plasma glucose (mmol/L) × fasting plasma insulin (μU/ml)/22.5. C, control; DM, diabetes mellitus; Met, metformin; EPE, Echinacea purpurea ethanol extract.

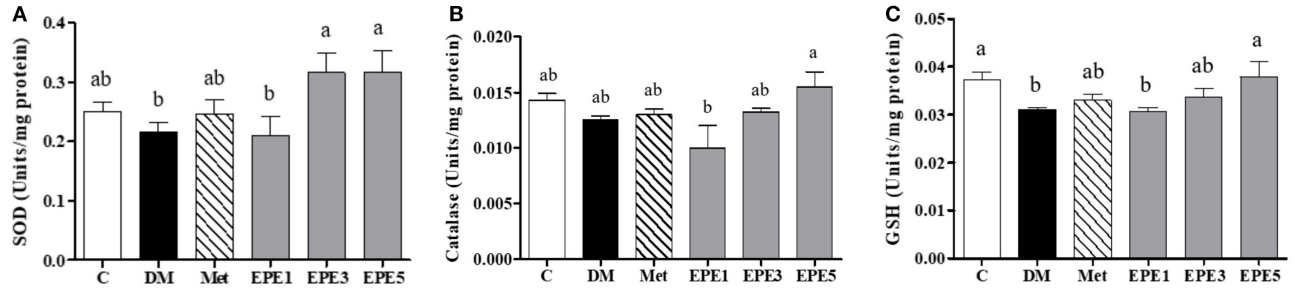


FIGURE 3 | The activities of **(A)** superoxide dismutase (SOD), **(B)** catalase, and **(C)** reduced type glutathione (GSH) of diabetic rats' sperm after 4 weeks of treatments. Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a, b) represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

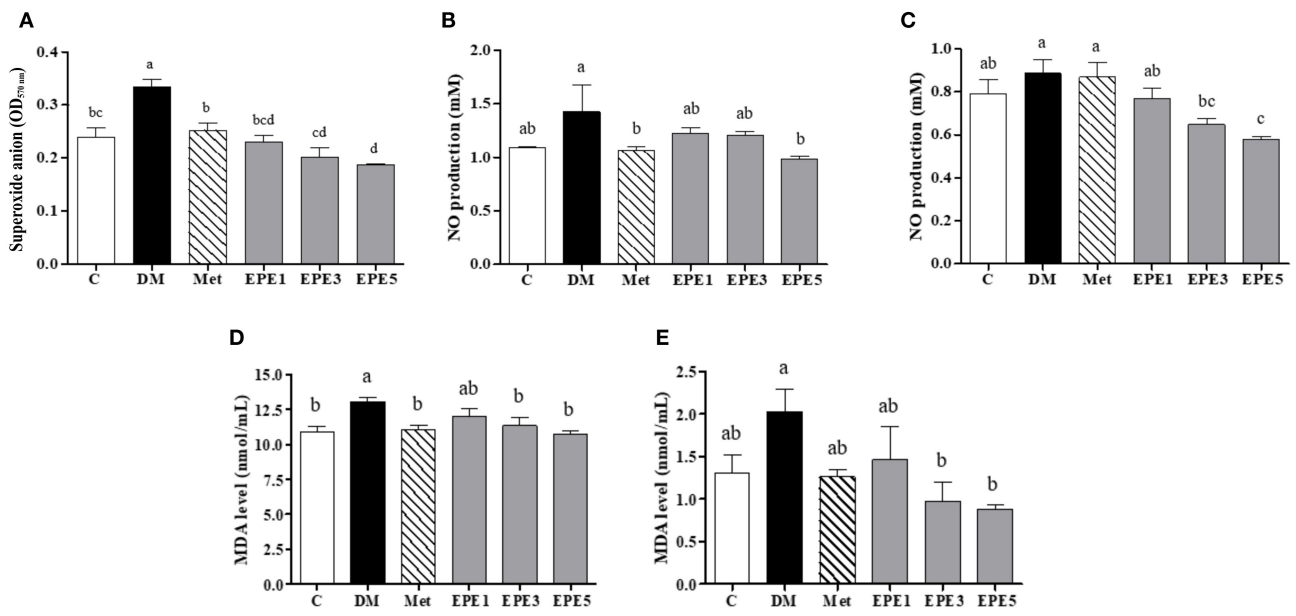


FIGURE 4 | The levels of **(A)** sperm superoxide anion, **(B)** plasma nitric oxide (NO), **(C)** sperm NO, **(D)** plasma malondialdehyde (MDA), and **(E)** sperm MDA in diabetic rats after 4 weeks of treatment. Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a–d) represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

significantly reduced phosphor-NF-κB p65 expression in the rats' testes.

Figure 6 showed that the levels of proinflammatory cytokine increased in the plasma and testes of the DM group, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. As shown in **Figures 6A–D**, these levels were significantly reduced after treatment with medium and high doses of EPE. As a positive control, metformin also significantly reduced the TNF-α level.

Kiss-1 Peptide Receptor, Testosterone Synthesis Enzymes, and Testosterone Expressions

Figure 7A showed that the relative expression of G-protein-coupled receptor (GPR54/Kiss-1 peptide receptor) decreased in the hypothalamus of the DM group. This level increased after treatment with metformin and EPE; however, there are no significant effects for the expression. The expressions of some testosterone synthesis enzymes, such as StAR, CYP11A1, and 17β-HSD proteins also reduced in the testes of the DM group (**Figures 7B–D**). There are no effects on the relative expression of the CYP11A1 protein after treatment with EPE. The expression of the 17β-HSD protein increased after treatment with EPE for 4 weeks; however, there are no significant effects. The StAR protein expression significantly restored after treatment with metformin, and medium and high doses of EPE (EPE3 and EPE5). Additionally, there are no significant effects on plasma testosterone level as shown in **Figure 7E**.

Mitochondria Membrane Potential and Sperm Properties

A low level of mitochondria membrane potential (MMP) was shown in the DM group (**Figure 8**). EPE and metformin

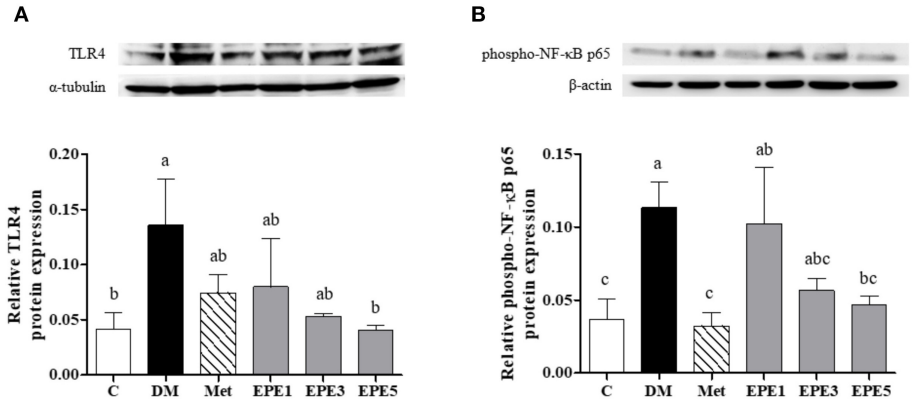


FIGURE 5 | Protein expression of **(A)** Toll-like receptor 4 (TLR4) and **(B)** phosphor-NF-κB p65 in the testes of diabetic rats after 4 weeks of treatments. Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a–c) represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

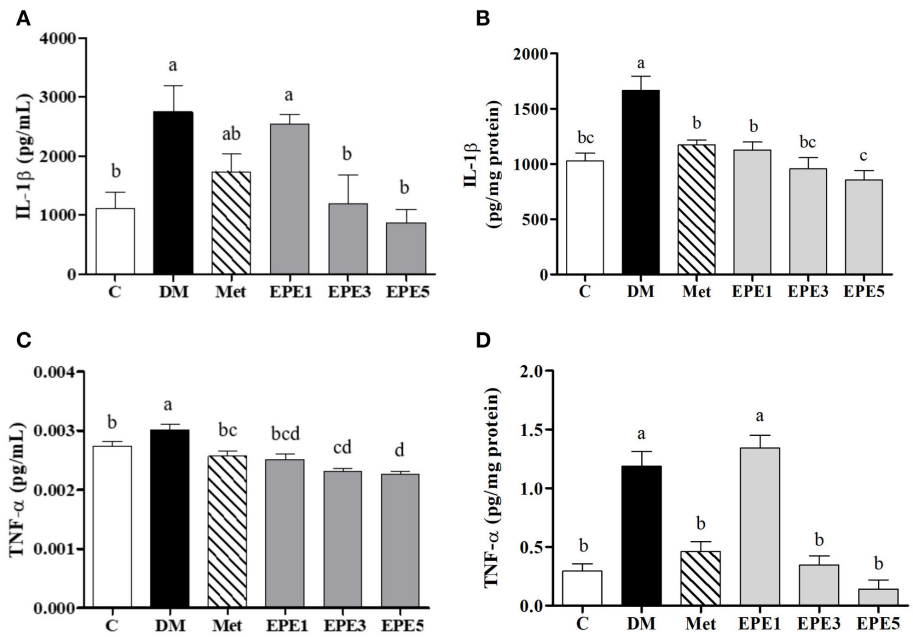


FIGURE 6 | The levels of **(A)** plasma interleukin (IL)-1β, **(B)** testis IL-1β, **(C)** plasma tumor necrosis factor (TNF)-α, and **(D)** testis TNF-α in diabetic rats after 4 weeks of treatments. Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a–d) represent significantly differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

treatment for 4 weeks significantly protected the mitochondrial function by restoring the level of MMP. **Table 3** shows that there are no effects on total sperm count after treatment with EPE for 4 weeks. However, EPE treatment significantly increased sperm progressive motility and decreased sperm abnormalities.

DISCUSSION

In this study, we succeeded in demonstrating the ameliorated effects of *Echinacea purpurea* ethanol extract (EPE) on oxidative stress, proinflammatory cytokines, and sperm properties associated with reproductive dysfunction of diabetic male rats. The diabetic rats were induced intraperitoneally by streptozotocin–nicotinamide (STZ-NA). Then the rats were treated with three doses of EPE and metformin (a positive control) for 4 weeks. Failure of insulin action is a characteristic of type 2 diabetes and also known as non-insulin-dependent diabetes mellitus (NIDDM) (32).

In this present study, the diabetes condition was confirmed by high levels of glucose and the homeostasis model assessment of insulin resistance (HOMA-IR) as shown in **Figure 2** and **Table 2**.

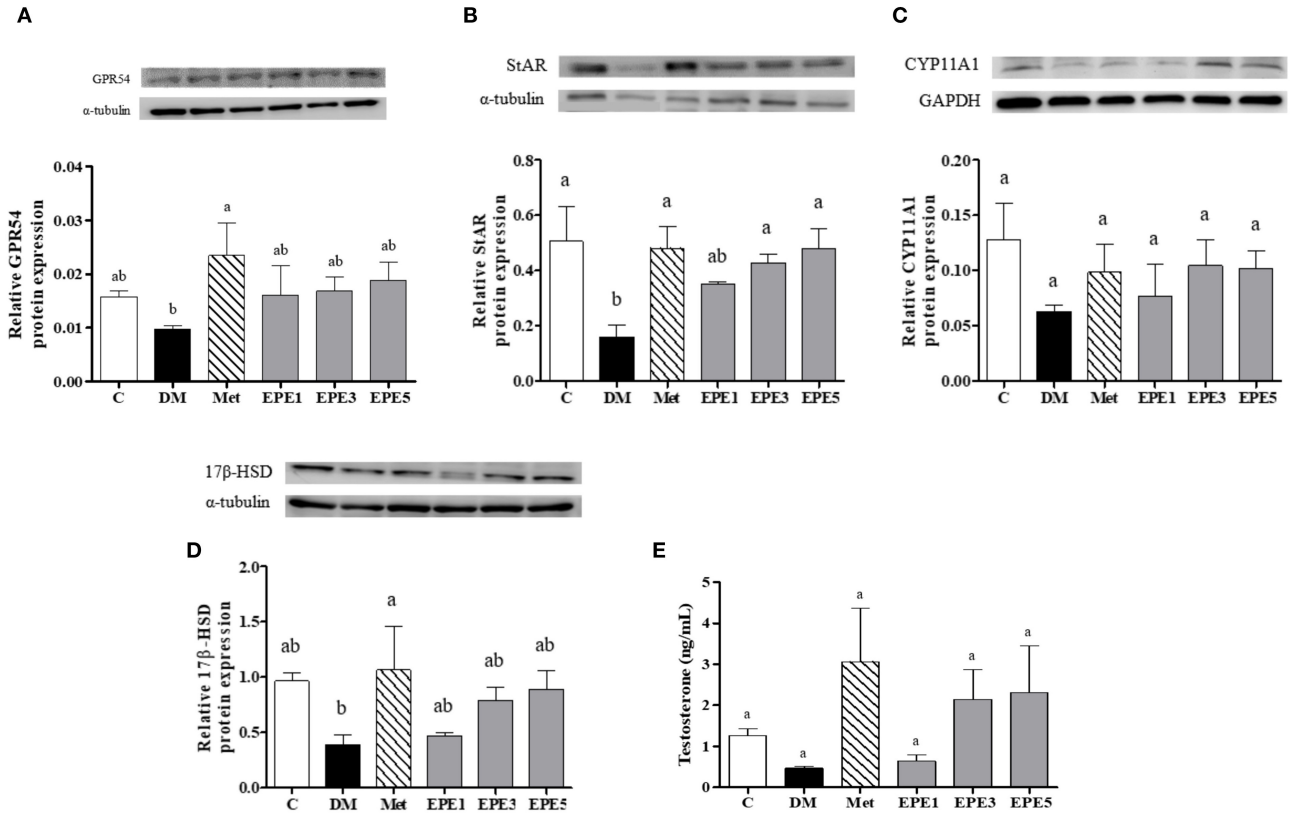


FIGURE 7 | Protein expression of **(A)** GPR54 (Kiss-1 receptor) in hypothalamus, **(B–D)** testosterone synthesis enzymes in testis, and **(E)** plasma testosterone level in diabetic rats after 4 weeks of treatment. Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a,b) represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

A previous reference reported that if the glucose concentration ≥200 mg/dl at 2 h post-load, glucose in the OGTT was confirmed as a provisional diagnosis of diabetes (17). The STZ–NA injection increases glucose levels in the diabetic animal model. This condition, due to STZ injection, triggers damage of the pancreatic β-cells, and the cell was partially protected from STZ by NA (15). An experiment with an STZ–NA to induce a diabetic condition also shows high levels of plasma glucose (2-h OGTT ≥200 mg/dl) and HOMA-IR in rat models as reported by a previous study (7). Additionally, a previous study also reported that 65 mg/kg of STZ with 230 mg/kg of NA injection was confirmed as a diabetes condition (15). The high dose of EPE administration successfully decreased plasma glucose and HOMA-IR levels of diabetic rats after 4 weeks of treatment.

Reducing enzymatic antioxidant activities especially superoxide dismutase (SOD) was observed in the untreated diabetes (DM) group. It also showed an increase in oxidative stress markers such as superoxide anion (O_2^-), nitric oxide (NO), and malondialdehyde (MDA) in plasma and sperm as shown in **Figures 3**, **4**. Reactive oxygen species (ROS) including O_2^- have been implicated in diabetes pathology, and they are involved in cell damage and insulin resistance (33). A high level of superoxide anion causes tissue damage. However, the presence of SOD protects tissues from oxidative damage by converting O_2^- to hydrogen peroxide (H_2O_2) (34). The SOD activity was enhanced by treatment with medium and high doses of EPE. This condition also results in a reduction of oxidative stress markers. Additionally, the reduced type of glutathione (GSH) also increased after high doses of EPE treatment. High levels of GSH confirmed that the EPE extract successfully ameliorates oxidative stress in the diabetic model. A low level of GSH is a marker for oxidative stress conditions (35).

A high level of Toll-like receptor 4 (TLR4) and phosphorylated p65 subunit of NF-κB (phospho-NF-κB p65) was observed in the untreated diabetes (DM) group (**Figure 5**). Toll-like receptor 4 is a cell surface receptor that involves immune responses by triggering activation of transcription factor and kinase cascade signaling. The TLR4 also involves insulin resistance (IR) and inflammation developments. TLR4 is also an upstream regulator of nuclear factor (NF)-κB activation. A high level of proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, was observed as the cascade signaling the TLR4 receptor (36). This present study also reported a high expression of phosphorylated p65 subunit of NF-κB and a high level of IL-1β and TNF-α (**Figure 6**). A previous study reported that activation of NF-kB transcription factor plays an important role in diabetes complications. Additionally, NF-kB activation is caused by oxidative stress (37). A high blood glucose

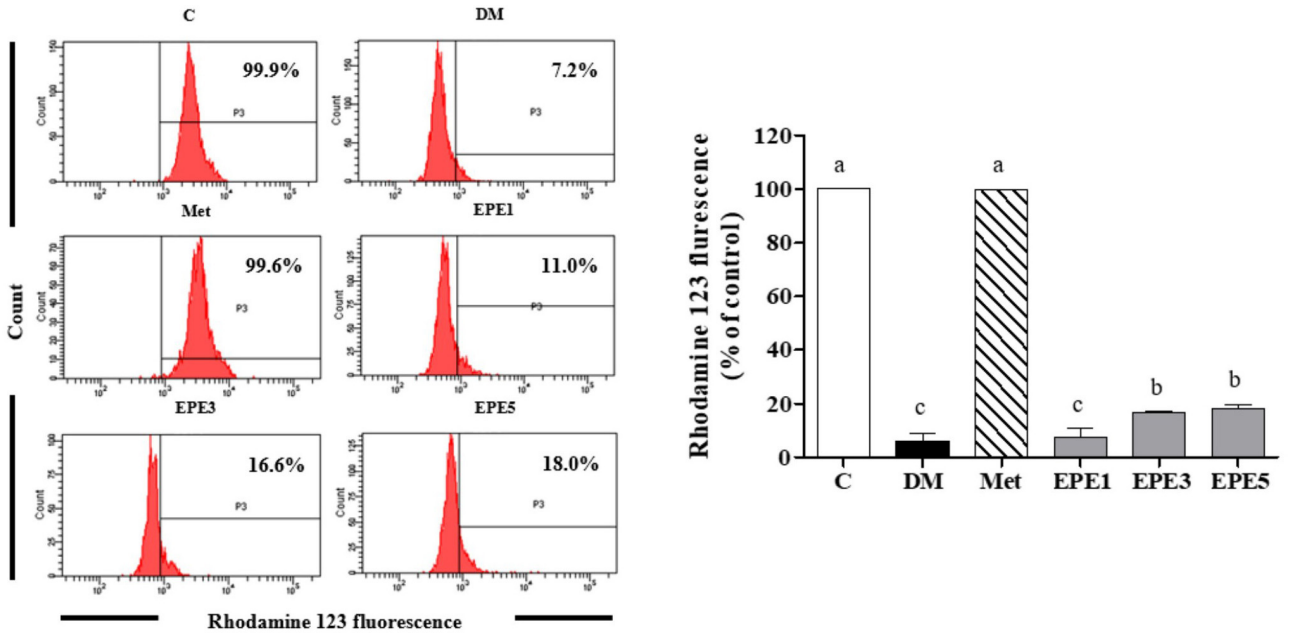


FIGURE 8 | The levels of mitochondria membrane potential (MMP) of sperm in diabetic rats after 4 weeks of treatments. Data are shown as the mean ± SEM ($n = 6$). The values with different superscript letters (a–c) represent significant differences ($p < 0.05$) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, *Echinacea purpurea* ethanol extract.

TABLE 3 | The total sperm count, sperm progressive motility, and sperm abnormalities of diabetic rats after 4 weeks of treatments.

Sperm properties	C	DM	Met	EPE1	EPE3	EPE5
Total count (10^5)	41.70 ± 3.21^{a}	38.80 ± 1.28^{a}	35.50 ± 4.40^{a}	37.60 ± 1.90^{a}	38.10 ± 3.08^{a}	41.2 ± 7.60^{a}
Progressive motility (%)	18.53 ± 1.18^{a}	9.03 ± 2.06^{c}	16.61 ± 2.06^{ab}	12.07 ± 1.70^{bc}	16.12 ± 0.62^{ab}	20.17 ± 1.71^{a}
Abnormalities (%)	2.10 ± 0.63^{d}	11.73 ± 0.91^{a}	4.74 ± 0.63^{c}	9.06 ± 1.11^{b}	4.24 ± 0.22^{cd}	3.30 ± 0.21^{cd}

Data are shown as the mean ± SEM (n = 6). The values with different superscript letters (a–d) in the same row represent significant differences (p < 0.05) as analyzed by Duncan's multiple range test. C, control; DM, diabetes mellitus; Met, metformin; EPE, Echinacea purpurea ethanol extract.

or a hyperglycemia condition has been considered to trigger oxidative stress and also increase proinflammatory cytokines, such as IL-1β and TNF-α (38, 39). These protein expressions were successfully increased by EPE administration after 4 weeks of treatment.

An untreated diabetes (DM) group showed a low expression of Kiss1 protein receptor (G-protein-coupled receptor 54, GPR54) (**Figure 7**). A low expression of this protein might be caused by the increasing level of proinflammatory cytokines. Additionally, a previous study reported that GPR54 expression was reduced by TNF-α (40). This condition improved by EPE treatment. In this study, we also observed some steroidogenesis-related genes, such as steroidogenic acute regulatory (StAR) protein, cytochrome P450 enzyme (CYP11A1), and 17β-hydroxysteroid dehydrogenase (HSD). A low expression of StAR protein was shown in the untreated diabetes (DM) group. The StAR protein plays an important role in the biosynthesis of steroid hormone. It acts as mediator of cholesterol transport across the mitochondrial membrane during steroidogenesis, whereas cholesterol has been known as a common precursor substrate of steroid hormones (41, 42). The StAR protein expression was successfully improved after EPE treatment.

This present study also reported a low level of mitochondrial membrane potential (MMP) in the untreated diabetes (DM) group. This condition was successfully ameliorated by EPE treatment (**Figure 8**). The MMP has been used to measure the mitochondrial function as an indicator of cell health (43). A previous study reported that male fertility can be affected by diabetes. This condition was characterized by low DNA integrity and sperm motility (44). The EPE treatment successfully increased sperm motility and reduced the sperm abnormalities (**Table 3**).

CONCLUSION

The streptozotocin–nicotinamide injection successfully induced diabetic conditions and is involved with male reproductive dysfunction. Diabetes disease was characterized by a high glucose level (hyperglycemia) and caused oxidative stress as well as regulated Toll-like receptor expression, increased expression of nuclear factor-kappa B transcription factor,

and proinflammatory cytokines, whereas male reproductive dysfunction was characterized by low expression of G-protein-coupled receptor, mitochondrial membrane receptor, and low quality of the sperm. However, after 4 weeks of oral administration of *Echinacea purpurea* ethanol extract, diabetes condition was successfully ameliorated and male reproductive dysfunction was also improved.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC Approval No. 103033) of the National Taiwan Ocean University.

AUTHOR CONTRIBUTIONS

Z-LK conceptualized the study. C-CL and C-FM conducted the formal analysis. C-CL and SS wrote the original draft. DT, SS, and Z-LK wrote, reviewed, and edited the article. All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. Wang P, Fiaschi-Taesch NM, Vasavada RC, Scott DK, García-Ocaña A, Stewart AF. Diabetes mellitus—advances and challenges in human β-cell proliferation. *Nat Rev Endocrinol.* (2015) 11:201. doi: 10.1038/nrendo.2015.9
2. Poitout V, Robertson RP. Minireview: secondary β-cell failure in type 2 diabetes—a convergence of glucotoxicity and lipotoxicity. *Endocrinology.* (2002) 143:339–42. doi: 10.1210/endo.143.2.8623
3. Alsharari SD, Al-Rejaie SS, Abuohashish HM, Aleisa AM, Parmar MY, Ahmed MM. Ameliorative potential of morin in streptozotocin-induced neuropathic pain in rats. *Trop J Pharm Res.* (2014) 13:1429–36. doi: 10.4314/tjpr.v13i9.8
4. Abd El-Twab SM, Mohamed HM, Mahmoud AM. Taurine and pioglitazone attenuate diabetes-induced testicular damage by abrogation of oxidative stress and up-regulation of the pituitary–gonadal axis. *Can J Physiol Pharmacol.* (2016) 94:651–61. doi: 10.1139/cjpp-2015-0503
5. Guneli E, Tugyan K, Ozturk H, Gumustekin M, Cilaker S, Uysal N. Effect of melatonin on testicular damage in streptozotocin-induced diabetes rats. *Euro Surg Res.* (2008) 40:354–60. doi: 10.1159/000118032
6. Marín-Peñalver JJ, Martín-Timón I, Sevillano-Collantes C, Cañizo-Gómez FJD. Update on the treatment of type 2 diabetes mellitus. *World J Diabetes.* (2016) 7:354–95. doi: 10.4239/wjd.v7.i17.354
7. Kong Z-L, Sudirman S, Hsu Y-C, Su C-Y, Kuo H-P. Fucoxanthin-rich brown algae extract improves male reproductive function on streptozotocin-nicotinamide-induced diabetic rat model. *Int J Mol Sci.* (2019) 20:4485. doi: 10.3390/ijms20184485
8. Kong Z-L, He J-L, Sudirman S, Kuo M-T, Miao S, Chang K-LB, et al. Nanoparticles of antroquinonol-rich extract from solid-state-cultured antrodia cinnamomea improve reproductive function in diabetic male rats. *Int J Nanomed Vol.* (2020) 15:4191–203. doi: 10.2147/IJN.S252885
9. Saeidnia S, Manayi A, Vazirian M. *Echinacea purpurea*: pharmacology, phytochemistry and analysis methods. *Pharmacogn Rev.* (2015) 9:63–72. doi: 10.4103/0973-7847.156353
10. Barrett B. Medicinal properties of *Echinacea*: a critical review. *Phytomedicine.* (2003) 10:66–86. doi: 10.1078/094471103321648692
11. Hudson JB. Applications of the phytomedicine *Echinacea purpurea* (purple coneflower) in infectious diseases. *J Biomed Biotechnology.* (2012) 2012:1–16. doi: 10.1155/2012/769896
12. Mao C-F, Zhang X-R, Johnson A, He J-L, Kong Z-L. Modulation of diabetes mellitus-induced male rat reproductive dysfunction with micro-nanoencapsulated *Echinacea purpurea* ethanol extract. *BioMed Res Int.* (2018) 2018:1–17. doi: 10.1155/2018/4237354
13. Chen Y-L, Sung J-M, Lin S-D. Effect of extraction methods on the active compounds and antioxidant properties of ethanolic extracts of *Echinacea purpurea* flower. *Am J Plant Sci.* (2015) 6:201–12. doi: 10.4236/ajps.2015.61023.y
14. Aarland RC, Bañuelos-Hernández AE, Fragoso-Serrano M, Sierra-Palacios EDC, Díaz De León-Sánchez F, Pérez-Flores LJ, et al. Studies on phytochemical, antioxidant, anti-inflammatory, hypoglycaemic and antiproliferative activities of *Echinacea purpurea* and *Echinacea angustifolia* extracts. *Pharm Biol.* (2017) 55:649–56. doi: 10.1080/13880209.2016.1265989
15. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D, et al. Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide. *Diabetes.* (1998) 47:224–9. doi: 10.2337/diab.47.2.224
16. Ghasemi A, Khalifi S, Jedi S. Streptozotocin-nicotinamide-induced rat model of type 2 diabetes (review). *Acta Physiol Hungarica.* (2014) 101:408–20. doi: 10.1556/APhysiol.101.2014.4.2
17. American Diabetes Association A. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* (2008) 32:S62–7. doi: 10.2337/dc08-S055
18. Kinaan M, Ding H, Triggle CR. Metformin: an old drug for the treatment of diabetes but a new drug for the protection of the endothelium. *Med Principles Prac.* (2015) 24:401–15. doi: 10.1159/000381643
19. Bartoli E, Fra GP, Schianca GPC. The oral glucose tolerance test (OGTT) revisited. *Euro J Internal Med.* (2011) 22:8–12. doi: 10.1016/j.ejim.2010.07.008
20. Mussbacher M, Schrottmaier WC, Salzmann M, Brostjan C, Schmid JA, Starlinger P, et al. Optimized plasma preparation is essential to monitor platelet-stored molecules in humans. *PLoS ONE.* (2017) 12:e0188921. doi: 10.1371/journal.pone.0188921
21. Younglai EV, Holt D, Brown P, Jurisicova A, Casper RF. Sperm swim-up techniques and DNA fragmentation. *Human Reprod.* (2001) 16:1950–3. doi: 10.1093/humrep/16.9.1950
22. Yashpal S, Mk G, Nikhil T, Kumar MR. A study of insulin resistance by HOMA-IR and its cut-off value to identify metabolic syndrome in urban Indian adolescents. *J Clin Res Pediatric Endocrinol.* (2013) 5:245–51. doi: 10.4274/Jcrpe.1127
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem..* (1976) 72:248–54. doi: 10.1016/0003-2697(76)90527-3
24. Iwase T, Tajima A, Sugimoto S, Okuda K-I, Hironaka I, Kamata Y, et al. A simple assay for measuring catalase activity: a visual approach. *Sci Rep.* (2013) 3:3081. doi: 10.1038/srep03081
25. Giustarini D, Fanti P, Matteucci E, Rossi R. Micro-method for the determination of glutathione in human blood. *J Chromatogr B.* (2014) 964:191–4. doi: 10.1016/j.jchromb.2014.02.018
26. Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Anal Biochem.* (1966) 16:359–64. doi: 10.1016/0003-2697(66)90167-9
27. Tunc O, Thompson J, Tremellen K. Development of the NBT assay as a marker of sperm oxidative stress. *Int J Androl.* (2010) 33:13–21. doi: 10.1111/j.1365-2605.2008.00941.x
28. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using griess reaction assay. *Sensors.* (2003) 3:276–84. doi: 10.3390/s30800276
29. Sönmez M, Türk G, Yüce A. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. *Theriogenology.* (2005) 63:2063–2072. doi: 10.1016/j.theriogenology.2004.10.003

30. Burruel VR, Yanagimachi R, Whitten WK. Normal mice develop from oocytes injected with spermatozoa with grossly misshapen heads1. *Biol Reprod.* (1996) 55:709–14. doi: 10.1095/biolreprod55.3.709
31. Palmeira CM, Moreno AJM, Madeira VMC, Wallace KB. Continuous monitoring of mitochondrial membrane potential in hepatocyte cell suspensions. *J Pharmacol Toxicol Methods.* (1996) 35:35–43. doi: 10.1016/1056-8719(95)00131-X
32. Olokoba AB, Obateru OA, Olokoba LB. Type 2 diabetes mellitus: a review of current trends. *Oman Med J.* (2012) 27:269–73. doi: 10.5001/omj.2012.68
33. Kaneto H, Katakami N, Matsuhisa M, Matsuoka T-A. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediators Inflamm.* (2010) 2010:1–11. doi: 10.1155/2010/453892
34. Shim S-Y, Kim H-S. Oxidative stress and the antioxidant enzyme system in the developing brain. *Korean J Pediatr.* (2013) 56:107–11. doi: 10.3345/kjp.2013.56.3.107
35. Calabrese V, Cornelius C, Leso V, Trovato-Salinaro A, Ventimiglia B, Cavallaro M, et al. Oxidative stress, glutathione status, sirtuin and cellular stress response in type 2 diabetes. *Biochim Biophys Acta Mol Basis Dis.* (2012) 1822:729–36. doi: 10.1016/j.bbadis.2011.12.003
36. Kim JJ, Sears DD. TLR4 and insulin resistance. *Gastroenterol Res Prac.* (2010) 2010:1–11. doi: 10.1155/2010/212563
37. Romeo G, Liu WH, Asnaghi V, Kern TS, Lorenzi M. Activation of nuclear factor- B induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. *Diabetes.* (2002) 51:2241–8. doi: 10.2337/diabetes.51.7.2241
38. Dogan Y, Akarsu S, Ustundag B, Yilmaz E, Gurgoze MK. Serum IL-1β, IL-2, and IL-6 in Insulin-dependent diabetic children. *Mediators Inflamm.* (2006) 2006:1–6. doi: 10.1155/MI/2006/59206
39. Gonzalez Y, Herrera MT, Soldevila G, Garcia-Garcia L, Fabián G, Pérez-Armendariz EM, et al. High glucose concentrations induce TNF-α production through the down-regulation of CD33 in primary human monocytes. *BMC Immunol.* (2012) 13:19. doi: 10.1186/1471-2172-13-19
40. Sarchielli E, Comeglio P, Squecco R, Ballerini L, Mello T, Guarnieri G, et al. Tumor necrosis factor α impairs Kisspeptin signaling in human gonadotropin-releasing hormone primary neurons. *J Clin Endocrinol Metab.* (2016) 102:46–56. doi: 10.1210/jc.2016-2115
41. Stocco DM. The role of the StAR protein in steroidogenesis: challenges for the future. *J Endocrinol.* (2000) 164:247–53. doi: 10.1677/joe.0.1640247
42. Falvo S, Chieffi Baccaria G, Spaziano G, Rosati L, Venditti M, Di Fiore MM, et al. StAR protein and steroidogenic enzyme expressions in the rat Harderian gland. *Comptes Rendus Biol.* (2018) 341:160–6. doi: 10.1016/j.crvi.2018.02.001
43. Sakamuru S, Attene-Ramos MS, Xia M. Chapter 2 mitochondrial membrane potential assay. In: Zhu H, Xia M, editors. *High-Throughput Screening Assays in Toxicology, Methods in Molecular Biology, Vol. 1473.* New York, NY: Springer Science+Business Media (2016). p. 17–22. doi: 10.1007/978-1-4939-6346-1_2
44. Roessner C, Paasch U, Kratzsch J, Glander H-J, Grunewald S. Sperm apoptosis signalling in diabetic men. *Reprod BioMed Online.* (2012) 25:292–9. doi: 10.1016/j.rbmo.2012.06.004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 07 May 2021
doi: 10.3389/fvets.2021.650572

Influence of Non-conventional Sperm Quality Parameters on Field Fertility in Ovine

Noelia Mendoza[1†], Adriana Casao[1†], Juan Domingo[1], Francisco Quintín[2], Adolfo Laviña[3], Enrique Fantova[4], José Álvaro Cebrián-Pérez[1], Teresa Muiño-Blanco[1] and Rosaura Pérez-Pe[1]*

[1] Grupo BIOFITER-Departamento de Bioquímica y Biología Molecular y Celular—Instituto Universitario de Investigación en Ciencias Ambientales de Aragón (IUCA), Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain, [2] Centro de Transferencia Agroalimentaria (Gobierno de Aragón), Zaragoza, Spain, [3] Asociación nacional de criadores de ganado ovino de la raza rasa aragonesa (ANGRA), Zaragoza, Spain, [4] Unión de Productores de Raza Rasa Aragonesa (UPRA)-Grupo Pastores, Zaragoza, Spain

The prediction of the fertilizing ability of a seminal dose continues to be a primary aim in the field of artificial insemination (AI). To achieve this goal, in this study we have included the evaluation of some non-conventional sperm quality markers. A total of 3,906 ewes from 52 different farms were inseminated with 357 refrigerated seminal doses obtained from 45 mature *Rasa Aragonesa* rams. The same samples were used for sperm quality analysis including membrane integrity, capacitation status, oxygen consumption and apoptotic-like markers such as phosphatidylserine translocation (PS), plasmalemma disorganization/mitochondrial membrane potential, caspase activation and DNA damage. Seminal doses from the breeding (B) season presented higher percentages of intact membrane (IM), non permeant (NP) membrane with high mitochondrial membrane potential ($\Delta\Psi$m) and IM without PS translocation spermatozoa than those from the non-breeding (NB) season. Therefore, we can conclude that there were less spermatozoa showing apoptotic-like features in the seminal doses from the B than the NB season, although these differences did not affect field fertility. Only the percentage of intact membrane, non-capacitated (IM-NC) spermatozoa showed a significant correlation with *in vivo* fertility ($P = 0.005$) and fecundity ($P = 0.007$) values obtained after cervical AI when all data were evaluated. When the data were sorted by season and distance to the farms where AI was performed, the correlation between the percentage of IM-NC spermatozoa and reproductive parameters increased in the NB season and progressively with remoteness from the farms. Some other sperm parameters, like NP with high $\Delta\Psi$m, IM sperm without active caspases and DNA-intact spermatozoa, also showed significant correlations with the reproductive parameters in the sorted data. Moreover, the increment in both the percentage of IM-NC and DNA-intact spermatozoa would increase the probability of obtaining a fertility higher than the mean (>52%), as revealed by a multiple logistic regression analysis. In conclusion, we have identified two seminal markers—the percentage of intact membrane, non-capacitated spermatozoa, and DNA intact spermatozoa—which could be used as a test to discard males in AI programs, which is highly important from an economic point of view and can contribute to achieving satisfactory fertility rates.

Keywords: ram, semen, quality, season, fertility, capacitation, apoptosis

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Jose Antonio Tapia, University of Extremadura, Spain
María Gracia Gervasi, University of Massachusetts Amherst, United States

***Correspondence:**
Rosaura Pérez-Pe
rosperez@unizar.es

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *07 January 2021*
Accepted: *06 April 2021*
Published: *07 May 2021*

Citation:
Mendoza N, Casao A, Domingo J, Quintín F, Laviña A, Fantova E, Cebrián-Pérez JÁ, Muiño-Blanco T and Pérez-Pe R (2021) Influence of Non-conventional Sperm Quality Parameters on Field Fertility in Ovine. Front. Vet. Sci. 8:650572. doi: 10.3389/fvets.2021.650572

INTRODUCTION

The use of spermatozoa with high fertilizing potential is critical for obtaining good results in reproductive technologies with domestic animals, but this implies the need to know in advance the fertilizing ability of a seminal dose. It is generally assumed that ejaculated spermatozoa with good fertilizing capacity must have progressive motility, normal morphology and intact plasma and acrosomal membranes. However, the standard semen analyses based only on these parameters do not permit an accurate assessment of all sperm functional characteristics, due to over- or under-estimating the fertilizing capacity of a given sample (1–3). Therefore, the consideration of new aspects for the assessment of sperm functionality might help to establish the quality of a seminal dose (4, 5). Fertilization is a process that requires several sperm capacities, and thus the combination of different analysis techniques of semen quality and/or sperm functionality would allow improving the predictive power for the fertility rate (6–8).

Capacitation is a prerequisite for achieving fertilization, but this sperm state must be attained in the oocyte's surroundings. It is well-known that cryopreservation induces an increment in the percentage of capacitated spermatozoa but, in certain species, this increment happens even during cooling to temperatures above freezing (9–12). So, this premature capacitation could diminish the fertility achieved with refrigerated seminal doses when artificial insemination is carried out. Refrigeration has also accounted for an increase in spermatozoa showing certain features related with apoptosis in somatic cells, such as phosphatidylserine translocation, DNA fragmentation, activation of caspases or mitochondrial impairment (13–15). All these types of sub-lethal damage could also impair the reproductive success of refrigerated seminal samples. However, the detrimental effect of apoptotic spermatozoa on field fertility in ovine has not been reported to date. Oxygen is essential for the generation of cellular energy (ATP) via oxidative phosphorylation, and thus oxygen consumption is a key indicator of metabolic activity within cells (16). However, there are scarce reports that link oxygen consumption and fertility (17).

Fertility results in ovine are influenced by seasonality. Seasonal changes in testicular size, sperm production, mating activity and fertility have been described in rams (18, 19). Likewise, differences between breeding and non-breeding seasons in some sperm quality parameters and seminal plasma composition have been shown (20–23).

This work's main objective was to investigate a possible correlation between non-conventional sperm quality parameters, including apoptosis-related markers, capacitation status and oxygen consumption, and field fertility results after artificial insemination (AI) with refrigerated ram semen samples. Moreover, differences in the parameters between samples obtained in the breeding and non-breeding seasons were examined.

MATERIALS AND METHODS

Sperm Collection and Seminal Dose Preparation

All the experiments were performed using semen samples obtained during the course of 3 years from 45 mature *Rasa Aragonesa* rams maintained at the Centro de Transferencia Agroalimentaria (CTA) in Zaragoza, Spain. Samples were obtained throughout the year, but for comparing results we divided the data between breeding (B, September to March) and non-breeding (NB, April to August) seasons. All animal procedures were performed in accordance with the Spanish Animal Protection Regulation RD1201/05, which conforms to European Union Regulation 2010/63. Approval from the Ethics Committee of the University of Zaragoza was not a prerequisite for this study since we worked with the obtained semen samples. The semen samples were collected using an artificial vagina, within the regular collecting schedule at the AI station. A total of 357 different ejaculates were used in this study. Sperm concentration was calculated in duplicate using a Neubauer chamber (Marienfeld, Germany). Progressive individual motility evaluations were assessed subjectively by visual estimation under a phase-contrast microscope at × 100 magnification, maintained at 37°C. All the samples used ranged between 80 and 85% motility. Semen was diluted to 4×10^8 cells/mL and seminal doses of 0.25 mL were prepared in straws and kept at 15°C until insemination time. Two straws of each sample were sent at the same temperature to our laboratory for analysis.

The artificial inseminations were carried out by the technical teams of ANGRA (National Association of *Rasa Aragonesa* Breeding) and UPRA-Grupo Pastores on different farms owned by these cooperative companies. A total of 3,906 ewes from 52 farms were inseminated. Data reported by the cooperatives were fertility (proportion of ewes that lambed per ewes inseminated), fecundity (number of lambs born per ewes inseminated) and prolificacy (number of lambs born per ewe lambed).

From now on these above-mentioned cooperatives will be named as Coop 1 and Coop 2. An essential difference in the reproductive management between both cooperatives was the use of melatonin implants in rams belonging to Coop 2 during the non-breeding season to avoid the reproductive effects of seasonality.

Preparation of Sperm Samples for Laboratory Analyses

In order to remove the dilution medium, 200 μL of each straw was diluted up to 1 mL with PBS and washed with 7 mL of sucrose buffer (10 mM NaCl, 222 mM sucrose, 2.5 mM KCl, 20 mM HEPES, 10 mM glucose, 1 mg/mL polyvinyl alcohol and polyvinyl pyrrolidone, pH 7.5) by centrifugation at 500 × g for 10 min. The supernatant was removed, leaving 1 mL to resuspend the pellet, and the sperm concentration was calculated using the Neubauer chamber (Marienfeld, Germany).

Flow Cytometry Analysis

All the measurements were performed on a Beckman Coulter FC 500 (Beckman Coulter Inc., Brea, CA) with CXP software, equipped with two lasers of excitation (Argon ion laser 488 nm and solid-state laser 633 nm) and 5 filters of absorbance (FL1-525, FL2- 575, FL3-610, FL4-675 and FL5-755, ± 5 nm each bandpass filter). A minimum of 20,000 events was recorded in all the experiments. The sperm population was gated for further analysis on the basis of its specific forward (FS) and side scatter (SS) properties; other non-sperm events were excluded. A flow rate stabilized at 200–300 cells/s was used.

Evaluation of Sperm Membrane Integrity

To determine plasma membrane integrity, 3 μL of each stain, 1 mM carboxyfluorescein diacetate (CFDA) and 1.5 mM propidium iodide (PI) (both from Sigma-Aldrich, subsidiary of Merck KGaA, Darmstadt, Germany), and 3 μL formaldehyde (0.005% final concentration) were added to 300 μL of sperm samples (final concentration of 5×10^6 cells/mL), based on a modification of the procedure described by Harrison and Vickers (24). Samples were incubated at 37°C in darkness for 15 min. The monitored parameters were FS log, SS log, FL1 (CFDA) and FL4 (PI). For the gated sperm cells, percentages of intact membrane (IM; CFDA+/PI-) spermatozoa were evaluated.

Evaluation of the Plasmalemma Stability and Mitochondrial Membrane Potential

A double stain technique (25, 26) was performed with slight modifications, using YO-PRO-1 (1 mM in DMSO) and MitoTracker Deep Red (MitoT 10 μM in DMSO), both from Thermo Fisher Scientific (Waltham, MA, USA). YO-PRO-1 is a DNA dye used as an apoptotic marker because it only permeates into cells that are beginning to undergo apoptosis. MitoTracker is a mitochondrion-selective stain that passively diffuses across the plasma membrane and accumulates in active mitochondria and is used to evaluate mitochondrial membrane potential ($\Delta\Psi$m). Two μL of each dye were added to 300 μl sperm samples (4×10^7 spermatozoa) that were incubated at RT in darkness for 15 min. The monitored parameters were FS log, SS log, FL1 (YO-PRO-1) and FL5 (MitoT).

The MitoTracker data were analyzed as suggested by Hallap et al. (27) considering spermatozoa with high fluorescence as cells with high $\Delta\Psi$m. Four different sperm populations were classified according to staining (28): YO-PRO-1 -/MitoT+, non-permeant (NP) membrane cells with high $\Delta\Psi$m; YO-PRO-1 -/MitoT-, NP cells with low $\Delta\Psi$m; YO-PRO-1 +/MitoT-, apoptotic cells that have lost the integrity of their plasmalemma and with low $\Delta\Psi$m; and YO-PRO-1 +/MitoT+, cells that have lost the integrity of their plasmalemma although with high $\Delta\Psi$m.

Detection of Membrane Phosphatidylserine (PS) Translocation

Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). We used the simultaneous staining with FITC- Annexin V (Thermo Fisher Scientific, Waltham, MA, USA) to detect PS translocation and propidium iodide (PI, Sigma-Aldrich, subsidiary of Merck KGaA, Darmstadt, Germany) to differentiate between live and dead cells, with or without PS translocation. Aliquots of 300 μL (4×10^6 cells) were stained with FITC-Annexin V (1 μL) combined with 7.5 μM PI and incubated at 37°C in the dark for 15 min in binding buffer. The monitored parameters were FS log, SS log, FL1 (FITC-Annexin V) and FL4 (PI). Four sperm subpopulations were distinguished: intact membrane (IM) cells with (Annx+/PI-) or without PS translocation (Annx-/PI-); and membrane-damaged cells with (Annx+/PI+) or without PS translocation (Annx-/PI+).

Detection of Activated Caspases

The caspase FITC-VAD-FMK *in situ* marker (Vybrant® FAM Caspase-3 and−7 Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) was used to detect active caspase-3 and−7. This cell-permeable caspase inhibitor peptide conjugated to FITC covalently binds to activated caspases, and functions as an *in situ* marker for apoptosis (29). Samples of 300 μL (1×10^6cells) containing FITC-VADFMK (5 nM) were incubated at 37°C and 5% CO_2 in the dark for 60 min. After washing twice with 100 μL of washing buffer (supplied with the kit) by centrifuging at 600 × g 8 min at RT, the pellet obtained was resuspended with 300 μL of washing buffer containing 7.3 μM ethidium homodimer (Eth), and flow cytometry was performed within 10 min. The monitored parameters were FS log, SS log, FL1 (FITC-VAD-FMK) and FL4 (Eth). With this technique, four sperm subpopulations were distinguished: intact membrane cells (IM) with (FITC-VAD+/Eth-) or without active caspase-3 and -7 (FITC-VAD-/Eth-); and dead with (FITC-VAD+/Eth+) or without (FITC-VAD-/Eth+) active caspases.

Evaluation of DNA Fragmentation

The presence of DNA strand breaks related to apoptosis in spermatozoa was evaluated by the TUNEL (terminal transferase-mediated dUDP nick end-labeling) assay using an *In Situ* Cell Death Detection Kit with fluorescein isothiocyanate (FITC)-labeled dUTP (Sigma-Aldrich, subsidiary of Merck KGaA, Darmstadt, Germany) (30). Sperm samples (4×10^7 cells/mL) were fixed with 4% paraformaldehyde in PBS at RT for 1 h. After two washes with 100 μL of PBS, the samples were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The reaction was performed by incubating the pellet obtained with 50 μL of labeling solution that contained the TdT enzyme and dUTP, for 1 h at 37°C in the dark. For each experimental set, a negative control was prepared by omitting TdT from the reaction mixture. Positive controls were simultaneously prepared by additional treatment with 10 IU DNase I for 10 min at 15–25°C before the labeling reaction. Two subsequent washes with PBS at 600 × g for 10 min at RT were performed to stop the reaction, and flow cytometry analysis was carried out. The monitored parameters were FS log, SS log and FL1 (TUNEL). TUNEL negative spermatozoa were considered DNA-intact.

Evaluation of the Capacitation Status

The sperm capacitation state was evaluated using the chlortetracycline (CTC) assay (31) that we previously validated for the evaluation of capacitation and acrosome reaction- like

changes in ram spermatozoa (32). For staining, washed samples were diluted with PBS (4×10^7 cells/mL). CTC solution (750 mM, Sigma-Aldrich) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 mM cysteine, pH 7.8, and passed through a 0.22 mm filter (Merck KGaA, Darmstadt, Germany). To 18 mL of sperm sample, 2 mL of ethidium homodimer-1 (EthD-1, 23.3 mM) were added and then incubated at 37°C in the dark for 10 min. Thereafter, 20 mL of CTC solution and 5 mL of 12.2% (w/v) paraformaldehyde in 0.5 M Tris–HCl, pH 7.8, were added. An aliquot of 4 mL of stained sample was placed onto a glass slide at room temperature, and mixed with 2 mL of 0.22 M Triethylenediamine (an antifade-reagent, Merck KGaA, Darmstadt, Germany) in glycerol:phosphate buffered saline (PBS, 9:1). The samples were covered with 24 × 48 mm coverslips, sealed with colorless enamel, and stored at 4°C in the dark.

For the evaluation of CTC patterns, the samples were observed under a Nikon Eclipse E-400 microscope with epifluorescence illumination using a V-2A filter at 1000 X magnification. At least 200 cells were counted in duplicate for each sample. Spermatozoa were classified into one of the following patterns (33): non-capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-reacted cells (showing no fluorescence on the head). The use of ethidium homodimer in this staining allows differentiating live and dead spermatozoa in these three types.

Determination of Oxygen Consumption

Sperm oxygen consumption was measured with a Clark oxygen electrode linked to a recorder system software (Oxygraph, Hansatech Instruments Ltd., Norfolk UK), as previously described (13). The zero point was set by adding sodium dithionite to the chamber filled with distilled water at 37°C and maintained with constant stirring to ensure a homogeneous distribution of O_2. Sperm samples were diluted with PBS up to 3×10^7 cells/mL and 1 mL of the sperm solution was loaded into the pre-warmed chamber. The plunger was inserted to expel air, and O_2 consumption was monitored for 3 min at 37°C with constant stirring. Results were expressed as fmol $O_2 \cdot mL^{-1} \cdot min^{-1}$ per million cells.

Statistical Analysis

Statistical analyses were undertaken using the IBM SPSS Statistics software v. 21 (Armonk, NY, USA) and GraphPad Prism v.9.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

Normality of the data was analyzed with the Kolmogorov-Smirnov test. Correlations between tested sperm parameters and field fertility were calculated using the Spearman rank correlation method. The Mann-Whitney test was carried out to determine whether there were significant differences between groups in some of the above-mentioned parameters. Finally, multiple logistic regression analysis was used for determining the dichotomous field fertility rate (higher or lower than the mean fertility) with sperm parameters. Due to the unbalanced data registry, only the parameters that allowed us to have more than 100 observations in the logistic regression analysis were included in the model.

RESULTS

Sperm Quality and Reproductive Parameters of Refrigerated Samples

The mean values of seminal quality and the reproductive parameters obtained with refrigerated seminal doses are summarized in **Table 1**. When these refrigerated samples reached our laboratory, and after sucrose buffer washing, the mean percentage of intact membrane (IM) spermatozoa, estimated as PI- was around 50% (47.63 ± 1.20%). However, percentages of IM spermatozoa that remained in a non-capacitated state (IM-NC), or without phosphatidylserine translocation or caspases activation, were relatively lower (19.12 ± 0.61%, 36.71 ± 0.90%, and 32.82 ± 1.05%, respectively). When the mitochondrial membrane potential was evaluated together with membrane permability, the percentage of non-permeant cells with high inner membrane potential ($\Delta\Psi$m) was also low (27.33 ± 1.39%). Regarding other apoptotic parameters, the mean value of spermatozoa without DNA injury in all evaluated seminal doses was 84.87 ± 0.44% (**Table 1A**). The oxygen consumption by these refrigerated samples was 0.59 ± 0.12 fmol $O_2 \cdot mL^{-1}$ $min^{-1}/10^6$ cells.

The reproductive results obtained after cervical inseminations were 51.97 ± 1.1% fertility, 0.83 ± 0.2 fecundity and 1.56 ± 0.2 prolificacy (**Table 1B**).

Influence of Season on the Analyzed Parameters

Comparing the results obtained in the breeding (B) and non-breeding (NB) season, we found significant differences in some of the analyzed sperm quality parameters (**Table 2**). The mean value of IN (PI-) spermatozoa was almost 10% higher in the B than in the NB season, this difference being highly significant ($P = 0.001$, **Table 2**). Regarding apoptotic-like markers, the percentage of NP spermatozoa with high $\Delta\Psi$m and IM sperm without PS translocation was also higher in the B than in the NB season ($P < 0.001$ and $P = 0.05$, respectively).

Moreover, spermatozoa in samples from the NB season showed significantly higher oxygen consumption than those in samples from the B season ($P < 0.05$). Regarding capacitation, the percentage of IM-NC spermatozoa was slightly higher in B than in NB, with a tendency to significance ($P = 0.087$).

Differences in the above-mentioned parameters did not lead to significant differences in the reproductive results between the two seasons. However, fertility, fecundity and prolificacy data were slightly higher in B than in NB (**Table 2**).

Differences Between Sires and Reproductive Management

The rams used in this study belonged to two farming cooperatives, named here as Coop 1 and Coop 2, so we also compared sperm quality parameters between seminal doses from animals belonging to both companies (**Table 3**). Percentages of

TABLE 1 | Mean, minimum and maximum values of (A) all analyzed parameters in refrigerated seminal doses, and (B) reproductive results obtained after artificial insemination with these seminal doses.

	n	Mean	S.E.M	Minimum	Maximum
A) Sperm quality parameters					
Intact membrane (IM) spermatozoa (PI-) (%)	286	47.63	1.20	3.00	95.00
IM non-capacitated (IM-NC) spermatozoa (%)	357	19.12	0.61	0.00	55.00
Non permeant (NP) sperm with high $\Delta\Psi$m (YO-PRO-1-/ MitoT+) (%)	138	27.33	1.39	0.10	68.90
IM sperm without PS translocation (Annx-/PI-) (%)	346	36.71	0.90	0.2	85.20
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	277	32.82	1.05	0.00	78.50
DNA-intact spermatozoa (TUNEL-) (%)	357	84.87	0.44	50.00	99.00
Oxygen consumption (fmol $O_2 \cdot mL^{-1} \cdot min^{-1}$ per million cells)	203	0.59	0.12	0.09	1.04
B) Reproductive parameters					
Fertility (ewes lambing per ewes inseminated, %)	357	51.97	1.10	0.00	100.00
Fecundity (number of lambs born per ewes inseminated)	357	0.83	0.20	0.00	2.14
Prolificacy (number of lambs born per ewe lambing)	357	1.56	0.20	0.00	3.00

N indicates the number of seminal doses evaluated and S.E.M. is the standard error of the mean.

TABLE 2 | Differences in analyzed parameters between seminal doses from the breeding and the non-breeding season.

Parameter	Breeding season	Non-breeding season	Significance level (*P*)
Intact membrane (IM) spermatozoa (PI-) (%)	51.97 ± 1.64	42.57 ± 1.66	$P = 0.001$
IM non-capacitated (IM-NC) spermatozoa (%)	19.92 ± 0.82	18.07 ± 0.90	$P = 0.087$
Non-permeant (NP) sperm with high $\Delta\Psi$m (YO-PRO-1 -/MitoT+) (%)	36.68 ± 1.77	16.21 ± 1.12	$P < 0.001$
IM sperm without PS translocation (Annx-/PI-) (%)	39.06 ± 1.16	33.82 ± 1.39	$P = 0.05$
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	32.07 ± 1.18	33.63 ± 1.79	n.s.
DNA-intact spermatozoa (TUNEL-) (%)	85.17 ± 0.59	84.49 ± 0.66	n.s.
Oxygen consumption (fmol $O_2 \cdot mL^{-1} \cdot min^{-1}$ per million cells)	0.55 ± 0.17	0.61 ± 0.17	$P = 0.019$
Fertility (ewes lambing per ewes inseminated, %)	53.01± 1.42	50.63 ± 1.72	n.s.
Fecundity (number of lambs born per ewes inseminated)	0.86 ± 0.02	0.79 ± 0.03	n.s.
Prolificacy (number of lambs born per ewe lambing)	1.59 ± 0.02	1.53 ± 0.03	n.s.

Results are expressed as a mean value ± standard error of the mean. Last column reflects the level of significance. n.s., non significant.

IM (PI-) and IM without PS translocation spermatozoa were significantly higher in samples from Coop 1 than Coop 2 ($P < 0.001$ and $P < 0.05$, respectively). In contrast, the Coop 2 seminal doses showed a higher value of DNA-intact spermatozoa ($P < 0.001$) than those from Coop 1. However, these differences did not lead to significant dissimilarities in the reproductive results of the two cooperatives (**Table 3**).

An important difference in the reproductive management between the cooperatives was the use of melatonin implants in rams belonging to Coop 2 during the NB season, in order to avoid the reproductive effects of seasonality. To investigate whether the observed differences between the two cooperatives were due to the melatonin treatment during the NB season, we compared the sperm quality values between cooperatives in the B and NB seasons. In NB, only the percentages of spermatozoa with intact DNA were significantly higher in Coop 2 (implanted animals) than in Coop 1 (non-implanted) (88.72 ± 0.96 vs. 83.11 ± 0.79%, $P < 0.001$, respectively). However, differences in this parameter were also significant ($P < 0.001$) in the B season (**Table 4**).

Relationship Between Reproductive and Sperm Quality Parameters

When the relationship between reproductive and sperm quality parameters was evaluated, only the percentage of IM-NC (intact membrane, non-capacitated) spermatozoa showed a significant correlation with field fertility ($P = 0.005$) and fecundity ($P = 0.007$), independently of the season, the cooperative and the distance (**Table 5**). When the effect of the season was evaluated, the percentage of IM-NC spermatozoa showed

TABLE 3 | Differences in analyzed parameters between cooperatives.

Parameter	COOP 1	COOP 2	Significance level (P)
Intact membrane (IM) spermatozoa (PI-) (%)	50.68 ± 1.50	41.41 ± 1.84	$P < 0.001$
IM non-capacitated (IM-NC) spermatozoa (%)	19.36 ± 0.74	18.45 ± 1.01	n.s.
Non-permeant (NP) sperm with high $\Delta\Psi$m (YO-PRO-1-/MitoT+) (%)	26.08 ± 1.57	31.65± 2.95	n.s
IM sperm without PS translocation (Annx-/PI-) (%)	37.92 ± 1.06	33.47 ± 1.70	$P = 0.024$
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	33.81 ± 1.29	30.97 ± 1.79	n.s.
DNA-intact spermatozoa (TUNEL-) (%)	83.63 ± 0.53	88.21 ± 0.66	$P < 0.001$
Oxygen consumption (fmol $O_2 \cdot mL^{-1} \cdot min^{-1}$ per million cells)	0.59 ± 0.14	0.58 ± 0.22	n.s.
Fertility (ewes lambing per ewes inseminated, %)	51.37 ± 1.28	53.60 ± 2.12	n.s.
Fecundity (number of lambs born per ewes inseminated)	0.82 ± 0.02	0.84 ± 0.03	n.s.
Prolificacy (number of lambs born per ewe lambing)	1.56 ± 0.02	1.56 ± 0.03	n.s.

Results are expressed as a mean value ± standard error of the mean. Last column reflects the level of significance. n.s., non significant.

TABLE 4 | Differences in analyzed parameters between seminal doses from the breeding and the non-breeding season for both cooperatives.

	Breeding season		Non-Breeding season	
Parameter	COOP 1	COOP 2	COOP 1	COOP 2
Intact membrane (IM) spermatozoa (PI-) (%)	56.71 ± 2.05	43.68 ± 2.40***	44.40 ± 2.02	38.06 ± 2.82
IM non-capacitated (IM-NC) spermatozoa (%)	20.23 ± 1.08	19.15 ± 1.01	18.23 ± 1.00	17.36 ± 2.07
Non-permeant (NP) sperm with high $\Delta\Psi$m (YO-PRO-1 -/MitoT+) (%)	37.90 ± 2.34	34.61 ± 2.69	16.82 ± 1.11	n.d.
IM sperm without PS translocation (Annx-/PI-) (%)	40.72 ± 1.34	35.05 ± 2.19*	34.68 ± 1.62	31.16 ± 2.68
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	33.23 ± 1.55	30.39 ± 1.81	32.45 ± 1.19	33.79 ± 2.05
DNA-intact spermatozoa (TUNEL-) (%)	84.06 ± 0.73	87.87 ± 0.90***	83.11 ± 0.79	88.72 ± 0.96***
Oxygen consumption (fmol $O_2 \cdot mL^{-1} \cdot min^{-1}$ per million cells)	0.56 ± 0.02	0.55 ± 0.02	0.61 ± 0.18	0.62 ± 0.42
Fertility (ewes lambing per ewes inseminated, %)	52.19 ± 1.72	54.99 ± 2.53	50.15 ± 1.96	51.46 ± 3.76
Fecundity (number of lambs born per ewes inseminated)	0.85 ± 0.03	0.88 ± 0.05	0.79 ± 0.03	0.79 ± 0.06
Prolificacy (number of lambs born per ewe lambing)	1.60 ± 0.03	1.57 ± 0.04	1.53 ± 0.03	1.55 ± 0.06

*Results are expressed as a mean value ± standard error of the mean. *P < 0.05 and ***P < 0.001 between cooperatives within season. n.d, not enough data to include in the comparative analysis.*

significant correlation with all the *in vivo* reproductive results during the NB season ($P = 0.025$, $P = 0.004$, and $P = 0.007$ for fertility, fecundity and prolificacy, respectively), but only a tendency to significance with fertility ($P = 0.082$) during the B season.

All seminal doses were prepared in the same AI centre and used in different farms distributed in a 250 km radius. The data were then divided in three categories according to the distance from the collection centre to the farm where the artificial insemination was carried out: short (<50 km), medium (50–100 km) and long (>100 km) distance. When the data were sorted by distance, the correlation between the percentage of IM-NC spermatozoa and reproductive parameters increased progressively with remoteness (**Table 5**).

Furthermore, three other sperm quality characteristics showed significant correlation with the reproductive parameters, but only in the B season: the oxygen consumption correlated significantly with fertility ($P < 0.05$), fecundity ($P < 0.01$) and prolificacy ($P < 0.05$); the percentage of spermatozoa with NP membrane and high $\Delta\Psi$m (YO-PRO-1-/MitoT+) with fertility and fecundity ($P < 0.05$); and the percentage of IM spermatozoa without active caspases with fecundity ($P < 0.05$). When the data were divided into categories according to the farm distance, TUNEL negative cells showed a positive correlation with fertility ($P < 0.1$) when the distance was > 100 km.

Multiple logistic regression analysis revealed that the increment in percentage of IM-NC ($P = 0.02$) together with the rise in DNA-intact spermatozoa ($P = 0.04$), would increase the probability of obtaining a fertility higher than the mean (>52%) (**Table 6**). ROC analysis (**Figure 1**) was used to identify the accuracy (area under the curve, which indicates discriminatory ability), sensitivity (true positive rate) and specificity (true negative rate) of the model. In our model, the area under the curve was 0.7328 ($P < 0.001$) and sensitivity and specificity

TABLE 5 | Spearman's rank correlation coefficient (Spearman's ρ) between some analyzed sperm parameters and the reproductive results obtained after artificial insemination, considering overall data and data separated by season or farm distance to the semen collection point.

	Parameter	Fertility	Fecundity	Prolificacy
Overall data	IM non-capacitated (IM-NC) spermatozoa (%)	0.147**	0.143**	n.s
In B season	IM non-capacitated (IM-NC) spermatozoa (%)	0.122†	n.s.	n.s.
	Oxygen consumption (fmol $O_2 \cdot mL^{-1} \cdot min^{-1}$ per million cells)	0.245*	0.346**	0.212*
	NP sperm with high $\Delta\Psi$m (YO-PRO-1-/MitoT+) (%)	0.246*	0.291*	n.s.
	IM sperm without active caspases (FITC-VAD-/Eth-) (%)	n.s.	0.163*	n.s.
In NB season	IM non-capacitated (IM-NC) spermatozoa (%)	0.180*	0.232**	0.191*
Distance < 50 km	IM non-capacitated (IM-NC) spermatozoa (%)	n.s.	n.s.	n.s
Distance 50–100 km	IM non-capacitated (IM-NC) spermatozoa (%)	0.195*	0.176†	n.s.
Distance >100 km	IM non-capacitated (IM-NC) spermatozoa (%)	0.158*	0.188*	0.146†
	DNA-intact spermatozoa (TUNEL-) (%)	0.144†	n.s.	n.s.

*n.s, non significant; $^{\dagger}P < 0.1$; *$P < 0.05$; and **$P < 0.01$. B, breeding; NB, non-breeding; IM, Intact membrane; NP, non permeant membrane.*

TABLE 6 | Multiple logistic regression results of fertility >52% ($n = 137$).

Variable	Beta	Odds ratios	95% CI for OR		*P*-value
			Lower limit	Upper limit	
Intercept	−9.478	7.65×10^{-5}	1.38×10^{-7}	0.02	0.002
Distance [<50 km]	–	1.000	–	–	–
Distance [50–100 km]	−0.917	2.503	0.865	7.516	0.09
Distance [>100 km]	1.207	3.344	1.314	9.018	0.01
Cooperative [Coop 1]	–	1.000	–	–	–
Cooperative [Coop 2]	0.044	1.045	0.349	3.189	0.94
Season [breeding]	–	1.000	–	–	–
Season [non-breeding]	0.844	2.325	0.778	7.345	0.14
Intact membrane (IM) spermatozoa (PI-) (%)	0.041	1.042	0.998	1.091	0.07
IM sperm without PS translocation (Annx-/PI-) (%)	−0.027	0.973	0.928	1.018	0.24
IM non-capacitated (IM-NC) spermatozoa (%)	0.056	1.058	1.011	1.110	0.02
DNA-intact spermatozoa (TUNEL-) (%)	0.061	1.063	1.006	1.129	0.04
IM sperm without active caspases (FITC-VAD-/Eth-) (%)	0.009	1.009	0.982	1.037	0.51
Non-permeant (NP) sperm with high $\Delta\Psi$m (YO-PRO-1-/ MitoT+) (%)	0.023	1.024	0.994	1.056	0.12

OR, Odds ratio; CI, confidence interval; (-) not applicable as the reference (most frequent value).

were 70.42 and 64.18%, respectively. The positive and negative predictive power was 67.57 and 67.19%, respectively.

DISCUSSION

In this study, we determined the relationship between *in vivo* fertility and some sperm quality parameters, including membrane integrity, capacitation state, mitochondria functionality, metabolic activity and apoptotic-like markers, in rams. We found that, when all data was evaluated, only one marker significantly correlated with *in vivo* fertility and with fecundity. This parameter was the percentage of intact membrane non-capacitated (IM-NC) spermatozoa, evaluated using the chlortetracycline (CTC) assay in combination with ethidium homodimer. We had previously validated this procedure for the evaluation of capacitation and acrosome reaction-like changes in ram spermatozoa (32) following a procedure reported earlier (34). This assay allows us to estimate simultaneously changes in the intracellular calcium distribution related to the capacitation status and plasma membrane integrity. Although some authors have also reported a correlation between capacitation status and *in vivo* fertility, their studies were conducted with frozen-thawed semen in other species (35, 36). However, to the best of our knowledge, this is the first study that correlates capacitation status with field fertility using refrigerated ram semen. Capacitation is a prerequisite for fertilization, but it has to occur in the female reproductive tract near the oocyte. If a high number of spermatozoa undergo capacitation during cryopreservation and transport, they will be less likely to survive until the time of insemination.

When the data from the seminal samples were divided into three categories according to the distance from the AI centre where the seminal doses were prepared to each farm, the relationship between the percentage of IM-NC spermatozoa

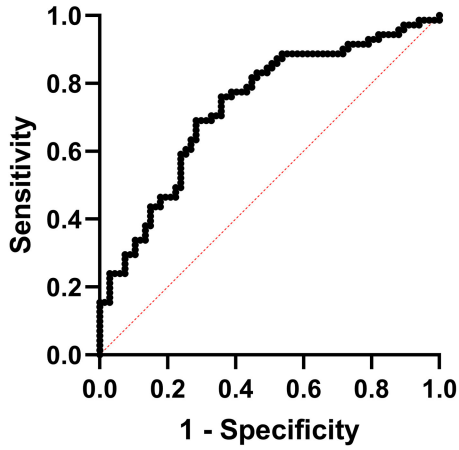


FIGURE 1 | Receiver operating characteristic (ROC) curve analysis of prediction model for fertility >52% after AI with refrigerated ram seminal doses.

and the reproductive results was lost for the nearest farms and increased progressively with remoteness. Furthermore, the percentage of spermatozoa without DNA damage also showed a tendency to significance in its positive correlation with field fertility when inseminations were done on remote farms. These results suggest that these parameters, the percentage of IM-NC and DNA-intact spermatozoa could be useful indicators of the sperm resistance over time. The increment of both the percentage of IM-NC and the DNA-intact spermatozoa would increase the probability of obtaining a fertility higher than the mean, as revealed by a multiple logistic regression analysis.

Sheep are a seasonal, short-day breeder species. Thus, when we compared sperm quality parameters between the breeding (B) and non-breeding (NB) seasons, we found that seminal doses from the B season presented higher percentages of IM spermatozoa, NP with high $\Delta\Psi$m spermatozoa, and IM without PS translocation sperm than in the NB season. Therefore, we can conclude that there are fewer spermatozoa showing apoptotic-like features in the seminal doses in the B than in the NB season. Although differences in sperm motility, morphology or viability between the two seasons have already been reported (20, 21, 37), this is the first time, to the best of our knowledge, that the effect of the season on apoptotic-like markers in ram ejaculates has been revealed. However, although fertility, fecundity and prolificacy results were slightly higher in the B than in the NB season, these differences in apoptotic-like parameters did not lead to significant differences in the reproductive results between the two seasons. Nonetheless, the correlation between the percentage of IM-NC spermatozoa and all the reproductive parameters, including prolificacy, increased when this was evaluated separately in the NB season data. This suggests that this marker would be more effective as a predictor of fertility when the sperm samples are "more stressed" or of less quality, as occurred when the time until insemination increased.

Plasma membrane integrity, evaluated as the non-permeability to certain dyes, is usually included in routine sperm analysis. However, this kind of evaluation does not detect subtle modifications in the sperm plasmalemma that reflect essential changes, such as the beginning of apoptosis, which lead the spermatozoa to certain death. The lack of correlation between membrane integrity and *in vivo* fertility has already been reported (2, 38–40) and is consistent with the results of the present study. Other authors found a significant, albeit limited, predictive capacity of this parameter on field fertility (41). However, it is worth pointing out that when PI was combined with an *in situ* activated caspase determination, or when we used more restrictive assays such as the double staining with YO-PRO-1 and Mitotracker Deep Red, which evaluates the beginning of plasmalemma destabilization and the loss of mitochondrial membrane potential, we found a significant correlation with the reproductive results in the B season.

The determination of sperm oxygen consumption using a Clark electrode has been used as a relatively simple method to evaluate the sperm mitochondrial function in several species such as stallion (42), human (43), boar (44), and ram (13). Oxygen consumption has often been considered a good indicator of the overall metabolic activity within cells, including spermatozoa (45, 46), and it has also been correlated with fertility in bulls (17). In this study, a positive correlation between this parameter and reproductive results was found, but only in the B season. Surprisingly, in samples from the NB season, the oxygen consumption was significantly higher than in those from the B season. This could be explained by the existence of a higher number of cells with mitochondrial membrane damage that could cause the membranes to become more "leaky" to protons, thereby increasing the oxygen consumption required for the maintenance of the proton gradient across the mitochondrial membrane and ATP synthesis (17).

We must highlight that the sires used in this study belonged to two farming cooperatives with different breeding schemes. Seminal doses from Coop 1, although presenting significantly higher membrane integrity values, also had higher DNA damage values than those from Coop 2. These results could be influenced by the use of melatonin implants in the NB season by Coop 2. We have already proved that melatonin implants during the NB season modify ram sperm motility characteristics and seem to improve fertilization results (47). However, in this study we found differences in sperm quality between the cooperatives in both seasons, not only during NB. Therefore, these differences could not be accurately attributed to the melatonin treatment. Furthermore, they did not lead to significant variations in the reproductive results achieved for both cooperatives.

In conclusion, this study reveals, for the first time, differences in apoptotic-like markers and oxygen consumption in ram seminal AI doses between the B and NB seasons. Moreover, we found two markers, the percentage of membrane intact non-capacitated spermatozoa, and the percentage of DNA-intact spermatozoa, that would allow discriminating between high and low fertility semen samples. Thus, these markers could be used to discard ejaculates with low fertilizing capacity and to test the value of particular males as semen donors after analyzing

an appropriate number of their ejaculates. Both procedures might improve fertility results obtained by artificial insemination in ovine.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the figshare repository (doi: 10.6084/m9.figshare.13521647).

ETHICS STATEMENT

All animal procedures were performed in accordance with the Spanish Animal Protection Regulation RD1201/05, which conforms to European Union Regulation 2010/63. Approval from the Ethics Committee of the University of Zaragoza was not a prerequisite for this study since we worked with the obtained semen samples. Written informed consent for participation was not obtained from the owners because ewes from farms associate to cooperatives were inseminated as always according to the breeding schedule by each cooperative, without any experimental procedure. Seminal doses for artificial insemination were collected at the Centro de Transferencia Agroalimentaria (CTA) in Zaragoza from rams belonging to cooperatives. We only analyzed in the lab an aliquot of the same seminal doses used for artificial inseminations, and veterinarians working for the cooperatives provide us results of fertility in order to study possible correlations.

AUTHOR CONTRIBUTIONS

RP-P and TM-B: conceptualization, experimental approach, and writing—original draft preparation. NM and JD: sperm quality analysis. FQ: semen samples preparation. AL and EF: responsible for artificial inseminations in farms. AC and NM: statistical analysis. AC and RP-P: data curation, writing—review, and editing. RP-P, JC-P, and TM-B: supervision. TM-B: project administration. TM-B and JC-P: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by Grant DGA A26 (Gobierno de Aragón). NM was financed by a FPI fellowship (Ministerio de Ciencia e Innovación). Publishing fees were financed by IUCA.

ACKNOWLEDGMENTS

The authors thank the Centro de Transferencia Agroalimentaria (Movera, Zaragoza) staff for their collaboration in the collection of semen samples and the preparation of doses for AI, veterinarians from the Technical Teams of ANGRA (Angel Macías and Elena Martín) and UPRA and Oviaragón for carrying out the artificial inseminations.

REFERENCES

1. Jeyendran RS, Caroppo E, Rouen A, Anderson A, Puscheck E. Selecting the most competent sperm for assisted reproductive technologies. *Fertil Steril.* (2019) 111:851–63. doi: 10.1016/j.fertnstert.2019.03.024
2. Meara CMO, Hanrahan JP, Prathalingam NS, Owen JS, Donovan A, Fair S, et al. Relationship between *in vitro* sperm functional tests and *in vivo* fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. *Theriogenology.* (2008) 69:513–22. doi: 10.1016/j.theriogenology.2007.12.003
3. Rodríguez-Martínez H. Can we increase the estimated value of semen assessment? *Reprod Domest Anim.* (2006) 41(Suppl. 2):2–10. doi: 10.1111/j.1439-0531.2006.00764.x
4. Baro Graf C, Ritagliati C, Torres-Monserrat V, Stival C, Carizza C, Buffone MG, et al. Membrane potential assessment by fluorimetry as a predictor tool of human sperm fertilizing capacity. *Front Cell Dev Biol.* (2020) 7:383. doi: 10.3389/fcell.2019.00383
5. Dutta S, Majzoub A, Agarwal A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab J Urol.* (2019) 17:87–97. doi: 10.1080/2090598X.2019.1599624
6. Amann RP, Hammerstedt RH. *In-vitro* evaluation of sperm quality - an opinion. *J Androl.* (1993) 14:397–406.
7. Kumaresan A, Johannisson A, Al-Essawe EM, Morrell JM. Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above- and below-average fertility bulls. *J Dairy Sci.* (2017) 100:5824–36. doi: 10.3168/jds.2016-12484
8. Sellem E, Broekhuijse ML, Chevrier L, Camugli S, Schmitt E, Schibler L, et al. Use of combinations of *in vitro* quality assessments to predict fertility of bovine semen. *Theriogenology.* (2015) 84:1447–54.e1445. doi: 10.1016/j.theriogenology.2015.07.035
9. Fuller SJ, Whittingham DG. Capacitation-like changes occur in mouse spermatozoa cooled to low temperatures. *Mol Reprod Dev.* (1997) 46:318–24. doi: 10.1002/(SICI)1098-2795(199703)46:3<318::AID-MRD10>3.0.CO;2-V
10. Gangwar C, Kharche SD, Mishra AK, Saraswat S, Kumar N, Sikarwar AK. Effect of diluent sugars on capacitation status and acrosome reaction of spermatozoa in buck semen at refrigerated temperature. *Trop Anim Health Prod.* (2020) 52:3409–15. doi: 10.1007/s11250-020-02374-8
11. Paulenz H, Söderquist L, Pérez-Pé R, Andersen Berg K. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. *Theriogenology.* (2002) 57:823–36. doi: 10.1016/S0093-691X(01)00683-5
12. Vadnais ML, Roberts KP. Effects of seminal plasma on cooling-induced capacitative changes in boar sperm. *J Androl.* (2007) 28:416–22. doi: 10.2164/jandrol.106.001826
13. Del Valle I, Mendoza N, Casao A, Cebrian-Perez JA, Perez-Pe R, Muino-Blanco T. Significance of non-conventional parameters in the evaluation of cooling-induced damage to ram spermatozoa diluted in three different media. *Reprod Domest Anim.* (2010) 45:e260–8. doi: 10.1111/j.1439-0531.2009.01552.x
14. Gallardo Bolaños JM, Miró Morán Á, Balao da Silva CM, Morillo Rodríguez A, Plaza Dávila M, Aparicio IM, et al. Autophagy and apoptosis have a role in the survival or death of stallion spermatozoa during conservation in refrigeration. *PLoS ONE.* (2012) 7:e30688. doi: 10.1371/journal.pone.0030688
15. Mendoza N, Casao A, Pérez-Pé R, Cebrián-Pérez JA, Muiño-Blanco T. New insights into the mechanisms of ram sperm protection by seminal plasma proteins. *Biol Reprod.* (2013) 88:1–15. doi: 10.1095/biolreprod.112.105650
16. Zhang J, Nuebel E, Wisidagama DR, Setoguchi K, Hong JS, Van Horn CM, et al. Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nat Protoc.* (2012) 7:1068–85. doi: 10.1038/nprot.2012.048
17. Garrett LJA, Revell SG, Leese HJ. Adenosine triphosphate production by bovine spermatozoa and its relationship to semen fertilizing ability. *J Androl.* (2008) 29:449–58. doi: 10.2164/jandrol.107.003533
18. Aller JF, Aguilar D, Vera T, Almeida GP, Alberio RH. Seasonal variation in sexual behavior, plasma testosterone and semen characteristics of Argentine Pampinta and Corriedale rams. *Span J Agric Res.* (2012) 10:345–52. doi: 10.5424/sjar/2012102-389-11

19. Zamiri MJ, Khalili B, Jafaroghli M, Farshad A. Seasonal variation in seminal parameters, testicular size, and plasma testosterone concentration in Iranian Moghani rams. *Small Ruminant Res.* (2010) 94:132–6. doi: 10.1016/j.smallrumres.2010.07.013
20. D'alessandro AG, Martemucci G. Evaluation of seasonal variations of semen freezability in Leccese ram. *Anim Reprod Sci.* (2003) 79:93–102. doi: 10.1016/S0378-4320(03)00113-1
21. Gundogan M, Serteser M. Some reproductive parameters and biochemical properties in Akkaraman and Awassi rams. *Turk J Vet Anim Sci.* (2005) 29:595–9.
22. Marti E, Mara L, Marti JI, Muino-Blanco T, Cebrian-Perez JA. Seasonal variations in antioxidant enzyme activity in ram seminal plasma. *Theriogenology.* (2007) 67:1446–54. doi: 10.1016/j.theriogenology.2007.03.002
23. Perez-Pe R, Barrios B, Muino-Blanco T, Cebrian-Perez JA. Seasonal differences in ram seminal plasma revealed by partition in an aqueous two-phase system. *J Chromatogr B.* (2001) 760:113–21. doi: 10.1016/S0378-4347(01)00259-6
24. Harrison RA, Vickers SE. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J Reprod Fertil.* (1990) 88:343–52. doi: 10.1530/jrf.0.0880343
25. Del Valle I, Gomez-Duran A, Holt WV, Muino-Blanco T, Cebrian-Perez JA. Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. *J Androl.* (2012) 33:717–25. doi: 10.2164/jandrol.111.014944
26. Martinez-Pastor F, Aisen E, Fernandez-Santos MR, Esteso MC, Maroto-Morales A, Garcia-Alvarez O, et al. Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. *Reproduction.* (2009) 137:225–35. doi: 10.1530/REP-08-0357
27. Hallap T, Nagy S, Jaakma U, Johannisson A, Rodriguez-Martinez H. Mitochondrial activity of frozen-thawed spermatozoa assessed by mitotracker deep red 633. *Theriogenology.* (2005) 63:2311–22. doi: 10.1016/j.theriogenology.2004.10.010
28. Garcia-Alvarez O, Maroto-Morales A, Ramon M, del Olmo E, Montoro V, Dominguez-Rebolledo AE, et al. Analysis of selected sperm by density gradient centrifugation might aid in the estimation of *in vivo* fertility of thawed ram spermatozoa. *Theriogenology.* (2010) 74:979–88. doi: 10.1016/j.theriogenology.2010.04.027
29. Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z. Apoptosis and beyond: cytometry in studies of programmed cell death. *Methods Cell Biol.* (2011) 103:55–98. doi: 10.1016/B978-0-12-385493-3.00004-8
30. Li X, Darzynkiewicz Z. Labelling DNA strand breaks with BrdUTP. Detection of apoptosis and cell proliferation. *Cell Proliferation.* (1995) 28:571–9. doi: 10.1111/j.1365-2184.1995.tb00045.x
31. Ward CR, Storey BT. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Dev Biol.* (1984) 104:287–96. doi: 10.1016/0012-1606(84)90084-8
32. Grasa P, Cebrián-Pérez JA, Muiño-Blanco T. Signal transduction mechanisms involved in *in vitro* ram sperm capacitation. *Reproduction.* (2006) 132:721–32. doi: 10.1530/rep.1.00770
33. Gillan L, Evans G, Maxwell WM. Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. *Reprod Fertil Dev.* (1997) 9:481–487. doi: 10.1071/R96046
34. Pérez-Pe R, Grasa P, Fernandez-Juan M, Peleato ML, Cebrián-Pérez JA, Muiño-Blanco T. Seminal plasma proteins reduce protein tyrosine phosphorylation in the plasma membrane of cold-shocked ram spermatozoa. *Mol Reprod Dev.* (2002) 61:226–33. doi: 10.1002/mrd.1152
35. Kuroda K, Fukushima M, Harayama H. Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese black cattle. *J Reprod Dev.* (2007) 53:1079–86. doi: 10.1262/jrd.19031
36. Thundathil J, Gil J, Januskauskas A, Larsson B, Soderquist L, Mapletoft R, et al. Relationship between the proportion of capacitated spermatozoa present in frozen-thawed bull semen and fertility with artificial insemination. *Int J Androl.* (1999) 22:366–73. doi: 10.1046/j.1365-2605.1999.00194.x
37. Marti JI, Aparicio IM, Leal CL, Garcia-Herreros M. Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. *Theriogenology.* (2012) 78:528–41. doi: 10.1016/j.theriogenology.2012.02.035
38. Gadea J, Selles E, Marco MA. The predictive value of porcine seminal parameters on fertility outcome under commercial conditions. *Reprod Domest Anim.* (2004) 39:303–8. doi: 10.1111/j.1439-0531.2004.00513.x
39. Perez-Pe R, Marti JI, Sevilla E, Fernandez-Sanchez M, Fantova E, Altarriba J, et al. Prediction of fertility by centrifugal countercurrent distribution (CCCD) analysis: correlation between viability and heterogeneity of ram semen and field fertility. *Reproduction.* (2002) 123:869–75. doi: 10.1530/reprod/123.6.869
40. Rodriguez-Martinez H. Laboratory semen assessment and prediction of fertility: still utopia? *Reprod Domest Anim.* (2003) 38:312–8. doi: 10.1046/j.1439-0531.2003.00436.x
41. Santolaria P, Vicente-Fiel S, Palacín I, Fantova E, Blasco ME, Silvestre MA, et al. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. *Anim Reprod Sci.* (2015) 163:82–8. doi: 10.1016/j.anireprosci.2015.10.001
42. Schober D, Aurich C, Nohl H, Gille L. Influence of cryopreservation on mitochondrial functions in equine spermatozoa. *Theriogenology.* (2007) 68:745–54. doi: 10.1016/j.theriogenology.2007.06.004
43. Ferramosca A, Focarelli R, Piomboni P, Coppola L, Zara V. Oxygen uptake by mitochondria in demembranated human spermatozoa: a reliable tool for the evaluation of sperm respiratory efficiency. *Int J Androl.* (2008) 31:337–45. doi: 10.1111/j.1365-2605.2007.00775.x
44. Ramio-Lluch L, Fernandez-Novell JM, Pena A, Colas C, Cebrian-Perez JA, Muino-Blanco T, et al. "*In vitro*" capacitation and acrosome reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational structure. *Reprod Domest Anim.* (2011) 46:664–73. doi: 10.1111/j.1439-0531.2010.01725.x
45. Darr CR, Cortopassi GA, Datta S, Varner DD, Meyers SA. Mitochondrial oxygen consumption is a unique indicator of stallion spermatozoal health and varies with cryopreservation media. *Theriogenology.* (2016) 86:1382–92. doi: 10.1016/j.theriogenology.2016.04.082
46. Magdanz V, Boryshpolets S, Ridzewski C, Eckel B, Reinhardt K. The motility-based swim-up technique separates bull sperm based on differences in metabolic rates and tail length. *PLoS ONE.* (2019) 14:e0223576. doi: 10.1371/journal.pone.0223576
47. Casao A, Vega S, Palacin I, Perez-Pe R, Lavina A, Quintin FJ, et al. Effects of melatonin implants during non-breeding season on sperm motility and reproductive parameters in rasa aragonesa rams. *Reprod Domest Anim.* (2010) 45:425–32. doi: 10.1111/j.1439-0531.2008.01215.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 07 May 2021
doi: 10.3389/fvets.2021.656937

Comparing the Effect of Different Antibiotics in Frozen-Thawed Ram Sperm: Is It Possible to Avoid Their Addition?

Luis Anel-Lopez[1,2], Marta F. Riesco[1,3], Rafael Montes-Garrido[1,2], Marta Neila-Montero[1,2], Juan C. Boixo[1,2], César Chamorro[1,2], Cristina Ortega-Ferrusola[4], Ana Carvajal[5], Jose R. Altonaga[2], Paulino de Paz[1,3], Mercedes Alvarez[1,2] and Luis Anel[1,2]*

[1] *Investigación en Técnicas de Reproducción Asistida - Universidad de León, Instituto de Desarrollo Ganadero y Sanidad Animal, University of León, León, Spain,* [2] *Department of Veterinary Medicine, Surgery and Anatomy, University of León, León, Spain,* [3] *Cellular Biology, Department of Molecular Biology, University of León, León, Spain,* [4] *Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain,* [5] *Department of Animal Health, Facultad de Veterinaria, Universidad de León, León, Spain*

It is crucial to perform a deep study about the most extensively used antibiotics in sperm extenders. Most of the protocols and concentrations used in ram are direct extrapolations from other species. It is important to establish species-specific antibiotic treatments to optimize their use and if it is possible to reduce the quantity. Previews studies have assessed some aspects of sperm quality *in vitro*, but this study aimed to go further and assess the effect of three different antibiotic treatments, which are the most extensively used, not only in sperm quality or assessing the inhibitory effect on bacterial growth but also assessing these important parameters of productivity such as fertility, prolificacy, fecundity, and sex-ratio during a freeze-thaw process. Gentamicyn (G) treatment showed the worst results, not only concerning sperm quality but also in the reproductive trials exhibiting a toxical effect at the experiment concentration, and being the most powerful inhibiting bacterial growth. For its part, Lincomicyn-spectinomycin (LS) showed similar results inhibiting bacterial growth but it did not show a detrimental effect either in sperm quality or in reproductive parameters. Penicillin-streptomycin (PS) showed good results in the sperm quality and in the reproductive *in vivo* trials, but it showed a very poor effect inhibiting bacterial growth probably due to some kind of antibiotic resistance. According to our results, there is not a significant positive relationship between the higher bacterial inhibitory activity of LS and PS samples, and the sperm quality respect Control samples (without antibiotics). In the case of G, which exhibited the most effective as antibacterial, we observed a toxic effect on sperm quality that could be translated on productivity parameters. Our results suggest that the bacterial contamination control in frozen-thawed semen may be possible without the use of antibiotics, although the effects of longer periods of cooling storage and different temperatures of storage need to be further investigated for animal semen. At this point, a reflection about a drastic reduction in the use of antibiotic treatments in sperm cryopreservation is mandatory, since freezing conditions could keep sperm doses contamination within the levels recommended by regulatory health agencies.

Keywords: ovine semen, artificial insemination, antibiotics, fertility, sex-ratio, prolificacy, antibiotic resistance

OPEN ACCESS

Edited by:
Szabolcs Nagy,
University of Pannonia, Hungary

Reviewed by:
Jane Morrell,
Swedish University of Agricultural Sciences, Sweden
María Ignacia Carretero,
University of Buenos Aires, Argentina

***Correspondence:**
Marta F. Riesco
mferrs@unileon.es

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *21 January 2021*
Accepted: *09 March 2021*
Published: *07 May 2021*

Citation:
Anel-Lopez L, Riesco MF, Montes-Garrido R, Neila-Montero M, Boixo JC, Chamorro C, Ortega-Ferrusola C, Carvajal A, Altonaga JR, de Paz P, Alvarez M and Anel L (2021) Comparing the Effect of Different Antibiotics in Frozen-Thawed Ram Sperm: Is It Possible to Avoid Their Addition? Front. Vet. Sci. 8:656937. doi: 10.3389/fvets.2021.656937

INTRODUCTION

Semen collection and sperm manipulation are not sterile processes, and bacterial contamination, especially environmental and non-pathological but also pathological species, of sperm samples cannot be avoided (1, 2). In ram, the final ejaculate collected is usually contaminated at least with bacteria from the technician, artificial vagina, penis, and prepuce. In this way, bacterial contamination could affect not only the sperm quality but also the final yield of the production. The bacteria-sperm interaction has been widely studied, especially in human sperm with *E. coli* as a model of contamination concluding that the spermicidal effect is concentration-dependent (3). Several negative effects have been reported from the bacterial contamination on the sperm quality such as the sperm motility impaired by bacterial adhesion and agglutination (4, 5), inducing morphological changes (6) altering the sperm function, increasing the phosphatidylserine translocation and the apoptosis activation (7). In addition, some studies suggest that bacterial contamination may increase the antibodies production affecting the glycocalyx complex in the sperm surface (3, 8). To prevent disease transmission in most domestic species, the addition of antibiotics to sperm extenders is mandatory in the European Union and so common in other countries outside (9). In this context, the antibiotic supplementation as an additive in most of the handling and preservative sperm extenders has been widely used in many species; in domestic ones such as ram (10), bull (11), stallion (12), or boar (13) and wild species: red deer (14) or brown bear (15). Many studies have demonstrated the beneficial effect of antibiotics inhibiting the bacterial proliferation in the sperm samples during their storage (1, 16–18). Most of these studies have been carried out in different species than ram, without species specific studies, which could generate unexpected effects on relevant production aspects such as fertility, prolificacy, fecundity, or sex-ratio. In the same way, most of these studies have been focused on the assessment of the effect of the antibiotics on the sperm quality assessed *in vitro* in buffalo, bull, and ram (10, 11, 19). It is important to take into account that the use of sperm insemination doses with contamination could be related to deleterious effects on the female reproductive tract and estrus status (20, 21), reducing the embryonic survival, or even reducing the litter size (22) in swine. However, the effects of antibiotics in important productive parameters such as fertility, prolificity, fecundity, or sex-ratio have not been widely studied in ram, usually being extrapolations and copies from other species. To get a deep knowledge about the effects of antibiotics not only on the sperm quality but also in the future production of the sperm doses is very important to optimize protocols and to reduce the use of antibiotics since the antimicrobial resistance supposes a global and serious danger not only for the human or animal health but also for the economics (23).

This study aimed to go further and assess the effect of three different antibiotic treatments, which are the most extensively used, not only in the sperm quality or assessing the inhibitory effect on the bacterial growth but also assessing these important parameters of productivity such as fertility, multiple lambing frequency, prolificacy, fecundity, and sex-ratio, which have a very important impact on the ovine livestock.

MATERIALS AND METHODS

Reagents and Media

All the products used in this paper were of at least reagent grade and were acquired from Sigma Aldrich (Madrid, Spain) unless otherwise stated. The medium for cytometry assessment was PBS.

Animals and Sperm Collection

Sperm samples were collected from mature ram males during the breeding season. The ejaculates were collected by artificial vagina at 40°C (Minitüb, Tiefenbach, Germany), and the tubes were maintained at 35°C during the initial evaluation of sperm quality. The volume was calculated using the graduation marks of the collection tubes. Mass motility was assessed by microscopy (warming stage at 38°C, 40x; score: 0–5; Labophot-2, Nikon, Tokyo, Japan), and the sperm concentration was assessed using a Nucleocounter n-100 (DADOS MARCAETC). Only ejaculates with good quality (volume: $\geq$0.5 mL; mass motility: $\geq$4; sperm concentration $\geq 3{,}000 \times 10^6$ mL^{-1}) were used and processed for the experiment.

Experimental Design

Before freezing, samples (1 valid ejaculate per male) were diluted down to a final concentration of 100×10^6 sperm/mL in their respective extender (Tes-Tris-Fructose 20% egg yolk_4% glycerol_320mOsm/Kg) as follows: without antibiotics (Control), with Penicillin-Streptomycin (PS) to a final concentration of 500 UI and 625 μg/mL, respectively, Lincomycin-Spectinomycin (LS) to a final concentration of 300 and 600 μg/mL, respectively, and Gentamicin (G) to a final concentration of 1,000 μg/mL. After this, samples were frozen and thawed as explained in point Sperm Cryopreservation. Samples were assessed *in vitro* just after thawing (T0) and after submitting them to a stress test of 2 h of incubation at 37°C (T2), except for the microbial assessment, which was carried out just after thawing (T0). For the *in vivo* trial (artificial insemination), samples were processed as described in statement Fertility Trials.

Sperm Cryopreservation

Sperm extended samples were cryopreserved following the protocol (24) modified by Alvarez et al. (25). Samples were refrigerated at −0.25°C/min from 30 to 5°C in the refrigerated chamber. After 2 h of equilibration at 5°C, samples were packed in 0.25 ml straws and then frozen in a programmable biofreezer (Kryo 10 Series III; Planer PLC, Sunbury-on-Thames, UK) at −20°C/min up to −100°C, transferred to liquid nitrogen containers, and stored for a minimum of 1 month. Thawing was performed by dropping straws in the water at 65°C for 6 s. One part of the samples was used to carry out the artificial inseminations; another part of the straws was used to carry out sperm *in vitro* assessment, just after thawing (T0) and after 2 h of incubation at 37°C as a stress test (T2). The last part of the samples was used to perform the microbial culture.

In vitro Sperm Evaluation

Straws from 9 males (one ejaculate per male) were used for the *in vitro* assessment as follows.

Computer-Assisted Sperm Analysis

Samples were diluted to 30 × 10^6 sperm/mL in their freezing extender to check the motility. A warmed Makler counting chamber was loaded with 5 μL of the sample. The analysis was carried out using a CASA system (Computer Assisted Sperm Analysis), consisting of an optical phase-contrast microscope (Nikon Labophot-2) (fitted with negative phase-contrast objectives and a warming stage at 37°C), a Basler A312fc camera (Basler, Germany), and a PC with the sperm Class Analyser software (ISAS v. 1.2; Proiser, Valencia, Spain). The magnification was 100×. At least five fields per sample were acquired at an acquisition rate of 25 images per second, recording a total of 200 motile sperm. The following parameters were used for the study: total motility (%; TM), progressive motility (%; PM), average path velocity (μm/sec; VAP) straight-line (rectilinear) velocity (μm/s; VSL), and amplitude of lateral head displacement (μm; AHL).

Image sequences were saved and analyzed afterward using the editing facilities provided by ISAS. Sperm were considered motile when VCL > 10 μm/s and progressive if VCL >10 and straightness (STR) >80%. The progressive sperm subpopulations were classified according to velocities as follows: Slow (VCL <25), Medium (VCL >25 and <65), and Rapid (VCL >65). Other events different from spermatozoa were removed, and settings were adjusted in each case to assure a correct track analysis.

Flow Cytometry

Flow cytometry acquisition was performed in a flow cytometer (MACSQuant Analyser 10, Miltenyi Biotech, Madrid, Spain) equipped with three lasers emitting at 405, 488, and 635 nm and 10 photomultiplier tubes (PMTs): V1 (excitation 405 nm, emission 450/50 nm), V2 (excitation 405 nm, emission 525/50 nm), B1 (excitation 488 nm, emission 525/50 nm), B2 (excitation 488 nm, emission 585/40 nm), B3 (excitation 488 nm, emission 655–730 nm; 655LP + split 730), B4 (excitation 499 nm, emission 750 LP), R1 (excitation 635 nm, emission 655–730 nm; 655LP+split 730), and R2 (excitation 635 nm, emission filter 750 LP). The system was controlled using MACS Quantify software (Miltenyi Biotech, Madrid, Spain). These excitation and emission wavelengths allowed us to find probe combinations that can simultaneously assess multiple parameters in a large number of sperm (a total of 40,000 events per sample and at least 20,000 sperm cells, at a flow rate of 200–300 cells per second, were acquired). Data were analyzed using FlowJo v.10.2 (Ashland, USA).

Simultaneous Flow Cytometric Assessment of the Viability, Caspase 3 and 7 Activity, and Metabolic Activity (ROS Generation)

Sperm samples of different experimental groups were diluted in PBS medium to obtain a total of 2 × 10^6 of sperm per sample; these samples were washed and centrifuged at 500 g for 10 min at room temperature (RT). Lyophilized Zombie Violet™ (Biolegend, San Diego, California, EEUU) dye was reconstituted in DMSO following the manufacturer's instructions (100 μl of DMSO to one vial of Zombie Violet™ dye). CellEvent™ Caspase-3/7 and CellROX™ Deep Red (Invitrogen, Eugene, Oregon, EEUU) were purchased as a 2 mM and 2.5 mM stabilized solution, respectively. Stock solutions of fluorescence probes were prepared at 1 μL and kept at −20°C in the dark until needed.

Zombie Violet™ stock solutions were resuspended in 1 mL of PBS while CellEvent™ Caspase-3/7 and CellROX™ in 10 μL. After samples centrifugation, the supernatant was discarded, and the sperm pellet was incubated at RT for 30 min in the dark with 96 μL of Zombie Violet™ (membrane integrity probe) (1:1,000 final dilution), 2 μL of CellEvent™ Caspase-3/7 (apoptosis marker) 4 μM final concentration, and 2 μL of CellROX® (ROS content labeling) 5 μM final concentration. After that, another washing step was performed to stop cell staining, and the pellet was resuspended in 1 mL of PBS, carrying out the analysis immediately by flow cytometry.

The interest sperm subpopulations assessed were plotted as follows: Non-Apoptotic Viable Sperm (Zombie low intensity, Caspase 3&7 negative), Apoptotic Sperm (Zombie low intensity -alive-, Caspase 3&7 positive), and High Metabolically Active Sperm (Zombie low intensity, CellROX positive).

Bacteriological Assessment

Straws from 8 males for each treatment, without antibiotics (control), with Penicillin-Streptomycin, with Lincomycin-Spectinomycin, or with Gentamicin, were thawed and 150 μL of each sample were plated onto blood agar plates (Oxoid, Wesel, Germany). The inoculum was spread rapidly over the entire agar surface with a sterile Digralsky spreader. Plates were incubated at 37°C under aerobic conditions and inspected after 48 h incubation. Bacterial growth was expressed as colony-forming units CFU/mL. Subcultures were performed until pure cultures were obtained. Primary identification was based on Gram staining and catalase and oxidase tests while confirmation was carried out using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker, Madrid, Spain).

Fertility Trials

For the fertility trial, sperm doses (25 x 10^6 sperm/straw), frozen-thawed as above described (2.4), from 10 mature males (Churra breed) were used. The experimental samples were randomly and sequentially distributed through 7 commercial farms following a commercial artificial insemination program (Churra breed improvement program) under the strict supervision of our research group. Adult Churra ewes (852 females between 2 and 5 years old lambed previously) were subjected to treatment for estrous induction and synchronization using intravaginal sponges with 20 mg fluorogestone acetate (Chronogest®, MSD) over 14 days. The sponges were removed and 500 IU of eCG were injected -IM- (Folligon®, MSD). Laparoscopic inseminations were performed by two vets with extensive experience, between 64 and 67 h after the removal of the sponges. The animals, having fasted for the previous 24 h, were tied to a special

cradle (IMV), placed on an inclined plane (45°), and the area in front of the teat was shaved and cleaned. Local anesthesia (mepivacaine HCL 2%, Braun™) was applied to the puncture points. Then two portals (for vision and manipulation/injection) were inserted by performing a pneumoperitoneum (CO_2). The semen, placed in a special applicator (Transcap®, IMV), was injected under visual inspection into each uterine horn (0.12 mL, 12.5×10^6 spz). Fertility [(lambing ewes/inseminated ewes) × 100] was calculated according to the births registered at 137–154 days post-insemination. Moreover, viable offspring and sex were registered, and prolificacy (lambs/lambed ewes), multiple lambing frequency [(multiple lambing/total lambing) × 100], fecundity (lambs/inseminated ewes) and sex-ratio [(female lambs/total lambs) × 100] were calculated.

Statistical Analysis

For the *in vitro* quality, data were analyzed using the SAS™ V.9.1 Package (SAS Institute Inc., Cary, NC, USA). Results are shown as means and standard errors of the mean. The normality of data was verified by Kolmogorov-Smirnov tests. Analyses of the data were carried out using linear mixed-effects models (MIXED procedure, ML method), including the type of antibiotic (C, PS, LS, and G) and incubation time after thawing (0 vs. 2 h) as fixed factors, and males as a random effect. Significant fixed effects were further analyzed using multiplecomparisons of means with Tukey contrasts. A significance level of $P < 0.05$ was used; $P < 0.1$ was considered as a trend.

Fertility, multiple lambing frequency, and sex ratio data were compared using a GENMOD procedure considering a binary response model. The statistical model included the type of antibiotic (C, PS, LS, and G) as a factor, and fertility, multiple lambing frequency, and sex-ratio as a response variable. Between-group differences in the frequency were tested using Wald Chi-Square. For sex-ratio, the study was completed comparing each experimental group to the "expected value" (50:50). Prolificacy and fecundity data were compared using a GLM procedure using the type of antibiotic as a factor; between-group differences were tested by Duncan test. The significance level was set at $P < 0.05$.

RESULTS

Sperm Motility

The mean, standard error, and male distribution for several sperm motility parameters are showed in **Figure 1**. Just after thawing non-significant differences ($P > 0.05$) were observed either for TM or for PM among the different antibiotic treatments (**Figures 1A,B**). After 2 h of incubation at 37°C, all the samples showed a significant decrease from 0 h, but no difference was observed between treatments. In contrast, when assessing the rapid PM, G samples showed significantly lower values ($P < 0.05$) than C just after thawing (**Figure 1C**). In the same way, when assessing ALH and VSL G samples showed significantly lower values ($P < 0.05$) than the C samples not only just after thawing but also after 2 h of incubation at 37°C (**Figures 1D,E**). Also, samples treated with PS showed significantly lower values of ALH than the C samples after submitting the samples to the incubation. Similarly, those samples treated with LS exhibited lower values ($P < 0.05$) of VSL respect to the control just after thawing. Finally, just after thawing, PS and G had significantly lower values of VAP ($P < 0.05$) than the C (**Figure 1F**). After the incubation, these significant differences disappeared.

Membrane Integrity (Sperm Viability), Caspase 3 and 7 Activity, and Metabolic Activity

The mean, standard error, and male distribution of the different evaluated sperm parameters are shown in **Figure 2**. No significant differences were found when assessing the viability just after thawing between the control and each treatment (**Figure 2A**). The viability of all groups was significantly lower ($P < 0.05$) after 2 h of incubation. After this incubation, PS kept higher values of non-apoptotic viable sperm than C ($P < 0.05$). Assessing the percentage of apoptotic cells, there were no significant differences among treatments either after thawing (T0) or after the incubation (T2) with respect to the C (**Figure 2B**). Finally, after assessing the metabolic status of sperm mitochondria via ROS generation, data indicated a higher tendency value ($P < 0.06$) in PS than the Control, not raising the significance, just after thawing. All treatments had a significant decrease ($P < 0.05$) after 2 h of incubation. However, PS showed significantly higher values of metabolic activity than the C after 2 h of incubation ($P < 0.05$). In contrast, LS and G did not show any significant differences ($P > 0.05$) compared to C (**Figure 2C**) for this parameter.

Bacteriological Assessment

The mean and standard error of the mean of CFU/mL recovered for each treatment is shown in **Figure 3**. A significant reduction in the number of viable bacteria after G and LS treatments as compared with the C without antibiotics was recorded ($P < 0.05$). PS treatment also decreases bacterial concentration although differences do not reach statistical significance when compared with control ($P = 0.345$; **Figure 3**).

Most of the samples resulted in the growth of a mixed microorganism population with up to 16 different species. The highest number of different bacterial species was identified in the C group without antibiotics, followed by PS, LS, and finally G with the lowest number of different bacterial species (**Figure 4**). A total of 10 genera and 16 bacterial species (**Figure 4**) were identified with *Pseudomonas* (3 isolates) and *Staphylococcus* (3 isolates) as the most common genera followed by *E. coli*. In this sense, G was the treatment with the highest antimicrobial spectra (4 survivor species) followed by LS (8 survivor species) and PS (10 survivor species) (**Figure 4**).

Fertility Trials

Samples treated with PS, the standard treatment, showed fertility of 52.0%. In the same way, samples treated with LS (50.5%) and C (52.9%) showed similar results ($P > 0.05$). In contrast, those samples treated with G exhibited the worst results (38.8%) being significantly lower ($P < 0.05$) than the other treatments (**Table 1**).

Similar prolificacy results were observed between C, G, and LS ($P > 0.05$), while PS (1.67 ± 0.07) showed significantly higher results ($P < 0.05$) than LS. Multiple lambing frequency followed

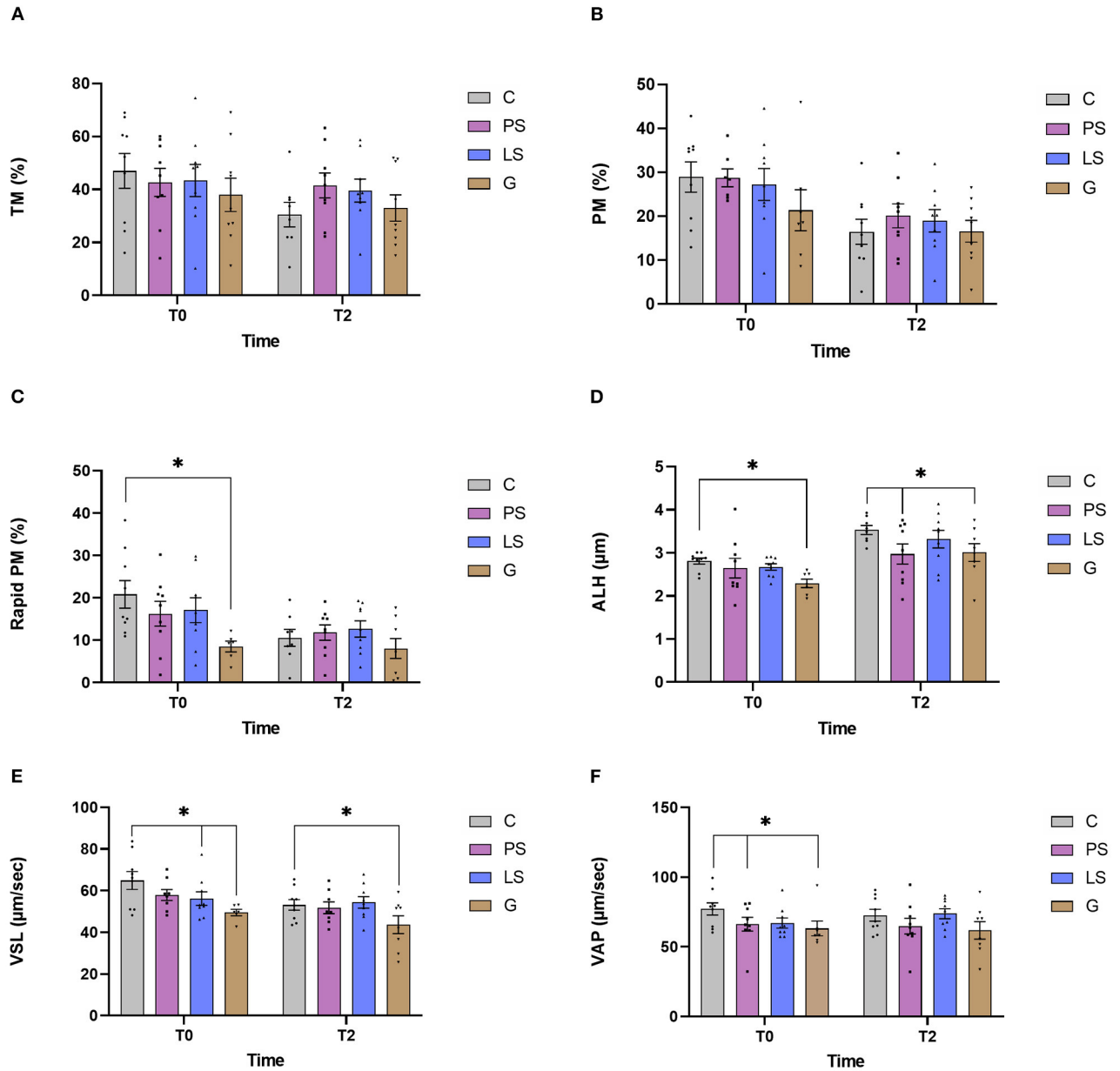


FIGURE 1 | Ram sperm motility data in the four experimental groups by time (just after thawing -T0- and after 2 h of incubation at 37°C -T2-): Control (C), without antibiotics; Penicillin-Streptomycin (PS); (500 UI)−625 µg/mL, respectively; Lincomycin-Spectinomycin (LS); 300–600 µg/mL respectively; and Gentamicin (G) 1,000 µg/mL. **(A)** Total motility (TM, %); **(B)** progressive motility (PM, %); **(C)** rapid progressive motile sperm (Rapid PM, %), **(D)** amplitude of the lateral head movement (ALH, µm), **(E)** velocity according to the straight path (VSL, µm/s), and **(F)** velocity according to the smoothed path (VAP, µm/s). Nine males were analyzed (1 ejaculate per male) including the same males in each experimental group. Graph dots represent individual male ejaculate. Significant differences ($P < 0.05$) are represented with an asterisk between the antibiotic treatment and the Control sample without antibiotics.

exactly the same significance distribution among treatments as prolificacy (**Table 2**). The results obtained in fecundity showed one more time the lowest rate in those samples assessed with G (0.59±0.06), being significantly lower ($P < 0.05$) than the C and PS (0.81 ± 0.06 and 0.83 ± 0.07, respectively). For its part, samples treated with LS (0.74 ± 0.06) did not show significant differences with respect to the other treatments (**Table 2**).

There was no significant sex ratio distortion ($P > 0.05$) with the expected value (50:50) in each experimental group. The C obtained 54.7% of females, being the treatment with the highest deviation of sex-ratio to females ($P < 0.05$). On the opposite, the percentage of females at birth was lower ($P < 0.05$) for those samples treated with G (42.4%) and LS (38.4%) than C (**Figure 5**). The result obtained for the samples treated with PS

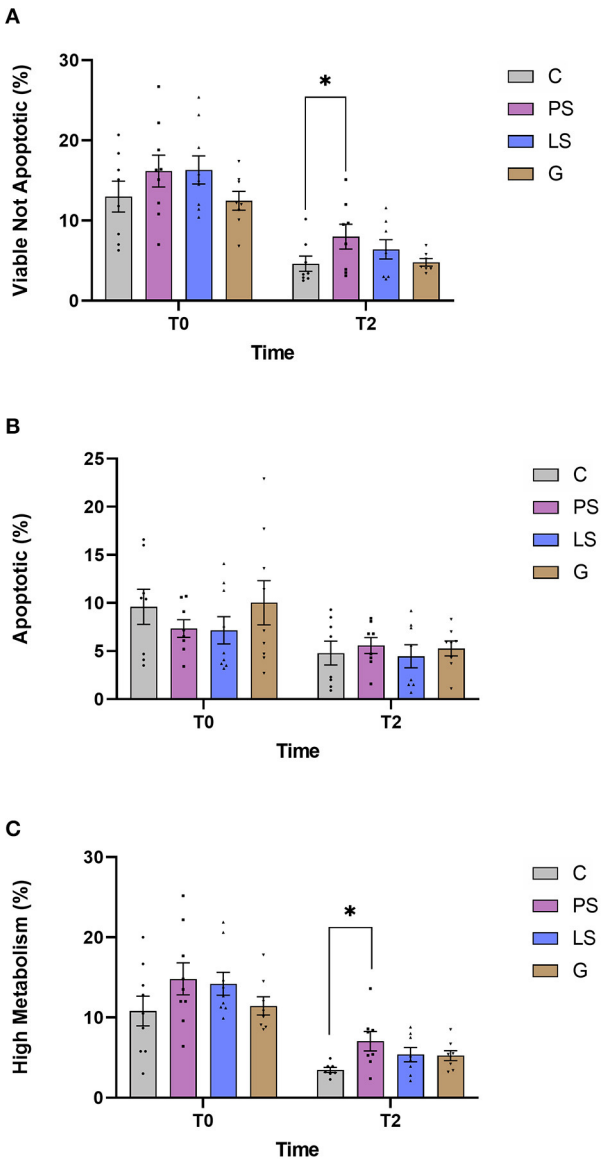


FIGURE 2 | Ram sperm multiparametric flow cytometry data in the four experimental groups by time (just after thawing -T0- and after 2 h of incubation at 37°C -T2-): Control (C), without antibiotics; Penicillin-Streptomycin (PS); (500 UI)−625 μg/mL, respectively; Lincomycin-Spectinomycin (LS); 300–600 μg/mL, respectively; and Gentamicin (G) 1,000 μg/mL. **(A)** Zombie low intensity cells and Caspase 3&7 negative cells (viable not apoptotic sperm, %); **(B)** Zombie low intensity cells and Caspase 3/7 positive cells (apoptosis, %); **(C)** Zombie low intensity cells and CellROX-positive cells (Sperm with high metabolic activity, %). Nine males were analyzed (1 ejaculate per male) including the same males in each experimental group. Graph dots represent individual male ejaculate. Significant differences ($P < 0.05$) are represented with an asterisk between the antibiotic treatment and the Control sample without antibiotics.

was the closest to a expected proportion of 50% (48.4% females) and not being significantly different ($P > 0.05$) than the other 3 treatments (**Figure 5**).

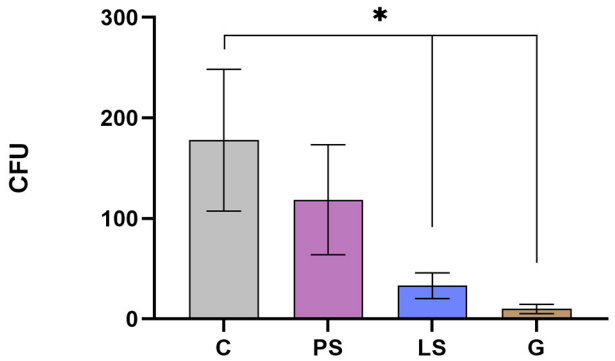


FIGURE 3 | Total viable aerobic bacterial (colony-forming units) in the four experimental groups: Control (C), without antibiotics; Penicillin-Streptomycin (PS); (500 UI)−625 μg/mL, respectively; Lincomycin-Spectinomycin (LS) 300–600 μg/mL, respectively; and Gentamicin (G) 1,000 μg/mL. Eight males were analyzed (1 ejaculate per male) including the same males in each experimental group. Significant differences ($P < 0.05$) are represented with an asterisk between the antibiotic treatment and the Control sample without antibiotics.

DISCUSSION

The increasing challenge to health care attributable to antimicrobial resistance, and the subsequent absence of access to effective treatments, is a worldwide concern. In the same way, the use of antibiotics as an additive in sperm extender is a standard nowadays. Semen is normally colonized by a high variety of microorganisms that may reduce sperm quality. However, contradictory results on the effect of bacterial flora can be found, as well as in the effect of different antibiotic families depending on such important factors as the species, the sperm extender composition, or even the cryopreservation protocols (26). In this context, it is important to carry out an intensive assessment, not only assessing *in vitro* sperm parameters but also assessing important productive parameters, of the main antibiotic families used nowadays in sperm extenders and get a clear idea about their effect and the suitability of their use. In this study, a total of 10 genera and 16 bacterial species were identified with *Pseudomonas* and *Staphylococcus* as the most common genera followed by *E. coli*. Other bacterial species were also isolated in a lower number such as *Sphingomonas paucimobilis*, *Deftia acidovorans*, *Mycrobacterium oxydans*, *Kocuria*, and *Corynebacterium*. Some of these bacteria were also found by Yániz et al. (10) in ram sperm. In the same way, *E.coli*, which is one of the most common species isolated in the current experiment, has also been detected as the most frequent bacterial species in human (6), equine (18), canine (27), and porcine (20) sperm doses.

As we have previously described, the effect of antibiotics on sperm quality have controversial results attending to factors such as species, type of extender, or cryopreservation protocol. Many authors recommended some antibiotics such as gentamicin as a suitable additive for semen storage (18, 28, 29). In the present study, gentamicin was the antibiotic exhibiting the most

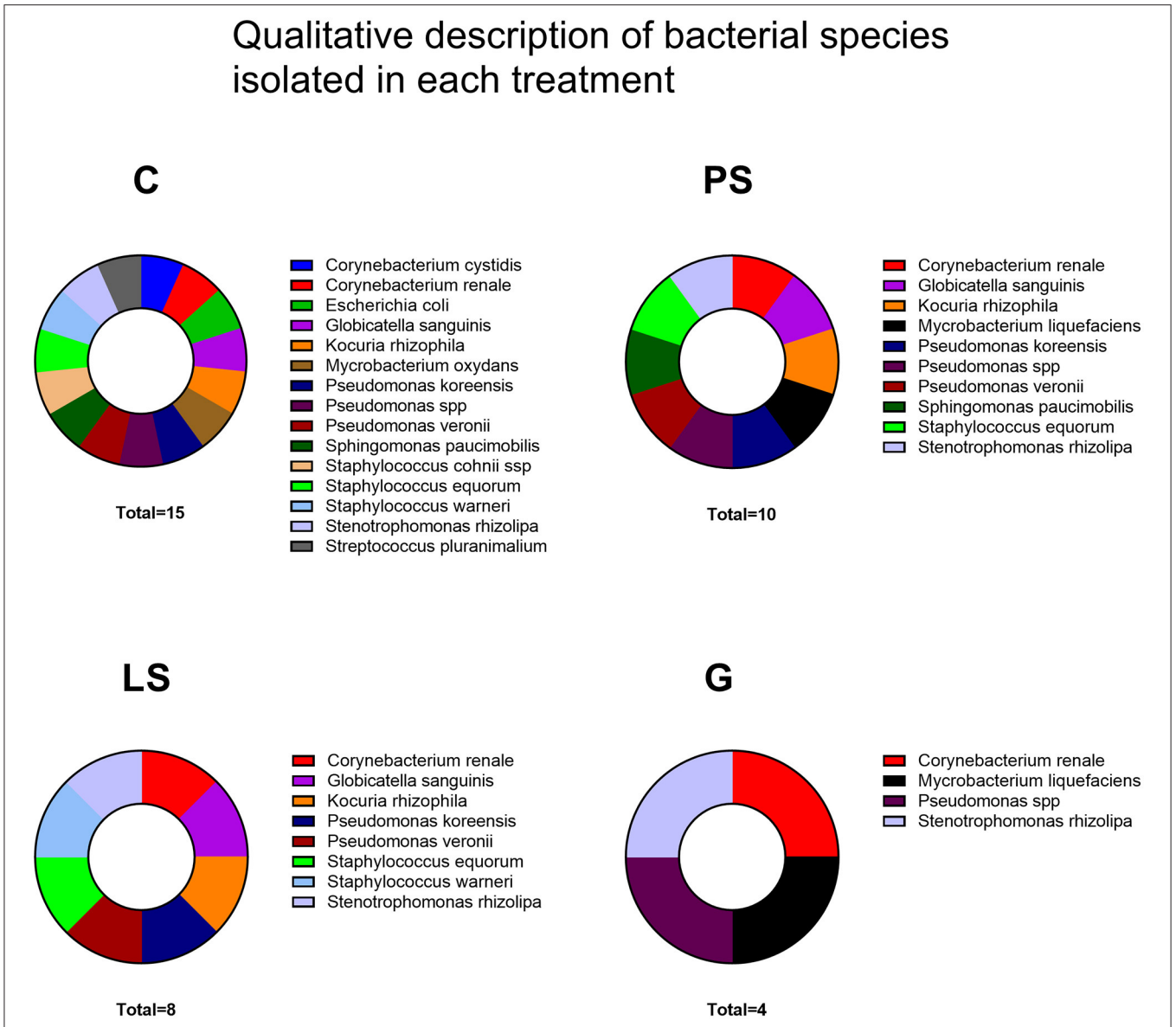


FIGURE 4 | Bacterial species isolated in each treatment after thawing: Control (C), without antibiotics; Penicillin-Streptomycin (PS), (500 UI)−625 μg/mL, respectively; Lincomycin-Spectinomycin (LS), 300–600 μg/mL, respectively; and Gentamicin (G) 1,000 μg/mL. Eight males were analyzed (1 ejaculate per male) including the same males in each experimental group.

powerful effect inhibiting bacterial growth together with LS. In contrast, Gentamicin was also the treatment with a lower sperm quality assessed *in vitro*. Jasko et al. (29) showed a negative effect on sperm motility when using gentamicin concentrations >1 mg/mL in equine sperm. On the contrary, Yániz et al. (10) found that the concentration of gentamicin in sperm extender up to 0.5 g/l did not affect sperm motility and viability after storage at 15°C for 48 h in ram. At this point, 1 g/L of gentamicin concentration looked like the safety limit for this type of antibiotic in equine. However, our results report a significant decrease in fertility and fecundity when this concentration of gentamicin was used. It could be possible that ram sperm are more sensitive to this type of antibiotic at this concentration than stallion. It should be taken into account that our study is the first time that a negative effect on fertility has been described in ram when gentamicin is used as an additive extender. With the same concentration (1 g/L) of Gentamicin, Aurich and Spergser (1) showed that sperm motility assessed *in vitro* was significantly reduced and the bacterial inhibition was not as high as expected in stallion sperm. This fact shows that there is a very important specie specific factor that can determine the efficiency of antibiotical substances in sperm extender among species. In this way, and after assessing our results, we can conclude that our concentration of gentamicin works greatly inhibiting the bacterial growth, but it resulted in a

TABLE 1 | Fertility results (lambed ewe/100 inseminated ewe) by treatment as follows: Control (without antibiotics), Penicillin-Streptomycin; with Penicillin (500 UI) and Streptomycin to a final concentration of 625 μg/mL; Lincomycin-Spectinomycin to a final concentration of 300 and 600 μg/mL, respectively; and Gentamicin to a final concentration of 1,000 μg/mL.

Treatment	Inseminated ewes (n)	Lambed ewes (n)	Fertility (%)
Control (without antibiotics)	221	117	52.9a
Penicillin-Streptomycin (500 UI–625 μg/mL)	211	105	49.7a
Lincomycin-Spectinomycin (300 and 600 μg/mL)	206	104	50.5a
Gentamicin (1,000 μg/mL)	214	83	38.8b

Ten males were used.
Different low case letters (a,b) indicate significant differences ($P < 0.05$) among treatments.

TABLE 2 | Multiple lambing frequency (%), prolificacy (lambs/lambed ewe), and fecundity results (lambs/inseminated ewes) by treatment as follows: Control (without antibiotics), Penicillin-Streptomycin; with Penicillin (500 UI) and Streptomycin to a final concentration of 625 μg/mL; Lincomycin-Spectinomycin to a final concentration of 300 and 600 μg/mL, respectively; and Gentamicin to a final concentration of 1,000 μg/mL.

Treatment	Multiple lambing frec. (%)	Prolificacy (MEAN±SE)	Fecundity (MEAN±SE)
Control (without antibiotics)	48.72ab	1.52 ± 0.05ab	0.81 ± 0.06a
Penicillin-Streptomycin (500 UI–625 μg/mL)	55.24a	1.67 ± 0.07a	0.83 ± 0.07a
Lincomycin-Spectinomycin (300 and 600 μg/mL)	41.35b	1.47 ± 0.06b	0.74 ± 0.06ab
Gentamicin (1,000 μg/mL)	45.78ab	1.53 ± 0.07ab	0.59 ± 0.06b

Ten males were used.
Different low case letters (a,b) indicate significant differences ($P < 0.05$) among treatments.

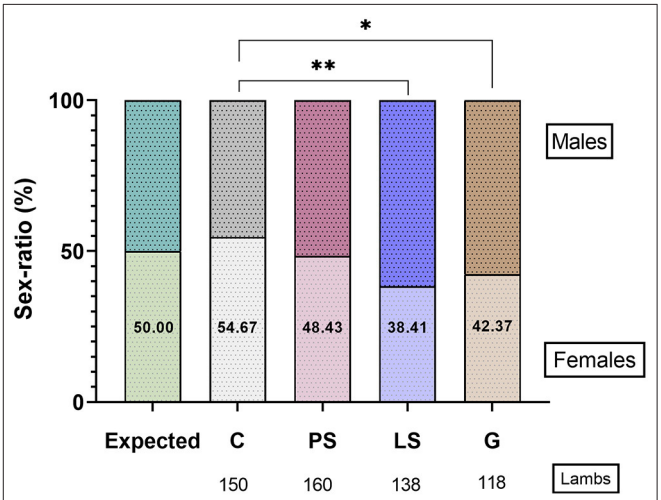


FIGURE 5 | The sex-ratio results for female (females/100 lambs) in the four experimental groups: Control (C), without antibiotics; Penicillin-Streptomycin (PS), (500 UI)–625 μg/mL, respectively; Lincomycin-Spectinomycin (LS), 300–600 μg/mL, respectively; and Gentamicin (G) 1,000 μg/mL. 371 ewes were inseminated with sperm doses from 10 males. *$P \leq 0.05$, **$P \leq 0.01$.

toxic effect on some sperm quality assesments (rapid progressive motility and different kinetic parameters: VAP, VSL, and ALH, **Figure 2**). This altered sperm quality could be affecting negatively important livestock parameters such as fertility. This fact is correlated to the decreased progressive motility assessed *in vitro*. In this context, high values of progressive motile sperm have been demonstrated as a good correlation factor with fertility in humans (30, 31). Probably this negative effect is due to some deleterious effects at the structural level since the sperm metabolism was not affected by the gentamicin respect to the C samples. A recent study carried out by Riesco et al. (32) demonstrated a novel sperm protein (ProAKAP4) as a promising diagnostic parameter of sperm quality in ram sperm, so long correlated to sperm motility. This protein is the most expressed protein of the sperm fibrous sheath being an important part of the flagellum (33, 34). This type of protein has also been related to sperm quality in several species such as humans (35), mice (36), or boar (37). In this sense, further investigations should be carried out to identify the exact mechanism of gentamicin toxicity. In any case, and in view of the results obtained in the present study, it is clear that gentamicin is not a suitable option, under these conditions, taking into account their negative effects, especially in the productive parameters.

The PS was the treatment with lower efficacy inhibiting bacterial growth. It gets a lower number of CFU but this decrease was not significant with respect to the control samples. This treatment (PS) has been one of the most common and extended antibiotics since it was discovered in 1928, so this intensive use for many decades could have produced a mechanism of resistance to this type of treatment (38, 39). In contrast, when the sperm samples were assessed *in vitro* those samples treated with PS showed the best results in the sperm physiology keeping higher values of viable sperm after submitting thawed samples to a stress test. The same effect was found when assessing the sperm with high mitochondrial activity, getting the best result of the experiment at both times 0 and 2 h. This fact could suggest that PS at this concentration is a suitable treatment since it inhibits in part the bacterial growth, not affecting the sperm quality. In the same way and after the field trials, PS keeps exhibiting similar results to Controls samples in fertility and prolificacy, not showing any adverse or toxic effect as the G in the fertility, or the LS in the prolificacy.

Finally, the LS treatment exhibited very interesting and complex results. After assessing sperm quality *in vitro*, LS did not show any deleterious effect either in the sperm physiology or in the sperm motility respect to the C samples. In addition, it was after the G, the treatment with the highest bacterial inhibitory activity, but not exhibiting adverse effects on the sperm quality *in vitro*. This fact could suggest that this treatment (LS) at this concentration has a perfect balance between the

bacterial inhibitory effects, not affecting the sperm physiology in ram. In this study, similar results to Azawi and Ismaeel (16), when assessing sperm quality *in vitro*, were obtained but opposite results when assessing the effect inhibiting bacterial growth, finding these authors a poor antimicrobial effect using a concentration of 1 mg/ml of lincomycin in ram. These differences could be due to several factors such as the use of Lincomicyn alone (not combined with spectinomycin) or the experimental design (refrigerated samples at 5°C till 96 h). In any case, Lincomicyn seems to be more efficient when using combined with spectinomycin, allowing us to use lower concentrations, getting similar results in the sperm quality *in vitro*, and performing a very high inhibition in the bacterial growth. In the same way, Akhter et al. (19) have demonstrated the superiority of LS compared to PS combinations inhibiting bacterial growth in buffalo sperm samples. These and our results are in concordance with those obtained by Aleem et al. (40) in buffalo, where those authors agree that several bacterial species exhibited antimicrobial resistance to PS combinations while LS and others showed a higher antimicrobial effect. After the field trials, some interesting effects were observed. Neither fertility nor prolificacy or fecundity were affected as expected. However, after assessing the prolificacy we can observe that this parameter was lower in respect to the samples without antibiotic. This effect could be due to some kind of sublethal effect, not being capable to detect it *in vitro* as previously occurred in species such as boar (41) or buffalo (19). On the other hand, when the fecundity was assessed this lower prolificacy was compensated because the fecundity is a complex parameter where fertility and prolificacy are combined. This is the first time that these antibiotic treatments have been tested under field conditions in ram with a large number of ewes inseminated.

It is known that the plasma membrane from X or Y-sperm are diferent. Korchunjit et al. (42) found that different defined combinations of cryomedia and sperm extender significantly alter the survivar ratio of frozen-thawed X-Y sperm. In this sense, some interesting results were observed in our study when the sex ratio was assessed. Samples without antibiotics have deviated to females but LS and G had deviated to males. Neither the experimental group nor the control group has differences concerning the expected sex ratio (50:50). If we observed the results obtained in CFU and sex ratio we can observe that sperm samples with higher microbial charge result in a higher number of females, and the treatments with the lower microbial contamination (LS and G) resulted in a significantly lower number of females, which is negative in dairy species where females have the main economical value. At this point, it looks like X-sperm has some kind of resistance to microbial contamination, or that the Y-sperm are more resistant to some possible deleterious or toxic effect of LS and G. To this concern, these results pave the way for further investigations about the interaction bacteria/sperm, and to assess the different effect of antibiotic treatments in the different sperm subpopulations (Y or X-sperm).

The sex ratio is a complex parameter that depends on many biological and environmental factors. Preview studies have found both pre- and post-fertilization factors. Moreover, sex ratio bias may be related to both semen and the uterine environment in humans (43). Studies linking sperm quality and sex ratio are controversial. Some studies carried out in humans did not find differences in the sex ratio between seminal samples with a normal quality or with moderate or severe alterations (oligozoospermia, asthenozoospermia, and teratozoospermia) (44) or at least, this relationship is weak. However, in humans, Bae et al. (45) observed that the percentage of bicephalic sperm was significantly associated with the increase of born females. In the same way Arikawa et al. (46), using IVF, observed that samples with low sperm motility (<40%) had a minor proportion of males compared to those samples with normal sperm motility. This variation in the sex ratio (less males) observed with low-quality semen could be associated with a lower proportion of Y-bearing sperm in the ejaculate, as suggested by Eisenberg et al. (47) in humans. Contrary, in our study the samples with lower VAP, ALH, VSL, and rapid PM increase the percentage of born males according to Mossman et al. (48) in humans. Group G had fewer rapid progressive sperm *in vitro* and this could impair the transport of X-bearing sperm *in vivo*. A study conducted by Balli et al. (49) in humans showed an association of higher sperm velocity in semen from patients that conceived predominantly female offsprings when compared with patients with male offsprings after assisted reproductive technology (ART) treatment.

Finally, it could be interesting to do a deep reflection about the use of antibiotics as additives in sperm extenders. As mentioned above, the reduction of using antibiotics is a worldwide concern. Among the main causes of bacterial resistance, there are some social issues such as overpopulation or global migration, but also the increasing use of antibiotics in clinics and animal production (50, 51). Recent studies carried out in boar sperm (52) are trying to optimize sperm handling protocols to avoid or reduce the use of antibiotics. Taking into account the results obtained in the present study, when using healthy semen donors under satisfactory hygiene-pathology conditions and when the samples are going to follow a frozen-thawing process, non-deleterious effects have been observed either in the sperm quality assessed *in vitro* or in the productive parameters (fertility, prolificacy, or fecundity) when using sperm samples without antibiotics. Our results suggest that the bacterial contamination control in frozen-thawed semen may be possible without the use of antibiotics, although the effects of longer periods of cooling storage and different temperatures of storage need to be further investigated for animal semen.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The current study was performed according to the Guidelines of the European Union Council (86/609/EU, modified by 2010/62/EU), following Spanish regulations (RD/1201/2005,

abrogated by RD/2013) for the use of laboratory animals. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of León (Spain) (ÉTICA-ULE-013-2018).

AUTHOR CONTRIBUTIONS

LA-L: conceptualization, methodology, formal analysis, investigation, resources, writing—original draft, data curation, writing—review & editing, visualization, supervision, and project administration. MR: methodology, investigation, supervision, data curation, formal analysis, and writing—review & editing. RM-G, MN-M, JB, CC, CO-F, and JA: methodology and investigation. AC: data curation, methodology, and investigation. PP: formal analysis, investigation, resources, writing—original draft, data curation, writing—review & editing, visualization, supervision, and funding acquisition. MA: conceptualization, methodology, investigation, resources, writing—original draft, data curation, writing–review & editing, visualization, and funding acquisition. LA: conceptualization, resources, formal analysis, resources, writing—original draft, data curation, writing—review & editing, visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

The authors acknowledge the JCyL-LE253P18 research contract and ORDEN EDU/556/2019 fellowship (Junta Castilla y León), FPU17/04142 (MCIU) and PRE2018-086400 (MEC) fellowships, the ITRA-ULE technicians (Ainoa Jordán, Pedro de Vega and Cecilia Pérez), ANCHE and the staff from Ovigén.

REFERENCES

1. Aurich C, Spergser J. Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. *Theriogenology.* (2007) 67:912–8. doi: 10.1016/j.theriogenology.2006.11.004
2. Bielanski A. Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. *Theriogenology.* (2007) 68:1–22. doi: 10.1016/j.theriogenology.2007.03.025
3. Auroux MR, Jacques L, Mathieu D, Auer J. Is the sperm bacterial ratio a determining factor in impairment of sperm motility: an *in-vitro* study in man with *Escherichia coli*. *Int J Androl.* (1991) 14:264–70. doi: 10.1111/j.1365-2605.1991.tb01091.x
4. Kaur S, Prabha V, Sarwal A. Receptor mediated agglutination of human spermatozoa by spermagglutinating factor isolated from *Staphylococcus aureus*. *J Urol.* (2010) 184:2586–90. doi: 10.1016/j.juro.2010.07.031
5. Monga M, Robertst JA. Spermagglutination by bacteria: receptor-specific interactions. *J Androl.* (1994) 15:151–6.
6. Diemer H, Michelmann M, Schiefer W. Escherichia coli-induced alterations of human spermatozoa. An electron microscopy analysis. *Int J Androl.* (2000) 23:178–86. doi: 10.1046/j.1365-2605.2000.00224.x
7. Villegas J, Schulz M, Soto L, Sanchez R. Bacteria induce expression of apoptosis in human spermatozoa. *Apoptosis.* (2005) 10:105–10. doi: 10.1007/s10495-005-6065-8
8. Kurpisz M, Alexander NJ. Carbohydrate moieties on sperm surface: physiological relevance. *Fertil Steril.* (1995) 63:158–65. doi: 10.1016/S0015-0282(16)57312-9
9. EUR-Lex−31992L0065–END–EUR-Lex [WWW Document]. Available online at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:31992L0065 (accessed April 9, 2020).
10. Yániz JL, Marco-Aguado MA, Mateos JA, Santolaria P. Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15ºC. *Anim Reprod Sci.* (2010) 122:142–9. doi: 10.1016/j.anireprosci.2010.08.006
11. Gloria A, Contri A, Wegher L, Vignola G, Dellamaria D, Carluccio A. The effects of antibiotic additions to extenders on fresh and frozen-thawed bull semen. *Anim Reprod Sci.* (2014) 150:15–23. doi: 10.1016/j.anireprosci.2014.08.012
12. Ortega-Ferrusola C, Johannisson A, Peña Vega FJ, Tapia JA, Rodriguez-Martinez H, Dalin AM, et al. Effect of different extenders seminal plasma on the susceptibility of equine spermatozoa to lipid peroxidation after single-layer centrifugation, through Androcoll-E. *J Equine Vet Sci*. (2011) 31:411–6. doi: 10.1016/j.jevs.2011.01.010
13. Schulze M, Grobbel M, Riesenbeck A, Brüning S, Schaefer J, Jung M, et al. Dose rates of antimicrobial substances in boar semen preservation-time to establish new protocols. *Reprod Domest Anim.* (2017) 52:397–402. doi: 10.1111/rda.12921
14. Anel-López L, García-Álvarez O, Parrilla I, Del Olmo D, Maroto-Morales A, Fernandez-Santos MRR, et al. Effect of sex-sorting and cryopreservation on the post-thaw sperm quality of iberian red deer spermatozoa. *Theriogenology.* (2017) 89:206–13. doi: 10.1016/j.theriogenology.2016.11.010
15. López-Urueña E, Alvarez M, Gomes-Alves S, Manrique P, Anel-López L, Chamorro CA, et al. Alternative procedures for the cryopreservation of brown bear ejaculates depending on the flexibility of the "in cooling" period (5°C). *Cryobiology.* (2014) 69:434–41. doi: 10.1016/j.cryobiol.2014.10.001
16. Azawi OI, Ismaeel MA. Influence of addition of different antibiotics in semen diluent on viable bacterial count and spermatozoal viability of awassi ram semen. *Vet World.* (2012) 5:75–9. doi: 10.5455/vetworld.2012.75-79
17. Madeira E, Goularte K, Pradieé J, Mondadori R, Lucia J, Bianchi I, et al. The use of antibiotics in cryopreservation of ram sperm. *Int J Vet Med Res Reports.* (2014) 2014:154947. doi: 10.5171/2014.154947
18. Varner DD, Scanlan CM, Thompson JA, Brumbaugh GW, Blanchard TL, Carlton CM, et al. Bacteriology of preserved stallion semen and antibiotics in semen extenders. *Theriogenology.* (1998) 50:559–73. doi: 10.1016/S0093-691X(98)00161-7
19. Akhter S, Ansari MS, Andrabi SMH, Ullah N, Qayyum M. Effect of antibiotics in extender on bacterial and spermatozoal quality of cooled buffalo (Bubalus bubalis) bull semen. *Reprod Domest Anim.* (2008) 43:272–8. doi: 10.1111/j.1439-0531.2007.00890.x
20. Althouse GC, Kuster CE, Clark SG, Weisiger RM. Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology.* (2000) 53:1167–76. doi: 10.1016/S0093-691X(00)00261-2
21. Maes D, Nauwynck H, Rijsselaere T, Mateusen B, Vyt P, de Kruif A, et al. Diseases in swine transmitted by artificial insemination: an overview. *Theriogenology.* (2008) 70:1337–45. doi: 10.1016/j.theriogenology.2008.06.018
22. Maroto Martín LO, Muñoz EC, De Cupere F, Van Driessche E, Echemendia-Blanco D, Rodríguez JMM, et al. Bacterial contamination of boar semen affects the litter size. *Anim Reprod Sci.* (2010) 120:95–104. doi: 10.1016/j.anireprosci.2010.03.008
23. Aslam B, Wang W, Arshad MI, Khurshid M, Muzammil S, Rasool MH, et al. Antibiotic resistance: a rundown of a global crisis. *Infect. Drug Resist*. (2018) 11:1645–58. doi: 10.2147/IDR.S173867
24. Anel L, de Paz P, Alvarez M, Chamorro C, Boixo J, Manso A, et al. Field and *in vitro* assay of three methods for freezing ram semen. *Theriogenology.* (2003) 60:1293–308. doi: 10.1016/S0093-691X(03)00140-7

25. Alvarez M, Chamorro CAA, Kaabi M, Anel-Lopez L, Boixo JCC, Anel E, et al. Design and "*in vivo*" evaluation of two adapted catheters for intrauterine transcervical insemination in sheep. *Anim Reprod Sci.* (2012) 131:153–9. doi: 10.1016/j.anireprosci.2012.03.001
26. Morrell JM, Wallgren M. Alternatives to antibiotics in semen extenders: a review. *Pathogens.* (2014) 3:934–46. doi: 10.3390/pathogen 3040934
27. Bjurström L, Linde-Forsberg C. Long-term study of aerobic bacteria of the genital tract in stud dogs. *Am J Vet Res.* (1992) 53:670–3.
28. Clément F, Vidament M, Guérin B. Microbial contamination of stallion semen. *Biol Reprod.* (1995) 52:779–86. doi: 10.1093/biolreprod/52.monograph_series1.779
29. Jasko DJ, Bedford SJ, Cook NL, Mumford EL, Squires EL, Pickett BW. Effect of antibiotics on motion characteristics of cooled stallion spermatozoa. *Theriogenology.* (1993) 40:885–93. doi: 10.1016/0093-691X(93) 90356-A
30. Vasan SS. Semen analysis and sperm function tests: how much to test? *Indian J Urol.* (2011) 27:41–48. doi: 10.4103/0970-1591.78424
31. Zhao Y, Vlahos N, Wyncott D, Petrella C, Garcia J, Zacur H, et al. Impact of semen characteristics on the success of intrauterine insemination. *J Assist Reprod Genet.* (2004) 21:143–8. doi: 10.1023/B:JARG.0000031246.76666.f6
32. Riesco MF, Anel-Lopez L, Neila-Montero M, Palacin-Martinez C, Montes-Garrido R, Alvarez M, et al. ProAKAP4 as novel molecular marker of sperm quality in ram: an integrative study in fresh, cooled and cryopreserved sperm. *Biomolecules.* (2020) 10:1046. doi: 10.3390/biom10071046
33. Huang Z, Somanath PR, Chakrabarti R, Eddy EM, Vijayaraghavan S. Changes in intracellular distribution and activity of protein phosphatase PP1γ2 and its regulating proteins in spermatozoa lacking AKAP41. *Biol Reprod.* (2005) 72:384–92. doi: 10.1095/biolreprod.104.034140
34. Pereira R, Sá R, Barros A, Sousa M. Major regulatory mechanisms involved in sperm motility. *Asian J Androl.* (2017) 19:5–14. doi: 10.4103/1008-682X.167716
35. Rahamim Ben-Navi L, Almog T, Yao Z, Seger R, Naor Z. A-Kinase anchoring protein 4 (AKAP4) is an ERK1/2 substrate and a switch molecule between cAMP/PKA and PKC/ERK1/2 in human spermatozoa. *Sci Rep.* (2016) 6:37922. doi: 10.1038/srep37922
36. Fang X, Huang LL, Xu J, Ma CQ, Chen ZH, Zhang Z, et al. Proteomics and single-cell RNA analysis of Akap4-knockout mice model confirm indispensable role of Akap4 in spermatogenesis. *Dev Biol.* (2019) 454:118–27. doi: 10.1016/j.ydbio.2019.06.017
37. Kwon WS, Oh SA, Kim YJ, Rahman MS, Park YJ, Pang MG. Proteomic approaches for profiling negative fertility markers in inferior boar spermatozoa. *Sci Rep.* (2015) 5:13821. doi: 10.1038/srep 13821
38. Sengupta S, Chattopadhyay MK, Grossart HP. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Front Microbiol.* (2013) 4:47. doi: 10.3389/fmicb.2013.00047
39. Spellberg B, Gilbert DN. The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clin Infect Dis.* (2014) 59:S71–5. doi: 10.1093/cid/ciu392
40. Aleem M, Chaudhry R, Khan N, Rizvi A, Ahmad R. Occurrence of pathogenic bacteria in buffalo semen. *Buffalo J.* (1990) 6:93–8.
41. Waberski D, Weitze KF, Meding S, Leiding C, Weiskopf S. Examination on sperm- and fertility-compatibility of lincospectinsterile solution as antibiotic additive in liquid boar semen. *Reprod Domest Anim.* (1990) 25:291–5. doi: 10.1111/j.1439-0531.1990.tb00476.x
42. Korchunjit W, Kaeoket K, Kitiyanant Y, Taylor J, Wongtawan T. Defined combinations of cryomedia and thawing extenders influence the viable X-Y boar sperm ratio *in vitro*. *Cryo Lett.* (2017) 38:160–5.
43. Graffelman J, Fugger EF, Keyvanfar K, Schulman JD. Human live birth and sperm-sex ratios compared. *Hum Reprod.* (1999) 14:2917–9. doi: 10.1093/humrep/14.11.2917
44. Jacobsen R, Bostofte E, Skakkebæk NE, Hansen J, Møller H. Offspring sex ratio of subfertile men and men with abnormal sperm characteristics. *Hum Reprod.* (2000) 15:2369–70. doi: 10.1093/humrep/15.11.2369
45. Bae J, Kim S, Chen Z, Eisenberg ML, Buck Louis GM. Human semen quality and the secondary sex ratio. *Asian J Androl.* (2016) 18:374–81. doi: 10.4103/1008-682X.173445
46. Arikawa M, Jwa SC, Kuwahara A, Irahara M, Saito H. Effect of semen quality on human sex ratio in in vitro fertilization and intracytoplasmic sperm injection: An analysis of 27,158 singleton infants born after fresh single-embryo transfer. *Fertil Steril.* (2016) 105:897–04. doi: 10.1016/j.fertnstert.2015.12.009
47. Eisenberg ML, Murthy L, Hwang K, Lamb DJ, Lipshultz LI. Sperm counts and sperm sex ratio in male infertility patients. *Asian J Androl.* (2012) 14:683–6. doi: 10.1038/aja.2012.58
48. Mossman JA, Slate J, Birkhead TR, Moore HD, Pacey AA. Sperm speed is associated with sex bias of siblings in a human population. *Asian J Androl.* (2013) 15:152–4. doi: 10.1038/aja.2012.109
49. Balli KS, Patton WC, Jacobson JD, Corselli J, King A, Chan PJ. Sperm velocity in seminal plasma and the association with gender of offspring. *Arch Androl.* (2004) 50:37–40. doi: 10.1080/01485010490250560
50. Marshall BM, Levy SB. Food animals and antimicrobials: Impacts on human health. *Clin Microbiol Rev*. (2011) 24:718–33. doi: 10.1128/CMR.00002-11
51. Singer AC, Shaw H, Rhodes V, Hart A. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Front Microbiol*. (2016) 7:1728. doi: 10.3389/fmicb.2016.01728
52. Waberski D, Luther AM, Grünther B, Jäkel H, Henning H, Vogel C, et al. Sperm function *in vitro* and fertility after antibiotic-free, hypothermic storage of liquid preserved boar semen. *Sci Rep.* (2019) 9:14748. doi: 10.1038/s41598-019-51319-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 24 May 2021
doi: 10.3389/fvets.2021.650946

Sperm Response to *in vitro* Stress Conditions in Wild and Domestic Species Measured by Functional Variables and ROS Production

Emma O'Brien[1], *Pedro García-Casado*[2], *Cristina Castaño*[1], *Adolfo Toledano-Díaz*[1], *Paula Bóveda*[1] *and Julián Santiago-Moreno*[1]*

[1] Department of Animal Reproduction, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain, [2] Zoitechlab (Arquimea Group), R&D Department, Madrid, Spain

The domestication process has resulted in profound changes in the reproductive physiology of the animals that might have affected the sperm characteristics and thus their sensitivity to handling and cryopreservation procedures. This work assesses the response of the sperm of domestic and wild ungulates to a cooling storage at 15°C for 20 h followed by incubation at 38.5°C, 5% CO_2, for 2 h. In addition, this paper examines the most representative sperm traits to assess their responsiveness to these stress conditions. Sperm samples were collected from domestic and their wild ancestor species: ram, mouflon, buck, Iberian ibex, domestic boar, and wild boar. Sperm motility, viability, mitochondrial membrane status, DNA fragmentation, and reactive oxygen species production were evaluated at the beginning of the experiment, after 20 h of refrigeration at 15°C, and, finally, at 2 h of incubation at 38.5°C. Sperm from all domestic species (ram, buck, and domestic boar) suffered more stress than their wild relatives (mouflon, Iberian Ibex, and wild boar). In pigs, the percentage of intact mitochondria was lower in the domestic species compared to wild boar. In sheep, we found a higher reactive oxygen species production in rams, while in goats, the curvilinear velocity was lower in the domestic species. The PCA (principal components analysis) showed that the motility and their kinetic variables were the most represented variables in the principal components of all species, indicating that they are essential biomarkers for evaluating the stress response. Sperm viability was highlighted as a representative variable for evaluating the stress response in domestic boar, mouflon, ram, and ibex.

Keywords: spermatozoa, cool-storage, mitochondrial membrane integrity, reactive oxygen species, DNA fragmentation

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Abouzar Najafi, University of Tehran, Iran
Bouchra El Amiri, Institut National de la Recherche Agronomique de Settat, Morocco

***Correspondence:**
Julián Santiago-Moreno
moreno@inia.es

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *08 January 2021*
Accepted: *07 April 2021*
Published: *24 May 2021*

Citation:
O'Brien E, García-Casado P, Castaño C, Toledano-Díaz A, Bóveda P and Santiago-Moreno J (2021) Sperm Response to in vitro Stress Conditions in Wild and Domestic Species Measured by Functional Variables and ROS Production. Front. Vet. Sci. 8:650946. doi: 10.3389/fvets.2021.650946

INTRODUCTION

Many factors affect sperm preserved under chilled conditions. Cold shock is associated with irreversible changes in capacitation, reduction of the sperm motility, and damage to the plasma membrane (1–3). During cooling, sperm cells are exposed to many harmful effects including ionic imbalance, activation of proteases, membrane phase transition, destabilization of the cytoskeleton, and production of free radicals or reactive oxygen species (ROS) (4). Moreover, there is much evidence indicating that sperm cell dysfunction is mainly induced by oxidative stress (5–8). The

increase of ROS levels disrupts the mitochondrial and plasma membranes of sperm cells, thus impairing their motility (9). This also causes DNA fragmentation (10), which affects the future sperm fusion with the oocyte.

The sperm sensitivity to cooling and freezing processes may vary among species. These differences may be attributable to variations in plasma membrane compositions (11), for instance, the content of lipids in the bilayer, degree of hydrocarbon chain saturation, cholesterol/phospholipids ratio, and protein/phospholipid ratio (12).

The domestication process in ungulate species has resulted in profound changes in the reproductive physiology (13, 14), such as the endocrine cycles and in the sexual accessory glands activity (15), that might have affected the sperm characteristics and thus their sensitivity to handling and cryopreservation procedures. It is well-known that the animal domestication involved drastic phenotypic changes driven by strong artificial selection to obtain new populations of breeds (16). This selection pressure strongly reduced the initial gene pool. More recently, the selection pressure was increased again via the use of artificial insemination, leading to a few industrial breeds with very high performances, but with low effective population sizes (17). Moreover, just 14 out of the about 30 domesticated mammalian and bird species provide 90% of human food supply from animals. Agriculture has concentrated in the past only on a very small number of breeds worldwide (18). This has led to substantive animal genetic erosion, which could ultimately have affected the resistance capacity of sperm to stress conditions. The questions that arise here are: Are there differences in the sperm cryoresistance between domestic species and their wild ancestors? Has domestication affected the resistance mechanisms of sperm to stress conditions?

To address these questions, we subjected sperm samples from six different species (domestic and their respective wild ancestors) to different incubation conditions (20 h at 15°C and then 2 h at 38.5°C, 5% CO_2) and we compared their response to these stress conditions. The comparative experimental design included boar (*Sus scrofa domestica*) vs. wild boar (*Sus scrofa*), ram (*Ovis aries*) vs. mouflon (*Ovis musimon*), and buck (*Capra hircus*) vs. ibex (*Capra pyrenaica*). In addition, this paper examines the most representative sperm traits to assess their responsiveness to these stress conditions. To our knowledge, this is the first study where the stress response of sperm is compared between wild and domestic species using diverse techniques to evaluate sperm quality.

MATERIALS AND METHODS

Experimental Animals and Semen Collection

Experimental animals were 31 adult males belonging to six species: ram (*Ovis aries*) aged 5–6 years ($n = 5$), mouflon (*Ovis musimon*) aged 4–8 years ($n = 6$), buck (*Capra hircus*) aged 5–6 years ($n = 5$), Iberian ibex (*Capra pyrenaica*) aged 4–11 years ($n = 6$), domestic boar (*Sus scrofa domestica*) aged 1–2 years ($n = 5$), and wild boar (*Sus scrofa*) aged 1–3 years ($n = 4$).

The rams, bucks, ibexes, and mouflons were housed under natural day length and temperature conditions at the Department of Animal Reproduction of INIA (Madrid, 40°25'N). Boars were housed under 12 h of artificial light and 18–22°C of ambient temperature at La Abadía, Stud Boar from Núcleos Genéticos 21, S. L. (Toledo). Two wild boars were housed at Iberian Kuna Fauna Center (Navas del Rey, Madrid) and two wild boars were captured at the Wildlife Park "El Pardo" (Madrid).

Semen ejaculates were collected from the domestic rams and bucks using an artificial vagina, as previously described Galarza et al. (19). Boar semen collection was performed with the gloved hand method, discarding gel fraction (20). The ejaculated samples from Iberian ibexes, mouflons, and wild boars were obtained by transrectal ultrasound-guided massage of the accessory sex glands (TUMASG), combined with electroejaculation when required (21, 22).

Animals were handled according to procedures approved by the INIA Ethics Committee that specifically approved the design of the current study (reference number PROEX 271/14) and were performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 2010/63/UE regarding the protection of animals used in scientific experiments.

Experimental Design

All reagents were purchased from Merck KGaA and/or its affiliates (Darmstadt, Germany) and Roche (Basel, Switzerland).

Immediately after sperm collection, each ejaculate was diluted with their species-specific solution (ACROMAX PLUS®, TTG or TCG, depending on the species) at 37°C and transported to the laboratory for initial assessment. Sperm-rich fraction of wild and domestic boar were diluted (1:1 v/v) in ACROMAX PLUS® (ZoitechLab, S.L., Madrid, Spain). Sperm from mouflon and ram were diluted (1:1 v/v) in TTG medium (210.59 mM Tes, 95.75 mM Tris, 10.09 mM glucose, 0.54 mM streptomycin, and 2.14 mM penicillin; 324 mOsm/kg, pH 7.1) (23). Sperm samples of Iberian ibex and buck were diluted (1:1 v/v) in Tris-citric acid-glucose (TCG) medium (313.7 mM Tris, 104.7 mM citric acid, and 30.3 mM glucose; 345 mOsm/kg, pH 6.8) (24).

Diluted samples were centrifuged (900 × *g* for 20 min) and the pellets were re-suspended in their corresponding medium [ACROMAX PLUS®, TTG + 6% egg yolk (EY), or TCG + 6% egg yolk (EY)], to a final concentration of 400 × 10^6 sperm/ml. Then, samples were subjected to stress: firstly, by refrigerating them at 15°C for 20 h, and then incubating at 38.5°C, 5% CO_2 for 2 h. The sperm traits of each sample were evaluated at the beginning of the experiment (0H), after 20 h of refrigeration at 15°C (20H 15°C), and, finally, at 2 h of incubation at 38.5°C (20H 15°C + 2H 38.5°C).

Assessment of Sperm Variables

Sperm viability were assessed using a seminal quality system SQS2® (ZoitechLab, S.L.—ARQUIMEA GROUP, Madrid, Spain) previously validated in our laboratory (see **Supplementary Material 1**).

Sperm motility analysis was assessed using a computer-aided sperm analysis (CASA) system coupled to a phase contrast

microscope (Nikon Eclipse model 50i; Nikon Instruments Europe B.V., Izasa S.A.; negative contrast) and employing a Sperm Class Analyzer (SCA®, Barcelona, Spain) v.4.0. software (Microptic S.L., Barcelona, Spain). Semen was diluted to a concentration of ∼40 million sperm/ml (25, 26) and loaded onto a warmed (37°C) 20-μm Leja® 8-chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). The percentage of motile sperm and the percentage showing progressive motility were recorded. A minimum of three fields and 500 sperm tracks were evaluated at a magnification of 100× for each sample (21). Motility kinetic variables—curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), and amplitude of lateral head displacement (ALH, μm)—were also recorded.

DNA integrity was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). For this, the kit "*In Situ* Cell Death Detection" (Roche, Basel, Switzerland) was used following the manufacturer's instructions with minor changes (27). Briefly, each sperm sample was diluted to 10×10^6 spermatozoa/ml in 4% paraformaldehyde. Subsequently, 10 μl of this dilution was placed on a glass slide and left to dry. Then, the spermatozoa were permeabilized with 0.1% of Triton X-100 in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). After a wash in PBS, fragmented DNA was nick end-labeled with tetramethylrhodamine-conjugated dUTP by adding 10 μl of the working solution provided by the kit—containing the substrates and the enzyme terminal transferase—on the sample. The reaction was conducted incubating the slides in a humid box for 1 h at 37°C. After a wash with PBS, the nucleus was counterstained with Hoechst at 0.1 mg/ml in PBS for 5 min in the dark. Following an additional wash with PBS, the slides were mounted using Fluoromount (Sigma-Aldrich, MO, USA) and observed under fluorescent microscopy (Eclipse E200, Nikon, Japan). A total of 200 sperm cells per slide were examined using an epifluorescence microscope with a triple band-pass filter (wavelength: 510–560 nm).

Sperm mitochondrial status was assessed using a Mitotracker Green FM® (MITO, Invitrogen M7514), according to Galarza et al. (19) with minor changes. Briefly, samples of 150 μl of semen diluted in TALP Stock (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH_2PO_4 H_2O, 1 mM Na-Lactate, 1.97 mM $CaCl_2$ $2H_2O$, 0.50 mM $MgCl_2$ $6H_2O$, 10 mM HEPES sodium, and 25 mM $NaHCO_3$; 320 mOsm/kg, pH 7.3) to a concentration of 25×10^6 sperm/ml were mixed with 2 μl of MITO (1 mM) and incubated in the dark at 38.5°C for 8 min. After incubation, the samples were transferred to a slide, covered with a cover slip, and examined immediately using an epifluorescence microscope with a triple band-pass filter (excitation: 450 nm, emission: 490 nm). Cells with a high green fluorescent signal in the middle piece were classified as Mitotracker$^+$ (Mito+). A total of 200 sperm cells per slide were examined.

Sperm ROS were detected by using CellROX® green (Thermo Fisher Scientific C10444) according to de Castro et al. (28) with minor changes. Samples of 150 μl of semen diluted in TALP Stock to a concentration of 25×10^6 sperm/ml were mixed with CellROX® green (final concentration, 5 μM) and incubated in the dark at 37°C for 30 min. After incubation, samples were washed with TALP Stock ones (10 min at 190 g) and were transferred to a slide, covered with a cover slip, and examined immediately using an epifluorescence microscope with a triple band-pass filter (excitation: 450 nm, emission: 490 nm). CellROX® green is a fluorescent probe that penetrates the cell and, when oxidized by intracellular free radicals, binds to DNA, emitting a more intense green fluorescence. Cells with a green fluorescent signal on the head were classified as low or high CellROX$^+$ signal. A total of 200 sperm cells per slide were examined.

The response to stress of each species was illustrated by calculating a stress resistance ratio (SR) for the sperm variables: SR = (value after stress/value before stress) × 100 (see **Supplementary Material 2**).

Statistical Analysis

Comparisons of sperm variables at 0H, refrigeration (20H 15°C), and incubation (20H 15°C + 2H 38.5°C) conditions for each species were made using an ANOVA repeated measures. The stress resistance ratio (SR) of domestic species and their wild relatives were compared between groups by the *t* test.

A principal components analysis (PCA) was used to identify which variables best explain the sperm stress response. We performed a PCA of all sperm variables [viability, motility, progressive motility, motility kinetic variables (VCL, VSL, VAP, and ALH), DNA fragmentation, mitochondrial membrane integrity, and low and high levels of ROS] for each six species and treatment period (0H, 20H at 15°C, and 20H at 15°C + 2H at 38.5°C). The optimal number of principal components was determined using the method of cross-validation, where the "optimal number" is defined as the number of principal components that achieves the best goodness of prediction Q^2X (see **Supplementary Material 3**).

Data were expressed as means ± SE (standard error of the mean). All statistical calculations were made using Statistica software for Windows v.12 (StatSoft Inc., Tulsa, OK, USA). The significant level was set at $p < 0.05$.

RESULTS

Effect of Stress Conditions (20H at 15°C + 2H at 38.5°C) on Sperm Traits of Wild and Domestic Species

For the wild boar sperm, total motility and integrity of mitochondrial membrane decreased ($p < 0.05$), while values of high ROS production increased significantly ($p < 0.05$) (**Table 1**). For the domestic boar sperm, values of sperm viability, total motility, VSL, VAP, and mitochondrial membrane integrity decreased ($p < 0.05$), while values of both low and high ROS levels increased significantly ($p < 0.05$).

For the mouflon sperm, values of ALH and mitochondrial membrane integrity reduced ($p < 0.05$), while percentage of DNA fragmentation and high ROS production increased ($p < 0.05$). Instead, the mitochondrial membrane integrity of ram

TABLE 1 | Wild (*Sus scrofa*) and domestic (*Sus scrofa domestica*) boar sperm quality variables (mean ± SE).

Sperm trait		Wild boar samples ($n = 4$)			Domestic boar samples ($n = 5$)		
		0H	20H 15°C	20H 15°C + 2H 38.5°C	0H	20H 15°C	20H 15°C + 2H 38.5°C
Viability (%)		73.7 ± 7.3	73.3 ± 2.7	71.3 ± 4.5	**82.8 ± 1.4 a**	**73.4 ± 2.9 ab**	**57.2 ± 6.9 b**
Motility (%)		**80.6 ± 8.7 a**	**48.4 ± 14.9 b**	**45.3 ± 15.3 b**	**64.1 ± 14.3 a**	**17.3 ± 8.9 b**	**20.8 ± 8.1 b**
Progressive motility (%)		23.4 ± 13.8	4.6 ± 3.6	4.9 ± 3.6	1.3 ± 0.4	0.7 ± 0.5	0.6 ± 0.4
Motility kinetic variables	VCL (μm/s)	49.3 ± 13.3	27.7 ± 4.5	26.8 ± 5.0	21.6 ± 1.2	22.5 ± 2.9	20.2 ± 3.2
	VSL (μm/s)	20.1 ± 4.9	6.4 ± 4.5	5.7 ± 4.3	**7.2 ± 1.3 a**	**2.3 ± 1.0 b**	**2.1 ± 0.5 b**
	VAP (μm/s)	36.6 ± 11.1	11.7 ± 5.2	10.6 ± 5.4	**11.1 ± 1.3 a**	**6.5 ± 1.8 ab**	**5.4 ± 1.1 b**
	ALH (μm)	1.9 ± 0.4	1.5 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.35 ± 0.1	1.2 ± 0.1
DNA fragmentation (%)		1.5 ± 0.3	3.7 ± 0.7	3.0 ± 0.9	1.0 ± 0.5	2.8 ± 0.6	3.4 ± 0.7
Mitochondrial membrane integrity (%)		**83.8 ± 4.6 a**	**75.0 ± 3.3 ab**	**69.3 ± 4.8 b**	**66.6 ± 4.5 a**	**37.0 ± 8.8 b**	**30.8 ± 4.2 b**
Low ROS level (%)		34.3 ± 3.2	43.3 ± 2.9	36.3 ± 4.8	**28.6 ± 2.7 b**	**38.4 ± 3.2 a**	**41.0 ± 3.6 a**
High ROS level (%)		**12.5 ± 3.1 b**	**27.3 ± 1.1 a**	**28.3 ± 4.3 a**	**26.0 ± 7.9 b**	**32.6 ± 4.7 ab**	**38.0 ± 5.8 a**

The sperm variables were evaluated before stress condition (0H), after refrigeration at 15°C for 20 h (20H 15°C), and subsequent incubation at 38.5°C for 2 h (20H 15°C + 2H 38.5°C). Sperm traits: viability, total motility, progressive motility, motility kinetic variables [curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and amplitude of lateral head displacement (ALH)], sperm DNA fragmentation, status of mitochondrial membranes, and oxidative stress level (% of low and high levels of ROS). Means within different letters are significantly different ($p < 0.05$) between groups (0H, 20H 15°C, and 2H 38.5°C) of each species. Bold values show statistically significant differences between groups.

TABLE 2 | Mouflon (*Ovis musimon*) and ram (*Ovis aries*) sperm quality variables (mean ± SE).

Sperm trait		Mouflon samples ($n = 6$)			Ram samples ($n = 5$)		
		0H	20H 15°C	20H 15°C + 2H 38.5°C	0H	20H 15°C	20H 15°C + 2H 38.5°C
Viability (%)		63.0 ± 5.3	58.0 ± 4.9	56.0 ± 6.4	70.8 ± 3.8	66.0 ± 5.2	62.4 ± 8.2
Motility (%)		67.9 ± 6.4	62.3 ± 4.5	58.2 ± 6.7	80.3 ± 2.6	87.8 ± 2.9	84.4 ± 5.6
Progressive motility (%)		42.1 ± 9.1	39.8 ± 6.2	33.9 ± 7.1	69.6 ± 4.2	79.2 ± 3.9	77.8 ± 5.9
Motility kinetic variables	VCL (μm/s)	84.1 ± 12.8	87.7 ± 8.4	77.2 ± 9.3	126.2 ± 7.9	131.2 ± 5.3	133.8 ± 5.4
	VSL (μm/s)	43.1 ± 5.9	37.9 ± 4.7	41.4 ± 5.4	74.7 ± 8.5	63.0 ± 1.9	75.3 ± 4.2
	VAP (μm/s)	59.7 ± 7.7	53.7 ± 5.9	56.1 ± 7.6	97.4 ± 9.6	97.2 ± 3.1	102.3 ± 5.0
	ALH (μm)	**2.9 ± 0.4 ab**	**3.5 ± 0.3 a**	**2.7 ± 0.2 b**	3.8 ± 0.2	4.2 ± 0.3	4.2 ± 0.1
DNA fragmentation (%)		**6.8 ± 1.7 b**	**21.5 ± 3.7 a**	**25.3 ± 5.3 a**	**0.6 ± 0.2 c**	**6.2 ± 0.7 b**	**15.0 ± 1.9 a**
Mitochondrial membrane integrity (%)		**81.2 ± 5.8 a**	**64.3 ± 7.1 b**	**63.2 ± 5.2 b**	**83.4 ± 3.4 a**	**74.4 ± 4.0 ab**	**66.4 ± 6.3 b**
Low ROS level (%)		35.3 ± 2.3	35.7 ± 3.8	34.2 ± 4.2	**24.4 ± 2.4 b**	**40.0 ± 3.8 a**	**38.0 ± 1.5 a**
High ROS level (%)		**23.2 ± 3.9 b**	**37.5 ± 5.5 a**	**39.0 ± 4.4 a**	**11.4 ± 1.6 b**	**40.2 ± 6.9 a**	**38.0 ± 2.8 a**

The sperm variables were evaluated before stress condition (0H), after refrigeration at 15°C for 20 h (20H 15°C), and subsequent incubation at 38.5°C for 2 h (20H 15°C + 2H 38.5°C). Sperm traits: viability, total motility, progressive motility, motility kinetic variables [curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and amplitude of lateral head displacement (ALH)], sperm DNA fragmentation, status of mitochondrial membranes, and oxidative stress level (% of low and high levels of ROS). Means within different letters are significantly different ($p < 0.05$) between groups (0H, 20H 15°C, and 2H 38.5°C) of each species. Bold values show statistically significant differences between groups.

sperm samples reduced ($p < 0.05$), while the percentage of DNA fragmentation and ROS levels increased ($p < 0.05$) (**Table 2**).

For the Iberian ibex sperm, the values of the mitochondrial membrane integrity decreased significantly ($p < 0.05$), while the percentage of DNA fragmentation increased ($p < 0.05$). For the buck sperm, values of VCL, VSL, and VAP reduced ($p < 0.05$), while levels of ROS production increased ($p < 0.05$) (**Table 3**).

Overall comparisons of the SR, for each sperm variable, between domestic and wild species are shown in the **Supplementary Table 3**. Only SR values showing significant differences are shown in **Figures 1–3**. The SR for mitochondrial membrane integrity was lower ($p < 0.05$) in domestic than in wild boar (**Figure 1**). The SR for ROS production was higher in ram ($p < 0.05$) than in mouflon (**Figure 2**). In buck, the SR for VCL showed lower values ($p < 0.05$) than in Iberian Ibex (**Figure 3**).

The Sperm Variables that Best Explain the Response to Stress

All values for PCA are shown in **Supplementary Material 3** (Tables 4–6) for each treatment period: 0H, refrigeration (20H

TABLE 3 | Iberian ibex (*Capra pyrenaica*) and buck (*Capra hircus*) sperm quality variables (mean ± SE).

Sperm trait		Iberian ibex samples ($n = 6$)			Buck samples ($n = 5$)		
		0H	20H 15°C	20H 15°C + 2H 38.5°C	0H	20H 15°C	20H 15°C + 2H 38.5°C
Viability (%)		64.7 ± 4.4	61.5 ± 4.6	62.5 ± 5.9	60.8 ± 5.8	62.4 ± 2.9	54.5 ± 2.6
Motility (%)		57.7 ± 7.8	52.9 ± 10.4	55.7 ± 8.6	81.9 ± 2.9	80.7 ± 4.5	81.9 ± 1.3
Progressive motility (%)		30.9 ± 11.1	31.2 ± 12.2	31.1 ± 11.8	74.8 ± 4.0	74.3 ± 5.2	72.6 ± 2.0
Motility kinetic variables	VCL (μm/s)	66.7 ± 12.4	62.8 ± 13.4	62.8 ± 12.9	**125.3 ± 10.3 a**	**99.5 ± 5.2 b**	**100.7 ± 6.1 b**
	VSL (μm/s)	44.6 ± 10.9	43.7 ± 12.8	34.8 ± 7.6	**89.9 ± 6.6 a**	**66.6 ± 5.5 b**	**67.1 ± 4.8 b**
	VAP (μm/s)	53.2 ± 12.2	50.2 ± 13.1	43.2 ± 9.5	**110.1 ± 9.6 a**	**79.3 ± 5.9 b**	**84.8 ± 5.8 b**
	ALH (μm)	2.1 ± 0.2	1.9 ± 0.2	2.4 ± 0.4	2.9 ± 0.2	3.1 ± 0.3	2.9 ± 0.1
DNA fragmentation (%)		**7.1 ± 0.8 b**	**9.7 ± 1.7 ab**	**10.7 ± 0.9 a**	0.6 ± 0.2	1.8 ± 0.4	1.4 ± 0.2
Mitochondrial membrane integrity (%)		**72.3 ± 4.8 a**	**63.0 ± 4.7 b**	**61.8 ± 3.5 b**	73.8 ± 7.7	66.6 ± 2.6	61.6 ± 3.4
Low ROS level (%)		39.2 ± 1.6	37.7 ± 3.0	38.0 ± 3.2	19.2 ± 3.3	19.8 ± 2.9	22.0 ± 1.9
High ROS level (%)		17.5 ± 4.4	24.5 ± 3.9	25.0 ± 3.7	**18.8 ± 2.4 b**	**44.6 ± 4.2 a**	**44.8 ± 3.6 a**

The sperm variables were evaluated before stress condition (0H), after refrigeration at 15°C for 20 h (20H 15°C), and subsequent incubation at 38.5°C for 2 h (20H 15°C + 2H 38.5°C). Sperm traits: viability, total motility, progressive motility, motility kinetic variables [curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and amplitude of lateral head displacement (ALH)], sperm DNA fragmentation, status of mitochondrial membranes, and oxidative stress level (% of low and high levels of ROS). Means within different letters are significantly different ($p < 0.05$) between groups (0H, 20H 15°C, and 2H 38.5°C) of each species. Bold values show statistically significant differences between groups.

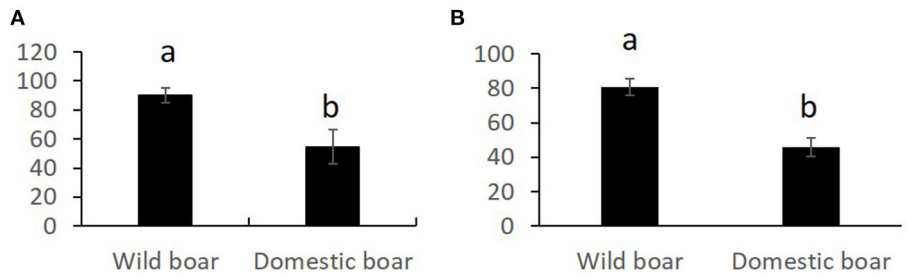


FIGURE 1 | Stress resistance factor (SR) (mean ± SE) for integrity of mitochondrial membrane after 20 h of refrigeration at 15°C **(A)** and after 20 h of refrigeration at 15°C and subsequent incubation at 38°C for 2 h **(B)** in wild and domestic boar sperm samples. Means within different letters are significantly different ($p < 0.05$) between species.

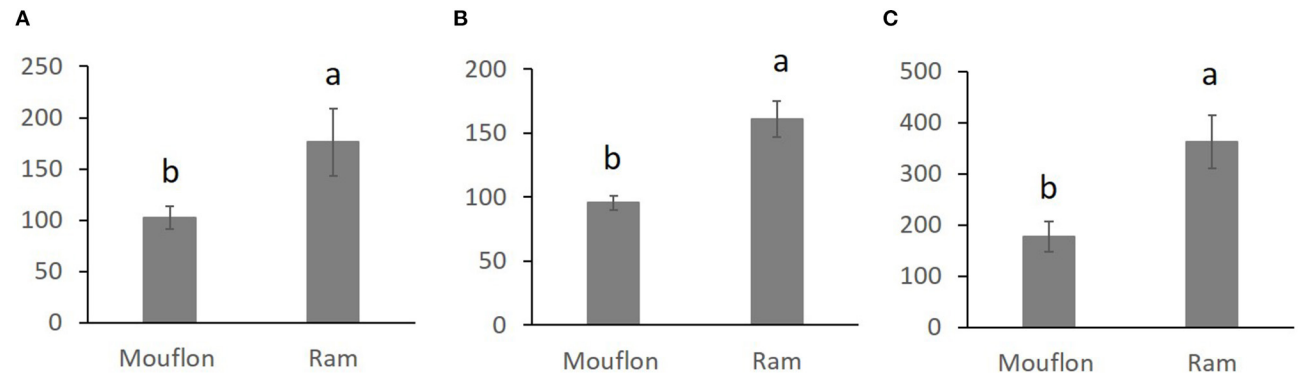


FIGURE 2 | Stress resistance factor (SR) (mean ± SE) for low levels of ROS after refrigeration at 15°C during 20 h **(A)** and after refrigeration at 15°C during 20 h and subsequent incubation at 38.5°C for 2 h **(B)** SR for high levels of ROS after refrigeration at 15°C during 20 h and subsequent incubation at 38.5°C for 2 h **(C)** in mouflon and ram sperm samples. Means within different letters are significantly different ($p < 0.05$) between species.

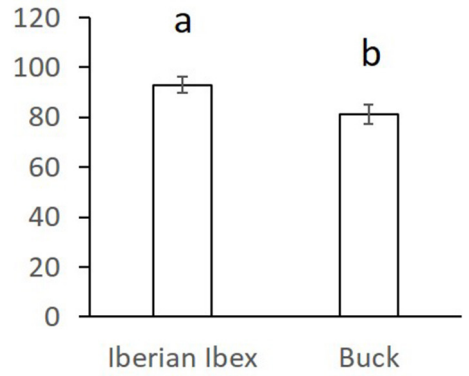


FIGURE 3 | Stress resistance factor (SR) (mean ± SE) for curvilinear velocity (VCL) after refrigeration at 15°C during 20 h and subsequent incubation at 38.5°C for 2 h in ibex and buck sperm samples. Means within different letters are significantly different ($p < 0.05$) between species.

at 15°C), and refrigeration followed by incubation (20H at 15°C + 2H at 38.5°C).

In wild boar, PCA rendered a single principal component (PC) for each period. In the PC for 0H samples (eigenvalue of 7.26), the most represented variables were VAP, VSL, VCL, ALH, total and progressive motility, and high ROS production, while in the PC for refrigerated and incubated samples (eigenvalue 5.85), the most represented variables were VAP, VSL, VCL, ALH, and total and progressive motility. In domestic boar, a single PC was also obtained for each period. In the PC for 0H samples (eigenvalue of 5.30), the most represented variables were VSL, progressive motility, VAP, and high and low ROS production, while in the PC for refrigerated and incubated samples (eigenvalue 6.59), the most represented were those related to motility (and its kinetics) and the sperm viability (**Supplementary Table 4**).

In mouflon, PCA indicated that two PCs (PC1 and PC2) explained the variability of the data for each period. In PC1 for 0H samples (eigenvalue 5.95), the most represented variables were the total and progressive motility and its kinetics variables (VCL, VSL, VAP, and AHL), while in PC2 (eigenvalue 2.40), the viability and state of the mitochondria were identified as the most represented variables. In PC1 for refrigerated and incubated samples (eigenvalue 5.26) the most represented variables were those related to kinetics motility and low ROS production, while in PC2 (eigenvalue 4.41), the motility, membrane mitochondrial status, and high ROS production were identified as the most represented variables. In ram, a single PC was obtained for each period. In the PC for 0H samples (eigenvalue of 5.78), the most represented variables were ROS production, DNA fragmentation, viability, and VCL, while in the PC for refrigerated and incubated samples (eigenvalue 7.58), the most represented variables were those related to motility (total and progressive motility, VCL, and ALH), viability, DNA fragmentation, and ROS production (**Supplementary Table 5**).

In Iberian ibex, two PCs (PC1 and PC2) were generated by applying PCA. In PC1 for 0H samples (eigenvalue 7.98), the most represented variables were the total and progressive motility, VCL, VSL, VAP, ALH, mitochondrial status, and high ROS production, while in PC2 (eigenvalue 1.51), the DNA fragmentation was identified as the most represented variables. In PC1 for refrigerated and incubated samples (eigenvalue 7.87), the most represented variables were motility and its kinetics variables, sperm viability and DNA fragmentation, while in PC2 (eigenvalue 1.75), the mitochondrial status and ROS production were identified as the most represented variables. In buck, a single PC was obtained for each period. In the PC for 0H samples (eigenvalue of 6.70), the most represented variables were total and progressive motility, VCL, VSL, VAP, and mitochondrial status. In the PC for refrigerated and incubated samples (eigenvalue 5.16), the most represented variables were those related to kinetics of motility (VCL, VSL, and VAP) and ROS production (**Supplementary Table 6**).

DISCUSSION

This work is the first to compare the stress response of sperm from domestic species and their wild ancestors using different semen evaluation techniques. The present findings revealed a species-specific sperm response to stress conditions related to chilling and subsequent incubation. Certainly, the sperm from domestic species appeared to be more sensitive to cooling storage followed by incubation. The percentage of sperm with intact mitochondria in domestic boar was lower than for wild boar. The ROS production was greater in domestic than in wild sheep, while in goats, the VCL was lower in domestic species.

The motility values for the diluted fresh semen in domestic boar were lower than those previously reported for this species: for instance, 88.7% in fresh samples (29) or 90% in samples diluted in ACROMAX® extender (30). After refrigeration and incubation treatments, the worst results for sperm motility, kinetic variables, sperm viability, and membrane mitochondrial status values were observed in porcine species. It is well-known that boar sperm are very susceptible to cold shock (1). The plasma membrane of porcine sperm contains less phosphatidylcholine and more phosphatidylethanolamine and sphingomyelin. In addition, boar sperm exhibits very low content of cholesterol, and therefore, boar sperm is more susceptible to cold damage (31).

Studies aiming at identifying the seminal characteristics of wild boar are scarce (32, 33). The values for sperm motility and viability were similar to those reported in these studies, but to our best knowledge, no studies have been conducted on chilling wild boar sperm. The SR for mitochondrial membrane integrity was higher in wild boar than domestic boar. ROS production increased after chilling and incubation in both wild and domestic boar, but the greatest values of high ROS in wild boar (28.3%) were similar than ROS values before the treatment (0H) in domestic boar (26.0%). Hence, a greater antioxidant capacity of seminal plasma of wild boar should not be ruled out.

The greatest total ROS production (low ROS level + high ROS level) was observed in domestic boar (79%), along with ram (76%), and mouflon (73.2%) samples, suggesting that the balance between ROS production and detoxification by antioxidants

was mainly disrupted in these species (11), generating a major oxidative stress. The ram sperm showed the lowest ROS values at 0H, revealing that the ROS production increased considerably during treatment. ROS production strongly increased after refrigeration and incubation in buck sperm, unlike ibex sperm, suggesting that wild goat sperm has a high antioxidant activity like described above for wild boar.

As it was expected, after the cooling period and subsequent incubation, the worst results of DNA fragmentation were observed in sheep samples. In mouflon's samples, the mean percentage of sperm with DNA fragmentation was 25.3%, while in ram, it was 15%. Previous studies in human sperm have already reported that there is a direct relationship between ROS production and DNA damage (34, 35); probably, the high levels of ROS production could be related with the high levels of DNA fragmentation in mouflon and ram samples. Conversely, high levels of fragmented DNA were not detected in boar sperm samples, despite the high production of ROS. Previous studies (30) reported that boar sperm samples diluted in a commercial extender showed very low levels of DNA fragmentation during the preservation (stored at 15°C, during 21 days), in comparison with undiluted semen.

The results confirmed the initial hypothesis that domestication and selection throughout long time ultimately seem to affect the resistance capacity of sperm to stress conditions. Sperm from wild species showed more resistance to stress caused by refrigeration and subsequent incubation than their domestic relatives. Hence, wild species appear to be an excellent model to identify molecular markers related with sperm resistance to stress conditions, such as cold storage (36). The techniques employed in this study allowed us to detect significant differences in some sperm traits; however, other biochemical, and molecular studies would have to be performed in the future, for instance, the characterization of plasma membrane fatty acids (37), the expression of sperm proteins involved in resistance to cold shock (38), the analysis of plasma seminal proteins with antioxidant activities (39), or even the evaluation of differential patterns in RNAm and RNAmi (36).

The PCA measures how well a variable is represented by the principal components and has been used in previous studies of human (40), ibex (41), puma (42), dog (43), turkey (44), and caiman (45) sperm. The aim of PCA is to reduce the dimensionality of a set of variables while retaining the maximum variability. After 22 h of stress, the motility (total or progressive) and their kinetic variables (VCL, VSL, VAP, or ALH) were the most represented variables in all PC of all species. This indicates that motility is an essential biomarker for evaluating the stress response in these species. Our data agree with previous studies performed in Iberian ibex sperm where the sperm motility and motility rate were the most represented variables of the PCA (eigenvalue 2) (41). In addition, sperm viability was the next most represented variable in domestic boar, ram, and Iberian ibex samples, and ROS production was the next most represented variable in mouflon, ram, and buck samples. Despite DNA fragmentation being a very useful variable to evaluate sperm quality and fertility capacity in other species, such as human sperm (46), it was not a substantial contributor to the evaluation of stress resistance in the most studied species, except in the ram and ibex.

In conclusion, motility variables were essential biomarkers for evaluating the stress response in all species. Sperm viability was highlighted as a representative variable for evaluating the stress response in domestic boar, mouflon, ram, and ibexes. The measurement of different sperm functional variables showed that sperm from wild ungulates showed more resistance to stress than sperm from domestic ones.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by INIA Ethics Committee.

AUTHOR CONTRIBUTIONS

EO'B: sperm collection, sperm analysis, data analysis, and drafted the manuscript. PG-C: discussion of results and drafted the manuscript. CC, AT-D, and PB: sperm collection and sperm analysis. JS-M: experimental design, data analysis, and drafted the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This research was funded by Zoitechlab S.L.-INIA contract CON18-141.

ACKNOWLEDGMENTS

We thank Jorge García Montaño from Zoitechlab S.L. and Iberian Kuna Fauna Center for their unfailing help in the provision of boar samples.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.650946/full#supplementary-material

REFERENCES

1. Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. *Anim Reprod Sci*. (2000) 62:143–72. doi: 10.1016/S0378-4320(00)00157-3
2. Salamon S, Maxwell WMC. Storage of ram semen. *Anim Reprod Sci*. (2000) 62:77–111. doi: 10.1016/S0378-4320(00)00155-X
3. Meyers SA. Spermatozoal response to osmotic stress. *Anim Reprod Sci*. (2005) 89:57–64. doi: 10.1016/j.anireprosci.2005.06.026
4. Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, et al. Advances in cryopreservation of bull sperm. *Front Vet Sci*. (2019) 6:268. doi: 10.3389/fvets.2019.00268
5. Aitken R, Koopman P, Lewis S. Seeds of concern. *Nature*. (2004) 432:48–52. doi: 10.1038/432048a
6. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. *Reprod Fertil Dev*. (2016) 28:1–10. doi: 10.1071/RD15325
7. Bennetts LE, Aitken RJ. A comparative study of oxidative damage in mammalian sperm. *Mol Reprod Dev*. (2005) 71:77–87. doi: 10.1002/mrd.20285
8. Kao SH, Chao HT, Chen HW, Hwang TIS, Liao TL, Wei YH. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril*. (2008) 89:1183–90. doi: 10.1016/j.fertnstert.2007.05.029
9. Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S. The role of antioxidants in sperm freezing: a review. *Cell Tissue Bank*. (2016) 17:745–56. doi: 10.1007/s10561-016-9566-5
10. Kasimanickam R, Pelzer KD, Kasimanickam V, Swecker WS, Thatcher CD. Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxidant enzymatic activity of semen in ram-lambs. *Theriogenology*. (2006) 65:1407–21. doi: 10.1016/j.theriogenology.2005.05.056
11. Bailey JL, Blodeau JF, Cormier N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon minireview. *J Androl*. (2000) 21:1–7.
12. Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*. (2000) 57:327–44. doi: 10.1016/S0093-691X(01)00674-4
13. Lincoln GA. Correlation with changes in horns and pelage, but not reproduction, of seasonal cycles in the secretion of prolactin in rams of wild, feral and domesticated breeds of sheep. *J Reprod Fertil*. (1990) 90:285–96. doi: 10.1530/jrf.0.0900285
14. Lincoln GA, Lincoln CE, McNeilly AS. Seasonal cycles in the blood plasma concentration of FSH, inhibin and testosterone, and testicular size in rams of wild, feral and domesticated breeds of sheep. *J Reprod Fertil*. (1990) 88:623–33. doi: 10.1530/jrf.0.0880623
15. Santiago-Moreno J, Gómez-Brunet A, González-Bulnes A, Toledano-Díaz A, Malpaux B, López-Sebastián A. Differences in reproductive pattern between wild and domestic rams are not associated with inter-specific annual variations in plasma prolactin and melatonin concentrations. *Domest Anim Endocrinol*. (2005) 28:416–29. doi: 10.1016/j.domaniend.2005.02.002
16. Moon S, Kim TH, Lee KT, Kwak W, Lee T, Lee SW, et al. A genome-wide scan for signatures of directional selection in domesticated pigs. *BMC Genom*. (2015) 16:130. doi: 10.1186/s12864-015-1330-x
17. Taberlet P, Coissac E, Pansu J, Pompanon F. Conservation genetics of cattle, sheep, and goats. *C R Biol*. (2011) 334:247–54. doi: 10.1016/j.crvi.2010.12.007
18. Food and Agriculture Organization of the United Nations. Domestic Animal Genetic Diversity (2004). Available online at: *http://www.fao.org/newsroom/en/news/2004/39892/index.html* (*accessed December,* 30, 2020).
19. Galarza DA, Ladrón de Guevara M, Beltrán-Breña P, Sánchez-Calabuig MJ, Rizos D, López-Sebastián A, et al. Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm. *Theriogenology*. (2019) 133:29–37. doi: 10.1016/j.theriogenology.2019.04.027
20. Awda BJ, Buhr MM. The effect of glove type on boar semen quality. *Theriogenology*. (2008) 70:1388. doi: 10.1016/j.theriogenology.2008.06.046
21. Santiago-Moreno J, Castaño C, Toledano-Díaz A, Esteso MC, López-Sebastián A, Guerra R, et al. Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. *Theriogenology*. (2013) 79:383–91. doi: 10.1016/j.theriogenology.2012.10.011
22. Pradiee J, O'Brien E, Esteso MC, Castaño C, Toledano-Díaz A, Lopez-Sebastián A, et al. Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (*Rupicapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates. *Anim Reprod Sci*. (2016) 171:121–8. doi: 10.1016/j.anireprosci.2016.06.007
23. Santiago-Moreno J, Esteso MC, Castaño C, Toledano-Díaz A, Rodríguez E, López-Sebastián A. Sperm selection by Capripure® density-gradient centrifugation versus the dextran swim-up procedure in wild mountain ruminants. *Anim Reprod Sci*. (2014) 149:178–86. doi: 10.1016/j.anireprosci.2014.07.003
24. Santiago-Moreno J, Esteso MC, Castaño C, Toledano-Díaz A, Delgadillo JA, López-Sebastián A. Seminal plasma removal by density-gradient centrifugation is superior for goat sperm preservation compared with classical sperm washing. *Anim Reprod Sci*. (2017) 181:141–50. doi: 10.1016/j.anireprosci.2017.04.002
25. Mortimer D, Aitken RJ, Mortimer ST, Pacey AA. Workshop report: clinical CASA—the quest for consensus. *Reprod Fertil Dev*. (1995) 7:951–9. doi: 10.1071/RD9950951
26. ESHRE Andrology Special Interest Group. Guidelines on the application of CASA technology in the analysis of spermatozoa. *Hum Reprod*. (1998) 13:142–5. doi: 10.1093/humrep/13.1.142
27. Santiago-Moreno J, Bernal B, Pérez-Cerezales S, Castaño C, Toledano-Díaz A, Esteso MC, et al. Seminal plasma amino acid profile in different breeds of chicken: role of seminal plasma on sperm cryoresistance. *PLoS ONE*. (2019) 14:e0209910. doi: 10.1371/journal.pone.0209910
28. de Castro LS, de Assis PM, Siqueira AF, Hamilton TR, Mendes CM, Losano JD, et al. Sperm oxidative stress is detrimental to embryo development: a dose-dependent study model and a new and more sensitive oxidative status evaluation. *Oxid Med Cell Longev*. (2016) 2016:8213071. doi: 10.1155/2016/8213071
29. Wasilewska K, Zasiadczyk Ł, Fraser L, Mogielnicka-Brzozowska M, Kordan W. The benefits of cooling boar semen in long-term extenders prior to cryopreservation on sperm quality characteristics. *Reprod Domest Anim*. (2016) 51:781–8. doi: 10.1111/rda.12751
30. Pérez-Llano B, Sala R, Reguera G, García-Casado P. Changes in subpopulations of boar sperm defined according to viability and plasma and acrosome membrane status observed during storage at 15 degrees C. *Theriogenology*. (2009) 71:311–7. doi: 10.1016/j.theriogenology.2008.07.023
31. Cerolini S, Maldjian A, Surai P, Noble R. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim Reprod Sci*. (2000) 58:99–111. doi: 10.1016/S0378-4320(99)00035-4
32. Fischman ML, Suhevic J, Rivolta MA, Cisale HO. Collection of wild boar semen by electroejaculation. *Vet Rec*. (2003) 153:365–6. doi: 10.1136/vr.153.12.365
33. Kozdrowski R, Dubiel A. The effect of season on the properties of wild boar (*Sus scrofa* L.) semen. *Anim Reprod Sci*. (2004) 80:281–9. doi: 10.1016/j.anireprosci.2003.08.006
34. Kodama H, Yamaguchi R, Fukuda J, Kasi H, Tanak T. Increased deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril*. (1997) 65:519–24. doi: 10.1016/S0015-0282(97)00236-7
35. Bejarano I, Monllor F, Marchena AM, Ortiz A, Lozano G, Jiménez MI, et al. Exogenous melatonin supplementation prevents oxidative stress-evoked DNA damage in human spermatozoa. *J Pineal Res*. (2014) 57:333–9. doi: 10.1111/jpi.12172
36. Ran MX, Zhou YM, Liang K, Wang WC, Zhang Y, Zhang M, et al. Comparative analysis of MicroRNA and mRNA profiles of sperm with different freeze tolerance capacities in boar (*Sus scrofa*) and Giant Panda (*Ailuropoda melanoleuca*). *Biomolecules*. (2019) 9:432. doi: 10.3390/biom9090432
37. Martínez-Soto JC, Landeras J, Gadea J. Spermatozoa and seminal plasma fatty acids as predictors of cryopreservation success. *Andrology*. (2013) 1:365–75. doi: 10.1111/j.2047-2927.2012.00040.x
38. Martínez-Fresneda L, Sylvester M, Shakeri F, Buness A, Del Pozo JC, García-Vázquez FA, et al. Proteomic analysis of ejaculated and epididymal sperm associated with freezability in Iberian ibex (*Capra pyrenaica*). 35th Annual Scientific Meeting of the Association of Embryo Technology in Europe

(AETE). *Proceedings*. (2019). p. 85. Abstract retrieved from Abstracts in Association of Embryo Technology in Europe.
39. Roca J, Broekhuijse ML, Parrilla I, Rodriguez-Martinez H, Martinez EA, Bolarin A. Boar differences in artificial insemination outcomes: can they be minimized? *Reprod Dom Anim*. (2015) 50:48–55. doi: 10.1111/rda.12530
40. Gómez-Torres MJ, Medrano L, Romero A, Fernández-Colom PJ, Aizpurúa J. Effectiveness of human spermatozoa biomarkers as indicators of structural damage during cryopreservation. *Cryobiology*. (2017) 78:90–4. doi: 10.1016/j.cryobiol.2017.06.008
41. Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Gómez-Brunet A, López-Sebastián A. Horn quality and postmortem sperm parameters in Spanish ibex (*Capra pyrenaica hispanica*). *Anim Reprod Sci*. (2007) 99:354–62. doi: 10.1016/j.anireprosci.2006.06.004
42. Cucho H, Alarcón V, Ordóñez C, Ampuero E, Meza A, Soler C. Puma (Puma concolor) epididymal sperm morphometry. *Asian J Androl*. (2016) 18:879–81. doi: 10.4103/1008-682X.187584
43. Soler C, Alambiaga A, Martí MA, García-Molina A, Valverde A, Contell J, et al. Dog sperm head morphometry: its diversity and evolution. *Asian J Androl*. (2017) 19:149–53. doi: 10.4103/1008-682X.189207
44. Iaffaldano N, Romagnoli L, Manchisi A, Rosato MP. Cryopreservation of turkey semen by the pellet method: effects of variables such as the extender, cryoprotectant concentration, cooling time and warming temperature on sperm quality determined through principal components analysis. *Theriogenology*. (2011) 76:794–801. doi: 10.1016/j.theriogenology.2011.04.012
45. Valverde A, Madrigal-Valverde M, Castro-Morales O, Gadea-Rivas A, Johnston S, Soler C. Kinematic and head morphometric characterisation of spermatozoa from the Brown Caiman (Caiman crocodilus fuscus). *Anim Reprod Sci*. (2019) 207:9–20. doi: 10.1016/j.anireprosci.2019.06.011
46. Esteves SC, Zini A, Coward RM, Evenson DP, Gosálvez J, Lewis SEM, et al. Sperm DNA fragmentation testing: summary evidence and clinical practice recommendations. *Andrologia*. (2020) 2020:e13874. doi: 10.1111/and.13874

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 May 2021
doi: 10.3389/fvets.2021.651477

A New Approach of Sperm Motility Subpopulation Structure in Donkey and Horse

Sabrina Gacem[1], Anthony Valverde[2], Jaime Catalán[1], Iván Yánez Ortiz[1], Carles Soler[3] and Jordi Miró[1]**

[1] Equine Reproduction Service, Autonomous University of Barcelona, Department of Animal Medicine and Surgery Veterinary Faculty, Catalonia, Spain, [2] Costa Rica Institute of Technology, School of Agronomy, Alajuela, Costa Rica, [3] Universitat de València, Departamento de Biología Celular, Biología Funcional y Antropología Física, Burjassot, Valencia, Spain

This study aimed to characterize the sperm kinematic values with high frames per second, to define the subpopulation structure of a horse and a donkey and compare them. A total of 57 fresh semen ejaculates (26 Spanish and 16 Arabian horse breeds and 10 donkeys) were collected and subsequently analyzed for kinematic parameters using the Computer-aided sperm motility analysis ISAS®v1.2 system and using a Spermtrack® 10-μm depth counting chamber. Sequences were recorded at 250 frames per second, and eight kinematic parameters were automatically evaluated. All kinematic parameters showed significant differences between a donkey and a horse and between horse breeds. All ejaculates evaluated showed excellent semen motility characteristics, with significantly higher values for all kinematic parameters for donkeys compared with horses except for beat-cross frequency. Donkey sperm was faster and linear than the horse. Regarding horse breeds differences, the Spanish horse had higher average path velocity, curvilinear velocity, and beat-cross frequency compared with the Arabian horse. Spanish horse sperm was rapid, but Arab horse was more linear. The principal component analysis showed three sperm subpopulations in the ejaculate of donkeys and horses with a significantly different motility characteristic between them. The dominant subpopulation for both donkey and horse was for rapid, straight, and linear with a high beat sperm (38.2 and 41.7%, respectively), whereas the lowest subpopulation was for the slowest and non-linear sperms. This, plus slight differences in the distribution of these subpopulations between Arabian and Spanish horses, were found. In conclusion, higher frames permitted to have a new interpretation of motile subpopulations with species and breed differences. More so, future works on donkey and horse breed spermatozoa should take into account differences between breeds that may interfere and alter the real analysis performed.

Keywords: equids, sperm motility, subpopulations, sperm kinematics, CASA-mot

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Abouzar Najafi, University of Tehran, Iran
Cecilia Dall'Aglio, University of Perugia, Italy

****Correspondence:***
Carles Soler
carles.soler@uv.edu
Jordi Miró
jordi.miro@uab.cat

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *09 January 2021*
Accepted: *07 April 2021*
Published: *25 May 2021*

Citation:
Gacem S, Valverde A, Catalán J, Yánez Ortiz I, Soler C and Miró J (2021) A New Approach of Sperm Motility Subpopulation Structure in Donkey and Horse. Front. Vet. Sci. 8:651477. doi: 10.3389/fvets.2021.651477

INTRODUCTION

All the present living species of equids are assigned to the genus *Equus*, sharing a common ancestor million years ago (1), being distributed worldwide in the most diverse environments. The *Equus* genus comprises two lineages: the caballine, which includes domesticated horses (*Equus ferus caballus*), and the wild endangered Przewalski's horse (*Equus ferus przewalskii*) of Mongolia, and

non-caballine grouping, which comprises the asses and zebras (2). The domestication process for horses was initiated approximately 450 generations ago, assuming an average generation time of 12 years for wild horses, whereas that for donkeys was around 6,000 years ago (3).

The domestication process started with the need of humans to shape animals' species according to their intentions, producing an artificial selection pressure. The last one has increased particularly since the establishment of studbooks and the development of clear breading objectives affecting the fertility potential (4). Consequently, all the domestic species are now quite far from the original in different aspects involving reproductive characteristics.

The sperm analysis is a prerequisite for breeding soundness examination, and the use of semen in artificial insemination in horses is like in all farm animals. The introduction of computer-assisted semen analysis (CASA) technology allowed for an unprecedented degree of sophistication in the study of sperm characteristics and particularly in reference to kinematics patterns (5, 6). Computer-aided sperm motility analysis (CASA-Mot) systems capture sequences from microscopic fields and automatically analyze all sperm trajectories (7, 8), offering a battery of quantitative kinematic parameters (9).

Improvement in CASA technology and especially the development of new software solutions and new high-performing cameras permitted the authors to analyze better the sperm trajectory (8, 10–12). Recent studies have suggested specific optimum video capture frame rates for a mathematically well-track definition in various species (12, 13). All the previous work using CASA-Mot systems was obtained using suboptimal kinematic data, which resulted in a low significance kinematic parameter. Those are some of the most important limitations in the general use of CASA technology in the past (5).

Furthermore, it was shown that spermatozoa could be grouped into coherent mathematical subpopulations in a wide variety of animals (14–16). These subpopulational structures were also observed for different morphological, biochemical, and physiological traits such as morphometry (17), DNA fragmentation (18), mitochondrial activity (19), osmotic properties (20), and functional status (21). What is clear nowadays is that the ejaculate is not composed of a homogeneous population of equivalent cells but a different subpopulation regarding various cellular properties. Its origin is related both to the testicular formation of the spermatozoa (22) and with their maturational process along the epididymis (23). Interestingly, it can be conditioned by external social interactions, almost in horses (24).

Our study aims to compare the distribution of these kinematic subpopulations between two close equid species horses and donkeys and two horse breeds Spanish and Arabian and that by using higher frames rate for sperm kinematic calculation.

MATERIALS AND METHODS

Animals

The study was conducted at the University Autonoma of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). Two ejaculates were collected from 13 pure Spanish breeds and eight pure Arabian horses, whereas three ejaculates were obtained from five Catalonian donkeys each, a total of 57 fresh ejaculates. Semen was collected from all animals three times a week throughout the year. All animals ranged from 3 to 15 years of age and fed three times a day with a standard diet (mixed hay and basic concentrate); water was also freely available. All the animals were housed in single boxes in the Equine Reproduction Service of the university. The housing facility is a European Union-approved semen collection center (authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. All animals were semen donors and underwent regular semen collection under CEE health conditions (free of equine arteritis, infectious anemia, and contagious metritis). Because this service already runs under the approval of the Regional Government of Catalonia (Spain) and because no manipulation of the animals other than semen collection was carried out, the ethics committee of our institution indicated that no further ethical approval was required.

Semen Collection

Ejaculates were collected through a pre-warmed artificial vagina model Hannover (Minitüb GmbH, Tiefenbach, Germany) with animals excited by an ovariectomized mare or jenny. An in-line nylon mesh filter was used to separate the gel fraction from the semen. Upon collection, gel-free semen was diluted immediately 1:5 (v:v) in skimmed milk (4.9% glucose, 2.4% skim milk, and 100-mL double-distilled water), previously preheated to 37°C.

Sperm analysis (morphology and concentration) was evaluated upon arrival of semen samples to the laboratory. Sperm concentration was determined using a hemocytometer (Neubauer Chamber; Paul Marienfeld, Germany). To this end, samples were previously diluted with a 4% formalin buffered solution, and the sperm count was adjusted for the dilution factor. Sperm morphology was evaluated by the eosin–nigrosin staining technique.

Semen Preparation and Computer-Assisted Semen Analysis

The remaining sample was diluted to a final concentration of 40 $\times$ 10^6 spermatozoa/mL, then a volume of 2 μL was mounted on standardized 10-μm depth counting chambers Spermtrack® (Proiser R+D S.L., Paterna, Spain). All chambers were pre-warmed and maintained at 37°C on a UB203 (Proiser R+D)-heated microscope stage throughout the analysis.

Sperm kinematic parameters were automatically assessed using the motility module of CASA system ISAS®v1 (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). The device is a combination of a Proiser HS640m digital camera mounted on the referred microscope. Images were captured by a 10$\times$ negative phase contrast objective (AN 0.25). For each analysis, up to 10 non-consecutive fields were recorded for 3 s at 250 frames per second (fps) in each analysis, permitting the identification of a minimum of 500 spermatozoa per ejaculate.

The settings of the CASA system were those recommended by the manufacturer: particle area >4 and <75 μm^2; connectivity: 6; cutoff values were VAP $\geq$ 10 μm/s for a sperm cell to be

considered as motile (10, 11). The following sperm motility parameters were determined, sperm velocity: the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP); and sperm movement trajectory: the frequency with which the actual track crossed the smoothed track in either direction [beat-cross frequency (BCF), hertz], and the maximum of the measured width of the head oscillation as the sperm cells swim (the amplitude of lateral head displacement [ALH]). Also, three progression proportions were calculated from the velocity measurements: (the linearity, LIN = VSL/ VCL), the departure of actual sperm track from linearity (wobble WOB = VAP/VCL), and linearity of the average path (straightness, STR = VSL/VAP).

Statistical Analysis

The data obtained from the analysis of all sperm variables were first tested for normality and homoscedasticity by using Shapiro–Wilks and Levene tests. A normal probability plot was used to check for a normal distribution. Multivariate procedures were performed to identify sperm subpopulations from the set of sperm motility data. All the values for kinematic variables were standardized to avoid any scaling effect.

Multivariate Procedures Analysis

Clustering procedures were performed to identify sperm subpopulations from the complete set of motility data. The first step was to perform a principal component analysis (PCA). The number of principal components (PCs) that should be used in the next step of the analysis was determined from the Kaiser criterion, namely selecting only those with an eigenvalue (variance extracted for that PC) > 1. Furthermore, Bartlett's sphericity test and the Kaiser–Meyer–Olkin were performed. As a rotation method, the varimax method with Kaiser normalization was used. The second process was to perform a clustering procedure. A two-step cluster procedure was performed, a hierarchical and a non-hierarchical analysis model, with the sperm-derived indices obtained after the PCA, that uses Euclidean distances from the quantitative variables after standardization of these data, so the cluster centers were the means of the observations assigned to each cluster. In the first step, to determine the optimal number of clusters, the final centroids were clustered hierarchically using the Ward method (25). All sperm cells within different breeds and species were clustered by using the multivariate k-means clustering procedure was made to classify the spermatozoa into a reduced number of subpopulations (clusters) according to their kinematic variables. The clustering procedure enables the identification of sperm subpopulations because each cluster contributed to a final cluster formed by the spermatozoa linked to the centroids. The analysis of variance and χ^2-test procedures were applied to evaluate statistical differences in the distributions of observations (individual spermatozoa) within subpopulations, and then a generalized linear model procedure was used to determine the effects of the breed and species on the mean kinematic variable values defining the different sperm subpopulations (i.e., the cluster centers). Differences between means were analyzed by the Bonferroni test. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was considered at $P <$ 0.05. All data were analyzed using the IBM SPSS package, version 23.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

All the kinematic parameters showed significant differences between both species horse and donkey, being higher for donkey unless BCF was higher for the horse. This means that donkey sperm motility was more linear than that of a horse (**Table 1**).

When comparing Arabian and Spanish horses, VAP, VCL, ALH, and BCF were higher in Spanish horses than in Arabian, being the contrary for LIN and WOB. VSL, STR, and ALH showed no differences among breeds. Following these results, Spanish horse sperm was faster but less linear than an Arabian horse (**Table 1**).

The PCA rendered three PCs for both species, explaining 90.9% for stallion and 89.8% for the donkey of the total variance (**Table 2**). The three PCs were equivalent for donkey and stallion, being PC1, named velocity, positively correlated to the velocity parameters (VCL and VAP) and sperm head oscillation ALH for both species; only BCF was also included for a stallion. PC2, named linearity, was positively correlated to progressivity parameters (LIN and STR) and to VSL. Finally, PC3, named oscillation, was positively correlated to WOB in both species and negatively correlated also to BCF for donkey (**Table 2**).

These PCs were used to identify three well-defined subpopulations (SP1, SP2, and SP3) in both stallion and donkey, showing differences for all the kinematic parameters among them (**Table 3**, **Figure 1**). SP1 had the lowest value of all kinematic parameters, being named as the slow and non-linear subpopulation. SP2 included spermatozoa characterized by the highest linear trajectories (LIN and STR) and high speed (VCL, VSL, and VAP), ALH, and BCF. This subpopulation included the fast, straight, and lineal with a high tail beat spermatozoa subpopulation. SP3 was characterized by the highest VCL, VAP, ALH, and BCF but low linear trajectories (LIN and STR), being defined as the fast with a high beat and non-linear subpopulation (**Table 3**).

The percentage of each subpopulation in reference to the total number of spermatozoa varied slightly between donkey and stallion. The stallion and donkey semen have the highest proportion of the fast, straight, and lineal with a high beat subpopulation (SP2: 38.2 and 41.7%, respectively). SP3 was in the middle with 33.6% for stallion and 31.4% for donkey. In contrast, the lowest proportion was for the slow and non-linear subpopulation SP1 (28.2% for stallion and 26.9% for donkey, **Table 3**).

The proportion of the three stallion subpopulations for Arabian and Spanish horse breeds was almost the same (**Table 4**).

DISCUSSION

Sperm competition plays an important role in sperm fertilization, so in male fertility and lead to a strong natural selection (26). Sperm competition occurs between sperm from two or more rival males making an attempt to fertilize a female within a sufficiently

TABLE 1 | Sperm kinematic variables (mean ± SEM) in horses and donkey.

Variable/Breed	Arabian	Spanish	Total horse	Donkey
VCL	214.52 ± 79.28^{a}	232.45 ± 89.55^{b}	224.96 ± 85.87^{x}	230.08 ± 91.45^{y}
VSL	60.14 ± 37.08^{a}	59.21 ± 34.83^{a}	59.60 ± 35.79^{x}	78.07 ± 48.01^{y}
VAP	161.59 ± 66.0^{a}	167.03 ± 68.89^{b}	164.76 ± 67.75^{x}	172.63.73 ± 63.08^{y}
LIN	26.99 ± 11.95^{b}	25.19 ± 11.23^{a}	25.94 ± 11.57^{x}	33.81 ± 19.26^{y}
STR	36.36 ± 15.50^{a}	35.49 ± 15.43^{a}	35.85 ± 15.46^{x}	42.75 ± 19.69^{y}
WOB	74.22 ± 9.91^{b}	71.21 ± 9.13^{a}	72.50 ± 9.58^{x}	74.89 ± 14.08^{y}
ALH	1.18 ± 0.26^{a}	1.30 ± 0.31^{b}	1.25 ± 0.30^{x}	1.41 ± 0.34^{y}
BCF	37.03 ± 15.69^{a}	39.25 ± 14.92^{b}	38.32 ± 15.29^{y}	33.99 ± 18.78^{x}

VCL (μm/s), curvilinear velocity; VSL (μm/s), straight-line velocity; VAP (μm/s), average path velocity; LIN (%), linearity; STR (%), straightness; WOB (%), wobble; ALH (μm), amplitude of lateral head displacement; BCF (Hz), beat-cross frequency. SEM = standard error of the mean. ^{a,b}Different superscripts mean significant statistical differences among horse breeds. Different letters (x, y) indicate differences among horse and donkey species $P < 0.05$.

TABLE 2 | Eigenvectors of the three principal components obtained in the study of sperm kinematics for horse and donkey.

	Horse			Donkey		
	PC1	PC2	PC3	PC1	PC2	PC3
VCL	0.96			0.86		
VSL		0.79			0.82	
VAP	0.90			0.93		
LIN		0.97			0.89	
STR		0.98			0.98	
WOB			0.97			0.90
ALH	0.90			0.84		
BCF	0.73					−0.67
Explained variation (%)	42.47	32.48	15.96	36.92	32.51	20.40

PC1, principal component designated "velocity;" PC2, principal component designated "linearity;" PC3, principal component designated "oscillation." VCL (μm/s), curvilinear velocity; VSL (μm/s), straight line velocity; VAP (μm/s), average path velocity; LIN (%), linearity; STR (%), straightness; WOB (%), wobble; ALH (μm), amplitude of lateral head displacement; BCF (Hz), beat-cross frequency. Only eigenvectors > 0.6 are presented for each principal component.

short period (27). This fact has been evidenced not only in mammalian but also in bird (28), fish (29), reptile (30), and insect (31) species. This relevance affects the sperm morphology leading to a production of longer sperms with larger mid-piece (32) and also affecting the head morphometry among close related camelids species (33).

The action of humans along domestication practice implied that natural selection had been replaced by strong artificial selection (34, 35). Domestication of equids took place in the Bronze Age for both horses (36) and donkeys (37). Artificial insemination practice meant a considerable advance in reproduction, and consequently in artificial selection, in all farm animals (38, 39) and recently started in donkey (40). The results presented here showed how close stallion and donkey species are, almost regarding sperm kinematics. In fact, there are more differences between the two considered stallion breeds than between one of them and the donkey. In some mammalian species, like camelids (33), offspring obtained by crossing different species remains fertile, but this is not the case in equids being expected to find higher differences in sperm kinematics. Effectively, the reproductive isolating mechanism is one of the most important speciation processes, and it is frequently related to sperm characteristics variation (41). In equid, species remain very close genetically, being able to produce hybrids viable but not fertile. So, a hinny is a domestic equine hybrid that is the offspring of a male horse (a stallion) and a female donkey (a jenny). It is the reciprocal cross to the more common mule, which is the product of a male donkey (a jack) and a female horse (a mare). Even more, both species can have hybrid offspring with zebras, indicating that evolutive divergency among this species is not enough to avoid reproduction among them.

From the methodological point of view, the results showed in the present work were obtained using the optimal frame rate for both species and stallion breeds (10, 11). Therefore, the subsequent subpopulation analyzed can be considered of high confidence.

Horse and donkey spermatozoa have a different way to move, as the donkey sperm is faster with a more linear trajectory compared with the horse. These differences were also appreciable at a frame rate of 25 fps (42).

To the best of our knowledge, only a few studies compared different horse breeds motility parameters and semen quality (43). Unfortunately, most of those works did not take into consideration kinematic variation between breeds. In the present study, we observed that Spanish breeds present higher sperm velocities (VCL and VAP) than Arab breeds, who showed higher linearity and sperm oscillation.

To complete the classical studies based on the comparison of the median values of each parameter as independent variables, the multivariate statistical procedures, including a reduction of dimensionality by PCA followed by clustering analysis, were developed to define sperm subpopulations (44, 45). During the last years, several studies have shown the universal presence of defined subpopulation structure inside the whole sperm population in the ejaculate (20, 46–48). This fact has changed the previously established paradigm that considered the ejaculate composed of "equivalent" cells

TABLE 3 | Descriptive statistics for the CASA-Mot variables (mean ± SD) for each sperm subpopulation species, horse, and donkey samples.

	Horse			Donkey		
	SP1	**SP 2**	**SP 3**	**SP 1**	**SP 2**	**SP 3**
n	5,606	7,626	6,714	4,851	7,549	5,678
% sperms	28.1	38.2	33.6	26.8	41.7	31.4
VCL	134.6 ± 48.17^{a}	233.45 ± 59.08^{b}	290.77 ± 69.08^{c}	142.33 ± 47.41^{a}	245.8 ± 75.51^{b}	286.73 ± 83.72^{c}
VSL	26.29 ± 15.67^{a}	85.59 ± 30.66^{c}	57.89 ± 28.88^{b}	26.18 ± 17.15^{a}	116.42 ± 32.80^{c}	71.41 ± 36.83^{b}
VAP	86.18 ± 30.08^{a}	176.51 ± 46.0^{b}	217.01 ± 49.33^{c}	92.65 ± 29.88^{a}	191.89 ± 39.25^{b}	215.36 ± 45.55^{c}
LIN	18.91 ± 8.14^{a}	36.6 ± 7.97^{c}	19.7 ± 7.80^{b}	18.35 ± 10.73^{a}	50.29 ± 15.50^{c}	25.1 ± 11.01^{b}
STR	29.7 ± 13.48^{b}	48.63 ± 10.86^{c}	26.48 ± 10.94^{a}	27.19 ± 14.18^{a}	60.67 ± 10.58^{c}	32.21 ± 12.96^{b}
WOB	64.67 ± 10.08^{a}	75.87 ± 7.47^{c}	75.21 ± 7.16^{b}	66.04 ± 14.83^{a}	79.21 ± 14.11^{c}	76.69 ± 13.26^{b}
ALH	1.03 ± 0.23^{a}	1.23 ± 0.22^{b}	1.46 ± 0.27^{c}	1.08 ± 0.21^{a}	1.46 ± 0.25^{b}	1.63 ± 0.29^{c}
BCF	22.64 ± 10.89^{a}	41.85 ± 13.11^{b}	47.41 ± 9.88^{c}	22.07 ± 11.66^{a}	36.8 ± 19.98^{b}	40.43 ± 17.39^{c}

n: total number of spermatozoa analyzed; SP1, fast and linear subpopulation; SP2, fast and non-linear subpopulation; SP3, slow and non-linear subpopulation. VCL (μm/s), curvilinear velocity; VSL (μm/s), straight-line velocity; VAP (μm/s), average path velocity; LIN (%), linearity; STR (%), straightness; WOB (%), wobble; ALH (μm), amplitude of lateral head displacement; BCF (Hz), beat-cross frequency. SD = standard deviation. $^{a-c}$*Values with different superscript letters differ significantly between sperm subpopulations of the same species.* $P < 0.05$.

competing for reaching the oocyte fertilization. Effectively, some kind of synergies must be present among sperm subpopulations for achieving the final goal of successful fertilization (6, 49).

In the present study, the whole collection of kinematic data was grouped into three PCs in donkey ejaculate, named velocity, progressiveness, and cell oscillation. Using this two-step approach, three subpopulations were obtained, showing that the most frequent SP was for the fastest with high linearity (42% of the total). In a previous study conducted on the Andalusian donkey, four subpopulations were observed, with the main subpopulation (36%) corresponding to low-velocity and high progressive spermatozoa and only 30% corresponding to progressive with high-velocity subpopulation (50). However, it is important to consider that the authors used a CASA-Mot system with only 25 fps, and the statistical procedure was a simple step clustering analysis.

Regarding the horse, the patter bot, both PCs and subpopulations were very similar to that observed in donkey, even if there were significant differences in the kinematic parameters between both species. Again, previous work showed four subpopulations but using a frame rate of just 16 fps (48). Even more, up to six subpopulations were found in other work using a frame rate of 25 fps and following a one-step statistical analysis (46).

All these differences can show the frame rate importance in a correct interpretation of sperm trajectories and the errors that can occur with fewer frames. These changes in sperm trajectories found between different frames also change the real distribution of sperm subpopulations of an ejaculate, all resulting in a misunderstanding of the real role of each subpopulation and its capacity to arrive at the oocyte and fertilize it.

Finally, in reference to the horse breeds considered here, there were no important differences in the sperm subpopulation structure, being little differences in the presence of some of the subpopulations. Instead of this, VCL and VAP for all subpopulations showed significant differences with higher velocities for the Spanish breed compared with Arabian. This could be explained by the history of the domestication of horses who spread out of western central Eurasia, place of origin, that started combined with the continued high genetic input from local wild populations; this hybridization increased genetic differentiation in population, which were accentuated by the human force that shaped their need for creating highly competing horses (1). This hybridization has affected the shape and the performance of the spermatozoa in different degrees depending on the breed, resulting in a decrease in per-cycle conception rates, at around 60% than those observed for other domestic livestock species (51). The Arabian horse is considered one of the most ancestral, with pure pedigree even if it was shown with the recent study of mitochondrial DNA sequences that there is heterogenicity and great diversity among this breed (52), whereas the pure Spanish horse is considered the first European "warmblood," a mixture of heavy European and lighter Oriental horses, taking its origin from the Andalusia Spanish region that is recognized as a distinct breed since the 15th century (53). Similar differences have been observed in other species like a bull (54), boar (55), and dog (56), showing how much artificial selection procedures conduces to processes close to the speciation process in natural selection (33).

In conclusion, a new performing camera permitted to acquire higher frames for better sperm motility analysis and therefore get more reliable results approachable to real sperm move and changing the old perspectives. The sperm had significantly higher values for all kinematic parameters for the donkey than the horse. Donkey sperm was faster and linear than the horse. Regarding horse breed differences, Spanish horse sperm is rapid, but Arab horse is more linear. The cluster analysis showed three sperm subpopulations; the predominant motile subpopulation in freshly ejaculated horse and donkey sperm had very fast velocity characteristics and a linear trajectory with a high beat frequency. Finally, the

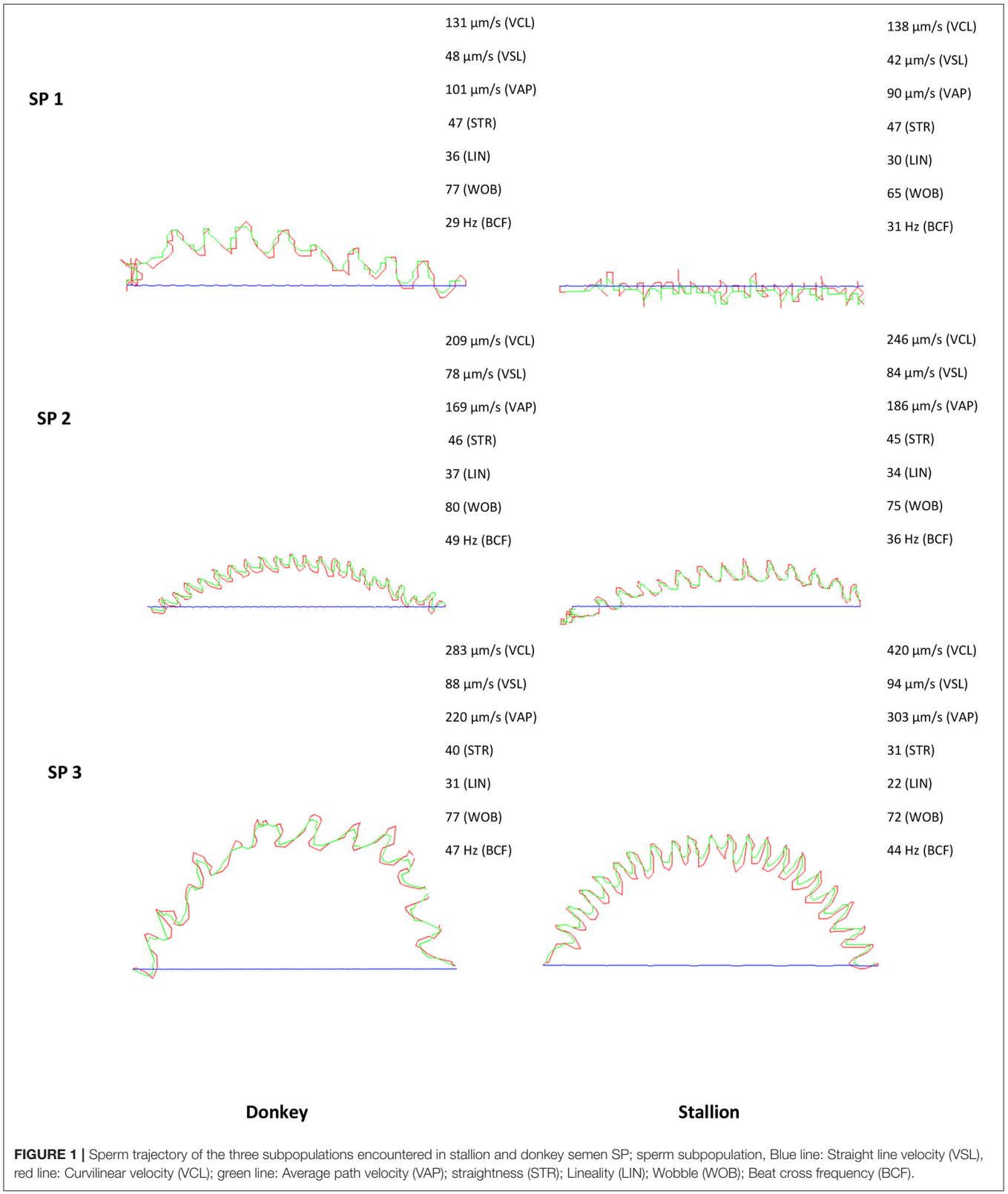


FIGURE 1 | Sperm trajectory of the three subpopulations encountered in stallion and donkey semen SP; sperm subpopulation, Blue line: Straight line velocity (VSL), red line: Curvilinear velocity (VCL); green line: Average path velocity (VAP); straightness (STR); Lineality (LIN); Wobble (WOB); Beat cross frequency (BCF).

identification and differentiation of the structure of functional sperm subpopulations seem to be an advantageous key element as a valuable alternative tool to successfully detect and improve critical handling of further treatment where the effect of the breed would be considered to avoid alterations in the interpretations of the analysis.

TABLE 4 | Descriptive statistics for the CASA-Mot variables (mean ± SEM) for each sperm subpopulation in two horse breeds, Arabian and Spanish.

	Arabian			Spanish		
	SP1	**SP 2**	**SP 3**	**SP 1**	**SP 2**	**SP 3**
n	2,831	3,366	2,128	3,942	4,917	2,762
% sperms	34%	40%	26%	34%	42%	24%
VCL	136.10 ± 47.84^{a}	263.81 ± 59.32^{c}	240.88 ± 57.11^{b}	142.41 ± 45.87^{a}	295.39 ± 69.05^{c}	248.89 ± 57.97^{b}
VSL	30.05 ± 17.47^{a}	61.34 ± 26.72^{b}	98.25 ± 34.41^{c}	31.51 ± 17.57^{a}	61.69 ± 29.51^{b}	94.35 ± 27.80^{c}
VAP	90.61 ± 32.52^{a}	206.71 ± 44.26^{c}	184.63 ± 45.64^{b}	93.47 ± 31.18^{a}	219.44 ± 48.70^{c}	178.69 ± 40.09^{b}
LIN	21.30 ± 9.45^{a}	23.04 ± 8.10^{b}	40.79 ± 8.82^{c}	21.28 ± 8.91^{a}	20.99 ± 8.66^{a}	38.25 ± 7.76^{b}
STR	32.11 ± 14.28^{b}	29.25 ± 10.12^{a}	53.24 ± 10.85^{c}	32.38 ± 13.34^{b}	28.14 ± 11.53^{a}	53.02 ± 9.68^{c}
WOB	66.86 ± 10.12^{a}	78.90 ± 6.82^{c}	76.89 ± 7.77^{b}	65.86 ± 10.01^{a}	74.82 ± 6.65^{c}	72.42 ± 7.95^{b}
ALH	1.02 ± 0.22^{a}	1.31 ± 0.23^{c}	1.19 ± 0.23^{b}	1.05 ± 0.21^{b}	1.47 ± 0.28^{c}	0.34 ± 0.25^{a}
BCF	23.84 ± 11.91^{a}	45.33 ± 11.11^{c}	41.46 ± 14.81^{b}	25.77 ± 11.94^{a}	47.39 ± 9.78^{c}	44.00 ± 12.79^{b}

n: total number of spermatozoa analyzed; SP1, fast and linear subpopulation; SP2, fast and non-linear subpopulation; SP3, slow and non-linear subpopulation. VCL (μm/s), curvilinear velocity; VSL (μm/s), straight-line velocity; VAP (μm/s), average path velocity; LIN (%), linearity; STR (%), straightness; WOB (%), wobble; ALH (μm), amplitude of lateral head displacement; BCF (Hz), beat-cross frequency. SEM = standard error of the mean. $^{a-c}$*Values with different superscript letters differ significantly between sperm subpopulations of the same breed. P < 0.05.*

Future work is needed to define the relationship of the observed sperm subpopulation structures and the fertility of the samples, considering the effect of different breeds.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the ethics committee considered no necessary as explained in the text.

AUTHOR CONTRIBUTIONS

CS and JM: conceptualization, validation, visualization, and supervision. SG, JC, IY, and JM: methodology. CS: software. AV: formal analysis. SG, JC, and JM: investigation. SG and CS: data curation. SG and AV: writing—original draft preparation. SG, CS, and JM: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was funded by the Equine Reproduction Service, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Spain. JC was funded by the National Agency for Research and Development (ANID), Ministry of Education, Chile (Scheme: Becas Chile Doctorado en el Extranjero, PFCHA; Grant: 2017/72180128). IY was funded by Secretary of Higher Education, Science, Technology and Innovation (SENESCYT), Ecuador (Scheme: Programa de Becas Internacionales de Posgrado 2019; Grant: CZ02-000507-2019).

REFERENCES

1. Orlando L, Ginolhac A, Zhang G, Froese D, Albrechtsen A, Stiller M, et al. Recalibrating equus evolution using the genome sequence of an early Middle Pleistocene horse. *Nature*. (2013) 499:74–8. doi: 10.1038/nature12323
2. Zeder MA, Bradley DG, Emshwiller E, Smith BD. *Documenting Domestication: New Genetic and Archaeological Paradigms*. Oakland, CA: University of California Press (2006). doi: 10.1525/9780520932425
3. Clutton-Brock J. Horse Power: *A History of the Horse and the Donkey in Human Societies*. Cambridge, MA: Harvard University Press (1992).
4. Thornton PK. Livestock production: recent trends, future prospects. *Philos Trans R Soc B Biol Sci*. (2010) 365:2853–67. doi: 10.1098/rstb.2010.0134
5. Amann RP, Waberski D. Computer-assisted sperm analysis (CASA): capabilities and potential developments. *Theriogenology*. (2014) 81:5–17. doi: 10.1016/j.theriogenology.2013.09.004
6. Valverde A, Castro-morales O, Madrigal-valverde M, Soler C. Sperm kinematics and morphometric subpopulations analysis with CASA systems : a review. *Rev Biol Trop*. (2019) 67:1473–87. doi: 10.15517/rbt.v67i6.35151
7. Gallagher MT, Smith DJ, Kirkman-Brown JC. CASA: tracking the past and plotting the future. *Reprod Fertil Dev*. (2018) 30:867. doi: 10.1071/RD17420
8. Gallagher MT, Cupples G, Ooi EH, Kirkman-Brown JC, Smith DJ. Rapid sperm capture: high-throughput flagellar waveform analysis. *Hum Reprod*. (2019) 34:1173–85. doi: 10.1101/551267
9. Soler C, Valverde A, Bompart D, Fereidounfar S, Sancho M, Yániz J, et al. New methods of semen analysis by casa. *Agric Biol*. (2017) 52:232–41. doi: 10.15389/agrobiology.2017.2.232eng
10. Gacem S, Bompart D, Valverde A, Catalán J, Miró J, Soler C. Optimal frame rate when there were stallion sperm motility evaluations and determinations for kinematic variables using CASA-Mot analysis in different counting chambers. *Anim Reprod Sci*. (2020) 223:10664. doi: 10.1016/j.anireprosci.2020.106643
11. Gacem S, Catalán J, Valverde A, Soler C, Miró J. Optimization of Casa-mot analysis of donkey sperm: optimum frame rate and values of kinematic variables for different counting chamber and fields. *Animals*. (2020) 10:1–16. doi: 10.3390/ani10111993

12. Bompart D, García-Molina A, Valverde A, Caldeira C, Yániz J, Núñez De Murga M, et al. CASA-Mot technology: how results are affected by the frame rate and counting chamber. *Reprod Fertil Dev*. (2018) 30:810–9. doi: 10.1071/RD17551
13. Valverde A, Madrigal M, Caldeira C, Bompart D, de Murga JN, Arnau S, et al. Effect of frame rate capture frequency on sperm kinematic parameters and subpopulation structure definition in boars, analysed with a CASA-Mot system. *Reprod Domest Anim*. (2019) 54:167–75. doi: 10.1111/rda.13320
14. Yániz J, Vicente-Fiel S, Soler C, Recreo P, Carretero T, Bono A, et al. Comparison of different statistical approaches to evaluate morphometric sperm subpopulations in man. *Asian J Androl*. (2016) 18:819–23. doi: 10.4103/1008-682X.186872
15. Abaigar T, Holt W, Harrison R, del Barrio G. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol Reprod*. (1999) 60:32–41. doi: 10.1095/biolreprod60.1.32
16. García-Molina A, Valverde A, Bompart D, Caldeira C, Vendrell A, Soler C. Updating semen analysis: a subpopulation approach. *Asian J Androl*. (2019) 22:118–9. doi: 10.4103/aja.aja_33_19
17. Yániz JL, Soler C, Santolaria P. Computer assisted sperm morphometry in mammals: a review. *Anim Reprod Sci*. (2015) 156:1–12. doi: 10.1016/j.anireprosci.2015.03.002
18. Urbano M, Ortiz I, Dorado J, Hidalgo M. Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity. *Reprod Domest Anim*. (2017) 52:468–76. doi: 10.1111/rda.12935
19. Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrín P, et al. Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. *PLoS ONE*. (2011) 6:e18112. doi: 10.1371/journal.pone.0018112
20. Oldenhof H, Blässe AK, Wolkers WF, Bollwein H, Sieme H. Osmotic properties of stallion sperm subpopulations determined by simultaneous assessment of cell volume and viability. *Theriogenology*. (2011) 76:386–91. doi: 10.1016/j.theriogenology.2011.02.027
21. Pérez-Llano B, Yenes-García P, García-Casado P. Four subpopulations of boar spermatozoa defined according to their response to the short hypoosmotic swelling test and acrosome status during incubation at 37°C. *Theriogenology*. (2003) 60:1401–7. doi: 10.1016/S0093-691X(03)00131-6
22. Borowsky R, Luk A, He X, Kim RS. Unique sperm haplotypes are associated with phenotypically different sperm subpopulations in Astyanax fish. *BMC Biol*. (2018) 16:72. doi: 10.1186/s12915-018-0538-z
23. Gutiérrez-Reinoso M, García-Herreros M. Normozoospermic versus teratozoospermic domestic cats: Differential testicular volume, sperm morphometry, and subpopulation structure during epididymal maturation. *Asian J Androl*. (2016) 18:871–8. doi: 10.4103/1008-682X.187583
24. Burger D, Dolivo G, Wedekind C. Ejaculate characteristics depend on social environment in the horse (*Equus caballus*). *PLoS ONE*. (2015) 10:e0143185. doi: 10.1371/journal.pone.0143185
25. Murtagh F, Legendre P. Ward's Hierarchical Agglomerative Clustering Method: Which Algorithms Implement Ward's Criterion? *J Classif*. (2014) 31:274–95. doi: 10.1007/s00357-014-9161-z
26. Tourmente M, Varea-Sánchez M, Roldan ERS. Faster and more efficient swimming: energy consumption of murine spermatozoa under sperm competition. *Biol Reprod*. (2019) 100:420–8. doi: 10.1093/biolre/ioy197
27. Tourmente M, Gomendio M, Roldan ER. Sperm competition and the evolution of sperm design in mammals. *Anim Reprod Sci*. (2011) 156:1–12. doi: 10.1186/1471-2148-11-12
28. Dunn PO, Whittingham LA, Pitcher TE. Mating systems, sperm competition, and the evolution of sexual dimorphism in birds. *Evolution*. (2001) 55:161–75. doi: 10.1111/j.0014-3820.2001.tb01281.x
29. Stoltz JA, Neff BD. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. *J Evol Biol*. (2006) 19:1873–81. doi: 10.1111/j.1420-9101.2006.01165.x
30. Friesen CR, Kahrl AF, Olsson M. Sperm competition in squamate reptiles. *Philos Trans R Soc B Biol Sci*. (2020) 375:20200079. doi: 10.1098/rstb.2020.0079
31. García-González F, Simmons LW. Sperm viability matters in insect sperm competition. *Curr Biol*. (2005) 15:271–5. doi: 10.1016/j.cub.2005.01.032
32. Firman RC, Simmons LW. Sperm midpiece length predicts sperm swimming velocity in house mice. *Biol Lett*. (2010) 6:513–6. doi: 10.1098/rsbl.2009.1027
33. Soler C, Sancho M, García A, Fuentes MC, Núñez J, Cucho H. Ejaculate fractioning effect on llama sperm head morphometry as assessed by the ISAS® CASA system. *Reprod Domest Anim*. (2014) 49:71–8. doi: 10.1111/rda.12226
34. Kowalczyk A, Czerniawska-Piatkowska E, Kuczaj M. Factors influencing the popularity of artificial insemination of Mares in Europe. *Animals*. (2019) 9:460. doi: 10.3390/ani9070460
35. Morrell JM. Artificial insemination: current and future trends. In: *Artificial Insemination in Farm Animals*. London: InTech (2011).
36. Guimaraes S, Arbuckle BS, Peters J, Adcock SE, Buitenhuis H, Chazin H, et al. Ancient DNA shows domestic horses were introduced in the southern caucasus and anatolia during the bronze age. *Sci Adv*. (2020) 6:eabb0030. doi: 10.1126/sciadv.abb0030
37. Greenfield HJ, Shai I, Greenfield TL, Arnold ER, Brown A, Eliyahu A, et al. Earliest evidence for equid bit wear in the ancient Near East: The "ass" from Early Bronze Age Tell eṣ-Ṣâfi/Gath, Israel. *PLoS ONE*. (2018) 13:e0196335. doi: 10.1371/journal.pone.0196335
38. Allen WR. The development and application of the modern reproductive technologies to horse breeding. *Reprod Domest* Anim. (2005) 40:310–29. doi: 10.1111/j.1439-0531.2005.00602.x
39. Samper JC, Plough T. Techniques for the insemination of low doses of stallion sperm. *Reprod Domest Anim*. (2010) 45:35–9. doi: 10.1111/j.1439-0531.2010.01632.x
40. Canisso IF, Panzani D, Miró J, Ellerbrock RE. Key aspects of donkey and mule reproduction. *Vet Clin North Am - Equine Pract*. (2019) 35:607–42. doi: 10.1016/j.cveq.2019.08.014
41. Howard DJ, Palumbi SR, Birge LM, Manier MK. Sperm and speciation. *Sperm Biol*. (2009) 367–403. doi: 10.1016/B978-0-12-372568-4.00009-4
42. Miró J, Lobo V, Quintero-Moreno A, Medrano A, Peña A, Rigau T. Sperm motility patterns and metabolism in Catalonian donkey semen. *Theriogenology*. (2005) 63:1706–16. doi: 10.1016/j.theriogenology.2004.07.022
43. Gottschalk M, Sieme H, Martinsson G, Distl O. Analysis of breed effects on semen traits in light horse, warmblood, and draught horse breeds. *Theriogenology*. (2016) 85:1375–81. doi: 10.1016/j.theriogenology.2015.11.030
44. Ibanescu I, Siuda M, Bollwein H. Motile sperm subpopulations in bull semen using different clustering approaches – associations with flow cytometric sperm characteristics and fertility. *Anim Reprod Sci*. (2020) 215:106329. doi: 10.1016/j.anireprosci.2020.106329
45. Ramón M, Martínez-Pastor F. Implementation of novel statistical procedures and other advanced approaches to improve analysis of CASA data. *Reprod Fertil Dev*. (2018) 30:860. doi: 10.1071/RD17479
46. Ortega-Ferrusola C, Macías García B, Suárez Rama V, Gallardo-Bolaños J, González-Fernández L, Tapia J, et al. Identification of sperm subpopulations in stallion ejaculates: changes after cryopreservation and comparison with traditional statistics. *Reprod Domest Anim*. (2009) 44:419–23. doi: 10.1111/j.1439-0531.2008.01097.x
47. Ortiz I, Dorado J, Morrell JM, Diaz-Jimenez MA, Pereira B, Consuegra C, et al. Comparison of sperm selection techniques in donkeys: motile subpopulations from a practical point of view. *Anim Reprod*. (2019) 16:282–9. doi: 10.21451/1984-3143-AR2018-0133
48. Quintero-Moreno A, Miró J, Teresa Rigau A, Rodríguez-Gil JE. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology*. (2003) 59:1973–90. doi: 10.1016/S0093-691X(02)01297-9
49. Víquez L, Barquero V, Soler C, Roldan ERS, Valverde A. Kinematic subpopulations in bull spermatozoa: A comparison of classical and bayesian approaches. *Biology*. (2020) 9:1–16. doi: 10.3390/biology9060138
50. Dorado J, Acha D, Gálvez MJ, Ortiz I, Carrasco JJ, Díaz B, et al. Sperm motility patterns in Andalusian donkey (Equus asinus) semen: effects of body weight, age, and semen quality. *Theriogenology*. (2013) 79:1100–9. doi: 10.1016/j.theriogenology.2013.02.006
51. Nath L, Anderson G, McKinnon A. Reproductive efficiency of thoroughbred and Standardbred horses in north-east Victoria. *Aust Vet J*. (2010) 88:169–75. doi: 10.1111/j.1751-0813.2010.00565.x
52. Głazewska I. Speculations on the origin of the Arabian horse breed. *Livest Sci*. (2010) 129:49–55. doi: 10.1016/j.livsci.2009.12.009

53. Royo LJ, Álvarez I, Beja-Pereira A, Molina A, Fernández I, Jordana J, et al. The origins of iberian horses assessed via mitochondrial DNA. *J Hered*. (2005) 96:663–9. doi: 10.1093/jhered/esi116
54. Valverde A, Areán H, Fernández A, Bompart D, García-Molina A, López-Viana J, et al. Combined effect of type and capture area of counting chamber and diluent on Holstein bull sperm kinematics. *Andrologia*. (2018) 51:1–10. doi: 10.1111/and.13223
55. Waterhouse KE, Hofmo PO, Tverdal A, Jr RRM. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction*. (2006) 131:887–94. doi: 10.1530/rep.1.01049
56. Valverde A, Arnau S, García-Molina A, Bompart D, Campos M, Roldán E, et al. Dog sperm swimming parameters analysed by computer-assisted semen analysis of motility reveal major breed differences. *Reprod Domest Anim*. (2019) 54:795–803. doi: 10.1111/rda.13420

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 26 May 2021
doi: 10.3389/fvets.2021.672058

The Role of Sperm Morphology Standards in the Laboratory Assessment of Bull Fertility in Australia

V.E.A. Perry [1,2,3]*

[1] Queensland Sperm Morphology Laboratory, Ruminant Reproduction Research Centre, Goondiwindi, QLD, Australia, [2] School of Veterinary Science, University of Queensland, Brisbane QLD, Australia, [3] Robinson Research Institute, University of Adelaide, Adelaide, SA, Australia

The lack of standardization in the laboratory assessment of semen questions the reliability of semen analysis, and makes meaningful interpretation of these evaluations impossible. We herein describe a standardization program for morphology assessment currently in place in Australia and outline the methods used, both for the categorization of the abnormalities, including newly described abnormalities, and those that permit standardized microscopic assessment between laboratories.

Keywords: sperm morphology laboratory assessment standardization, bull fertility correlation, sperm laboratory standardization, correlation of fertility with sperm abnormalities, bull

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
John Kastelic, University of Calgary, Canada
Arlindo A. Moura, Federal University of Ceara, Brazil

***Correspondence:**
V.E.A. Perry
viv.perry@adelaide.edu.au

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *25 February 2021*
Accepted: *22 April 2021*
Published: *26 May 2021*

Citation:
Perry VEA (2021) The Role of Sperm Morphology Standards in the Laboratory Assessment of Bull Fertility in Australia. Front. Vet. Sci. 8:672058. doi: 10.3389/fvets.2021.672058

INTRODUCTION

The determination of the percentage morphologically normal bull sperm is highly repeatable and is strongly correlated with days to conception and calf output in both dairy herds (1–3) and beef herds (4). Increasingly, however, bull semen, in collection centers, is often evaluated only for total sperm numbers (concentration) and sperm motility (3). Furthermore, flow cytometry and measures of membrane intact sperm are replacing microscopy due to the faster turn over of these techniques (5).

The lack of standardization of laboratory semen assessment, including morphology, is highlighted by Brito (6). He observed significant differences in classifications and results in studies conducted between eight semen processing centers, laboratory and processing centers and five veterinary University laboratories. He concluded that: These observations question the reliability of semen analysis and make it impossible to meaningfully interpret evaluation.

The term "morphology assessed" may also be abused as it can be performed using improper protocols. For example, examination of sperm under low power microscopy with the inability to detect important abnormalities The ASMA (automated computer assisted sperm morphology analysis) tool for example can increase standardization but its inability to report all but basic head measurement and surface features means that many abnormalities are undetected (7) with little relationship with fertility reported (8).

The importance of a standardized approach led to the Australian standardization UQSMSP (University of Queensland Sperm Morphology Standardization Program) developed in 2018. It furthered the standardized morphology assessment reported in 2006 (9), as part of the standardized BBSE to be used by the Australian Cattle Veterinarian (ACV). This scheme uses central laboratories to provide unbiased expert analysis of sperm morphology (10–13). The progress of this standardization scheme along with advances in our knowledge of bull sperm abnormalities is reported here.

DISCUSSION

In the US, Canada and the UK morphology examination is usually completed crush side using vital stains such as nigrosin eosin which enable assessment of morphology under bright field microscopy. This method has been shown to be less accurate in its assessment of morphological abnormalities, particularly head abnormalities, in many studies (1, 14–17) when compared to the assessment of wet mounts under DIC (Differential Interference Contrast) or phase contrast microscopy usually completed in a specialized laboratory.

The considered professional gold standard for both the assessment of bovine and equine sperm morphology is DIC microscopy at x1,000 magnification, the recommended standard for Australian laboratories. Samples are sent to the central laboratories in buffered formal saline, which enables high quality wet preparations to be examined by the morphologist. Even with this level of microscope it is still necessary to focus up and down on each sperm to accurately assess abnormalities at the limit of resolution. This, however, is less the case than is necessary with Phase contrast microscopy (13). In recent years the advance of the DIC microscope has enabled even difficult to detect abnormalities of the DNA e.g., pale centers, to be viewed without the aid of Feulgen staining, although the latter is instigated as a base check.

Fertile bulls have a spermiogram, which contains <30% abnormal sperm (4, 18, 19). This threshold level is accepted as standard in Australia as in many other countries, importantly, however, individual thresholds for each abnormality (10) vary and are based upon the currently known effect upon fertility. Each defect on each abnormal sperm is recorded; that is, more than one defect may be recorded per sperm. This is important in a standardized program as one morphologist may count a different abnormality to another. All counts are completed using the online morphology counting system developed by the ACV. This system allocates numbers to each trait (**Figure 1**). These numbers are entered into the keyboard and the software collates the number of abnormalities which creates the morphology report.

Morphologists are required to update their skills at an annual workshop and implement skills which maximize both the number and randomization of fields of view, e.g., only sperm in the middle of the field of view are counted. A minimum of 100 sperm are counted per spermiogram (12). This is however, increased to 200 in the case of borderline (62–77) counts (20).

Evenson (21) divided sperm into those with compensable or uncompensable traits. This concept is simplified if we consider that the female tract (22) and finally the vestments of the ovum act as a filtration system for the sperm population. They act as barriers to the progress of sperm such that only the fittest arrive at the ovum (23). Compensable traits preclude affected sperm from fertilizing the ovum, i.e., the abnormality does not allow them either to reach the ova or attach to the ova. A compensable abnormality, therefore, is one that can be compensated for by increasing the number of spermatozoa in the ejaculate; that is the fertility of the bull will increase with increasing numbers of spermatozoa. These include traits, which cause, for example, abnormal or nil motility (these are filtered out in the female tract), and abnormal head shape (filtered out crossing the zona as these interfere with hyperactive motility required at this juncture). The threshold for such abnormalities is set at 30% (12, 24, 25). Increasing numbers of spermatozoa, however, cannot compensate for uncompensable traits. Sperm with these traits are able to reach the ovum and initiate fertilization (thereby blocking polyspermy) and/or embryo development but that development is unsustainable. The cow therefore returns to oestrus. Such traits include, nuclear vacuoles and pyriform heads. They tend to be the subtler more difficult to detect abnormalities yet cause the biggest decrease in conception rates. The suggested threshold of such abnormalities is therefore 20% (12, 25). In general it could be said that sperm with abnormalities that do not allow them to reach the ova or attach are considered compensable traits. Those abnormalities, which allow the sperm to fertilize the ova but result in early embryonic death or abnormal development, are considered uncompensable.

UQSMSP issues guidelines on the equipment and protocols required to be upheld by members. The eight main sperm categories with tolerance levels are, in order; normal sperm-(which includes abnormalities observed but that are considered not to effect conception rates), proximal droplets (PD) (20%), midpiece abnormalities (MP) (30%), loose heads and principle piece (tail) abnormalities (HT) (30%), pyriform heads (PY) (20%), knobbed acrosomes (KA) (30%), vacuoles and teratoids (VT) (including abnormalities of DNA condensation) (30%), swollen acrosomes (SA) (including those sperm with lost acrosomes) (30%) (9). These main categories are further divided into sub categories in the advanced sheet view, for example; differentiation between flat acrosomes, beaded acrosomes and indented knobbed acrosomes, and the various categories of vacuolation (13). All morphologists must complete their counts using this advanced sheet with all subcategories to enable comparisons between morphologists if any queries arise on the counts completed.

The maintenance of standardization is emphasized by the requirement that samples are kept by morphology laboratories for 3 years and that these samples were made available to UQSMSP examiners where a disagreement arises. Each morphologist is required to perform competency checks on five samples per annum which are sent out from UQSMSP and results submitted back for analysis within 2 weeks. Unacceptable variation from the median results in second round of test samples being completed with the option of additional tuition supplied by UQSMSP.

When conducting the assessment of the spermiogram the morphologists must be cognisant of both the stages of spermatogenesis as well as the environmental and developmental effects upon sperm morphology. It is established that any environmental stress sufficient to cause elevation in circulating cortisol is sufficient to affect sperm morphology (4, 26). A cascade of linked developmental processes occur during spermiogenesis that are testosterone dependent, such as;formation of the acrosome from the golgi apparatus, compaction of sperm chromatin, mitochondrial and centriole organization to form the flagellum and initiation of cytoplasm resorption. This is neatly shown in the publication by Callaghan et al. (27)

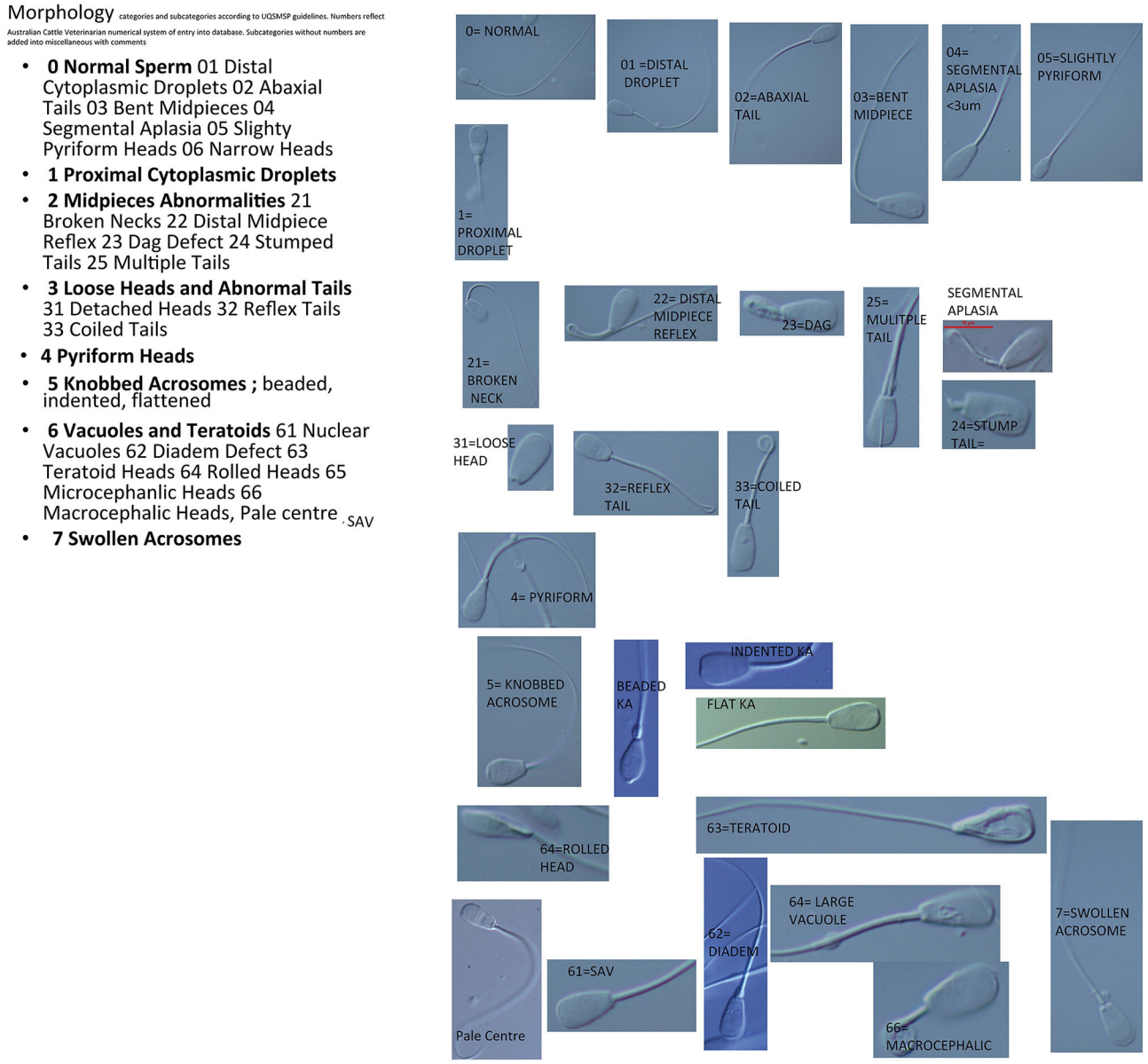


FIGURE 1 | Images of sperm defects as categorized for the UQSMSP.

where a single acidotic event was followed sequentially by elevated cortisol, reduced FSH and testosterone consequent with increased sperm abnormalities in the subsequent weeks. Similar sequential appearance of spermatozoal abnormalities were observed following transport and relocation (13) or dexamethasone (28).

Analogous abnormalities have been observed following exposure to heat whether due to obesity, scrotal abnormality, climate or fever (29). Mechanisms that maintain testis homeothermy include the cremaster and dartos muscles and the testicular vascular cone (30). This latter consists of the coiled veins of the pampiniform plexus and the incoming testicular artery. A distinct scrotal neck is necessary for the adequate functioning of the testicular vascular cone, where heat exchange occurs between the venous and arterial blood. This may be absent in the obese animal. The testis are particularly susceptible to heat as testicular function occurs in a marginally hypoxic environment where an increase in temperature may increase metabolic rate, but there is no corresponding increase in blood flow. Tissues are therefore susceptible to hypoxia (28). Alternately, a detailed study (31) of testicular blood flow in sheep and mice recently suggests that it is heat itself rather than hypoxia that affects testicular function. A recent study, (32), found the number of bulls passing the sperm morphology test at

70% were reduced in Far Northern Australia, although, climatic region had less effect than breed. Equally season may affect morphology either via temperature or nutritional intake (11) with one study showing an elevation in bulls failing the knobbed acrosome and vacuole thresholds in the summer months (32).

Nutritional deficiencies during development, whether prenatal (33), pre weaning (34–36) or pre sale (27, 37, 38) have been shown to affect maturation of the spermiogram. During adulthood nutritional restriction and/or dietary change may have deleterious effects particularly in bulls predisposed to developing certain sperm abnormalities such as nuclear vacuoles (27). Immature spermiograms in pubertal and peri pubertal bulls display particularly high levels of proximal droplets which may vary between ejaculates collected on the same day (33, 38).

Dietary intake of toxic substances such as gossypol in cotton seed has been shown to affect morphology in some studies (39) but not in others (40). The dietary supply of metallic cations (e.g., calcium, iron) is thought to cause this differential effect as these bind gossypol in the rumen (40) and may be present in, for example, the mineral content of bore water or when lime is added to the diet.

These environmental effects overlay inherited conditions such as the knobbed acrosome (41–43), and the Dag defect (44). Relatedly breed has a significant effect upon morphology: For example the Belgian blue compared to the Friesian (45) and Bos indicus breed bulls compared to Bos taurus breeds (32). A Canadian study (46), however, reported no effect of breed on the spermiogram between the Bos taurus breeds used.

STANDARDIZATION UNDER UQSMSP

Images of each category are given in **Figure 1**.

Normal Sperm

This category includes normal variations of form and those sperm with abnormal forms that are recognized as having no effect upon fertility in the bull. This includes: abaxial tail, minor segmental aplasia, distal droplets, slightly bent midpiece, slightly pyriform. The reasoning behind their inclusion as normal is listed below.

Proximal Droplets

These are normally observed in the pubertal bull with incidence decreasing with age (33, 47). In the mature bull they indicate abnormal spermiogenesis (and/or epididymal function). They were observed 7–10 days following a temperature or stress event (28) and 15 days following ruminal acidosis (27).

The prognosis depends upon the type of abnormalities associated with the proximal droplets. Counts of 10–15% proximal droplets (35) have been associated with decreased fertility. This trait is considered uncompensable as the sperm fail to bind to the ova and furthermore that sperm associated with high numbers of proximal droplet sperm have impaired ability to bind with the ova (48). Amann et al. (47) also reported that in bulls with >30% proximal droplets that the associated apparently normal spermatozoa displayed immaturity and reduced ability to fertilize ova. PDs are also associated with decreased membrane integrity and increased chromatin damage post-thaw (49). This defect has a threshold of 20% as studies show proximal droplets are associated with poor pregnancy rates (50).

Distal Droplets

Unlike the boar there are no reports of distal droplets being associated with infertility in the bull. Sperm with distal droplets will lose the droplet if left in a water bath for 15–30 min or if gently agitated. The number of sperm within the ejaculate with distal droplets, also vary widely between sequential ejaculates (12). Case studies using bulls with high numbers of distal droplets in natural service achieve normal pregnancy rates (51). For this reason distal droplets in isolation are not generally considered to be a defect by the author or by other researchers (24, 51) and are placed in the normal category.

Cause

The Sertoli cell effects elongation of the spherical spermatid from stage 8–12 along with the exertions of the manchette (52). The surplus cytoplasm and organelles from this process remain attached to the spermatid as a residual body attached at the sperm neck. All sperm entering the caput epididymis therefore, have this droplet, however, only 10% remain by the time sperm leave the cauda epididymis The presence of a cytoplasmic droplet whether in the proximal or distal position may be an indication that the sperm has not acquired essential binding proteins from the seminal vesicle fluid (53). These binding proteins are essential for the sperm to bind to the zona pellucida. For this reason it is important that massage of the ampullae and seminal vesicles is sufficient to illicit a quantity of seminal fluid during the collection process (12).

Pyriform Heads

Narrow in the postacrosomal region. Young bulls up to 2 years old and in good condition display a greater likelihood of recovery from this condition than do older bulls. This condition is particularly seen in young over fat bulls (51). It is very important to note, that there is variation in the degree of this abnormality: In a series of experiments Barth et al. (54) reported that fertility was related to the severity of pyriformity of the head. A moderate degree of pyriformity, in the absence of other signs of disturbed spermatogenesis, is not detrimental to fertility. However, extreme tapering in the postacrosomal region results in significant reductions in fertility. Pyriformity is considered only partially compensable (55). As in this study some pyriform sperm were able to fertilize oocytes but these had a reduced ability to cleave. The threshold of not more than 20% is therefore applied to this abnormality.

Cause

Pyriform heads are induced in bulls following stress such as dexamethasone treatment and scrotal insulation (28) 20d post insult with some bulls showing predisposition to this abnormality following a stress event (51). Pyriform heads are differentially excluded from advancement in the female reproductive tract at the specifically precluded from the cervix, uterus, utero-tubule junction (22).

Knobbed Acrosomes

This abnormality may be heritable or arise following a stress event (27) and is often observed in the peripubertal bull (33, 38) prior to the adult spermiogram. It was observed to rise 30 d after a single acidotic event (27).

Two forms are regularly observed; beaded and indented, however a third form; flattened, a subcategory of the indented form (56, 57) is essential to differentiate due to the fertility prognosis of each.

The beaded form is considered inherited by an autosomal recessive gene (41, 42). The beaded form is often associated with sterility and usually occurs as a high percentage of the ejaculate.

The indented form is described as an enlargement of the apical ridge that then folds back on the apex of the sperm head and is much more common than the beaded form. In the pig this form is also associated with gene aberrations on chromosome 15 (43).

As stated, indented or flattened acrosomes vary in their effect upon fertility. In non-competitive matings such bulls may achieve near normal fertility however this may reflect that normal sperm coexisting with these sperm are in sufficient numbers to achieve conception as sperm with the flattened or indented form were unable to penetrate the zona pellucida (56). This abnormality is therefore considered a compensable defect and is given a 30% threshold.

This is supported by work by Andersson et al. (58) who reported that when present in <25% of sperm there was no decrease in fertility. In bulls with a high percentage of this abnormality (>80%) the indented acrosome defect may not be compensable as in such sperm did not bind to the zona pellucida and other sperm present in the ejaculate that appeared normal could bind to the zona the resulting zygotes had a reduced ability to cleave (59).

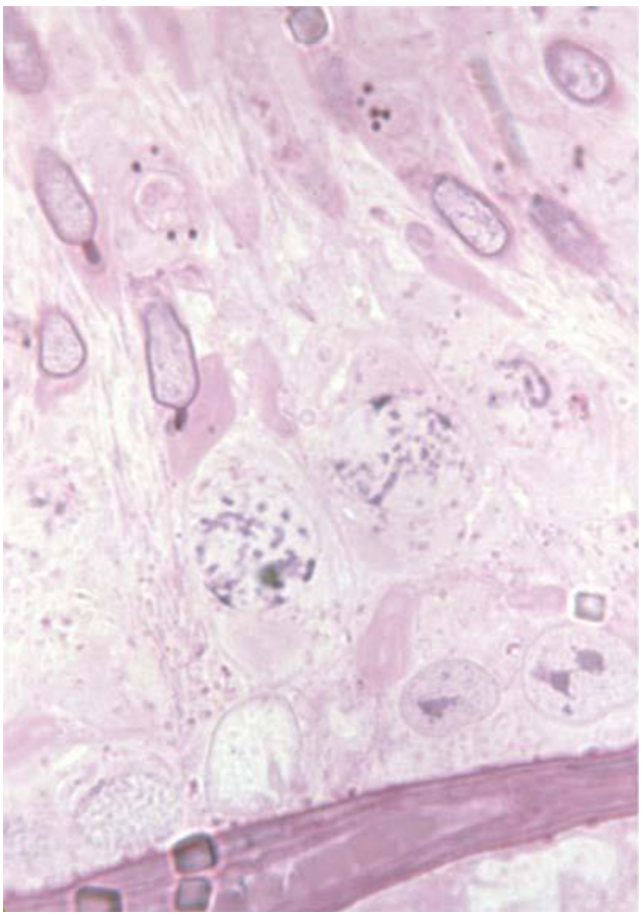


FIGURE 2 | Images of the developing knobbed acrosome defect. Courtesy of Professor Albert Barth.

Cause

The acrosome develops from the Golgi-Endoplasmic reticulum system in the very early spermatid with the knobbed acrosome defect observed from the stage 7 spermatid (**Figure 2**). In the pig the KA defect has been linked to genes associated with ubiquitination; a prerequisite for both chromatin remodeling and acrosome formation (43). The KA defect is also actively selected against in the female reproductive tract such that normal sperm within the ejaculate are more likely to reach the ova (22).

Swollen Acrosomes

These are given a separate category to knobbed as swelling and sloughing of the acrosome is a normal progression during sperm aging. The problem can be associated with "rusty load/ accumulated sperm" (12). Aging of the sperm causes the acrosome to undergo a similar reaction to capacitation resulting in the lifting of the acrosome, such sperm will be unable to attach to the oocyte. Swollen acrosomes are often seen in conjunction with other head abnormalities such as knobbed acrosomes. This is because the KA abnormality causes premature initiation of the acrosome reaction (59). The swollen acrosome in these cases may hide the knobbed defect in initial observations. In these cases spermiogenesis has obviously been disrupted. This abnormality is compensable and seldom occurs in very high numbers except in accumulators or when examining frozen thawed sperm.

Vacuoles and Teratoids

Nuclear vacuolation occurs during spermiogenesis and may be caused be caused by an environmental stress although some there is some evidence for the heritability of this trait in some bulls (27, 32). The abnormality is more commonly observed in Bos indicus cross bulls than in Bos taurus breeds (32). Some bulls are predisposed to this condition (perhaps due to a hormone imbalance in the testis) following a stress event (27, 51). Vacuoles appeared 30 d after acidotic (27) event or 20 d after dexamethasone treatment (28). Three forms of vacuolation occur; large confluent vacuoles, diadem defect, small apical vacuoles.

Large confluent vacuoles (LCV) or craters can be so large as to be a "bite" size piece missing from the side of the head. This abnormality may occur after disruption to spermiogenesis, for example following a ruminal acidosis (27). It has also been reported as an inherited abnormality in a Santa Gertrudis herd (60). Smaller craters were also seen on other sperm in the

ejaculate. Bulls with a high percentage of this abnormality were infertile. Canadian studies concur with this effect finding levels of >20% reduce pregnancy rates (51).

Apical vacuoles are commonly associated with the diadem defect or with multiple small vacuoles scattered throughout the nucleus. Unlike LCV or diadem however, they appear to be more transient than the other forms. Ejaculates with high numbers of apical vacuoles (80%) have reduced conception rates and in an IVF study no sperm with these vacuoles were observed inside the zona pellucida (51).

The diadem defect, an arrangement of vacuoles along the equatorial region of the sperm appears a serious cause of infertility in the bull. Fluctuations in the prevalence of this defect occurs between ejaculates (61) with stress being a predisposing factor.

A high incidence of the vacuole defect, >60% (62, 63) is known to cause severe reduction in fertility. There is debate as to whether this abnormality is compensable as some sperm with this defect could bind to the zona and did initiate fertilization (but it could not be determined if this fertilization produced viable zygotes) (63). Further, most of the sperm with this abnormality did not reach the zona. However, Pilip et al. (62) reported that sperm with multiple nuclear vacuoles had a reduced ability to fertilize ova.

In view of this uncertainty nuclear vacuoles are given a threshold level of not more than 20% (59).

Abnormal DNA Condensation

This abnormality can be detected by SCSA- a flow cytometric assay that uses the metachromatic properties of acridine orange to measure the susceptibility of chromatin to denaturisation (29) or by Feulgen staining under x1,000 phase contrast or DIC microscopy. Feulgen and SCSA methods correlate and both enable assessment of affected sperm (64). Advances in DIC microscopy also now permit the examination of abnormal DNA condensation to a limited extent. When this or pale centers are considered the major reason for bull sperm falling below threshold Feulgen staining is often completed as a check.

Pale Centers

Analogous to the DNA condensation this abnormality may be observed under Feulgen staining at x1,000 phase (or DIC) as the gold standard. However, it may also be observed under good DIC at x1,000. The abnormality is displayed as a narrowing of the sperm head in the PAS region. This abnormality has been reported to cause decreased fertility in a case study in Canada (Barth pers com) and in Australia by the author. This condition is currently under study by the author and colleagues. The abnormality may appear in conjunction with vacuole abnormalities or as the only abnormality present.

Rolled Head Nuclear Crest-Giant Head Syndrome

This abnormality is also included under the vacuole/teratoid category as it is uncommon. It is thought to be an inherited condition. The prognosis for recovery is very poor (65). The number tolerated in the ejaculate is at 20% because of the ability to penetrate the zona pellucida but the inability to produce a viable embryo. Reports upon its effect, when present at 20–30% of the ejaculate, on conception rates vary between 27 and 74% (51).

Teratoid Sperm

These are sperm that are so grossly abnormal in structure as to be barely recognizable as a sperm cell. The sperm nucleus varies from normal to grossly misshapen, may be vacuolated and the tail is often coiled up completely and lies superimposed on the head. These cells are indicative of severe disturbance to spermatogenesis and spermiogenesis. They often occur at very low levels in the spermiogram (1%) but when seen at higher levels the prognosis is poor. There should be no more than 15% of this type of sperm in an ejaculate and they should be associated with at least 70% normal sperm.

Multinuclear - Multiflagellar Sperm Defect

Multiflagellar sperm are sometimes observed but this abnormality where the sperm have multiple nuclei, no acrosome and multiple tails has not been reported again in bulls to the authors knowledge (66).

Midpiece Defects

Distal Reflex Midpieces

This is the most common defect seen in bull ejaculates (10, 46) not to be confused with a simple bent tail as the midpiece is also involved in the bend. This defect can occasionally arise as an artifact due to prolonged contact with a hypotonic solution (e.g., Nigrosin-Eosin stain), cold-shock, or solutions >pH 7. It is one of the first abnormalities to appear after a stress event as it occurs in the cauda epididymis [16 days after an acidotic event (27) or only 4–11 days after dexamethasone treatment (28)].

It is usually of a transient nature with recovery likely within 16 days. The presence of a cytoplasmic droplet at the tail bend identifies the problem as one occurring mainly in the distal half of the cauda epididymis. The prognosis varies with circumstance and the types of other abnormalities present. Where it occurs with abnormalities such as a fracture at the tail bend, aplasia of the midpiece or Dag-like defects there may be an underlying cause such as disturbed spermiogenesis. Some bulls have a predisposition for this defect with fluctuations in the percentage of affected sperm throughout the year. Up to 30% of this abnormality is tolerated in the ejaculate as these cells display reverse motility and would therefore be unable to penetrate the zona pellucida so other normal cells would be able to participate in ovum fertilization (65).

Dag-Like Defect

This can be an inherited defect with a serious effect upon fertility when present in large numbers (>50%) (65, 67). It can reflect disturbance in the testis or epididymis and is not normally present at >4%. It is a compensable trait as the sperm are not forwardly motile (25). Fertility is therefore only impaired once >30% of this defect is identified in the ejaculate with <70% normal sperm. Presences of fractured

axonemal elements, with filaments protruding from the sheath are observed.

Segmental Aplasia of the Mitochondrial Sheath and the Pseudodroplet

In a case study bull with 90% segmental aplasia was reported to have normal fertility over 3 breeding seasons (29). This would indicate that the condition has little effect on fertility. This condition can be permanent or transient; if the defect is seen to occur in two tests done 10 weeks apart it suggests a permanent condition. Gossypol in the diet (39) and a viral disease (Bovine Ephemeral fever) (68), have both been shown to have an affect on the mitochondrial sheath. If gaps in the midpiece are larger than 3 microns these may result in fractures of the midpiece and sperm showing such severe segmental aplasia are considered under midpiece abnormalities, however, sperm observed with slight gaps are considered under the normal category (12).

A report of a new abnormality of the mitochondrial sheath which did not affect bull fertility (69) is similar to the previously described pseudodroplet both under light microscope and TEM images (51, 65, 70). The difference between the observations is that the effect upon the mitochondrial sheath is apical (69) compared to points along the midpiece (51) and that, at least in the Blom study this defect did affect fertility via an effect on motility of the sperm. The defect is observed as a thickening of the midpiece often associated with a bend or fracture. TEM reveals an accumulation of dense granules within these thickened areas. The light microscope images of the apical defect are similar to a broken neck appearance.

Abaxial Tails

The prognosis for this abnormality is determined by the presence or absence of an accessory tail. Ejaculates containing 60–100% spermatozoa with abaxial tails alone (71) cause no decrease in fertility. However, abaxial tails seen in an ejaculate with other spermatozoa with accessory tails (72) can cause a significant drop in fertility. The cause of this difference lies in the formation of the tail within the spermatid. Tail formation begins with the migration of the proximal and distal centriole to the base of the nucleus. The distal centriole gives rise to the tail with the proximal centriole forming the neck of the midpiece. Normally in spermatids replication of the centrioles is suppressed so that one flagella is formed. Lack of this suppression may allow the formation of additional tails. The presence of additional fossa and/or tails therefore may indicate the presence of additional centrioles. These structures are critical to the separation of chromosomes during the first cleavage of the ovum. This being the case, abaxial tails should not be considered a defect if present on their own. However, if abaxial tails are present at relatively low numbers (12–20%) with >17% accessory tails the bull would be considered of questionable fertility (65). Abaxial tails with accessory tails are considered within midpiece defect category, however, within the normal category if present on their own.

Tail Stump Defect

This condition is hereditary inherited via a recessive gene and has a poor prognosis. It is a compensable defect, as the sperm cannot journey to the fertilization site, bulls with 30–40% of this defect have been found to be fertile. It should be noted that care should be taken to differentiate this from detached heads as a cytoplasmic droplet often covers the vestigial midpiece portion.

Loose Heads/Tail Abnormalities

Loose/Detached Heads

This is a problem that can arise with testicular degeneration or hypoplasia, inflamed ampullae or epididymis, heat stress and more usually, as a sign of a "rusty load." If the motility is low in the initial crush side motility assessment of the semen then further ejaculates (up to 3) should be taken so that sperm that may have "accumulated" in storage can be eliminated and a representative sample collected. In the representative sample, fertility can be related to the percentage of detached heads found: the bull can still be considered "fertile" with 30–40% of this defect, but if the ejaculate contains 70% of this abnormality the bull would have severely decreased fertility. This is considered to be a minor abnormality and some latitude is allowed as it is considered to be a compensable effect; these sperm cannot participate in fertilization, as they cannot swim up the female tract.

Decapitated Head

The decapitated head defect has been reported in Guernsey and Hereford bulls. This may be an inherited problem. It can be differentiated from detached loose head by the large number of vigorously moving tails in the fresh specimen and the presence of the proximal droplet still attached to the tail. This trait when it occurs affects 80–100% of sperm in the ejaculate.

Principal Piece/Tail Defects

These are seldom seen in high numbers and may be caused by temperature shock or stress event during passage through the epididymis (28), therefore levels of this defect may decrease after 8–11 days. Levels of 30% are acceptable with 70% normal sperm as this is a compensable abnormality.

CONCLUSION

It is important to firstly establish what we consider to be normal when we examine the ejaculate. We accept that a fertile bull should be >70% normal (4, 18, 19), however, this figure should be interpreted according to the type of abnormalities contained within the sample (9, 12, 29). Simply listing all of the abnormalities present is not helpful in forming a prognosis. Analagous to this, the laboratory should have the ability to give a prognosis based upon their knowledge of spermatogenesis together with information such as that provided by a full bull breeding soundness examination where environmental

stressors are recorded (e.g., vaccination history, puberty, age, body condition etc.).

This standardized Australian model (9) has enabled increased accuracy of prognosis for practitioners and is well-regarded both in Australia and overseas (73, 74). The updated 2018 UQSMSP standardization scheme involving skill updates and training of morphology laboratories along with annual examination of work should result in reliable analysis which is easily interpreted.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

REFERENCES

1. Al-Makhzoomi A, Lundeheim N, Håård M, Rodríguez-Martínez H. Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology*. (2008) 70:682–91. doi: 10.1016/j.theriogenology.2008.04.049
2. Nagy S, Johannisson A, Wahlsten T, Ijäs R, Andersson M, Rodriguez-Martinez H. Sperm chromatin structure and sperm morphology: their association with fertility in AI-dairy Ayrshire sires. *Theriogenology*. (2013) 79:1153–61. doi: 10.1016/j.theriogenology.2013.02.011
3. Attia S, Katila T, Andersson M. The effect of sperm morphology and sire fertility on calving rate of finnish Ayrshire AI bulls. *Reprod Domest Anim*. (2016) 51:54–8. doi: 10.1111/rda.12645
4. Holroyd RG, Doogan W, De Faveri J, Fordyce G, McGowan MR, Bertram JD, et al. Bull selection and use in northern Australia. 4. Calf output and predictors of fertility of bulls in multiple-sire herds. *Anim Reprod Sci*. (2002) 71:67–79. doi: 10.1016/S0378-4320(02)00026-X
5. Hossain MS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodriguez-Martinez H. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J Androl*. (2011) 13:406–19. doi: 10.1038/aja.2011.15
6. Brito LFC. A multilaboratory study on the variability of bovine semen analysis. *Theriogenology*. (2016) 85:254–266. doi: 10.1016/j.theriogenology.2015.05.027
7. Auger J. Assessing human sperm morphology: top models, underdogs or biometrics? *Asian J Androl*. (2010) 12:36–46. doi: 10.1038/aja.2009.8
8. Gravance CG, Casey ME, Casey PJ. Pre-freeze bull sperm head morphometry related to post-thaw fertility. *Anim Reprod Sci*. (2009) 114:81–8. doi: 10.1016/j.anireprosci.2008.09.014
9. Fordyce G, Entwistle K, Norman S, Perry V, Gardiner B, Fordyce P. Standardising bull breeding soundness evaluations and reporting in Australia. *Theriogenology*. (2006) 66:1140–8. doi: 10.1016/j.theriogenology.2006.03.009
10. Perry VE, Phillips N, Fordyce G, Gardiner B, Entwistle K, Chenoweth P, et al. Semen collection and evaluation. In: *Bull Fertility: Selection & Management in Australia* Brisbane, QLD: Australian Veterinary Association. (2002). p. 1−19.
11. Entwistle KW, Fordyce G. *Evaluating and Reporting Bull Fertility*. Eight Mile Plains, QLD: Australian Cattle Veterinarians Australia (2003).
12. McAuliffe P, Johnston P H, Johnston, Perry VEA. *Ejaculators, Morphology and Microscopes*. Barton, ACT: Australian Cattle Veterinarian (2010).
13. Perry VEA. *Increasing standardisation in sperm morphology Australian Cattle Veterinarians*. Sydney, ACT: AVA (2017).
14. Sekoni VO, Gustafsson BK, Mather EC. Influence of wet fixation, staining techniques, and storage time on bull sperm morphology. *Nordisk Vet*. (1981) 33:161–6. doi: 10.1080/00291463.1981.10636780
15. Sprecher DJ, Coe PH. Differences in bull spermiograms using eosin-nigrosin stain, feulgen stain, and phase contrast microscopy methods. *Theriogenology*. (1996) 45:757–64. doi: 10.1016/0093-691X(96)00005-2
16. Freneau GE, Chenoweth PJ, Ellis R, Rupp G. Sperm morphology of beef bulls evaluated by two different methods. *Anim Reprod Sci*. (2010) 118:176–81. doi: 10.1016/j.anireprosci.2009.08.015
17. Palmer CW, Persson Y, Söderquist L. Classification of the potential breeding ability of range beef bulls based on semen quality parameters in samples collected by transrectal massage—A comparison of the Swedish and Canadian systems. *Anim Reprod Sci*. (2013) 140:124–30. doi: 10.1016/j.anireprosci.2013.06.001
18. Wiltbank JN, Parish NR. Pregnancy rate in cows and heifers bred to bulls selected for semen quality. *Theriogenology*. (1986) 25:779–83. doi: 10.1016/0093-691X(86)90093-2
19. Fitzpatrick LA, Fordyce G, McGowan MR, Bertram JD, Doogan VJ, De Faveri J, et al. Bull selection and use in northern Australia. Part 2. Semen traits. *Anim Reprod Sci*. (2002) 71:39–49. doi: 10.1016/S0378-4320(02)00024-6
20. Kuster CE, Singer RS, Althouse GC. Determining sample size for the morphological assessment of sperm. *Theriogenology*. (2004) 61:691–703. doi: 10.1016/S0093-691X(03)00240-1
21. Evenson DP. Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. *Reprod Fertility Dev*. (1999) 11:1–16. doi: 10.1071/RD98023
22. Mitchell JR, Senger PL, Rosenberger JL. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract2. *J Anim Scie*. (1985) 61:956–67. doi: 10.2527/jas1985.614956x
23. Saacke RG, Dejarnette JM, Bame JH, Karabinus DS, Whitman SS. Can spermatozoa with abnormal heads gain access to the ovum in artificially inseminated super- and single-ovulating cattle? *Theriogenology*. (1998) 50:117–28. doi: 10.1016/S0093-691X(98)00119-8
24. Johnson WH. The significance to bull fertility of morphologically abnormal sperm. *Vet Clin Food Anim Pract*. (1997) 13:255–70. doi: 10.1016/S0749-0720(15)30339-X
25. Perry VEA. Semen collection and evaluation. In: Fordyce G, Entwistle KW, editors. *Bull Fertility: Selection and Management No. 1*. Brisbane, QLD: Australian Veterinaria Association (2002). p. 5.1–19.
26. Perry VE, Chenoweth PJ, Post TB, Munro RK. Patterns of development of gonads, sex-drive and hormonal responses in tropical beef bulls. *Theriogenology*. (1991) 35:473–86. doi: 10.1016/0093-691X(91)90297-Q
27. Callaghan MJ, McAuliffe P, Rodgers RJ, Hernandez-Medrano J, Perry VEA. Subacute ruminal acidosis reduces sperm quality in beef bulls1. *J Anim Sci*. (2016) 94:3215–28. doi: 10.2527/jas.2015-0235
28. Barth AD, Bowman PA. The sequential appearance of sperm abnormalities after scrotal insulation or dexamethasone treatment in bulls. *Can Vet J*. (1994) 35:93–102.
29. Barth AD. *Bull Breeding Soundness*. 3rd ed. Western Canadian Association of Bovine Practitioners (2013).
30. Hees H, Leiser R, Kohler T, Wrobel H. Vascular morphology of the bovine spermatic cord testis - Light- I. and scanning electron-microscopic studies on the testicular artery and pampiniform plexus. *Cell Tissue Res*. (1984) 237 K:31–8. doi: 10.1007/BF00229196
31. Rizzoto G, Kastelic JP. A new paradigm regarding testicular thermoregulation in ruminants? *Theriogenology*. (2020) 147:166–75. doi: 10.1016/j.theriogenology.2019.11.019
32. Felton-Taylor J, Prosser KA, Hernandez-Medrano JH, Gentili S, Copping KJ, Macrossan PE, et al. Effect of breed, age, season and region on sperm morphology in 11,387 bulls submitted to breeding soundness evaluation in Australia. *Theriogenology*. (2020) 142:1–7. doi: 10.1016/j.theriogenology.2019.09.001
33. Copping KJ, Ruiz-Diaz MD, Rutland CS, Mongan NP, Callaghan MJ, McMillen IC, et al. Peri-conception and first trimester diet modifies reproductive development in bulls. *Reprod Fertil Dev*. (2018) 30:703–20. doi: 10.1071/RD17102
34. Brito LFC, Barth AD, Rawlings NC, Wilde RE, Crews DHJ, Boisclair YR, et al. Effect of feed restriction during calfhood on serum concentrations of metabolic hormones, gonadotropins, testosterone, and on sexual development in bulls. *Reproduction*. (2007) 134:171–81. doi: 10.1530/REP-06-0353

35. Barth AD, Brito LFC, Kastelic JP. The effect of nutrition on sexual development of bulls. *Theriogenology*. (2008) 70:485–94. doi: 10.1016/j.theriogenology.2008.05.031
36. Callaghan M, Perry VEA. Restricted pre-weaning nutrition suppresses reproductive development in beef bulls. In: *Proceedings Association of Applied Animal Andrology*. Sydney, NSW (2010).
37. Callaghan MJ, Rodgers RJ, Perry VEA. Supplementation of rangeland primiparous Bos indicus x Bos taurus beef heifers during lactation. 1. Effects on dam milk production and liveweight, bull calf growth, live carcass characteristics and metabolic hormone concentrations. *Theriogenology*. (2020) 152:69–82. doi: 10.1016/j.theriogenology.2020.04.030
38. Callaghan MJ, Rodgers RJ, Perry VEA. Supplementation of rangeland primiparous Bos indicus x Bos taurus beef heifers during lactation. 2. Effects upon the reproductive development of bull calf progeny. *Theriogenology*. (2020) 152:83–93. doi: 10.1016/j.theriogenology.2020.04.032
39. Chenoweth PJ, Chase, Jr CC, Risco CA, Larsen RE. Characterization of gossypol-induced sperm abnormalities in bulls. *Theriogenology*. (2000) 53:11193–2003. doi: 10.1016/S0093-691X(00)00264-8
40. Cusack PMV, Perry V. The effect of feeding whole cottonseed on the fertility of bulls. *Aust Vet J*. (1995) 72:463–6. doi: 10.1111/j.1751-0813.1995.tb03490.x
41. Hancock JL. The spermatozoa of sterile bulls. *J Exp Biol*. (1953) 30:50–8.
42. Barth AD. The knobbed acrosome defect in beef bulls. *Can Vet J*. (1986) 27:379–84.
43. Sironen A, Uimari P, Nagy S, Paku S, Andersson M, Vilkki J. Knobbed acrosome defect is associated with a region containing the genes STK17b and HECW2 on porcine chromosome 15. *BMC Genomics*. (2010) 11:699. doi: 10.1186/1471-2164-11-699
44. Blom E. A new sterilizing and hereditary defect (the 'dag defect') located in the bull sperm tail. *Nature*. (1966) 209:739. doi: 10.1038/209739a0
45. Hoflack G, Opsomer G, Van Soom A, Maes D, de Kruif A, Duchateau L. Comparison of sperm quality of Belgian Blue and Holstein Friesian bulls. *Theriogenology*. (2006) 66:1834–46. doi: 10.1016/j.theriogenology.2006.05.007
46. Menon AG, Barkema HW, Wilde R, Kastelic JP, Thundathil JC. Associations between sperm abnormalities, breed, age, and scrotal circumference in beef bulls. *Can J Vet Res*. (2011) 75:241–7.
47. Amann R, Seidel G, Mortimer R. Fertilizing potential *in vitro* of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. *Theriogenology*. (2000) 54:1499–515. doi: 10.1016/S0093-691X(00)00470-2
48. Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. The use of *in vitro* fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. *Anim Reprod Sci*. (2001) 65:181–92. doi: 10.1016/S0378-4320(00)00231-1
49. Carreira JT, Mingoti GZ, Rodrigues LH, Silva C, Perri SH, Koivisto MB. Impact of proximal cytoplasmic droplets on quality traits and *in-vitro* embryo production efficiency of cryopreserved bull spermatozoa. *Acta Vet Scand*. (2012) 54:1. doi: 10.1186/1751-0147-54-1
50. Söderquist L, Janson L, Larsson K, Einarsson S. Sperm morphology and fertility in A. I. Bulls. *J Vet Med Ser A*. (1991) 38:534–43. doi: 10.1111/j.1439-0442.1991.tb01045.x
51. Barth A. *Bull Breeding Soundness*. 3rd ed. Saskatoon, SK: WCABP, Western Canadian Association of Bovine Practitioners (2013).
52. Meistrich ML, Trostle-Weige PK, Russell LD. Abnormal manchette development in spermatids of azh/azh mutant mice. *Am J Anat*. (1990) 188:74–86. doi: 10.1002/aja.1001880109
53. Kysilka C. Bull seminal vesicle hemolytic factor. A new phospholipid binding protein. *FEBS Lett*. (1975) 56:202–4. doi: 10.1016/0014-5793(75)81091-X
54. Barth AD, Bowman PA, Bo GA, Mapletoft RJ. Effect of narrow sperm head shape on fertility in cattle. *Canad Veterinary J*. (1992) 33:31–39.
55. Thundathil J, Barth AD, Palasz AT, Mapletoft RJ. Fertilization characteristics of bovine sperm with pyriform heads. *Theriogenology*. (1998) 49:374. doi: 10.1016/S0093-691X(98)90727-0
56. Thundathil J, Meyer R, Palasz AT, Barth AD, Mapletoft RJ. Effect of the knobbed acrosome defect in bovine sperm on IVF and embryo production. *Theriogenology*. (2000) 54:921–34. doi: 10.1016/S0093-691X(00)00402-7
57. Meyer RA, Barth AD. Effect of acrosomal defects on fertility of bulls used in artificial insemination and natural breeding. *Can Vet J*. (2001) 42:627–34.
58. Andersson M, Vierula M, Alanko M. Three types of acrosomal aberrations of bull spermatozoa and their relation to fertility. *Acta Vet Scand*. (1990) 31:175–9.
59. Thundathil J, Palomino J A, Barth, Mapletoft R, Barros C. Fertilizing characteristics of bovine sperm with flattened or indented acrosomes. *Anim Reprod Sci*. (2001) 67:231–43. doi: 10.1016/S0378-4320(01)00127-0
60. Olley T. *Nuclear Vacuole Defects in Bovine Spermatozoa*. Honours, Brisbane, QLD: University of Queensland (2001).
61. Larsen RE, Chenoweth PJ. Diadem/crater defects in spermatozoa from two related angus bulls. *Mol Reprod Dev*. (1990) 25:87–96. doi: 10.1002/mrd.1080250115
62. Pilip R, Del Campo MR, Barth AD, Mapletoft RJ. *In vitro* fertilizing characteristics of bovine spermatozoa with multiple nuclear vacuoles: a case study. *Theriogenology*. (1996) 46:1–12. doi: 10.1016/0093-691X(96)00136-7
63. Thundathil J, Palasz AT, Barth AD, Mapletoft RJ. Fertilization characteristics and *in vitro* embryo production with bovine sperm containing multiple nuclear vacuoles. *Mol Reprod Dev*. (1998) 50:328–33. doi: 10.1002/(SICI)1098-2795(199807)50:3<328::AID-MRD9>3.0.CO;2-L
64. Dobrinski I, Hughes HPA, Barth AD. Flow cytometric and microscopic evaluation and effect on fertility of abnormal chromatin condensation in bovine sperm nuclei. *J Reprod Fertil*. (1994) 101:531–8. doi: 10.1530/jrf.0.1010531
65. Barth AD, Oko RJ. *Abnormal Morphology of Bovine Spermatozoa*. 1st ed. Ames, IA: Iowa State University Press (1989).
66. Kopp C, Sukura A, Tuunainen E, Gustavsson I, Parvinen M, Andersson M. Multinuclear–multiflagellar sperm defect in a bull – a new sterilizing sperm defect. *Reprod Domest Anim*. (2007) 42:208–13. doi: 10.1111/j.1439-0531.2006.00754.x
67. Koefoed-Johnsen H, Andersen J, Andresen E, Blom E, Philipsen H. The dag defect of the tail of the bull sperm. Studies on the inheritance and pathogenesis. *Theriogenology*. (1980) 14:471–5. doi: 10.1016/0093-691X(80)90059-X
68. Chenoweth PJ, Burgess GW. Mid-piece abnormalities in bovine semen following ephemeral fever. *Aust Vet J*. (1972) 48:37–8. doi: 10.1111/j.1751-0813.1972.tb02214.x
69. Rocha A, Oliveira E, Vilhena MJ, Diaz J, Sousa M. A novel apical midpiece defect in the spermatozoa of a bull without an apparent decrease in motility and fertility. A case study. *Theriogenology*. (2006) 66:913–22. doi: 10.1016/j.theriogenology.2006.02.032
70. Blom E. *A New Sperm Defect - "Pseudo-Droplets" - in the Middle Piece of the Bull Sperm*. Copenhagen: State Veterinary Serum Laboratory (1968).
71. Barth AD. Abaxial tail attachment of bovine spermatozoa and its effect on fertility. *Can Vet J*. (1989) 30:656–62.
72. Aughey E, Renton JP. Abnormal spermatozoa in an Ayrshire bull. *Vet Record*. (1968) 82:129–31.
73. Irons PC, Nöthling JO, Bertschinger HJ. Bull breeding soundness evaluation in Southern Africa. *Theriogenology*. (2007) 68:842–7. doi: 10.1016/j.theriogenology.2007.06.013
74. Penny CD. Case reports: bull pre-breeding examination. *Livestock*. (2010) 15:15–8. doi: 10.1111/j.2044-3870.2010.tb00321.x

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

MINI REVIEW
published: 22 June 2021
doi: 10.3389/fvets.2021.610888

Sperm Functional Genome Associated With Bull Fertility

Memmet Özbek[1], *Mustafa Hitit*[2], *Abdullah Kaya*[3], *Frank Dean Jousan*[4] *and Erdogan Memili*[4]*

[1] Department of Histology and Embryology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Turkey, [2] Department of Genetics, Faculty of Veterinary Medicine, Kastamonu University, Kastamonu, Turkey, [3] Department of Artificial Insemination and Reproduction, Faculty of Veterinary Medicine, Selcuk University, Konya, Turkey, [4] Department of Animal and Dairy Sciences, Mississippi State University, Starkville, MS, United States

Bull fertility is an important economic trait in sustainable cattle production, as infertile or subfertile bulls give rise to large economic losses. Current methods to assess bull fertility are tedious and not totally accurate. The massive collection of functional data analyses, including genomics, proteomics, metabolomics, transcriptomics, and epigenomics, helps researchers generate extensive knowledge to better understand the unraveling physiological mechanisms underlying subpar male fertility. This review focuses on the sperm phenomes of the functional genome and epigenome that are associated with bull fertility. Findings from multiple sources were integrated to generate new knowledge that is transferable to applied andrology. Diverse methods encompassing analyses of molecular and cellular dynamics in the fertility-associated molecules and conventional sperm parameters can be considered an effective approach to determine bull fertility for efficient and sustainable cattle production. In addition to gene expression information, we also provide methodological information, which is important for the rigor and reliability of the studies. Fertility is a complex trait influenced by several factors and has low heritability, although heritability of scrotal circumference is high and that it is a known fertility maker. There is a need for new knowledge on the expression levels and functions of sperm RNA, proteins, and metabolites. The new knowledge can shed light on additional fertility markers that can be used in combination with scrotal circumference to predict the fertility of breeding bulls. This review provides a comprehensive review of sperm functional characteristics or phenotypes associated with bull fertility.

Keywords: proteomics, metabolomics, transcriptomics, DNA methylome, chromatin dynamics, bull fertility

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Szabolcs Nagy, University of Pannonia, Hungary
Marina Rufino Salinas Fortes, The University of Queensland, Australia

***Correspondence:**
Erdogan Memili
em149@msstate.edu

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *27 September 2020*
Accepted: *05 May 2021*
Published: *22 June 2021*

Citation:
Özbek M, Hitit M, Kaya A, Jousan FD and Memili E (2021) Sperm Functional Genome Associated With Bull Fertility. Front. Vet. Sci. 8:610888. doi: 10.3389/fvets.2021.610888

INTRODUCTION

Projections indicate that the world population will rise to 9 billion people by 2,050, requiring a 50% increase in food production (1) to satisfy the demands of a growing population. Animal agriculture will benefit from technological advances to produce livestock and their byproducts more efficiently and economically. Biotechnology is crucial in promoting sustainability of livestock production in order to meet these demands for high-quality food products with less environmental impact. Important advances in livestock production have been achieved through reproductive biotechnology (2). Bull fertility, defined as the ability of a spermatozoa to fertilize an oocyte and support embryonic development (3), and accurate evaluation of semen quality parameters

used as predictors of bull fertility, remain as important research imperatives to further enhance improvements in genetic selection in cattle (4). Generally, in studies, bull fertility is calculated based on the conception rate. For each bull, at least 100 insemination records are considered reliable data to evaluate the correct conception rate. Pregnancy diagnosis is controlled by trans-rectal ultrasonographic examination within 45–50 days following artificial insemination. The conception rate of each bull was plotted in a graph, and the standard deviation (SD) and the mean were calculated. The criterion for selecting high-fertile bulls was conception rate more than "mean + 1 or 2 SD," while those below "mean – 1 or 2 SD" were considered as low-fertile bulls (5, 6).

Bulls are evaluated based on a breeding soundness exam (BSE) that is composed of an inspection of semen characteristics (phenotypes) combined with phenotypic features. Despite great efforts put into evaluating bulls using BSE, bull fertility is deemed suboptimal under field conditions, with a conception rate varying from 20 to 40% (7). Such differences may be due to the presence of subtle sperm abnormalities that might not be determined using current, established techniques. Semen evaluation tests, such as abnormalities, concentration, volume, membrane integrity, and motility, are now being conducted to predict the quality of semen samples for cryopreservation and subsequent use for artificial insemination. Although the standard semen evaluation procedures can help visually recognize poor-quality sperm, they are not enough to detect potential markers of subfertile bulls (7, 8). Since spermatogenesis in the bovine bull takes 61 days from spermatogonia to fully matured spermatozoa (9), there is ample time for molecular, cellular, and physiological errors to occur that can hamper sperm production and render infertility. Defects in the male germ cells during fetal life may be more probable causes of infertility than defects incurred in later phases of development, such as neonatal and postnatal periods (10). Therefore, more comprehensive studies spanning developmental stages and robust methods are needed to accurately ascertain semen quality and predict bull fertility for precision animal agriculture (11).

Genome-wide association studies (GWAS) have been effective in applying dense genetic markers, such as single-nucleotide polymorphism (SNP) markers, to determine genomic regions associated with economically important phenotype such as fertility (12). There are several studies showing a relationship between genomic regions and quantitative trait loci (QTL) and male reproductive traits in cattle (**Table 1**). Using a comprehensive genomic analysis on bulls, Han and Peñagaricano (25) demonstrated approximately eight genomic regions that are highly associated with bull fertility where most of these genomic regions contain genes including *Ckb*, *Kat8*, *Igf1r*, and *Tdrd9*, which are associated with sperm physiology, such as sperm motility and sperm–egg interaction. Feugang et al. (15) reported that polymorphisms in two bovine genes encoding sperm head proteins, collagen type I alpha 2 chain, and integrin subunit beta 5 are associated with bull fertility. In addition, Tüttelmann et al. (26) showed that polymorphisms in *Prm1* and *Prm2* genes were associated with human sperm quality. An SNP in *Spata1*, a gene implicated in sperm head structure, has been shown to be related to stallion fertility (27). Because the inheritance of fertility is low and is influenced by environmental and epigenetic factors, there are fewer genetic markers associated with fertility.

Epigenetics refers to molecular processes that may regulate gene expression (active vs. inactive genes) without alterations in the DNA sequence. Epigenetic modifications, including DNA methylation, histone modifications, and nucleosome positioning, can be transmitted to the daughter cells through cell divisions. Aberrant alterations in the epigenetic profiles may give rise to abnormal gene silencing or activation (28). Transformation of male germ cells into functional spermatozoa requires a specific order involving the accumulation of specific non-coding RNA, substitution of protamines for most histones, and large-scale DNA methylation changes (29, 30). Although transcription is hardly observable in the mature sperm cells, the differentiation program in the male germline is regulated through a series of transcriptional modulations that depend directly on epigenetic reprogramming (31, 32).

PROTEOMICS, TRANSCRIPTOMICS, AND METABOLOMICS OF SPERM CELLS

Sperm Proteins and Bull Fertility

Sperm contains diverse proteins present in the sperm membrane, flagellum, cytoplasm, acrosome, and nucleus that play key roles in sperm physiology (33). Of these proteins, some are energy-related enzymes involved in sperm motility, both signaling and structural. For example, the outer dense fiber protein (ODF) has been implicated in the protection of the sperm tail against shear forces and motility in the mouse (34). Zhao et al. (35) stated that ODF2 might bind to and maintain acetylated levels of α-tubulin in HEK293T cell lines exposed to cold environment. In humans, energy-related proteins isocitrate dehydrogenase subunit alpha and phosphoglycerate mutase 2 are down- or upregulated in asthenozoospermia, respectively (36). Sperm postacrosomal sheath WW domain-binding protein (PAWP) and PLC zeta are involved in oocyte activation and embryogenesis in mice and humans (37, 38). However, Satouh et al. (39), using real-time PCR, immunoblotting, and electron microscopy, asserted that PAWP does not play an essential role in the formation of mouse sperm head or spermatogenesis in PAWP null mice. Compared with other studies, the differences in the findings of Satouh et al. (39) may arise from methodological approaches used and, perhaps, the functional interaction of PAWP with other proteins.

Using Western blotting and real-time PCR, Velho et al. (40) postulated that expression of integrin subunit beta 5 (ITGβ5) in germ cells and resultant embryos is important for fertilization and embryonic development in bovine. The fertility prediction for each bull was obtained using the Probit.F90 software (41) and expressed as the percent deviation of its conception rate from the average conception rate of all bulls. Moreover, IZUMO and fertilin subunit beta (ADAM 2) is considered to play a crucial role in the interactions between the sperm and zona pellucida, and in acrosome reactions. IZUMO1 binds to Juno, a receptor present on the egg, and facilitates gamete recognition during fertilization (42). Using 2D-PAGE, Park et al. (42) showed that ATP synthase H+ transporting mitochondrial

TABLE 1 | Genomic regions and quantitative trait loci demonstrated to be associated with bovine male reproductive traits.

Chromosome	Positions[a] (Mb BTAU4.0)	*N* Markers[b]	Phenotype	*N* Animals[c]	Breed	References
8	93 cM (MCM64–71 Mb)	263 MS	Dystocia and stillbirth	888	Holstein	(13)
5	70 cM	130	FSH serum concentration	126	MARC herd	(14)
29	44 cM	130	Age at puberty in males	126	MARC herd	(14)
29	44 cM	130	Testicular weight and volume	126	MARC herd	(14)
1	70.3 Mb	8,207 SNP	Noncompensatory fertility in bulls (semen)	221	Holstein	(15)
4	12.0 Mb	8,207 SNP	Noncompensatory fertility in bulls (semen)	221	Holstein	(15)
14	22 Mb	43,863 SNP	Paternal calving ease	1,800	German Fleckvieh	(16)
21	3.1 Mb	43,863 SNP	Paternal calving ease	1,800	German Fleckvieh	(16)
15	74.7 Mb	45,878 SNP	Daughter stillbirth	1,654	Holstein	(17)
3	30.28 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
4	17.35 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
4	76.89 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
4	109.13 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
5	47.38 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
6	39.71 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
8	89.91 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
10	81.45 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
12	29.53 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
12	45.01 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
13	36.68 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
14	5.93 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
15	46.78 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
17	58.73 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
19	62.00 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
22	38.91 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
22	58.96 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
X	31.67 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
X	43.13 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
X	101.55 Mb	38,416 SNP	Noncompensatory fertility in bulls (semen)	795	Holstein	(18)
1	95 cM (BMS4031–91.3 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
4	46 cM (BMS1840–51 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
4	96 cM (RM088–108.5 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
5	12 cM (BMS610–13 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
5	101 cM (BM315–104 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
5	127 cM (BMS597)	390 MS	Scrotal circumference	1,769	Angus	(19)
6	102 cM (BM8124)	390 MS	Scrotal circumference	1,769	Angus	(19)
7	10 cM (RM012–0.5 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
7	28 cM (RM006–16 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
7	41 cM (BM6105–22 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
8	12 cM (IDVGA11–10 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
9	68 cM (BMS2377–72.7 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
9	110 cM (BMS1967–92 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
10	99 cM (BMS614–94 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
10	118 cM (BL1134–102 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
11	12 cM (INRA044–6 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
11	29 cM (BMS2325–11.8 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
11	93 cM (BMS989–86.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
12	13 cM (BMS2252–10.4 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
13	41 cM (BMS1352–28.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)

(Continued)

TABLE 1 | Continued

Chromosome	Positions[a] (Mb BTAU4.0)	*N* Markers[b]	Phenotype	*N* Animals[c]	Breed	References
15	21 cM (ADCY2-BTA20 at 69.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
15	34 cM (JAB8−29.3 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
16	73 cM (INRA048)	390 MS	Scrotal circumference	1,769	Angus	(19)
17	94 cM (BM1233-BTA18 54.7 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
18	77 cM (BM2078−62.0 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	12 cM (BMS745−11.8 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	56 cM (BMS650−36.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	80 cM (IDVGA44−56.7 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
19	98 cM (RM388−59.4 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
20	1 cM (RM106−1.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
21	30 cM (BM103−20.0 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
22	27 cM (DIK2694−21.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
22	65 cM (BMS875−46.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
23	35 cM (BOLADRB1−26.3 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
25	59 cM (BMS1353−32.9 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
26	15 cM (FASMC2−11.1 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
27	61 cM (BMS1675−46.2 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
28	30 cM (BMS510−21.8 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
28	49 cM (BMS1714−34.6 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
29	13 cM (BMS764−10.0 Mb)	390 MS	Scrotal circumference	1,769	Angus	(19)
2	108–109 Mb	43,821 SNP	Serum inhibin at 4 months	786	Brahman	(20)
14	22–26 Mb	43,821 SNP	Scrotal circumference at 12 months	1,112	Brahman	(20)
28	18 Mb	43,821 SNP	Luteinizing hormone levels at 4 months	537	Brahman	(20)
X	4 Mb	43,821 SNP	Percent normal sperm at 24 months	964	Brahman	(20)
X	40–55 Mb	43,821 SNP	Percent normal sperm at 24 months	964	Brahman	(20)
X	97 Mb	43,821 SNP	Percent normal sperm at 24 months	964	Brahman	(20)
X	62–96 Mb	43,821 SNP	Scrotal circumference at 12 months	1,112	Brahman	(20)
14	22–28 Mb	43,821 SNP	Age at puberty	1,118	Brahman	(21)
X	86 Mb	43,821 SNP	Age at puberty	1,118	Brahman	(21)
2	25.6 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
5	119.4 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
18	54.3 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
25	1.4 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
25	2.8 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
25	4.8 Mb	38,650 SNP	Sire conception rate	1,755	Holstein	(22)
13	8.42 Mb	46,035 SNP	Tail stump sperm defect	321	Swedish Red	(23)
25	2.98 Mb	54,001 SNP	Asthenospermia		Nordic Red	(24)

[a] *Chromosomal positions are represented in centiMorgans (cM). The microsatellite marker location was employed to translate cM into Mb positions, according to the BTAU4.0 assembly.*
[b] *Indicates the number of gene markers used in the relevant study (SNP, single nucleotide polymorphisms; MS, microsatellite).*
[c] *Represents how many experimental animals were used.*

F1 complex beta subunit (ATP5B), alpha-2-HS-glycoprotein 2 (AHSG), enolase 1 (ENO1), apoptosis-stimulating of p53 protein (ASPP2), and phospholipid hydroperoxide glutathione peroxide (GPx4) were more abundant in sperm from high-fertility bulls, whereas ubiquinol–cytochrome c reductase complex core protein 2 (UQCRC2), ropporin-1, and voltage-dependent anion channel 2 (VDAC2) were in greater amounts in sperm from low-fertility bulls (**Table 2**).

Currently, proteomic approaches are widely used to explore male reproductive physiology (43). Aslam et al. (5) analyzed the bull sperm proteome using 2D-DIGE and MALDI-TOF-MS techniques, and validated these proteomic studies using Western blotting. The authors reported that malate dehydrogenase 2 (MD2), enolase 1 (ENO1), calpain-7 like protein (CAPN7), N-acetyllactosaminide beta-1,6-N-acetylglucosaminyl transferase isoform C (GCNT2), RIB43A

domain with coiled-coils 1 (RIBC1), condensen-2 complex subunit D3 (NCAPD3), 2,4-dienoyl CoA reductase-1 (DECR1), beta galactosidase-1-like protein-2 like (LacA-like protein-2 like), GDP dissociation inhibitor 2 (GDI2), chain D, F-1 ATPase (ATP5D), ubiquitin carboxyl terminal hydrolase-12 (USP12), and thimet oligopeptidase-1 (TOP) are over expressed in sperm from high-fertility bulls, whereas binder of sperm-1 (BSP1), transmembrane protein-43 (TMEM43), and dystonin-like isoform-1(DST like isoform 1) are more abundant in sperm from low-fertility bulls (**Table 2**).

The MDH2 catalyzes the reversible oxidation of malate to oxaloacetate using NAD+/NADH as a cofactor in the citric acid cycle (45). Aslam et al. (5) suggested that the reduction of MDH2 has a negative impact on energy metabolism of spermatozoa, disrupting sperm motility, capacitation, and ultimately fertilizing ability. ENO1, a multifunctional enzyme, is found mainly in the motile sperm tail. In addition to regulating the constant provision of energy for motility, it assists in the protection of the sperm from oxidative stress (42). The RibC is a ribbon protein that is vital for sperm motility and structural integrity of sperm tails, suggesting that low expression of RibC in bull sperm reduces fertility by disrupting sperm motility (5). Calpains in mammalian sperm are involved during the acrosome reaction and capacitation (46). The Rab are small GTP-binding proteins that are critical in vesicular trafficking of molecules. The GDI keeps the function of Rab proteins under control by freeing it from membranes and preventing the GDP dissociation (47). The USP12 plays a crucial role in maintaining the androgen receptors steady and improving their cellular functions (48). The TOP are highly expressed enzymes in testes and exert their functions by catalyzing the hydrolysis of gonadotropin-releasing hormone (49). The LacA-like protein-2, which is produced and secreted from the epididymis, binds to sperm membranes during the maturation process in rats (50). Aslam et al. (5) suggested that low levels of expression of this enzymatic protein are considered to have a significant role in sperm physiology and led to a reduction in functional competence of the sperm in low-fertility bulls.

Using 2D-DIGE analysis of bull sperm, it was shown that ALB, TIMP, spermadhesin-1, and binder of sperm proteins (BSP)-1, 3, and 5, PEBP1, and AKI in sperm and seminal plasma were more abundant in sperm from high fertility bulls, while PSMA6, ELSPbP1, CCT5, CCT8, and CLU were in greater amounts in seminal plasma and in sperm from low-fertility bulls. The expression levels of ZFP34, HSP90, BCL62, IFNRF4, NADHD, histone H1, and TUBB3 were higher in high-fertility bull sperm (44) (**Table 2**). Matrix metalloproteinases (MMPs) belonging to a group of proteolytic zinc-dependent enzymes are crucial components of semen (51). The MMPs and other proteases participate in semen liquefaction in the female genital tracts, and they are needed for sperm viability during capacitation in humans (52).

Spermadhesin family members interact with carbohydrates, phospholipids, and zona pellucida glycoproteins and participate in sperm–egg binding (53–55). Spermadhesin-1 is a nonglycosylated protein produced by the epithelium of the epididymis, ampulla, and seminal vesicle, and is secreted into the seminal fluid (56, 57). Furthermore, it has been suggested that recombinant spermadhesin-1 influences sperm mitochondrial activity through its binding ability to the sperm midpiece (58).

Albumin has been reported to facilitate cholesterol outflow from sperm membranes and mediates sperm capacitation in the female reproductive tract (59, 60). Moreover, albumin preserves sperm against lipid peroxidation by binding to free radicals (44). Adenylate kinase isoenzyme 1 (AK1), a ubiquitous enzyme related to cellular energy homeostasis, is expressed in murine and bovine sperm flagella, suggesting its participation in sperm motility (61–63). Furthermore, AK1 has been reported to be active when spermatozoa are highly motile (62). Phosphatidylethanolamine-binding protein 1 (PEBP1) is an evolutionarily conserved protein in mammals and reported to be present in the acrosome, the postacrosomal region, and the tail of both human and mouse sperm. The PEBP1 seems to promote inhibition of sperm capacitation because it serves either as a decapacitation factor released throughout capacitation or as a membrane-bound, glycophosphatidylinositol (GPI)-anchored receptor for a decapacitation factor (64, 65). Binder of sperm proteins (BSP) are synthesized in the male accessory sex glands and bind to sperm via choline phospholipids upon ejaculation, which prevent premature initiation of the capacitation and acrosome reaction (66). Among the BSP proteins, BSP1, BSP3, and BSP5 are predominant proteins secreted into bovine seminal plasma, all of which contain two tandem repeated fibronectin type 2 (Fn2) domains (67, 68).

Cholesterol and phospholipids contribute to the regulation of sperm membrane bilayer stability and fluidity. The BSP proteins promote efflux of phospholipids and cholesterol from sperm membranes, thereby, disrupting sperm membrane architecture, resulting in capacitation (66). Moreover, BSP proteins promote the binding of sperm to the epithelium of the oviduct, contributing to maintain sperm viability and motility in the oviduct (69). Studies on BSPs have reported different results. Some studies (5, 68, 69) showed that BSP protein expression in semen was negatively correlated with bull fertility, unlike the findings of Kasimanickam et al. (44) who reported a positive correlation between BSP expression and bull fertility. These differences were attributed to degenerated and fragmented sperm membrane wastes in semen. A single-cell analysis approach may be required to obtain a reliable result. Furthermore, due to structural similarities to BSP, epididymal sperm-binding protein E12 (ELSPbP1) can induce lipid efflux and perturb the membrane stability (70).

Proteasome subunit alpha type-6 (PSMA6) belongs to proteasome multicatalytic protease degrading polyubiquitinated proteins into small peptides and amino acids (71). Proteasomes are localized in the acrosomal region, connecting head and tail (33, 72). The presence and expression levels of PSMA6 are associated with sperm DNA fragmentation in bulls (68). T-complex protein 1 subunit 3 (CCT3) and 8 (CCT8) are parts of the class II chaperonins (73). Cytoplasmic CCT expression has been shown to localize in the centrosomes and microtubules of the manchette during spermatogenesis and assumed to be discarded during spermiation. Hence, it is considered that the abundance of CCT subunits in sperm from low-fertility bulls reflects uncompleted developmental processes

TABLE 2 | Fertility-associated proteins of sperm from low- and high-fertility bulls.

Protein name	Abbreviation	High fertility	Low fertility	Function	Methods	Breed	References
ATP synthase H+ transporting mitochondrial F1 complex beta subunit	ATP5B	Upregulated		Energy metabolism	2D-PAGE	Hanwoo	(42)
Alpha-2-HS-glycoprotein 2	AHSG	Upregulated		Immune system	2D-PAGE	Hanwoo	(42)
Enolase 1	ENO1	Upregulated		Energy metabolism	2D-PAGE	Hanwoo	(42)
Apoptosis-stimulating of p53 protein	ASPP2	Upregulated		Oxidative stress	2D-PAGE	Hanwoo	(42)
Phospholipid hydro peroxide glutathione peroxide	GPx4	Upregulated		Oxidative stress	2D-PAGE	Hanwoo	(42)
Ubiquinol-cytochrome c reductase complex core protein 2	UQCRC2		Upregulated	Oxidative stress	2D-PAGE	Hanwoo	(42)
Ropporin-1			Upregulated	Cell signaling	2D-PAGE	Hanwoo	(42)
Voltage-dependent anion channel 2	VDAC2		Upregulated	Ion transport	2D-PAGE	Hanwoo	(42)
Malate dehydrogenase 2	MD2	Upregulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Enolase 1	ENO1	Upregulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Calpain-7-like protein	CAPN7	Upregulated		Acrosome reaction and capacitation	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
N-Acetyllactosaminide beta-1,6 N acetylglucosaminyl transferase isoform C	GCNT2	Upregulated		Development and maturation of erythroid cells	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
RIB43A domain with coiled-coils 1	RIBC1	Upregulated		Sperm motility and the structural integrity of sperm tail	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Condensen-2 complex subunit D3	NCAPD3	Upregulated			2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
2,4-Dienoyl CoA reductase-1	DECR1	Up regulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Beta galactosidase-1-like protein-2	LacA like protein-2	Upregulated		Maturation of spermatozoa	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
GDP dissociation inhibitor 2	GDI2	Upregulated		Preventing membrane integrity	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Chain D, F-1 ATPase	ATP5D	Upregulated		Energy metabolism	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Ubiquitin carboxyl terminal hydrolase-12	USP12	Upregulated		Cell signaling	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Thimet oligopeptidase-1	TOP	Upregulated		Catalyze the hydrolysis of gonadotropin-releasing hormone	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)

(Continued)

TABLE 2 | Continued

Protein name	Abbreviation	High fertility	Low fertility	Function	Methods	Breed	References
Binder of sperm-1	BSP1		Upregulated	Prevent premature acrosome reaction and capacitation	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Transmembrane protein-43	TMEM43		Upregulated	Maintain nuclear envelope structure	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Dystonin-like isoform-1	DST like isoform 1		Upregulated	An integrator of intermediate filaments, actin, and microtubule cytoskeleton networks	2D-DIGE and MALDI-TOF-MS	Holstein x Tharparkar crossbred	(5)
Albumin	ALB	Upregulated		Ease cholesterol outflow from sperm membranes and preserves sperm against lipid peroxidation	2D-DIGE	Holstein	(44)
The tissue inhibitors of metalloproteinase	TIMP	Upregulated		Inhibit MMPs by binding to their catalytic Zn cofactor	2D-DIGE	Holstein	(44)
Spermadhesin-1	SPADH1	Upregulated		Participate in sperm–egg binding	2D-DIGE	Holstein	(44)
Binder of sperm proteins 1, 3, and 5	BSP1, 3, 5	Upregulated		Prevent premature acrosome reaction and capacitation	2D-DIGE	Holstein	(44)
Phosphatidylethanolamine-binding protein 1	PEBP1	Upregulated		Promote inhibition of early sperm capacitation	2D-DIGE	Holstein	(44)
Adenylate kinase isoenzyme 1	AK1	Upregulated		Energy metabolism	2D-DIGE	Holstein	(44)
Heat shock protein 90	HSP90	Upregulated		Stabilizes proteins against heat stress	2D-DIGE	Holstein	(44)
B-cell lymphoma-62	BCL62	Upregulated		Antiapoptotic	2D-DIGE	Holstein	(44)
NADH dehydrogenase	NADHD	Upregulated		Energy metabolism	2D-DIGE	Holstein	(44)
Interferon regulatory factor 4	IFNRF4	Upregulated		Immune system	2D-DIGE	Holstein	(44)
Class III β-tubulin	TUBB3	Upregulated		Sperm motility	2D-DIGE	Holstein	(44)
Proteasome subunit alpha type-6	PSMA6		Upregulated	Associated with sperm DNA fragmentation	2D-DIGE	Holstein	(44)
Phosphatidylethanolamine-binding protein 1	PEBP1		Upregulated	Inhibition of sperm capacitation	2D-DIGE	Holstein	(44)
T-complex protein 1 subunits 3 and 8	CCT3, CCT8		Upregulated	Reflecting incomplete developmental processes	2D-DIGE	Holstein	(44)
Clusterin	CLU		Upregulated	Oxidative stress	2D-DIGE	Holstein	(44)
The tissue inhibitors of metalloproteinase-2	TIMP-2	Upregulated		Inhibit MMPs by binding to their catalytic Zn cofactor	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
C-type natriuretic peptide	NPPC	Upregulated		Stimulating intracellular cGMP and sperm motility	Mass spectrometry coupled with Nano HPLC	Holstein	(4)

(Continued)

TABLE 2 | Continued

Protein name	Abbreviation	High fertility	Low fertility	Function	Methods	Breed	References
Sulfhydryl oxidase	QSOX1	Upregulated		Oxidative stress	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Binder of sperm-5	BSP5	Upregulated		Participate in sperm–egg binding	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Galectin-3-binding protein	LGALS3BP		Upregulated	Inhibiting cell signaling	Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Tissue factor pathway inhibitor 2	TFPI2		Upregulated		Mass spectrometry coupled with Nano HPLC	Holstein	(4)
Clusterin	CLU		Upregulated	Oxidative stress	Mass spectrometry coupled with Nano HPLC	Holstein	(4)

throughout spermatogenesis (74). Clusterin (CLU), a 75- to 80-kDa disulfide-linked heterodimeric protein, is produced in the testis, epididymis, and seminal vesicles and has been speculated to be an alternative oxidative stress marker for seminal plasma in humans (75). The CLU is localized mainly on the abnormal sperm surface (76). Furthermore, increased levels of CLU expression in semen are positively correlated with sperm DNA defects (75).

Using mass spectrometry coupled with nano HPLC, a total of 1,159 proteins were detected in the bull seminal plasma, of which 29 were abundant in low-fertility bulls, whereas 50 were abundant in high-fertility bulls (77). While TIMP-2, C-type natriuretic peptide, sulfhydryl oxidase, and BSP5 revealed a relationship with high-fertility bulls, galectin-3-binding protein, tissue factor pathway inhibitor 2, clusterin, and 5′-nucleotidase were associated with low-fertile bulls based on multivariate analysis. Furthermore, high levels of transmembrane protein 2, prosaposin, and NAD (P) (+)-arginine ADP ribosyltransferase proteins had the highest positive correlations with fertility ranking, whereas quantities of nucleotide exchange factor SIL1, galectin-3-binding protein, and vitamin D-binding protein exhibited the highest negative correlations with fertility ranking (77) (**Table 2**). The C-type natriuretic peptide (NPPC) is a member of natriuretic peptides that exerts its physiological functions through binding to two distinct membrane-bound guanylyl cyclases and activating cyclic guanosine monophosphate signaling pathways (78, 79). In addition to being synthesized by cardiomyocytes and known to modulate vascular permeability and dilation/constriction, NPPC is also produced locally by Sertoli cells in the testis and serves in an autocrine manner (80). Also, NPPC is more abundantly expressed in male reproductive tissues than in other tissues (81, 82). In rats, NPPC was intensely expressed in Leydig cells and epididymal epithelium, and its expression dramatically increased after puberty (83). Furthermore, NPPC receptor (NPR-B) has been shown to localize in the acrosome and tail of human sperm, suggesting that NPPC binds to NPR-B, thus, stimulating intracellular cGMP and sperm motility (84). In the male reproductive tract, QSOX1 maintains the structure and function of sperm through the oxidization of sulfhydryl groups that might damage the cell (85). Sulfhydryl oxidase (QSOX1) is involved in the reduction of an oxygen molecule to hydrogen peroxide; thus, it creates disulfide bonds in peptides and proteins (86). It has been proposed that QSOX is essential for sperm physiology, and its dysregulation is attributed to defects that may occur during spermatogenesis in hamsters (87) and rats (88).

Galectins (Gals) belong to members of ß-galactoside-binding lectins, which can be localized in extracellular spaces and in cellular components such as cell membrane, cytoplasm, and nucleus (89). They are implicated in cell-to-cell interactions, cell–extracellular matrix interactions, receptor crosslinking or lattice formation, intracellular signaling, and posttranscriptional splicing (90). Gal-3 has antiapoptotic effects, unlike most members of the galectin family (91). Gal-3 expression has been observed in the epithelium of corpus and cauda epididymis but not in initial segment and caput epididymis, suggesting that Gal-3 participates in maturation and storage of rat sperm (92). Previously, Gal-3-binding protein has been observed in bovine epidydimal fluid (57) and shown to participate in sperm motility, semen liquefaction, and angiogenesis in the female reproductive tract (93).

Gomes et al. (94) examined the proteome and posttranslational modifications in bovine seminal plasma with the aid of a top–down mass spectrometry (TDMS) strategy to uncover more comprehensive information. They separated plasma proteins using sheathless capillary zone electrophoresis (CZE)-MS and reversed-phase liquid chromatography (LC)-MS. Then, the proteins were fragmented using electron-transfer/higher-energy collisional dissociation and 213-nm ultraviolet photodissociation. The use of the sheathless CZE-MS method helped identify 417 proteoforms, including 170 unique species, whereas 3,090 proteoforms, including 1,707 unique species were detected by using LC-MS. The researchers identified 1,433 proteoforms (EThcD) and 2,151 proteoforms (213 nm UVPD) with 612 species for EThcD and 1,021 for 213-nm UVPD (94).

Sperm Transcriptome and Bull Fertility

Sperm delivers, not just the paternal DNA, but other factors, such as cell signaling molecules, RNA, and transcription factors, into the oocyte at the time of fertilization (95). New cutting-edge technologies, such as RNA sequencing (RNA-seq) and microarray analysis have enabled characterization of various types of sperm RNAs, including transfer RNA (tRNA), ribosomal RNA (rRNA), messenger RNA (mRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), small non-coding RNA (sncRNA), long non-coding RNA (lncRNA), and mitochondrial RNA (mt-RNA), which are present in bovine spermatozoa (96). The miRNAs, piRNAs, and tRNAs are grouped as "small non-coding" RNAs (sRNAs) (97).

There are several transcriptomic studies on bull sperm using different techniques. Wang et al. (98) used strand-specific RNA sequencing to profile the semen transcriptome (lncRNA and mRNA) and to ascertain the functions of lncRNA and mRNA in bull sperm motility. They detected 20,875 transcripts of protein-encoding genes in semen and found 19 different mRNAs between high- and low-motility sperm. They also detected five differentially expressed genes, such as *Efna1*, *Rbmx*, *Mlph*, *Rpl30*, and *Aqp2*, which participate in "extracellular exosome" GO term. Among them, the ephrin A1 (Efna1) protein that is localized on cell surfaces participates in membrane integrity and sperm morphology, and it has been reported that *Efna1* is highly present in both seminal plasma and sperm (44, 99) and possibly influences sperm motility (98). Heterogeneous nuclear ribonucleoprotein G (*Rbmx*) has been proposed to be a possible splicing factor that modulates spermatogenesis (100). Based on immunohistochemical methods, aquaporin 2 (*Aqp2*) is expressed in male germ cells, seminiferous epithelium, Leydig cells, and in the male reproductive tract (101), suggesting that *Aqp2* directly or indirectly participates in male fertility.

Moreover, Wang et al. (98) also identified 11,561 lncRNA in bull sperm, of which 2,517 were distinctly expressed between the low- and high-motility sperm. They also determined that TCONS_00041733 lncRNA targets the node gene *ephrin A1* and participates in the physiology of the male reproductive system. Card et al. (102) detected 6,166 transcripts in bull sperm, most of which were full-length transcripts that *Plcz1* and *Crisp2* transcripts are associated with bull fertility. Furthermore, a comprehensive microarray analysis revealed 415 transcripts to be differentially expressed in sperm from high- and low-fertility bulls (103). Légaré et al. (104) showed that 10 mRNA transcripts (*Smcp*, *Akap4*, *Tcp11*, *Spata3*, *Ctcfl*, *Odf1*, *Adam28*, *Spata18*, *Fam161a*, and *Sord*) in bovine sperm were associated with reproductive system functions. They also found that five mRNA transcripts (*Cyst11*, *Dead*, *Mx1*, *Defb124*, and *Defb119*) are related to the immune defense response.

Sperm miRNA content is dynamic, and the factors affecting spermatogenesis and epididymal maturation influence sperm miRNA composition (105, 106). Microarray and RNA-Seq-based gene expression profiling studies showed that *miR-10a*, *miR-10b*, *miR-34c*, *miR-100*, *miR-103*, *miR-196b*, *miR-365-2*, and *miR-2478* consistently exist in bovine spermatozoa (96, 105, 107). Interestingly, RT-qPCR studies determined that *miR-19b-3p*, *miR-34c-3p*, *miR-148b-3p*, *miR-320a*, and *miRNAs miR-1249* were detectable at low levels, whereas *miR-27a-5p* and *502-5p* were not detectable in sperm from most high-fertility bulls (108). These miRNA transcripts, such as *miR-34*, *miR-34b/c*, and *miR-449*, modulate spermatogenesis and possibly embryogenesis (109, 110). Liu et al. (111) have suggested that *miR-34c-5p* is involved in mRNA degradation and translational repression.

Two different RNA-seq platforms were Illumina and Ion Proton, and they provided evidence that the most abundant miRNA in the bovine sperm is *miR-196b* and is more abundantly expressed in the zygote than the oocyte. *miR-196b* targets transcripts of *Hoxa7*, *Hoxa9*, and *Hoxc8* genes. In addition, protein products of these genes play crucial roles in the meiotic phases of spermatogenesis and are present at high levels in spermatocytes (96, 111, 112). Menezes et al. (113) examined the dynamics of *miR-15a*, *miR-29b*, and *miR-34a* in low- and high-fertility bull sperm using RT-qPCR (113). They reported that *miR-15a* and *miR-29* were more abundantly present in sperm from low-fertility bulls than those of high fertility bulls. However, *miR-34a* expression levels did not differ in sperm from the two groups. In addition, results of several studies suggest that lncRNAs may be involved in the regulation of testis development and spermatogenesis. For example, Zhang et al. (114) showed that *Dmrt1* was involved in the transition of germ cells from mitosis to meiosis using transfection, Western blotting, and Northern and Southern blotting hybridizations. Based on proteomic, immunostaining, and microarray approaches, *HongrES2* has been reported to modulate sperm maturation, and *Mrhl* lncRNA influences spermatogenesis (115, 116). The *Tsx*, which is specifically expressed in pachytene spermatocytes, has a crucial role in the progression of spermatocyte meiosis (117).

Sperm Metabolome and Bull Fertility

Metabolites are also associated with physiological events via a cascade of complex biochemical networks (118, 119) and may provide insights of an individual's phenom (119). Metabolomic methods are used to detect low molecular weight compounds that may offer deep insights into the regulatory pathways within spermatozoa as well (120, 121). In this regard, the mounting evidence shows that mature sperm metabolize a wide range of exogenous substrates that modulate the signaling pathways implicated in key aspects of sperm physiology, including the acrosome reaction, capacitation, hyperactivation, motility, and fusion of spermatozoon and egg (122). The latest improvements in methods of metabolite profiling of infertile individuals offer better insights into the development of useful fertility markers (123). Several metabolite biomarkers have been discovered by untargeted metabolic profiling of sperm samples from healthy individuals and infertile patients using different analytical techniques such as nuclear magnetic resonance (NMR) (124) and mass spectrometry (MS) (125).

There were 22 distinct metabolites detectable in bull sperm employing gas chromatography-mass spectrometry (GC-MS) analysis (126) where major metabolites were fatty acids/conjugates and organic acids/derivatives. The researchers also showed that the levels of five sperm metabolites that differed between high- *vs.* low-fertility groups were benzoic

acid, gamma-aminobutyric acid (GABA), palmitic acid, carbamate, and lactic acid. In addition, four metabolic pathways were found to be associated with differential metabolites, namely, glycolysis or gluconeogenesis, aspartate and glutamate metabolism, pyruvate metabolism, alanine, and β-alanine metabolism. GABA plays an essential role in sperm physiology by inducing the acrosome reaction and sperm hyperactivation. Furthermore, benzoic acid participates in GABA regulation and is highly expressed in high-fertility bull sperm. Because of its participation in lipid metabolism to generate energy, palmitic acid production may be more abundant in high-fertility bull sperm. Higher levels of lactic acid in high-fertility bull sperm might be because anaerobic glycolysis is more efficiently utilized in high-fertility sperm compared with those in low-fertility sperm. Functions of carbamate are considered as potential regulators of intracellular pH in sperm (127).

Velho et al. (128) studied seminal plasma metabolomes of Holstein bulls using GC-MS. They reported that the most abundant metabolites were fructose followed by urea, citric acid, phosphoric acid, and lactic acid. Erythronic acid, 4-ketoglucose, 2-oxoglutaric acid, androstenedione, and D-xylofuranose represented the least predominant metabolites in bull seminal fluid. They demonstrated that levels of 2-oxoglutaric acid were low, whereas the levels of fructose were greater in high-fertility bulls compared with low-fertility bulls. Sperm metabolism can oxidize fructose and convert it to lactic acid (129), supporting both fructose and lactic acid as necessary for fertile sperm as energy sources. Therefore, in many species, fructose is the main monosaccharide abundantly present in semen (124, 130–132).

Citric acid influences the acrosome reaction, sperm transport, and fertilization by being an energy source and regulating semen pH as a chelator for calcium, magnesium, and zinc (133, 134), suggesting that citric acid is a candidate fertility marker in seminal plasma. However, roles of urea and phosphoric acid in seminal plasma on bull fertility remain mostly unclear. Velho et al. (128) speculated that phosphoric acid in seminal plasma may result from catalysis of inorganic phosphate. Hydrolysis of inorganic pyrophosphate to two phosphate ions yields energy (135) that may be utilized for sperm motility or fertilization. Urea in seminal plasma is considered as a metabolite resulting from protein degradation (136). High concentrations of urea in seminal plasma suggest that fertile sperm contain enough protein sources, and some of these proteins are metabolized for biological processes associated with fertility. Using MS, Soggiu et al. (137) demonstrated that isocitrate dehydrogenase, triose phosphate isomerase, and alpha enolase were fertility-associated molecules in bull sperm. Recently, amino acid contents in seminal plasma were shown to be associated with bull sperm freezability (138). Using GC-MS, the researchers also showed that the most abundant amino acid in bull seminal fluid was glutamic acid. Furthermore, phenylalanine concentration in seminal plasma was significantly associated with post-thaw viability.

SPERM EPIGENOME AND BULL FERTILITY

Sperm DNA Methylation and Bull Fertility

DNA methylation has been the most studied epigenetic mechanism in sperm and is presumed to fulfill a major role in the non-genetic information transfer across generations. Sperm DNA methylation participates in many physiological processes, such as silencing of transposable elements (139), paternal genomic imprinting (30), DNA compaction (140), and chromosome inactivation in females (141). In combination with histone modifications, DNA methylation has a fundamental role in modulating gene expression in germ cells by inhibiting the binding of transcription factors to enhancers or by recruiting the binding of proteins that facilitate the deacetylation or methylation of histones, thereby stabilizing the nucleosomes (142). Advances in technologies offered quantitative and base-level ultra-resolution methylome maps. DNA methylation involves the addition of a methyl group to the carbon-5 position of cytosine in the context of cytosine followed by guanine (CpG dinucleotides), referred to as 5-methylcytosine (5mC), although to a lesser extent, DNA methylation also occurs at cytosine bases in a non-CpG context (143, 144).

Gametic DNA methylation is archived in a progressive manner via the activity of the *de novo* methyltransferases DNMT3A, DNMT3B, and their cofactor DNMT3L. Significant levels of DNA methylation are present at birth and must be sustained by DNMT1 during adulthood across different phases of spermatogenesis (145, 146). However, DNA methylation can be reversible, mediated by the ten-eleven translocation (TET) family of DNA dioxygenases that progressively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (147–151). The CpG islands experience differential methylation during gametogenesis and early embryonic development (152). Exposure to harmful environmental conditions may alter DNA methylation patterns in male germ cells and inhibit differentiation into functional mature spermatozoa, thereby causing infertility (153, 154).

Employing whole-genome bisulfite sequencing (WGBS) data (486 × coverage) from neighboring CpG sites among 28 distinct bull sperm samples, Liu et al. (155) identified 31,272 methylation haplotype blocks (MHB) based on the correlation analysis of methylation levels. Of these MHBs, they defined highly variably methylated, variably methylated, and conserved methylated regions. By integrating evidence from traditional and molecular quantitative trait loci, they revealed that highly variably methylated regions may play roles in transcriptional regulation and function in variations in complex traits. Furthermore, they detected 46 variably methylated regions significantly related to reproduction traits, nine of which were modulated by cis-SNP. These variably methylated regions were colocalized with fertility-associated genes, such as *Crisp2*, *Hgf*, and *Zfp36l1*. Sperm protein CRISP2 has important roles in spermatogenesis, modulation of flagellar motility, acrosome reaction, and gamete fusion. Naz et al. (156) showed that HGF was distinctly expressed in the vas deferens and epididymis in mice. Moreover, Herness and Naz (157) implicated that HGF is involved in the process of

acquisition of the potential for sperm motility as sperm mature during epididymal transit, as when immotile mouse sperm from the caput epididymis were incubated with HGF, motility of these spermatozoa was increased by 5–15%. Therefore, it is plausible that there is a relationship between expression patterns of these genes and fertility.

Using GWAS, Fang et al. (158) compared sperm DNA methylomes between cattle and humans, finding that genes with conserved hypermethylated promoters (e.g., Cd80 and Tcap) have been shown to be involved in immune responses, whereas genes with conserved non-methylated promoters (e.g., Anks1a and Wnt7a) participated in embryonic and fetal development. They also found that genes with cattle-specific hypomethylated promoters (e.g., *Dgat2* and *Ldhb*) predominantly engaged in lipid storage and metabolism (158). Using WGBS, Zhou et al. (159) compared methylomes of sperm DNA with those of three somatic tissues in bulls. They detected large differences in the methylation patterns of global CpGs, hypomethylated regions (HMR), partially methylated domains (PMD), common repeats, and pericentromeric satellites between sperm and somatic tissues. Moreover, they observed high methylation in the active gene bodies and low methylation in the promoter regions. Interestingly, meiosis-related genes including *Kif2b* and *Repin1* have been shown to be hypermethylated in somatic cells but hypomethylated in sperm. It has been reported that a broad range of kinesins have important functions in spermatogenesis. Kinesin-13 proteins, mitotic centromere-associated kinesin (MCAK), KIF2A, and KIF2B are involved in spindle bipolarity through induction of depolymerization of microtubules to modulate mitotic dynamics during spermatogenesis (160). In addition, REPIN1 could be regarded as the possible key transcription factors in spermatids (112). Therefore, previous studies support the positive correlation between hypomethylated *Kif2b and Repin1* genes with fertility. Therefore, there is a need for further studies on the functional associations between sperm DNA methylation and bull fertility and early development.

Sperm Chromatin Dynamics and Bull Fertility

During spermiogenesis, chromatin structure and cellular morphology of round spermatids undergo dramatic reconfigurations, giving rise to an extremely condensed chromatin state and transcriptional quiescence in spermatozoa. During this period, histone hyperacetylation occurs increasingly in round and elongating spermatids, resulting in chromatin destabilization and loosening of chromatin structure to facilitate histone eviction (161, 162). In the early post-meiotic phase, most of the nucleosomal canonical histones are gradually replaced with testis-specific histone variants (noncanonical) (77). The linker histone H1 and H2A, H2B, and H3 have testis-specific histone variants. However, no histone H4 variant is known in mammals (161) (**Figure 1**).

Oliveira et al. (163) examined the differences in expression of two core histones (H2B and H4) and a histone variant (H3.3) in bull sperm using immunocytochemistry staining and Western blotting. However, they did not observe any differences in the levels of H2B, H3.3 or H4 in sperm from high-fertile *vs.* low-fertile bulls. Using immunofluorescence, Western blotting, and flow cytometry, Kutchy et al. (164) determined the associations between expression of the testis-specific histone variant 2B in sperm and bull fertility. Moreover, sperm chromatin damage and abnormal protamination were reported to be associated with reduced fertility in bulls using immunofluorescence, Western blotting, and chromatin dispersion tests (165, 166). In addition, using flow cytometry and immunocytochemistry, methylation and acetylation of sperm histone 3 lysine 27 (H3K27me3 and H3K27ac) were shown to be associated with bull fertility (167). Verma et al. (168) examined tri-methylated H3K27 (H3K27me3)- and di-methylated H3K4 (H3K4me2)-enriched genes in sperm of water buffalo bulls (*Bubalus bubalis*) with different fertility by using a custom ChIP on-chip array. For H3K27me3- and H3K4me2-enriched genes, they detected 80 and 84 genes, respectively. Among the H3K4me2-enriched genes, *Cct5*, *Cdc45*, *Dmc1*, *Meg3*, *Mlh1*, *Prdm14*, *Pax3*, *Sox4*, *Sox14*, and *Tbx15* have crucial roles in spermatogenesis and embryogenesis. While *Cct5*, *Cdc45*, *Dmc1*, *Mlh1*, *Prdm14*, *Pax3*, *Sox4*, *Sox14*, and *Tbx15* genes were in greater amounts in sperm from high fertility bulls, *Meg3* was enriched in sperm from subfertile bulls.

Considering that the H3K4me2 epigenetic modification activates gene transcription, the appearance of some H3K4me2-enriched genes in high-fertility bulls raises contradictions with previous studies. For example, CCT5 has been reported to be expressed in the microtubules of the manchette and centrosomes of spermatids and is discarded at later stages of development in mice (74). Also, CCT5 is highly expressed in sperm from low-fertility bulls (44). This might be due to the presence of seminal plasma in the samples analyzed because discarded CCT5 may be seen in the ejaculate. *Pax3*, *Sox4*, and *Sox14* are genes encoding for transcription factors that participate in supporting embryonic development (169, 170). The DMC1 and CDC45 are involved in meiotic recombination and initiation of chromosomal DNA replication, respectively (171, 172). Sancar (173) reported that MLH1 prevents exonuclease-mediated DNA degradation by repairing mismatched DNA pairs. Furthermore, Ji et al. (174) demonstrated that SNP in *Mlh1* gene gave rise to reduced fertility in humans. PRDM14 functions as a transcriptional regulator during germ cell development (175, 176). It also has a critical role in epigenetic modification by both recruiting DNA demethylases of the TET family and by repressing DNA methyltransferases in primordial germ cells and naïve pluripotent stem cells (177–179). Some studies reported that *Prdm14* knockout in mice gave rise to misregulation of H3K27me3 in primordial germ cells and embryonic stem cells, thereby being involved in histone modification (180, 181).

The product of *Meg3(Gtl2)* gene acts as a long non-coding RNA; therefore, it does not encode a protein. Researchers claimed that *Meg3* is involved in p53-mediated transactivation and its suppression of cell proliferation (182). Moreover, abnormal methylation of *Meg3* gene gave rise to deterioration of spermatogenesis (183). Nine of H3K27me3-enriched genes, including *Cdkn2c*, *Fancl*, *Foxa1*, *Gfra1*, *Lhx3*, *Rpl3*, *Six6*, *Sox4*,

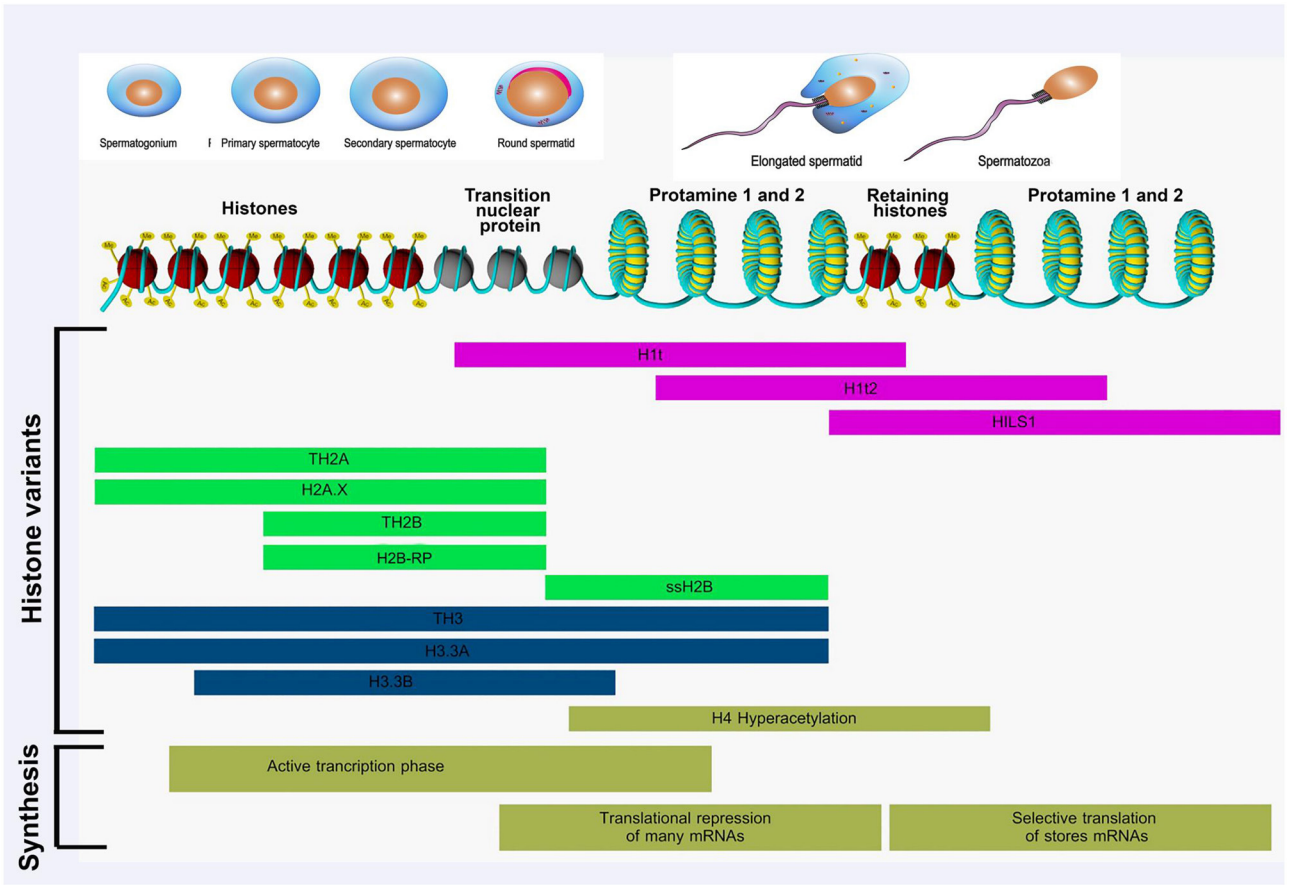


FIGURE 1 | Histone modifications occurring during spermatogenesis from spermatogonia to spermatozoa. Different histone variants are transcribed and translated in this process. Active transcription is observed at the beginning of spermatogenesis. Subsequently, translation of many mRNA is repressed, stored mRNAs are repressed, and stored mRNAs are selectively translated to relevant proteins. Adapted from Kimmins and Sassone-Corsi (77) and Rathke et al. (161).

and *Sox14*, were speculated to be participating in sperm function and embryonic development. While H3K27me3-enriched *Foxa1* gene was in greater amounts in sperm from subfertility bulls, the others were enriched in sperm from high-fertility bulls (168). Hammoud et al. (183) showed that increase in H3K27me3 in sperm genome gives rise to inactivation of gene promoters in early embryo development. Interestingly, *Sox4* and *Sox14* genes have bivalent chromatin structure marks, both of which bear H3K27me3 and H3K4me2. Bernstein et al. (184) reported that bivalent chromatin structure marks were critical in embryonic development. On the other hand, *Cdkn2c* gene suppression is required for effective modulation of spermatogenesis in mice (185), which agrees with the reports by Verma et al. (168). There is a need for further research aimed at demystifying the functional underpinnings of suppression of these genes associated with H3K27me3 modification and fertility.

CONCLUSIONS AND PROSPECTS

The major advancements in the *-omic* technologies (metabolomics, proteomics, transcriptomics, and genomics) have enabled high-throughput screening of a wide range of molecular and cellular dynamics in fertility molecules. These approaches also provide means of detecting minute amounts of changes in molecules due to their higher sensitivity. Such attributes of the advanced methods are vitally important for innovative studies to produce new knowledge with transformational and translational values. However, an ejaculate contains many spermatozoa with different phenotypes. Therefore, each spermatozoon should be examined using new high technologies of single-cell analyses such as single-cell metabolomics, proteomics, transcriptomics, and genomics. In addition, these methods should be combined with conventional techniques, such as sperm chromatin structure assay, computer-assisted sperm analyses (CASA), integrity of membranes, flow cytometry, and reactive oxidation stress levels, to determine semen quality using system biology approaches. As an economically important trait, fertility has become more important as there is an urgent need for more efficient, sustainable, and profitable production for food animals to feed the ever-increasing human population in the world. The bull is a unique model for the study of male fertility because of the availability of large amounts of sperm from bulls with reliable fertility phenotypes, and the significant similarities between the bull and other mammals in both sperm biology and genetics.

AUTHOR CONTRIBUTIONS

All authors assisted in the conception of the study, contributed to manuscript revision, read, and approved the submitted version.

FUNDING

The authors declare that this study received funding from Alta Genetics, Inc. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. It was also funded by Agriculture and Food Research Initiative Competitive Grant no. 2017-67016-26507. Partial funding was provided by Mississippi Agricultural Forestry Experiment Station. MH was funded through a competitive international postdoctoral fellowship by the Scientific and Technological Research Council of Turkey (TUBITAK-1059B191800016).

REFERENCES

1. FAO. *Food and Agriculture Organization of the United Nations. OECD-FAO Agric Outlook*. Rome: FAO (2012).
2. Gifford JAH, Gifford CA. Role of reproductive biotechnologies in enhancing food security and sustainability. *Anim Front.* (2013) 3:14–9. doi: 10.2527/af.2013-0019
3. Kaya A, Memili E. Sperm macromolecules associated with bull fertility. *Anim Reprod Sci.* (2016) 169:88–94. doi: 10.1016/j.anireprosci.2016.02.015
4. Viana AGA, Martins AMA, Pontes AH, Fontes W, Castro MS, Ricart CAO, et al. Proteomic landscape of seminal plasma associated with dairy bull fertility. *Sci Rep.* (2018) 8:16323. doi: 10.1038/s41598-018-34152-w
5. Aslam MMK, Sharma VK, Pandey S, Kumaresan A, Srinivasan A, Datta TK, et al. Identification of biomarker candidates for fertility in spermatozoa of crossbred bulls through comparative proteomics. *Theriogenology.* (2018) 119:43–51. doi: 10.1016/j.theriogenology.2018.06.021
6. Ugur MR, Kutchy NA, Menezes EB, Ul-Husna A, Haynes BP, Uzun A, et al. Retained acetylated histone four in bull sperm associated with fertility. *Front Vet Sci.* (2019) 6:223. doi: 10.3389/fvets.2019.00223
7. Kastelic JP, Thundathil JC. Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod Domest Anim.* (2008) 43:368–73. doi: 10.1111/j.1439-0531.2008.01186.x
8. Selvaraju S, Parthipan S, Somashekar L, Krishnan Binsila B, Kolte AP, Arangasamy A, et al. Systems Biology in Reproductive Medicine Current status of sperm functional genomics and its diagnostic potential of fertility in bovine (Bos taurus). *Reprod Med.* (2018) 64:484–501. doi: 10.1080/19396368.2018.1444816
9. Staub C, Johnson L. Review: spermatogenesis in the bull. *Animal.* (2018) 12:S27–35. doi: 10.1017/S1751731118000435
10. De Jonge CJ, Barratt C eds. *The Sperm Cell Production, Maturation, Fertilization, Regeneration*. 2nd ed. Cambridge: Cambridge University Press (2017).
11. Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI et al. Advances in cryopreservation of bull sperm. *Front Vet Sci.* (2019) 6:268. doi: 10.3389/fvets.2019.00268
12. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, et al. Finding the missing heritability of complex diseases. *Nature.* (2009) 461:747–53. doi: 10.1038/nature08494
13. Kühn C, Bennewitz J, Reinsch N, Xu N, Thomsen H, Looft C, et al. Quantitative trait loci mapping of functional traits in the German Holstein cattle population. *J Dairy Sci.* (2003) 86:360–8. doi: 10.3168/jds.S0022-0302(03)73614-5
14. Casas E, Lunstra DD, Stone RT. Quantitative trait loci for male reproductive traits in beef cattle. *Anim Genet.* (2004) 35:451–3. doi: 10.1111/j.1365-2052.2004.01190.x
15. Feugang JM, Kaya A, Page GP, Chen L, Mehta T, Hirani K, et al. Two-stage genome-wide association study identifies integrin beta 5 as having potential role in bull fertility. *BMC Genomics.* (2009) 10:176. doi: 10.1186/1471-2164-10-176
16. Pausch H, Flisikowski K, Jung S, Emmerling R, Edel C, Götz K-U, et al. Genome-wide association study identifies two major loci affecting calving ease and growth-related traits in cattle. *Genetics.* (2011) 187:289–97. doi: 10.1534/genetics.110.124057
17. Cole JB, Wiggans GR, Ma L, Sonstegard TS, Lawlor TJ, Crooker BA, et al. Genome-wide association analysis of thirty one production, health, reproduction and body conformation traits in contemporary US Holstein cows. *BMC Genomics.* (2011) 12:408. doi: 10.1186/1471-2164-12-408
18. Blaschek M, Kaya A, Zwald N, Memili E, Kirkpatrick BW. A whole-genome association analysis of noncompensatory fertility in Holstein bulls. *J Dairy Sci.* (2011) 94:4695–9. doi: 10.3168/jds.2010-3728
19. McClure MC, Morsci NS, Schnabel RD, Kim JW, Yao P, Rolf MM, et al. A genome scan for quantitative trait loci influencing carcass, post-natal growth and reproductive traits in commercial Angus cattle. *Anim Genet.* (2010) 41:597–607. doi: 10.1111/j.1365-2052.2010.02063.x
20. Fortes MRS, Reverter A, Hawken RJ, Bolormaa S, Lehnert SA. Candidate genes associated with testicular development, sperm quality, and hormone levels of inhibin, luteinizing hormone, and insulin-like growth factor 1 in Brahman bulls. *Biol Reprod.* (2012) 87:51–8. doi: 10.1095/biolreprod.112.101089
21. Fortes MRS, Lehnert SA, Bolormaa S, Reich C, Fordyce G, Corbet NJ, et al. Finding genes for economically important traits: Brahman cattle puberty. *Anim Prod Sci.* (2012) 52:143–50. doi: 10.1071/AN11165
22. Peñagaricano F, Weigel KA, Khatib H. Genome-wide association study identifies candidate markers for bull fertility in Holstein dairy cattle. *Anim Genet.* (2012) 43:65–71. doi: 10.1111/j.1365-2052.2012.02350.x
23. Pausch H, Venhoranta H, Wurmser C, Hakala K, Iso-Touru T, Sironen A, et al. A frameshift mutation in ARMC3 is associated with a tail stump sperm defect in Swedish Red (Bos taurus) cattle. *BMC Genet.* (2016) 17:49. doi: 10.1186/s12863-016-0356-7
24. Iso-Touru T, Wurmser C, Venhoranta H, Hiltpold M, Savolainen T, Sironen A, et al. A splice donor variant in CCDC189 is associated with asthenospermia in Nordic Red dairy cattle. *BMC Genomics.* (2019) 20:286. doi: 10.1186/s12864-019-5628-y
25. Han Y, Peñagaricano F. Unravelling the genomic architecture of bull fertility in Holstein cattle. *BMC Genet.* (2016) 17:143. doi: 10.1186/s12863-016-0454-6
26. Tüttelmann F, Krenková P, Römer S, Nestorovic AR, Ljujic M, Štambergová A, et al. A common haplotype of protamine 1 and 2 genes is associated with higher sperm counts. *Int J Androl.* (2010) 33:e240–8. doi: 10.1111/j.1365-2605.2009.01003.x
27. Giesecke K, Hamann H, Stock KF, Woehlke A, Sieme H, Distl O. Evaluation of SPATA1-associated markers for stallion fertility. *Anim Genet.* (2009) 40:359–65. doi: 10.1111/j.1365-2052.2008.01844.x
28. Pang Y-Y, Lu RJ-H, Chen P-Y. Behavioral epigenetics: perspectives based on experience-dependent epigenetic inheritance. *Epigenomes.* (2019) 3:18. doi: 10.3390/epigenomes3030018
29. Seisenberger S, Andrews S, Krueger F, Arand J, Walter J, Santos F, et al. The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Mol Cell.* (2012) 48:849–62. doi: 10.1016/j.molcel.2012.11.001
30. Stewart KR, Veselovska L, Kelsey G. Establishment and functions of DNA methylation in the germline. *Epigenomics.* (2016) 8:1399–413. doi: 10.2217/epi-2016-0056
31. Hammoud SS, Low DHP, Yi C, Lee CL, Oatley JM, Payne CJ, et al. Transcription and imprinting dynamics in developing

postnatal male germline stem cells. *Genes Dev.* (2015) 29:2312–24. doi: 10.1101/gad.261925.115
32. Hill PWS, Leitch HG, Requena CE, Sun Z, Amouroux R, Roman-Trufero M, et al. Epigenetic reprogramming enables the transition from primordial germ cell to gonocyte. *Nature.* (2018) 555:392–6. doi: 10.1038/nature25964
33. Rawe VY, Díaz ES, Abdelmassih R, Wójcik C, Morales P, Sutovsky P, et al. The role of sperm proteasomes during sperm aster formation and early zygote development: implications for fertilization failure in humans. *Hum Reprod.* (2008) 23:573–80. doi: 10.1093/humrep/dem385
34. Tarnasky H, Cheng M, Ou Y, Thundathil JC, Oko R, Van Der Hoorn FA. Gene trap mutation of murine Outer dense fiber protein-2 gene can result in sperm tail abnormalities in mice with high percentage chimaerism. *BMC Dev Biol.* (2010) 10:67. doi: 10.1186/1471-213X-10-67
35. Zhao W, Li Z, Ping P, Wang G, Yuan X, Sun F. Outer dense fibers stabilize the axoneme to maintain sperm motility. *J Cell Mol Med.* (2018) 22:1755–68. doi: 10.1111/jcmm.13457
36. Zhao C, Huo R, Wang FQ, Lin M, Zhou ZM, Sha JH. Identification of several proteins involved in regulation of sperm motility by proteomic analysis. *Fertil Steril.* (2007) 87:436–8. doi: 10.1016/j.fertnstert.2006.06.057
37. Ito J, Parrington J, Fissore RA. PLCζ and its role as a trigger of development in vertebrates. *Mol Reprod Dev.* (2011) 78:846–53. doi: 10.1002/mrd.21359
38. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, et al. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J.* (2014) 28:4434–40. doi: 10.1096/fj.14-256495
39. Satouh Y, Nozawa K, Ikawa M. Sperm postacrosomal WW domain-binding protein is not required for mouse egg activation. *Biol Reprod.* (2015) 93:94. doi: 10.1095/biolreprod.115.131441
40. Velho A, Wang H, Koenig L, Grant KE, Menezes ES, Kaya A, et al. Expression dynamics of Integrin Subunit Beta 5 in bovine gametes and embryos imply functions in male fertility and early embryonic development. *Andrologia.* (2019) 51:e13305. doi: 10.1111/and.13305
41. Chang YM, Gianola D, Heringstad B, Klemetsdal G. Effects of trait definition on genetic parameter estimates and sire evaluation for clinical mastitis with threshold models. *Anim Sci.* (2004) 79:355–63. doi: 10.1017/S1357729800090226
42. Park Y-J, Kwon W-S, Oh S-A, Pang M-G. Fertility-related proteomic profiling bull spermatozoa separated by percoll. *J Proteome Res.* (2012) 11:4162–8. doi: 10.1021/pr300248s
43. Calven P, Rolland AD, Jégou B, Pineau C. Testicular postgenomics: targeting the regulation of spermatogenesis. *Philos Trans R Soc B Biol Sci.* (2010) 365:1481–500. doi: 10.1098/rstb.2009.0294
44. Kasimanickam RK, Kasimanickam VR, Arangasamy A, Kastelic JP. Sperm and seminal plasma proteomics of high- versus low-fertility Holstein bulls. *Theriogenology.* (2019) 126:41–8. doi: 10.1016/j.theriogenology.2018.11.032
45. Sharma R, Agarwal A, Mohanty G, Hamada AJ, Gopalan B, Willard B, et al. Proteomic analysis of human spermatozoa proteins with oxidative stress. *Reprod Biol Endocrinol.* (2013) 11:48. doi: 10.1186/1477-7827-11-48
46. Bastián Y, Roa-Espitia AL, Mújica A, Hernández-González EO. Calpain modulates capacitation and acrosome reaction through cleavage of the spectrin cytoskeleton. *Reproduction.* (2010) 140:673–84. doi: 10.1530/REP-09-0545
47. Shisheva A, Südhof TC, Czech MP. Cloning, characterization, and expression of a novel GDP dissociation inhibitor isoform from skeletal muscle. *Mol Cell Biol.* (1994) 14:3459–68. doi: 10.1128/MCB.14.5.3459
48. Burska UL, Harle VJ, Coffey K, Darby S, Ramsey H, O'Neill D, et al. Deubiquitinating enzyme Usp12 is a novel co-activator of the androgen receptor. *J Biol Chem.* (2013) 288:32641–50. doi: 10.1074/jbc.M113.485912
49. Orlowski M, Reznik S, Ayala J, Pierotti AR. Endopeptidase 24.15 from rat testes. Isolation of the enzyme and its specificity toward synthetic and natural peptides, including enkephalin-containing peptides. *Biochem J.* (1989) 261:951–8. doi: 10.1042/bj2610951
50. Sosa MA, Barbieri MA, Bertini F. Binding of β-galactosidase from rat epididymal fluid to the sperm surface by high-affinity sites different from phosphomannosyl receptors. *Reproduction.* (1991) 93:279–85. doi: 10.1530/jrf.0.0930279
51. Baumgart E, Lenk SV, Loening SA, Jung K. Tissue inhibitors of metalloproteinases 1 and 2 in human seminal plasma and their association with spermatozoa. *Int J Androl.* (2002) 25:369–71. doi: 10.1046/j.1365-2605.2002.00383.x
52. Shimokawa KI, Katayama M, Matsuda Y, Takahashi H, Hara I, Sato H, et al. Matrix metalloproteinase (MMP)-2 and MMP-9 activities in human seminal plasma. *Mol Hum Reprod.* (2002) 8:32–6. doi: 10.1093/molehr/8.1.32
53. Sanz L, Calvete JJ, Mann K, Schäfer W, Schmid ER, Amselgruber W, et al. The complete primary structure of the spermadhesin AWN, a zona pellucida-binding protein isolated from boar spermatozoa. *FEBS Lett.* (1992) 300:213–8. doi: 10.1016/0014-5793(92)80848-B
54. Romero A, Romao MJ, Varela PF, Kölln I, Dias JM, Carvalho AL, et al. The crystal structures of two spermadhesins reveal the CUB domain fold. *Nat Struct Biol.* (1997) 4:783–8. doi: 10.1038/nsb1097-783
55. Solís D, Romero A, Jiménez M, Díaz-Mauriño T, Calvete JJ. Binding of mannose-6-phosphate and heparin by boar seminal plasma PSP-II, a member of the spermadhesin protein family. *FEBS Lett.* (1998) 431:273–8. doi: 10.1016/S0014-5793(98)00772-8
56. Dostálová Z, Calvete JJ, Sanz L, Hettel C, Riedel D, Töpfer-Petersen E, et al. Immunolocalization and Quantitation of Acidic Seminal Fluid Protein (aSFP) in ejaculated, swim-up, and capacitated bull spermatozoa. *Biol Chem Hoppe Seyler.* (1994) 375:457–62. doi: 10.1515/bchm3.1994.375.7.457
57. Moura AA, Souza CE, Stanley BA, Chapman DA, Killian GJ. Proteomics of cauda epididymal fluid from mature Holstein bulls. *J Proteomics.* (2010) 73:2006–20. doi: 10.1016/j.jprot.2010.06.005
58. Bustamante-Filho IC, Salton GD, Munari FM, Schneider MR, Mattos RC, Laurino JP, et al. Recombinant expression and purification of the bovine acidic seminal fluid protein. *Anim Reprod.* (2014) 11:96–103.
59. Go KJ, Wolf DP. Albumin-mediated changes in sperm sterol content during capacitation. *Biol Reprod.* (1985) 32:145–53. doi: 10.1095/biolreprod32.1.145
60. Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod.* (1998) 59:1–6. doi: 10.1095/biolreprod59.1.1
61. Atkinson DE. The energy charge of the adenylate pool as a regulatory parameter. Interaction with Feedback Modifiers. *Biochemistry.* (1968) 7:4030–4. doi: 10.1021/bi00851a033
62. Schoff PK, Cheetham J, Lardy HA. Adenylate kinase activity in ejaculated bovine sperm flagella. *J Biol Chem.* (1989) 264:6086–91. doi: 10.1016/S0021-9258(18)83316-6
63. Cao W, Haig-Ladewig L, Gerton GL, Moss SB. Adenylate kinases 1 and 2 are part of the accessory structures in the mouse sperm flagellum. *Biol Reprod.* (2006) 75:492–500. doi: 10.1095/biolreprod.106.053512
64. Gibbons R, Adeoya-Osiguwa SA, Fraser LR. A mouse sperm decapacitation factor receptor is phosphatidylethanolamine-binding protein 1. *Reproduction.* (2005) 130:497–508. doi: 10.1530/rep.1.00792
65. Nixon B, MacIntyre DA, Mitchell LA, Gibbs GM, O'Bryan M, Aitken RJ. The identification of mouse sperm-surface-associated proteins and characterization of their ability to act as decapacitation factors. *Biol Reprod.* (2006) 74:275–87. doi: 10.1095/biolreprod.105.044644
66. Desnoyers L, Manjunath P. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. *J Biol Chem.* (1992) 267:10149–55. doi: 10.1016/S0021-9258(19)50212-5
67. Manjunath P, Lefebvre J, Jois PS, Fan J, Wright MW. New nomenclature for mammalian BSP genes. *Biol Reprod.* (2009) 80:394–7. doi: 10.1095/biolreprod.108.074088
68. D'Amours O, Frenette G, Fortier M, Leclerc P, Sullivan R. Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes. *Reproduction.* (2010) 139:545–56. doi: 10.1530/REP-09-0375
69. Gwathmey TYM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod.* (2006) 75:501–7. doi: 10.1095/biolreprod.106.053306
70. Saalmann A, Mnz S, Ellerbrock K, Ivell R, Kirchhoff C. Novel sperm-binding proteins of epididymal origin contain four fibronectin type II-modules. *Mol Reprod Dev.* (2001) 58:88–100. doi: 10.1002/1098-2795(200101)58:1<88::AID-MRD12>3.0.CO;2-D
71. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev.* (2002) 82:373–428. doi: 10.1152/physrev.00027.2001
72. Morales P, Kong M, Pizarro E, Pasten C. Participation of the sperm proteasome in human fertilization.

Hum Reprod. (2003) 18:1010–7. doi: 10.1093/humrep/deg111
73. Gómez-Puertas P, Martín-Benito J, Carrascosa JL, Willison KR, Valpuesta JM. The substrate recognition mechanisms in chaperonins. *J Mol Recognit.* (2004) 17:85–94. doi: 10.1002/jmr.654
74. Souès S, Kann ML, Fouquet JP, Melki R. The cytosolic chaperonin CCT associates to cytoplasmic microtubular structures during mammalian spermiogenesis and to heterochromatin in germline and somatic cells. *Exp Cell Res.* (2003) 288:363–73. doi: 10.1016/S0014-4827(03)00248-9
75. Zalata A, El-Samanoudy AZ, Shaalan D, El-Baiomy Y, Taymour M, Mostafa T. Seminal clusterin gene expression associated with seminal variables in fertile and infertile men. *J Urol.* (2012) 188:1260–4. doi: 10.1016/j.juro.2012.06.012
76. O'bryan MK, Murphy BF, Liu DY, Clarke GN, Baker HWG. The use of anticlusterin monoclonal antibodies for the combined assesment of human sperm morphology and acrosome integrity. *Hum Reprod.* (1994) 9:1490–6. doi: 10.1093/oxfordjournals.humrep.a138736
77. Kimmins S, Sassone-Corsi P. Chromatin remodelling and epigenetic features of germ cells. *Nature.* (2005) 434:583–9. doi: 10.1038/nature03368
78. Levin ER, Gardner DG, Samson WK. Natriuretic peptides. *N Engl J Med.* (1998) 339:321–8. doi: 10.1056/NEJM199807303390507
79. Öztop M, Cinar K, Turk S. Immunolocalization of natriuretic peptides and their receptors in goat (Capra hircus) heart. *Biotech Histochem.* (2018) 93:389–404. doi: 10.1080/10520295.2018.1425911
80. Sellitti DF, Koles N, Mendona MC. Regulation of C-type natriuretic peptide expression. *Peptides.* (2011) 32:1964–71. doi: 10.1016/j.peptides.2011.07.013
81. Chrisman TD, Schulz S, Potter LR, Garbers DL. Seminal plasma factors that cause large elevations in cellular cyclic GMP are C-type natriuretic peptides. *J Biol Chem.* (1993) 268:3698–703. doi: 10.1016/S0021-9258(18)53749-2
82. Nielsen SJ, Gøtze JP, Jensen HL, Rehfeld JF. ProCNP and CNP are expressed primarily in male genital organs. *Regul Pept.* (2008) 146:204–12. doi: 10.1016/j.regpep.2007.09.022
83. Özbek M, Hitit M, Öztop M, Beyaz F, Ergün E, Ergün L. Spatiotemporal expression patterns of natriuretic peptides in rat testis and epididymis during postnatal development. *Andrologia.* (2019) 51: e13387. doi: 10.1111/and.13387
84. Xia H, Chen Y, Wu KJ, Zhao H, Xiong CL, Huang DH. Role of C-type natriuretic peptide in the function of normal human sperm. *Asian J Androl.* (2016) 18:80–4. doi: 10.4103/1008-682X.150254
85. Chang TSK, Morton B. Epididymal sulfhydryl oxidase: a sperm-protective enzyme from the male reproductive tract. *Biochem Biophys Res Commun.* (1975) 66:309–15. doi: 10.1016/S0006-291X(75)80329-9
86. Ostrowski MC, Kistler WS, Williams-Ashman HG. A flavoprotein responsible for the intense sulfhydryl oxidase activity of rat seminal vesicle secretion. *Biochem Biophys Res Commun.* (1979) 87:171–6. doi: 10.1016/0006-291X(79)91662-0
87. Cornwall GA, Vindivich D, Tillman S, Chang TS. The effect of sulfhydryl oxidation on the morphology of immature hamster epididymal spermatozoa induced to acquire motility in vitro. *Biol Reprod.* (1988) 39:141–55. doi: 10.1095/biolreprod39.1.141
88. Tury A, Mairet-Coello G, Esnard-Fève A, Benayoun B, Risold PY, Griffond B, et al. Cell-specific localization of the sulphydryl oxidase QSOX in rat peripheral tissues. *Cell Tissue Res.* (2006) 323:91–103. doi: 10.1007/s00441-005-0043-x
89. Barondes SH, Cooper DNW, Gitt MA, Leffler H. Galectins. Structure and function of a large family of animal lectins. *J Biol Chem.* (1994) 269:20807–10. doi: 10.1016/S0021-9258(17)31891-4
90. Thijssen VLJL, Poirier F, Baum LG, Griffioen AW. Galectins in the tumor endothelium: opportunities for combined cancer therapy. *Blood.* (2007) 110:2819–27. doi: 10.1182/blood-2007-03-077792
91. Akahani S, Nangia-Makker P, Inohara H, Kim H-RC, Raz A. Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res.* (1997) 57:5272–6.
92. Özbek M, Hitit M, Yildirim N, Özgenç Ö, Ergün E, Ergün L, et al. Expression pattern of galectin-1 and galectin-3 in rat testes and epididymis during postnatal development. *Acta Histochem.* (2018) 120:814–27. doi: 10.1016/j.acthis.2018.09.006
93. Kovak MR, Saraswati S, Schoen DJ, Diekman AB. Investigation of galectin-3 function in the reproductive tract by identification of binding ligands in human seminal plasma. *Am J Reprod Immunol.* (2014) 72:403–12. doi: 10.1111/aji.12273
94. Gomes FP, Diedrich JK, Saviola AJ, Memili E, Moura AA, Yates III JR. EThcD and 213 nm UVPD for top-down analysis of bovine seminal plasma proteoforms on electrophoretic and chromatographic time frames. *Anal Chem.* (2020) 92:2979–87. doi: 10.1021/acs.analchem.9b03856
95. Krawetz SA. Paternal contribution: new insights and future challenges. *Nat Rev Genet.* (2005) 6:633–42. doi: 10.1038/nrg1654
96. Selvaraju S, Parthipan S, Somashekar L, Kolte AP, Binsila BK, Arangasamy A, et al. Occurrence and functional significance of the transcriptome in bovine (Bos taurus) spermatozoa. *Sci Rep.* (2017) 7:42392. doi: 10.1038/srep42392
97. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* (2004) 116:281–97. doi: 10.1016/S0092-8674(04)00045-5
98. Wang X, Yang C, Guo F, Zhang Y, Ju Z, Jiang Q, et al. Integrated analysis of mRNAs and long noncoding RNAs in the semen from Holstein bulls with high and low sperm motility. *Sci Rep.* (2019) 9:2092. doi: 10.1038/s41598-018-38462-x
99. Rego JPA, Martins JM, Wolf CA, Van Tilburg M, Moreno F, Monteiro-Moreira AC, et al. Proteomic analysis of seminal plasma and sperm cells and their associations with semen freezability in Guzerat bulls. *J Anim Sci.* (2016) 94:5308–20. doi: 10.2527/jas.2016-0811
100. Delbridge ML, Lingenfelter PA, Disteche CM, Graves JAM. The candidate spermatogenesis gene RBMY has a homologue on the human X chromosome. *Nat Genet.* (1999) 22:223–4. doi: 10.1038/10279
101. Yeste M, Morató R, Rodríguez-Gil JE, Bonet S, Prieto-Martínez N. Aquaporins in the male reproductive tract and sperm: functional implications and cryobiology. *Reprod Domest Anim.* (2017) 52:12–27. doi: 10.1111/rda.13082
102. Card CJ, Anderson EJ, Zamberlan S, Krieger KE, Kaproth M, Sartini BL. Cryopreserved bovine spermatozoal transcript profile as revealed by high-throughput ribonucleic acid sequencing. *Biol Reprod.* (2013) 88:41–9. doi: 10.1095/biolreprod.112.103788
103. Feugang JM, Rodriguez-Osorio N, Kaya A, Wang H, Page G, Ostermeier GC, et al. Transcriptome analysis of bull spermatozoa: implications for male fertility. *Reprod Biomed Online.* (2010) 21:312–24. doi: 10.1016/j.rbmo.2010.06.022
104. Légaré C, Akintayo A, Blondin P, Calvo E, Sullivan R. Impact of male fertility status on the transcriptome of the bovine epididymis. *MHR Basic Sci Reprod Med.* (2017) 23:355–69. doi: 10.1093/molehr/gax019
105. Stowe HM, Calcatera SM, Dimmick MA, Andrae JG, Duckett SK, Pratt SL. The bull sperm microRNAome and the effect of fescue toxicosis on sperm microRNA expression. *PLoS ONE.* (2014) 9:e113163. doi: 10.1371/journal.pone.0113163
106. Nixon B, Stanger SJ, Mihalas BP, Reilly JN, Anderson AL, Tyagi S, et al. The microRNA signature of mouse spermatozoa is substantially modified during epididymal maturation. *Biol Reprod.* (2015) 93:91. doi: 10.1095/biolreprod.115.132209
107. Capra E, Turri F, Lazzari B, Cremonesi P, Gliozzi TM, Fojadelli I, et al. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between High-and Low-motile sperm populations. *BMC Genomics.* (2017) 18:14. doi: 10.1186/s12864-016-3394-7
108. Fagerlind M, Stålhammar H, Olsson B, Klinga-Levan K. Expression of mi RNA s in bull spermatozoa correlates with fertility rates. *Reprod Domest Anim.* (2015) 50:587–94. doi: 10.1111/rda.12531
109. Tscherner A, Gilchrist G, Smith N, Blondin P, Gillis D, LaMarre J. MicroRNA-34 family expression in bovine gametes and preimplantation embryos. *Reprod Biol Endocrinol.* (2014) 12:85. doi: 10.1186/1477-7827-12-85
110. Yuan S, Tang C, Zhang Y, Wu J, Bao J, Zheng H, et al. mir-34b/c and mir-449a/b/c are required for spermatogenesis, but not for the first cleavage division in mice. *Biol Open.* (2015) 4:212–23. doi: 10.1242/bio.201410959
111. Liu W-M, Pang RTK, Chiu PCN, Wong BPC, Lao K, Lee K-F, et al. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc Natl Acad Sci.* (2012) 109:490–4. doi: 10.1073/pnas.1110368109

112. Zhu Z, Li C, Yang S, Tian R, Wang J, Yuan Q, et al. Dynamics of the transcriptome during human spermatogenesis: predicting the potential key genes regulating male gametes generation. *Sci Rep.* (2016) 6:19069. doi: 10.1038/srep19069
113. Menezes ESB, Badial PR, El Debaky H, Husna AU, Ugur MR, Kaya A, et al. Sperm miR-15a and miR-29b are associated with bull fertility. *Andrologia.* (2020) 52:e13412. doi: 10.1111/and.13412
114. Zhang L, Lu H, Xin D, Cheng H, Zhou R. A novel ncRNA gene from mouse chromosome 5 trans-splices with Dmrt1 on chromosome 19. *Biochem Biophys Res Commun.* (2010) 400:696–700. doi: 10.1016/j.bbrc.2010.08.130
115. Ni M-J, Hu Z-H, Liu Q, Liu M-F, Lu M, Zhang J-S, et al. Identification and characterization of a novel non-coding RNA involved in sperm maturation. *PLoS ONE.* (2011) 6:e26053. doi: 10.1371/journal.pone.0026053
116. Arun G, Akhade VS, Donakonda S, Rao MRS. mrhl RNA, a long noncoding RNA, negatively regulates Wnt signaling through its protein partner Ddx5/p68 in mouse spermatogonial cells. *Mol Cell Biol.* (2012) 32:3140–52. doi: 10.1128/MCB.00006-12
117. Anguera MC, Ma W, Clift D, Namekawa S, Kelleher III RJ, Lee JT. Tsx produces a long noncoding RNA and has general functions in the germline, stem cells, and brain. *PLoS Genet.* (2011) 7:e1002248. doi: 10.1371/journal.pgen.1002248
118. Fukusaki E. Application of metabolomics for high resolution phenotype analysis. *Mass Spectrom.* (2014) 3:S0045. doi: 10.5702/massspectrometry.S0045
119. Guijas C, Montenegro-Burke JR, Warth B, Spilker ME, Siuzdak G. Metabolomics activity screening for identifying metabolites that modulate phenotype. *Nat Biotechnol.* (2018) 36:316–20. doi: 10.1038/nbt.4101
120. Gromski PS, Muhamadali H, Ellis DI, Xu Y, Correa E, Turner ML, et al. A tutorial review: metabolomics and partial least squares-discriminant analysis–a marriage of convenience or a shotgun wedding. *Anal Chim Acta.* (2015) 879:10–23. doi: 10.1016/j.aca.2015.02.012
121. Dipresa S, De Toni L, Garolla A. New markers for predicting fertility of the male gametes in the post genomic era. *Protein Pept Lett.* (2018) 25:434–9. doi: 10.2174/0929866525666180418120635
122. Odet F, Gabel S, London RE, Goldberg E, Eddy EM. Glycolysis and mitochondrial respiration in mouse LDHC-null sperm. *Biol Reprod.* (2013) 88:91–5. doi: 10.1095/biolreprod.113.108530
123. Chen X, Hu C, Dai J, Chen L. Metabolomics analysis of seminal plasma in infertile males with kidney-yang deficiency: a preliminary study. *Evid Based Complement Altern Med.* (2015) 2015:892930. doi: 10.1155/2015/892930
124. Jayaraman V, Ghosh S, Sengupta A, Srivastava S, Sonawat HM, Narayan PK. Identification of biochemical differences between different forms of male infertility by nuclear magnetic resonance (NMR) spectroscopy. *J Assist Reprod Genet.* (2014) 31:1195–204. doi: 10.1007/s10815-014-0282-4
125. Engel KM, Baumann S, Rolle-Kampczyk U, Schiller J, von Bergen M, Grunewald S. Metabolomic profiling reveals correlations between spermiogram parameters and the metabolites present in human spermatozoa and seminal plasma. *PLoS ONE.* (2019) 14:e0211679. doi: 10.1371/journal.pone.0211679
126. Menezes EB, Velho ALC, Santos F, Dinh T, Kaya A, Topper E, et al. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genomics.* (2019) 20:714. doi: 10.1186/s12864-019-6074-6
127. Memili E, Moura AA, Kaya A. Metabolomes of sperm and seminal plasma associated with bull fertility. *Anim Reprod Sci.* (2020) 220:106355. doi: 10.1016/j.anireprosci.2020.106355
128. Velho ALC, Menezes E, Dinh T, Kaya A, Topper E, Moura AA, et al. Metabolomic markers of fertility in bull seminal plasma. *PLoS ONE.* (2018) 13:e0195279. doi: 10.1371/journal.pone.0195279
129. Mann T. Studies on the metabolism of semen3. Fructose as a normal constituent of seminal plasma. Site of formation and function of fructose in semen. *Biochem J.* (1946) 40:481–91.
130. Baronos S. Seminal carbohydrate in boar and stallion. *Reproduction.* (1971) 24:303–5. doi: 10.1530/jrf.0.0240303
131. Mendoza G, White IG, Chow P. Studies of chemical components of Angora goat seminal plasma. *Theriogenology.* (1989) 32:455–66. doi: 10.1016/0093-691X(89)90012-5
132. Matsuoka T, Imai H, Asakuma S, Kohno H, Fukui Y. Changes of fructose concentrations in seminal plasma and glucose and testosterone concentrations in blood plasma in rams over the course of a year. *J Reprod Dev.* (2006) 52:805–10. doi: 10.1262/jrd.18039
133. Kamp G, Lauterwein J. Multinuclear magnetic resonance studies of boar seminal plasma. *Biochim Biophys Acta.* (1995) 1243:101–9. doi: 10.1016/0304-4165(94)00117-G
134. Sørensen MB, Bergdahl IA, Hjøllund NHI, Bonde JPE, Stoltenberg M, Ernst E. Zinc, magnesium and calcium in human seminal fluid: relations to other semen parameters and fertility. *Mol Hum Reprod.* (1999) 5:331–7. doi: 10.1093/molehr/5.4.331
135. Yi Y-J, Sutovsky M, Kennedy C, Sutovsky P. Identification of the inorganic pyrophosphate metabolizing, ATP substituting pathway in mammalian spermatozoa. *PLoS ONE.* (2012) 7:e34524. doi: 10.1371/journal.pone.0034524
136. Newairy A-SA, Salama AF, Hussien HM, Yousef MI. Propolis alleviates aluminium-induced lipid peroxidation and biochemical parameters in male rats. *Food Chem Toxicol.* (2009) 47:1093–8. doi: 10.1016/j.fct.2009.01.032
137. Soggiu A, Piras C, Hussein HA, De Canio M, Gaviraghi A, Galli A, et al. Unravelling the bull fertility proteome. *Mol Biosyst.* (2013) 9:1188–95. doi: 10.1039/c3mb25494a
138. Ugur MR, Dinh T, Hitit M, Kaya A, Topper E, Didion B, et al. Amino acids of seminal plasma associated with freezability of bull sperm. *Front cell Dev Biol.* (2020) 7:347. doi: 10.3389/fcell.2019.00347
139. Zamudio N, Barau J, Teissandier A, Walter M, Borsos M, Servant N, Bourc'his D. DNA methylation restrains transposons from adopting a chromatin signature permissive for meiotic recombination. *Genes Dev.* (2015) 29:1256–70. doi: 10.1101/gad.257840.114
140. Miller D, Brinkworth M, Iles D. Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction.* (2010) 139:287–301. doi: 10.1530/REP-09-0281
141. Larson EL, Vanderpool D, Keeble S, Zhou M, Sarver BAJ, Smith AD, et al. Contrasting levels of molecular evolution on the mouse X chromosome. *Genetics.* (2016) 203:1841–57. doi: 10.1534/genetics.116.186825
142. Allegrucci C, Thurston A, Lucas E, Young L. Epigenetics and the germline. *Reproduction.* (2005) 129:137–49. doi: 10.1530/rep.1.00360
143. Yoder JA, Soman NS, Verdine GL, Bestor TH. DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *J Mol Biol.* (1997) 270:385–95. doi: 10.1006/jmbi.1997.1125
144. Jang HS, Shin WJ, Lee JE, Do JT. CpG and non-CpG methylation in epigenetic gene regulation and brain function. *Genes (Basel).* (2017) 8:148. doi: 10.3390/genes8060148
145. Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, Kohara Y, et al. Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Hum Mol Genet.* (2007) 16:2272–80. doi: 10.1093/hmg/ddm179
146. Seisenberger S, Peat JR, Hore TA, Santos F, Dean W, Reik W. Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers. *Philos Trans R Soc B Biol Sci.* (2013) 368:20110330. doi: 10.1098/rstb.2011.0330
147. Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science.* (2009) 324:929–30. doi: 10.1126/science.1169786
148. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science.* (2009) 324:930–35. doi: 10.1126/science.1170116
149. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science.* (2011) 333:1300–3. doi: 10.1126/science.1210597
150. Wu H, Zhang Y. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell.* (2014) 156:45–68. doi: 10.1016/j.cell.2013.12.019
151. Neri F, Incarnato D, Krepelova A, Parlato C, Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* (2016) 11:1191–205. doi: 10.1038/nprot.2016.063

152. Moore LD, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology.* (2013) 38:23–38. doi: 10.1038/npp.2012.112
153. Donkin I, Barrès R. Sperm epigenetics and influence of environmental factors. *Mol Metab.* (2018) 14:1–11. doi: 10.1016/j.molmet.2018.02.006
154. Kiefer H, Perrier J-P. DNA methylation in bull spermatozoa: evolutionary impacts, interindividual variability, and contribution to the embryo. *Can J Anim Sci.* (2019) 100:1–16. doi: 10.1139/cjas-2019-0071
155. Liu S, Fang L, Zhou Y, Santos DJA, Xiang R, Daetwyler HD, et al. Analyses of inter-individual variations of sperm DNA methylation and their potential implications in cattle. *BMC Genomics.* (2019) 20:888. doi: 10.1186/s12864-019-6228-6
156. Naz RK, Joseph A, Lee Y, Ahmad K, Bhargava MM. Expression of scatter factor/hepatocyte growth factor is regionally correlated with the initiation of sperm motility in murine male genital tract: is scatter factor/hepatocyte growth factor involved in initiation of sperm motility? *Mol Reprod Dev.* (1994) 38:431–9. doi: 10.1002/mrd.1080380411
157. Herness EA, Naz RK. Presence and tyrosine phosphorylation of c-met receptor in human sperm. *J Androl.* (1999) 20:640–7.
158. Fang L, Zhou Y, Liu S, Jiang J, Bickhart DM, Null DJ, et al. Comparative analyses of sperm DNA methylomes among human, mouse and cattle provide insights into epigenomic evolution and complex traits. *Epigenetics.* (2019) 14:260–76. doi: 10.1080/15592294.2019.1582217
159. Zhou Y, Connor EE, Bickhart DM, Li C, Baldwin RL, Schroeder SG, et al. Comparative whole genome DNA methylation profiling of cattle sperm and somatic tissues reveals striking hypomethylated patterns in sperm. *Gigascience.* (2018) 7:giy039. doi: 10.1093/gigascience/giy039
160. Ma D-D, Wang D-H, Yang W-X. Kinesins in spermatogenesis. *Biol Reprod.* (2017) 96:267–76. doi: 10.1095/biolreprod.116.144113
161. Rathke C, Baarends WM, Awe S, Renkawitz-Pohl R. Chromatin dynamics during spermiogenesis. *Biochim Biophys Acta.* (2014) 1839:155–68. doi: 10.1016/j.bbagrm.2013.08.004
162. Govin J, Caron C, Lestrat C, Rousseaux S, Khochbin S. The role of histones in chromatin remodelling during mammalian spermiogenesis. *Eur J Biochem.* (2004) 271:3459–69. doi: 10.1111/j.1432-1033.2004.04266.x
163. de Oliveira R V, Dogan S, Belser LE, Kaya A, Topper E, Moura A, et al. Molecular morphology and function of bull spermatozoa linked to histones and associated with fertility. *Reproduction.* (2013) 146:263–72. doi: 10.1530/REP-12-0399
164. Kutchy NA, Velho A, Menezes ESB, Jacobsen M, Thibaudeau G, Wills RW, et al. Testis specific histone 2B is associated with sperm chromatin dynamics and bull fertility-a pilot study. *Reprod Biol Endocrinol.* (2017) 15:59. doi: 10.1186/s12958-017-0274-1
165. Dogan S, Vargovic P, Oliveira R, Belser LE, Kaya A, Moura A, et al. Sperm protamine-status correlates to the fertility of breeding bulls. *Biol Reprod.* (2015) 92:91–2. doi: 10.1095/biolreprod.114.124255
166. Kutchy NA, Menezes ESB, Ugur MR, Husna AU, ElDebaky H, Evans HC, et al. Sperm cellular and nuclear dynamics associated with bull fertility. *Anim Reprod Sci.* (2019) 211:106203. doi: 10.1016/j.anireprosci.2019.106203
167. Kutchy NA, Menezes ESB, Chiappetta A, Tan W, Wills RW, Kaya A, et al. Acetylation and methylation of sperm histone 3 lysine 27 (H3K27ac and H3K27me3) are associated with bull fertility. *Andrologia.* (2018) 50:e12915. doi: 10.1111/and.12915
168. Verma A, Rajput S, Kumar S, De S, Chakravarty AK, Kumar R, et al. Differential histone modification status of spermatozoa in relation to fertility of buffalo bulls. *J Cell Biochem.* (2015) 116:743–53. doi: 10.1002/jcb.25029
169. Underhill DA. Genetic and biochemical diversity in the Pax gene family. *Biochem Cell Biol.* (2000) 78:629–38. doi: 10.1139/bcb-78-5-629
170. Kamachi Y, Uchikawa M, Kondoh H. Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet.* (2000) 16:182–7. doi: 10.1016/S0168-9525(99)01955-1
171. Owens JC, Detweiler CS, Li JJ. CDC45 is required in conjunction with CDC7/DBF4 to trigger the initiation of DNA replication. *Proc Natl Acad Sci.* (1997) 94:12521–6. doi: 10.1073/pnas.94.23.12521
172. Slotman JA, Paul MW, Carofiglio F, de Gruiter HM, Vergroesen T, Koornneef L, et al. Super-resolution imaging of RAD51 and DMC1 in DNA repair foci reveals dynamic distribution patterns in meiotic prophase. *PLoS Genet.* (2020) 16:e1008595. doi: 10.1371/journal.pgen.1008595
173. Sancar A. Excision repair invades the territory of mismatch repair. *Nat Genet.* (1999) 21:247–9. doi: 10.1038/6753
174. Ji G, Long Y, Zhou Y, Huang C, Gu A, Wang X. Common variants in mismatch repair genes associated with increased risk of sperm DNA damage and male infertility. *BMC Med.* (2012) 10:49. doi: 10.1186/1741-7015-10-49
175. Nakaki F, Hayashi K, Ohta H, Kurimoto K, Yabuta Y, Saitou M. Induction of mouse germ-cell fate by transcription factors in vitro. *Nature.* (2013) 501:222–6. doi: 10.1038/nature12417
176. Seki Y. PRDM14 is a unique epigenetic regulator stabilizing transcriptional networks for pluripotency. *Front Cell Dev Biol.* (2018) 6:12. doi: 10.3389/fcell.2018.00012
177. Okashita N, Kumaki Y, Ebi K, Nishi M, Okamoto Y, Nakayama M, et al. PRDM14 promotes active DNA demethylation through the ten-eleven translocation (TET)-mediated base excision repair pathway in embryonic stem cells. *Development.* (2014) 141:269–80. doi: 10.1242/dev.099622
178. Grabole N, Tischler J, Hackett JA, Kim S, Tang F, Leitch HG, et al. Prdm14 promotes germline fate and naive pluripotency by repressing FGF signalling and DNA methylation. *EMBO Rep.* (2013) 14:629–37. doi: 10.1038/embor.2013.67
179. Mallol A, Guirola M, Payer B. PRDM14 controls X-chromosomal and global epigenetic reprogramming of H3K27me3 in migrating mouse primordial germ cells. *Epigenetics Chromatin.* (2019) 12:38. doi: 10.1186/s13072-019-0284-7
180. Yamaji M, Seki Y, Kurimoto K, Yabuta Y, Yuasa M, Shigeta M, et al. Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat Genet.* (2008) 40:1016. doi: 10.1038/ng.186
181. Yamaji M, Ueda J, Hayashi K, Ohta H, Yabuta Y, Kurimoto K, et al. PRDM14 ensures naive pluripotency through dual regulation of signaling and epigenetic pathways in mouse embryonic stem cells. *Cell Stem Cell.* (2013) 12:368–82. doi: 10.1016/j.stem.2012.12.012
182. Zhang X, Rice K, Wang Y, Chen W, Zhong Y, Nakayama Y, et al. Maternally expressed gene 3 (MEG3) noncoding ribonucleic acid: isoform structure, expression, and functions. *Endocrinology.* (2010) 151:939–47. doi: 10.1210/en.2009-0657
183. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature.* (2009) 460:473–8. doi: 10.1038/nature08162
184. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell.* (2006) 125:315–26. doi: 10.1016/j.cell.2006.02.041
185. Zindy F, den Besten W, Chen B, Rehg JE, Latres E, Barbacid M, Pollard JW, Sherr CJ, Cohen PE, Roussel MF. Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors p18(Ink4c) and p19(Ink4d). *Mol Cell Biol.* (2001) 21:3244–55. doi: 10.1128/MCB.21.9.3244-3255.2001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 09 December 2021
doi: 10.3389/fvets.2021.692874

Effects of Oral Administration of *Lepidium meyenii* on Morphology of Mice Testis and Motility of Epididymal Sperm Cells After Tetrahydrocannabinol Exposure

Adelaide Greco[1,2], Chiara Del Prete[3], Davide De Biase[4], Veronica Palumbo[3], Sandra Albanese[2], Dario Bruzzese[5], Domenico Carotenuto[6], Francesca Ciani[3], Simona Tafuri[3], Leonardo Meomartino[1], Marcello Mancini[2], Orlando Paciello[3] and Natascia Cocchia[3]*

[1] *Interdepartmental Center of Veterinary Radiology, University of Naples Federico II, Naples, Italy,* [2] *Institute of Biostructures and Bioimaging of the National Council of Research, Naples, Italy,* [3] *Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples, Italy,* [4] *Department of Pharmacy, University of Salerno, Fisciano, Italy,* [5] *Department of Public Sanity, University of Naples Federico II, Naples, Italy,* [6] *Universidad Nacional Mayor San Marcos, Lima, Peru*

OPEN ACCESS

Edited by:
Manuel Alvarez Rodriguez, Linköping University, Sweden

Reviewed by:
Pier Paolo Claudio, University of Mississippi, United States
Ali Shalizar-Jalali, Urmia University, Iran

***Correspondence:**
Veronica Palumbo
veronica.palumbo@unina.it

Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *09 April 2021*
Accepted: *17 November 2021*
Published: *09 December 2021*

Citation:
Greco A, Del Prete C, De Biase D, Palumbo V, Albanese S, Bruzzese D, Carotenuto D, Ciani F, Tafuri S, Meomartino L, Mancini M, Paciello O and Cocchia N (2021) Effects of Oral Administration of Lepidium meyenii on Morphology of Mice Testis and Motility of Epididymal Sperm Cells After Tetrahydrocannabinol Exposure. Front. Vet. Sci. 8:692874. doi: 10.3389/fvets.2021.692874

Background: Tetrahydrocannabinol (THC) administration is associated with testicular damage and reduced semen quality. Oral administration of *Lepidium Meyenii* (maca) improves spermatogenesis and sperm motility and count and reduces spermatogenic damage.

Objectives: The aim of this study was to evaluate the effect of administration of THC, maca, and their combination on testicular tissue and semen parameters.

Materials and Methods: Thirty-six-week-old male mice were classified into control, THC, Maca, and THC + Maca groups. The mice were subjected to Eco Color Doppler ultrasound examination of the testicles before and after treatment. After euthanasia, the epididymis, testes, liver, and kidney were collected for histological examination. For morphometry of the testis, tubular diameters and seminiferous epithelium height were measured. Sperm concentration and sperm motilities were assessed. Differences among the groups were assessed using the Kruskal–Wallis and Dunn's *post-hoc* test.

Results: In all the groups, there were no significant changes in testicular morphology before and after treatment. Histological assessment of the testes showed no alterations in control, no significant alterations in Maca, mild to moderate alterations in THC, and mild alterations in THC + Maca groups. Histological examination of the other organs showed no significant differences among the groups. Tubular diameter showed significantly increased thickening for THC and THC + Maca compared with that for Maca and control. Moreover, seminiferous epithelium height decreased for THC compared with that in the control, Maca, and THC + Maca groups. No statistically significant reduction in the spermatogenic index was observed for THC compared with that for Maca

and THC + Maca. Epididymal cross-sections of the groups showed no significant alterations. Sperm concentration and motility were higher for control and THC + Maca groups than in group THC and Maca.

Conclusion: *In vivo* maca administration reduced the deleterious effect of THC on testicular parenchyma and semen production.

Keywords: *Lepidium meyenii* (maca), sperm cells, THC, antioxidant, ultrasound color Doppler

INTRODUCTION

The medical properties of marijuana and cannabinoids have been widely recognized (1, 2). *Cannabis*-based medicines have proven useful in alleviating autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, and other inflammatory diseases and also play an important role in the treatment of certain neurological diseases such as Alzheimer's disease and amyotrophic sclerosis lateral (2–5). More recent research has shown the ability of *Cannabis*-based medicine to reduce the spread of neoplastic cells (4). These cannabinoids have a high safety profile in relation to the risks of acute toxicity, but not in chronic use (4). Chronic toxic effects have been recognized in reproductive performance (6, 7), and it has been scientifically demonstrated that *Cannabis sativa* and Ruta Graveolens induce hypofertility (8). Currently, the illegal use of *Cannabis sativa* and cannabinoids is widespread and growing, especially in individuals of reproductive age, for recreational, social, medical, and spiritual reasons. Simultaneously, the problems of couple hypofertility is increasing, one-third of which are due to male factors (9). Numerous studies have shown the negative effect of daily marijuana intake on male fertility in both laboratory animals (10) and humans (7).

The direct effect of prolonged exposure to cannabinoids on reproductive organs in various animal species, which interferes with the normal anatomy, histology, and function of male reproductive organs, has been evaluated. Few studies have examined the physical (morphological and histological) effects of the use of exogenous cannabinoids on reproductive organs in humans. Although Kolodny et al. (11) concluded that the chronic use of marijuana in humans does not induce changes in testicular size and histological features of the testicles themselves, numerous studies have shown the opposite. Since endocannabinoid receptor endocannabinoid signaling system (ECSs) are involved in the regulation of the male reproductive system, numerous studies have been conducted to evaluate the effect of cannabinoids on various quality parameters of the semen (7–11). The correlation between cannabinoid exposure and sperm morphological alterations has been poorly studied (7); only one study has shown that this exposure represents a risk factor for the decay of the morphological characteristics of sperm (12).

Both in humans and animals, regular exposure to *Cannabis* induces reduction in sperm concentration (SC) in the ejaculate. Furthermore, the reduction in the number of spermatozoa per ejaculate is dose-dependent (7, 13, 14).

Finally, the literature clearly demonstrates the correlation between exposure to cannabinoids and motility and sperm vitality, both *in vivo* in men and animals and *in vitro*.

Therefore, exogenous cannabinoids, which disturb the physiological homeostasis of ECS receptors, induce harmful energy-dependent effects capable of affecting potential sperm fertility (7, 13, 14).

It is widely documented that oxidative stress plays an important role in the development of hypofertility. A recent study in rats showed that the administration of antioxidants such as melatonin and vitamin C together with the intake of cannabinoids reduces the spermiotoxic effect of the latter (15).

Among the various phytotherapeutics, a tuber, *Lepidium meyenii*, known in common parlance as maca, is recognized by the Andean people and used by the Inca people for its antioxidant power and ability to improve both male and female reproductive functions (16). The aphrodisiac effect of maca as well as its power to increase the reproductive capacity of those who consume it have been scientifically verified (7, 17). Maca has been consumed in Peru for 400 years, both as food and as a medicine. In fact, it has found use in the treatment of rheumatism, respiratory problems, and hormonal imbalances, in the stimulation of metabolism and memory, as a laxative, and finally for the treatment of depression, anemia, leukemia, aids, cancer, alcoholism, and reproduction (18, 19). Other studies have shown that maca can improve the quality of Stallone seed and its refrigerability (9, 20). In addition, maca counteracts the spermiotoxic effects induced by lead acetate in rats (21). The scientific recognition of its properties has led scientists and clinicians to officially include maca as a drug for the treatment of human male hypofertility (7). The aim of this study was to explore *in vivo* the effects of Δ-9-tetrahydrocannabinol (THC) in inducing morphological and histological changes in mouse testes, evaluate sperm motility and concentration, and explore the use of maca in mitigating or boosting the *in vivo* effect of cannabinoids in mice fertility.

MATERIALS AND METHODS

Animal Procedures

The animal protocols used in this work were evaluated and approved by the Animal Use and Ethical Committee (OPBA) of CEINGE, Biotecnologie Avanzate s.c.a.r.l. (Naples, Italy) and by the Italian Ministery of Health [number of authorization 659 del 31.08.17, in accordance with FELASA guidelines and the guidelines defined by the European Communities Council Directive (2010/63/EU)]. Twenty-four C57BL/6 male mice at 6 weeks of age were purchased from Charles River Laboratories

International, Inc. and were allowed to acclimate for 2 weeks before the experiments. Mice were divided into four groups: control group (six mice) without any treatment, the first group (nine mice) received 10 mg/kg di Δ^9-THC in 0.1 ml of sesame oil subcutaneously for 30 days; the second group (10 mice) received 50 mg/kg maca *via* oral administration for 30 days, and the third group (5 mice) received 10 mg/kg di Δ^9-THC subcutaneously and 50 mg/kg maca by oral administration.

High-Frequency Ultrasound

High-frequency ultrasound equipment (Vevo 2100, VisualSonics Inc., Toronto, Ontario, Canada) with a multifrequency (30–50 MHz) probe (MicroScan™ MS550D, VisualSonics Inc., Toronto, Ontario, Canada) was used in all procedures.

Mice were divided into three groups based on the established treatment and subjected to ultrasound examination of the testicles before and after treatment. Ultrasound examination was performed under general anesthesia with isoflurane in oxygen (induction phase: 5% isoflurane in 2 L/min oxygen; maintenance phase: 2% isoflurane in 2 L/min oxygen). All ultrasound examinations were performed before treatment and one day after treatment.

Each examined animal was placed in a dorsal decubitus position on the handling table of the Vevo imaging station (Vevo Integrated Rail System III; VisualSonics Inc., Toronto, Ontario, Canada), and vital signs (temperature, heart rate, and respiratory rate) were recorded using a dedicated monitoring system. Body temperature was maintained at 36 ± 5°C *via* an infrared lamp. After positioning, the animal was tricotomized in the pubic and abdominal regions. Each ultrasound session lasted ∼45 min. For each testicle, the mediolateral, dorsoventral, and craniocaudal diameters were measured. The volume (mm^3) of each testicle was calculated using the ellipsoid formula (width × depth × length × xπ/6). Thereafter, a 3D acquisition of mouse testes was performed: a set of consecutive 2D image planes of the testicles were acquired and then automatically reconstructed into 3D views.

Vascularization of tissues within the testicles was assessed using 2D and 3D color-Doppler (36.1 mm/s velocity, 25 dB Doppler gain), and a percent vascularity value (PV%) was provided after the volume had been created (mm^3). The PV% provides the percentage of the volume that contains flow detected from the color Doppler image. All ultrasonographic assessments were performed by the same trained physician (S.A.), who was unaware of the results obtained in the previous evaluation and blinded to the mice group and pathological results.

Histopathology and Morphometry

After treatment and the last ultrasound examination, mice were euthanized with overdose of Isoflurane: Isoflurane (Isovet®, 1,000 mg/ml, EDRA S.p.A., Italy) were delivered *via* a custom fitted anesthetic machine (Vet-Equipe, Inc., Livermore, CA, USA) that allowed the direct introduction of the gas into the anesthetic chamber. Afterwards mice were subjected to cervical dislocation according to the European rules about animal experimentation.

The testes, liver, kidney, and colon were harvested and preserved in 10% neutral buffered formalin (code no. 05-01007Q, Bio-Optica, Milan, Italy), dehydrated, and embedded in paraffin (code no. 06-7920, Bio-Optica, Milan, Italy). Paraffin blocks were cut into 4-μm-thick sections and stained with hematoxylin and eosin for analysis of morphology.

For the liver, kidney, and colon histologic assessment, several parameters were semiquantitatively evaluated separately by two independent, experienced pathologists (O.P. and D.DB.) in a blinded fashion, with good concordance (Cohen's $\kappa = 0.913$, $P < 0.001$).

For the liver histological examination, three main broad categories of histological features were analyzed: steatosis, inflammation, and necrosis. The grading system was adapted from Kleiner et al. (22), as previously described (23). Kleiner's grading system considers the following histological variables: severity of steatosis (quantified by the evaluation of parenchymal involvement by steatosis): score 0, <5%; score 1, 5–33%; score 2, >33–66%, score 3, >66%; location (predominant distribution pattern): zone 3, score 0; zone 1, score 1; azonal, score 2; inflammation: lobular inflammation (overall assessment of all inflammatory foci): score 0, no foci; score 1, <2 foci per ×200 magnification field; score 2, 2–4 foci per ×200 magnification field; score 3, >4 foci per ×200 magnification field; necrosis: score 0, present; score 1, absent.

For the kidney, the examined histologic features were: (1) epithelial degeneration, (2) glomerular atrophy, (3) vascular changes, (4) stromal fibrosis, and (5) tubular atrophy. When present, the damage was evaluated semiquantitatively as 0: none, 1: mild, 2: moderate, or 3: severe (24).

For the colon, the histologic scoring system was adapted from Coretti et al. (25) as follows: (a) the severity of inflammatory cell infiltration was evaluated based on the percentage of leukocyte density in the lamina propria area and estimated in a high-power field representative of the section (0 for no signs of inflammation, 1 for minimal <10%, 2 for mild 10–25% with scattered neutrophils, 3 for moderate 26–50%, 4 for marked >51% with dense infiltrate); (b) The extent of the inflammation was estimated as expansion of leukocyte infiltration (0 for none, 1 for mucosal, 2 for mucosal and submucosal, and 3 for mucosal, submucosal, and transmural levels).

Morphometry of the testis was carried out as previously described by other authors (26, 27), with modifications. Micrographs of experimental and control animals were acquired under a light microscope (Nikon Eclipse E600) attached to a microphotography system (Nikon digital camera DMX1200). For morphometric analysis, setting scale and conversion of values from pixels to micrometers were obtained from a picture with known distance in micrometer. Transverse sections of testes with at least 20 round or nearly round seminiferous tubules were chosen randomly to measure tubular diameters and seminiferous epithelium height for each animal regardless of the stage of the seminiferous epithelium cycle (26) using images obtained at ×100 magnification. The diameter (D) of the seminiferous tubules was measured across the minor and major axes of the tubules by calculating the average of two diameters, D1 and D2. The same tissue section used for measuring tubular diameters

was used to measure the seminiferous epithelium height. For this analysis, two perpendicular lines in each field were drawn from the basement membrane (tunica propria) to the tubule lumen (luminal border). The mean of these two values was considered as the height of the seminiferous tubule.

For tubular spermatogenesis index evaluation and quantification, we applied a ten-point scoring system formulated by Johnsen (28) and used both in human and experimental pathology because of its good reproducibility (29). The Johnsen criteria were established according to the profile of the cells encountered along the seminiferous tubules, ranging from no cells to complete spermatogenesis.

Semen Collection and Evaluation

Immediately after euthanasia, the cauda epididymis and the vasa deferentia were excised. The tissues were incised and placed into a 2-ml Eppendorf with 500 μL of pre-warmed Dulbecco's phosphate-buffered saline solution (Sigma-Aldrich, Milan, Italy). Spermatozoa were allowed to swim up into the medium for at least 30 min at 35°C.

SC was determined using a Bürker chamber at phase contrast (400× magnification), and the results are presented in sperm cells/mL. Sperm motility was evaluated by placing 10 μL of pre-warmed (37°C) semen suspension between a pre-warmed slide and a coverslip. The slides were examined for total motility (%), as well as rapid and slow progressive motile sperm (%) by a blinded investigator using a phase contrast microscope (Leitz Laborlux K Microscope, Leitz, Italy) at 100× magnification and heating stage (37°C). For each sample, 10 different randomly selected fields were evaluated.

Numerical variables are reported as medians with interquartile ranges (25th, 75 percentile). Differences among groups were assessed using the Kruskal–Wallis test, followed by Dunn's *post-hoc* test. Statistical significance was set at $p < 0.05$. All analyses were conducted using the statistical platform R (ver. 4.0.1).

RESULTS

B-mode acquisition in the transverse and longitudinal planes, followed by a motor 3D-B-mode and -Color Doppler Mode reconstruction of both testicles was performed in all 24 mice before and after treatment (**Figure 1**). Mice imaged before treatment were considered as controls.

In all the groups, there were no significant changes in testicular morphology before and after treatment, nor in the echogenic structures evaluated. The testicles had a testicular volume ranging from 58.157 to 84.205 mm^3 pre-treatment and a volume of 54.959 to 85.883 mm^3 post-treatment. No significant differences were evident among the groups and between all mice pre- and post-treatment.

Similar to the tridimensional analysis, we reported the percentage of vascularization (PV%) in all the groups pre- and post-treatment (**Figure 2**). No significant differences were found among pre-treatment groups (**Figure 2**). After treatment, the THC group showed a significantly higher PV% than the Maca ($p = 0.032$) and Maca + THC group ($p = 0.004$; **Figure 2**).

Histopathology and Morphometry

Morphological analysis was performed for the 24 treated and 4 untreated C57BL/6 mice. Histological examination of formalin-fixed and paraffin-embedded sections of the liver, kidney, and cecum showed no evident histopathological changes for the selected parameters and no statistically significant difference among mice groups.

Histological assessment of testes from mice of the control group showed no alterations with a normal histoarchitecture that consisted of uniform, well-organized seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue. Seminiferous tubules had an intact epithelium with a full complement of spermatogenic cells. Mature spermatozoa filled with tubule lumens and interstitial tissue had a normal distribution of Leydig cells.

In the control group and experimental group 3 (THC + Maca), no severe and significant alterations were observed in testicular parenchyma or spermatogenesis. In experimental group 1 (THC), transverse sections of the testis showed mild to moderate pathologic modifications accounting for almost 45% of the testicular parenchyma. Pathologic findings consisted mostly of multifocal detachment of the germinal epithelium, irregular and buckled basement membrane, tubular deformation and degeneration, several shrunken seminiferous tubules, and multifocally increased luminal diameter. In experimental group 2 (Maca), transverse sections of the testis showed an overall normal histoarchitecture of the testicular parenchyma with scattered seminiferous tubules lined by intact epithelium and normal spermatogenesis. A small number of seminiferous tubules, accounting for ∼25% of the testicular parenchyma, showed mild alterations such as detachment of the germinal epithelium and a reduced population of mature spermatozoa. Representative pictures of testicular morphology in the control and experimental groups are shown in **Figures 3A–D**.

Morphometric results are summarized in **Table 1**. Morphometric measurements showed that tubular diameter significantly decreased in experimental groups 1 and 2 compared with control group and experimental group 3 ($p < 0.05$). Moreover, seminiferous epithelium height decreased significantly in experimental group 1 compared with control group and experimental groups 2 and 3 ($p < 0.01$). The spermatogenic index had a level of 10 (complete spermatogenesis with many spermatozoa) in the control group and experimental group 3, but shifted from 10 to 9 (many spermatozoa present, but germinal epithelium disorganized with marked sloughing or obliteration of lumen) in experimental groups 1 and 2. Therefore, a slight but not statistically significant reduction in the spermatogenic index was observed in experimental group 1 ($p < 0.001$) compared with experimental groups 2 and 3.

Epididymal cross-sections of control groups, as well as experimental groups 1, 2, and 3, showed no significant alterations. The epididymal lumen was filled with spermatozoa, and the epithelium showed an intact basement membrane, epididymal tubules, pseudostratified columnar epithelium, and interstitial areas.

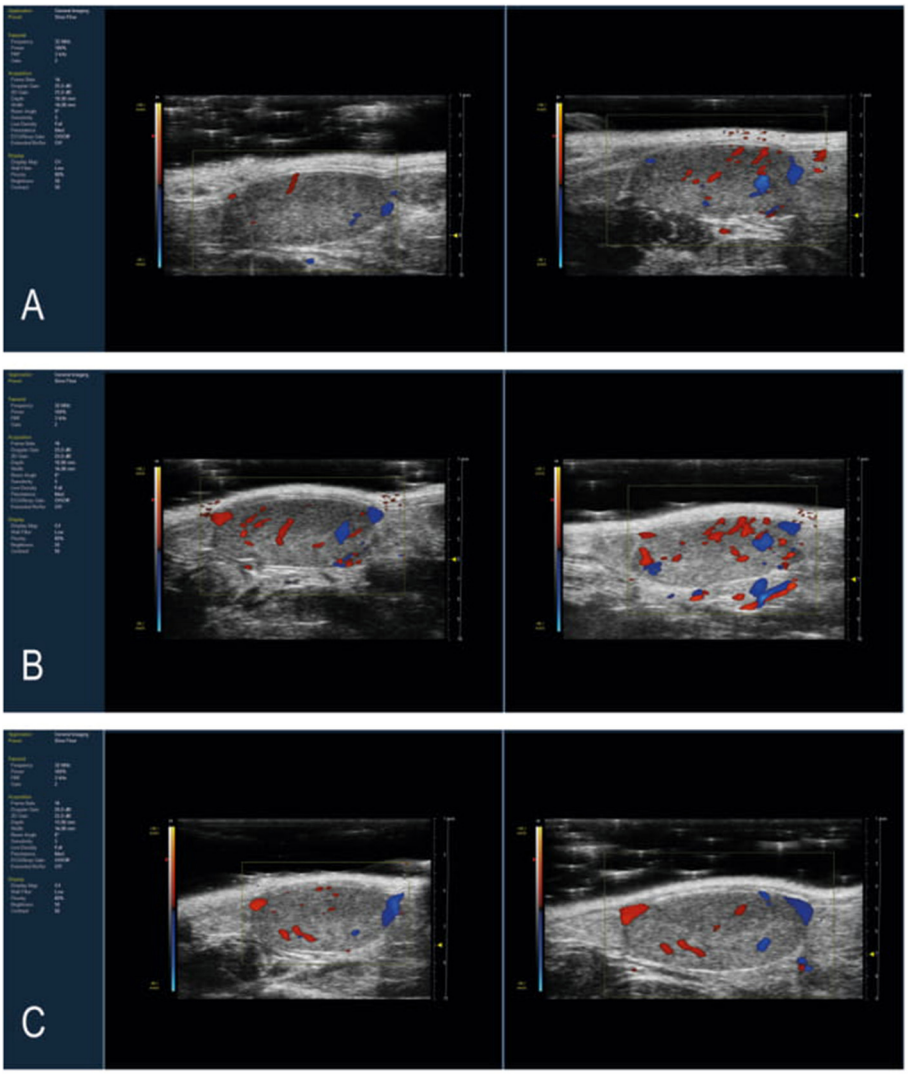


FIGURE 1 | Representative Longitudinal Scan of mouse testis with color Doppler HFUS image. Images of pre-treatment and post-treatment mice testis with **(A)** 10 mg/kg THC, **(B)** 50 mg/Kg maca, and **(C)** 10 mg/kg Δ^9-THC and 50 mg/Kg maca. After 30 days of treatment, the THC group showed more intense vascularization than the baseline. In contrast, the Maca and Maca + THC groups showed less differences in pre- and post-treatment vascularization.

Semen Parameters

Semen evaluation was performed on the 24 treated mice and in 6 untreated c57/BL/6 mice used as controls. Significant differences in all semen-related variables were found among the groups (**Figure 4**). The THC group showed a significantly lower semen concentration (23 [20; 26.5] $\times$ 10^6 spz/ml) than the Maca group (36.5 [31.5; 43.2] $\times$ 10^6 spz/ml; p = 0.015), THC + Maca (52 [46.5; 62.5] $\times$ 10^6 spz/ml; $p < 0.01$) and control groups (53 [43.5; 56.2] $\times$ 10^6 spz/ml; $p < 0.001$). Maca administration resulted in lower semen concentrations in the THC + Maca group ($p = 0.032$).

Total motility was significantly reduced in the THC group [34 (30; 35.5)] and Maca group [55.5 (50.2; 60.8)] compared with that in the control group (75 [75; 80]; $p < 0.001$ and $p = 0.036$, respectively). The THC group also showed a significantly reduced total motility compared to the Maca group ($p = 0.018$) and THC + Maca group (80 [75; 83.5]; $p < 0.001$).

The percentage of rapid progressive motile sperms was significantly reduced in the THC group (15 (10, 23)) and Maca group (39 [34; 41.5]) than in the control group (70 [65; 71.2]; $p < 0.001$ and $p = 0.004$, respectively). The THC group showed a significantly reduced percentage of rapid progressive motile sperm with respect to the Maca ($p = 0.019$) and THC + Maca group (50 [47.5; 56.5]; $p < 0.001$).

With respect to slow progressive motile sperms (%), both the control (5 [5; 6.2]) and THC groups (5 [5; 6.5]) showed significantly reduced percentages when compared with the Maca (13.5 [10; 20.8]; $p < 0.001$ for both) and THC + Maca (15 [11; 15.5]) groups ($p = 0.006$ and 0.003, respectively).

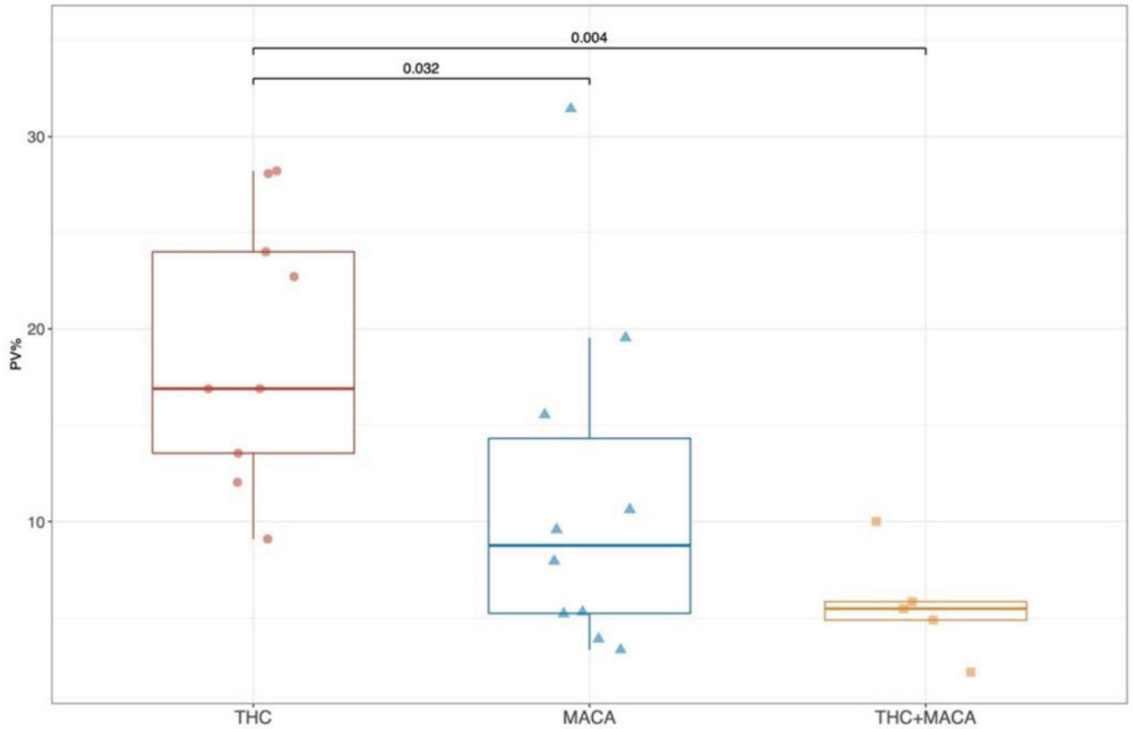


FIGURE 2 | Percentage of vascularization from color Doppler images. Boxplot showing the distribution of post-treatment PV (%) stratified by experimental groups. Boxes represent the 25th to 75th percentile, the line represents the median and the whisker represents the min and max aside outliers that are reported outside the whiskers.

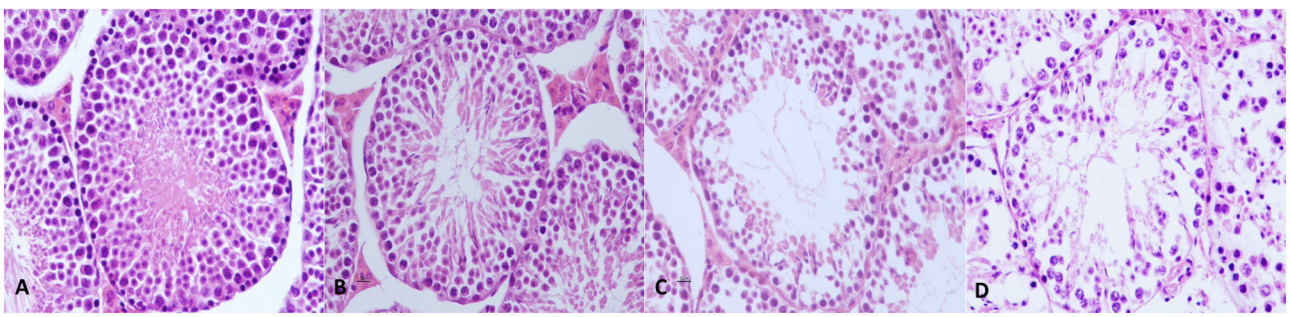


FIGURE 3 | Mouse testis from control and experimental groups. **(A)** Testes from mice of the control group showed normal histoarchitecture with uniform, well-organized seminiferous tubules, and complete spermatogenesis. **(B)** In experimental group 1, no severe and significant alterations were observed in testicular parenchyma nor in spermatogenesis. **(C)** In experimental group 2, transverse sections of the testis showed scattered mild to moderate alterations, which were present mostly in the multifocal detachment of germinal epithelium, irregular and buckled basement membrane, tubular deformation and degeneration, shrunken seminiferous tubules, and increased luminal diameter. **(D)** In experimental group 3, mild alterations such as the detachment of germinal epithelium and reduced population of mature spermatozoa are shown. Hematoxylin and eosin, original magnification 40×.

DISCUSSION

Studies on human reproduction are challenging, given ethical considerations, and the results on the impact of marijuana are confounded by socioeconomic factors and drug variability (9). Strong efforts have been made during the years to elucidate the effect of marijuana on reproduction in human and animal models (9, 30). One of the purposes of this study was to verify the effect of THC administration *in vivo* on the characteristics of epididymal mouse sperm cells.

Furthermore, studies in humans have suggested that dietary supplementation with antioxidants reduces seminal oxidative stress and improves semen quality, particularly in subfertile males (9, 31–33). Many studies have focused on the use of natural antioxidants from terrestrial plants to prevent sperm damage caused by reactive oxygen species (ROS) (34–36). Maca is a

TABLE 1 | Morphometry of the testis of control and experimental group 1 (THC), group 2 (Maca), and group 3 (THC + Maca).

Parameter	Control	Group 1 (THC)	Group 2 (Maca)	Group 3 (THC + Maca)
Seminiferous tubular diameter	215.05 ±27.6[a]	167.8 ±27.4[b]	178.8 ±15.2[b]	209.3 ±29.5[a]
Seminiferous epithelial height	72.35 ±9.87[a]	43.16 ±0.69[b]	59.8 ±2.7[a]	66.15 ±3.4[a]

Different subscript letters indicate significant differences between groups ($p < 0.05$).

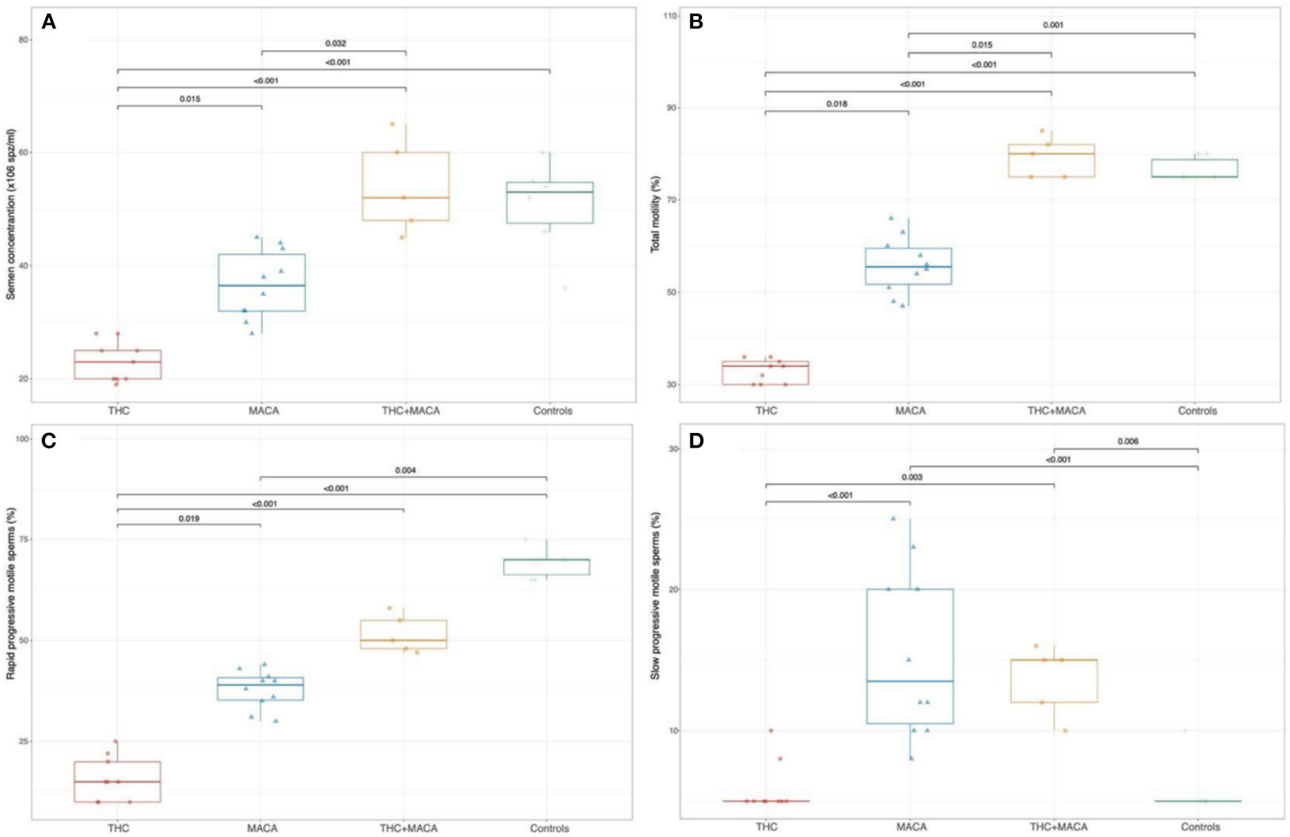


FIGURE 4 | Semen parameters in control and experimental groups. Boxplot showing the distribution of semen parameters stratified by experimental groups. Semen concentration **(A)**, total motility **(B)**, the percentage of rapid and slow progressive motile sperm **(C,D)**. Boxes represent the 25th–75th percentile, the line represents the median and the whisker represents the min and max aside outliers that are reported outside the whiskers.

traditional Andean crop used as a nutraceutical for the fertility-enhancing properties associated with its antioxidant activity (37, 38). Moreover, horses with maca dietary supplementation showed an improvement in semen quality during cooling by protecting testicular cell membranes and mitochondria from oxidative stress (9). To date, the *in vivo* effects of THC combined with maca have not been explored. Ultrasound examination is the imaging technique of choice to explore *in vivo* morphology and vascularization of soft tissue (39), including the testis, in experimental animal models of disease (7, 40).

In our study, *in vivo* ultrasound examination showed the absence of grossly morphologic alterations in mice treated with THC, maca, and the combination of the two treatments. However, we found a significant improvement in the percentage of vascularization in mice treated with THC. This could be attributed to the vascular congestion in the seminiferous tubules of testes, also reported by others (41). We speculate that the administration of THC in animal models leads to an early stage of vascular congestion and subsequently, to vascular damage, especially for a prolonged administration of up to 6 months. However, the increase in the percentage of vascularization by itself can induce hyperthermia and consecutively hypofertility. The PV % decreases when mice are treated with maca, and this is even more evident when mice are treated with THC and maca. Maca is confirmed to have a beneficial effect related to the decrease in oxidative stress, which could explain the improvement of the fertility of mice treated with both maca and THC. Finally, maca used alone did not demonstrate the same effect in improving vascularization *in vivo* (42).

Histological evaluations was performed in order to identify possible alterations subsequent to the oral administration of THC and/or maca. The analysis of the selected organs other than testis did not reveal any abnormalities. The effect of THC and Maca on spermatogenesis were evaluated by morphometric parameters and morphological evaluation of testis histology using tubular spermatogenesis index, already reported in literature (26, 27). Those analysis revealed very mild to moderate alterations in parenchymal cytoarchitecture and spermatogenesis in experimental groups compared to the controls. The administration of THC affected the spermatogenesis mostly at the stage of spermiation, showing detachment of the germinal epithelium, exfoliation of spermatocytes, multifocally increased luminal diameter, and a slight reduction in spermatogenesis. However, maca administration seems to reverse the effect of THC on spermatogenesis. Similar results were found after administration of lead acetate plus maca (21).

In line with the morphometric evaluation of seminiferous tubules, the *in vitro* semen evaluation showed a drastic reduction in semen concentration and a loss of sperm motility, confirming the negative effect of THC on male fertility. Despite a large number of recent studies, the results of whether THC affects the ability of sperm to fertilize and generate embryos remains unclear, and the effects of cannabinoids are controversial. The association between the chronic use of THC and abnormalities in sperm count, concentration, motility, and morphology, as well as structural changes in the testis in humans, has been widely reported and reviewed in the literature (9, 43, 44). However, a recent study conducted in male mice showed opposite results, with no negative effect of THC on the male reproduction process (45). Furthermore, the reduction in motility and ATP in sperm treated with THC was dose-dependent (45–48). The mechanism by which THC induces sperm damage is still under investigation. THC activates cannabinoid receptors, which are part of the endogenous endocannabinoid system. This system is a relatively novel system located in the hypothalamus, pituitary, and gonads in both sexes and is involved in spermatogenesis and sperm function (44, 49). The negative effects of THC on testicular morphology and spermatogenesis may depend on the modulation of cannabinoid receptors that are present on Sertoli and Leydig cells and that modulate the balance of molecular signaling and nurturing the microenvironment (50). Modulation of cannabinoid receptors such as CB1 (on Leydig cells) and CB2 (on Sertoli cells) have been suggested to induce local reduction of testosterone production and apoptosis of Sertoli cells, respectively, hence affecting sperm development (51, 52). In the last few years, a growing amount of data has underlined the potential role of oxidative stress in the mechanism of action of THC (53, 54). The risk of stroke in young *Cannabis* users has recently been correlated with the generation of reactive ROS, leading to oxidative stress (53). Moreover, a recent study conducted *in vivo* on rats showed that THC induced cerebral mitochondrial dysfunction and increased hydrogen peroxide production (54). Since oxidative stress is involved in male infertility, different studies have examined the role of this stress in *Cannabis*-associated sperm alterations (55, 56). These studies confirmed the implication of oxidative stress in *Cannabis*-induced spermatotoxicity (55, 56). In our study, oral administration of maca (group 2) and the combination of THC and maca (group 3) interestingly showed little to no pathologic effect on testis and spermatogenesis. However, these data were not completely corroborated by the *in vitro* evaluation of semen, which revealed a harmful effect of maca on SC and sperm motility. Indeed, other investigators have observed a beneficial effect of maca administration on spermatogenesis in mice, improving sperm count and motility (7, 9, 16, 57). Meanwhile, the use of only maca reduced sperm motility and concentration, and the supplementation of mice receiving THC with maca improved sperm characteristics.

Based on results, authors can hypothesize different mechanisms used by Maca to reverse the deleterious effect of THC. Since mice treated with THC plus Maca have similar histological results to control group, maca could protect the testis from spermatogenic disruption caused by THC preventing apoptosis of the developing germ cells and improving the number of cells progress through the spermatogenesis. Previous study suggests that Maca reversed deleterious effects due lead acetate on spermatogenesis by protecting onset of mitosis and spermiation (21).

Positive effect of Maca is correlated with its antioxidant effect that reduces THC-associated sperm damage caused by oxidative stress. Similar ameliorative effects in *Cannabis sativa*-associated spermiotoxicity were reported with the use of other antioxidants, such as a combination of melatonin and vitamin C (15, 55). The negative effect of the administration of maca on semen can be due to an alteration of the endogenous antioxidant systems by this antioxidant. Oxidative stress caused by ROS is physiologically balanced by endogenous antioxidant systems. The authors' hypothesis is that in these mice, under physiological conditions, antioxidant supplementation is not necessary because the balance between pro-oxidants and antioxidants is already in place for the natural evolution of sperm physiology. The addition of antioxidants in the diet or in the semen-targeted improvement of semen production and quality should take into account the endogenous production of antioxidants, which varies greatly between individuals (9, 58, 59). There is a limitation of the study and potential bias caused by the subjective evaluation of sperm motility.

In conclusion, this study confirmed that the oral administration of maca prevents the harmful effect of THC on mouse spermatogenesis and spermatozoa features, and it lends further credibility to the hypothesis that Maca could be an alternative treatment for male infertility. In order to evaluate the biological activity of maca during oral supplementation, successive studies should be carried out on redox status measurements and reproductive hormonal modifications in treated mice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Use and Ethical Committee (OPBA) of CEINGE, Biotecnologie Avanzate s.c.a.r.l. (Na-ples, Italy) and by the Italian Ministry of Health [number of authorization 659 del 31.08.17, in accordance with FELASA guidelines and the guidelines defined by the European Communities Council Directive (201 0/63/EU)].

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This study was supported by a grant from the Department of Veterinary Medicine and Animal Productions University of Naples Federico II, Italy, in partnership with the Interdepartmental Center of Veterinary Radiology, University of Naples Federico II, and CEINGE, Advanced Biotechnology, University of Naples Federico II.

ACKNOWLEDGMENTS

We thank the Euro-BioImaging facility at the Institute of Biostructures and Bioimaging (CNR), Naples for help with Imaging experiments.

REFERENCES

1. Birdsall SM, Birdsall TC, Tims LA. The use of medical marijuana in cancer. *Curr Oncol Rep.* (2016) 18:40. doi: 10.1007/s11912-016-0530-0
2. Hill KP. Medical marijuana for treatment of chronic pain and other medical and psychiatric problems: a clinical review. *JAMA.* (2015) 313:2474–83. doi: 10.1001/jama.2015.6199
3. Solimini R, Rotolo MC, Pichini S, Pacifici R. Neurological disorders in medical use of Cannabis: an update. *CNS Neurol Disord Drug Targets.* (2017) 16:527–33. doi: 10.2174/1871527316666170413105421
4. Tkaczyk M, Florek E, Piekoszewski W. Marihuana and cannabinoids as medicaments. *Przegladlekarski.* (2011) 69:1095–7.
5. Whiting PF, Wolff RF, Deshpande S, Di Nisio M, Duffy S, Hernandez AV, et al. Cannabinoids for medical use: a systematic review and meta-analysis. *JAMA.* (2015) 313:2456–73. doi: 10.1001/jama.2015.6358
6. Brents LK. Marijuana, the endocannabinoid system and the female reproductive system. *Yale J Biol Med.* (2016) 89:175–91.
7. Gonzales GF, Gonzales-Castañeda C, Gasco M. A mixture of extracts from Peruvian plants (black maca and yacon) improves sperm count and reduced glycemia in mice with streptozotocin-induced diabetes. *Toxicol Mech Methods.* (2013) 23:509–18. doi: 10.3109/15376516.2013.785656
8. Sailani MR, Moeini H. Effect of Ruta graveolens and *Cannabis sativa* alcoholic extract on spermatogenesis in the adult wistar male rats. *Indian J Urol.* (2007) 23:257–60. doi: 10.4103/0970-1591.33720
9. Fronczak CM, Kim ED, Barqawi AB. The insults of illicit drug use on male fertility. *J Androl.* (2012) 33:515–28. doi: 10.2164/jandrol.110.011874
10. Park B, McPartland JM, Glass M. Cannabis, cannabinoids and reproduction. *Prostaglandins Leukot Essent Fatty Acids.* (2004) 70:189–97. doi: 10.1016/j.plefa.2003.04.007
11. Kolodny RC, Masters WH, Kolodner RM, Toro G, Depression of plasma testosterone levels after chronic intensive marihuana use. *N Engl J Med.* (1974) 290:872–4. doi: 10.1056/NEJM197404182901602
12. Amoako AA, Marczylo TH, Marczylo EL, Elson J, Willets JM, Taylor AH, et al. Anandamide modulates human sperm motility: implications for men with asthenozoospermia and oligoasthenoteratozoospermia. *Hum Reprod.* (2013) 28:2058–66. doi: 10.1093/humrep/det232
13. Hsiao P, Clavijo RI. Adverse effects of cannabis on male reproduction. *Eur Urol Focus.* (2018) 4:324–8. doi: 10.1016/j.euf.2018.08.006
14. Bari M, Battista N, Pirazzi V, Maccarrone M. The manifold actions of endocannabinoids on female and male reproductive events. *Front Biosci (Landmark Ed).* (2011) 16:498–516. doi: 10.2741/3701
15. Alagbonsi IA, Olayaki LA, Salman TM. Melatonin and vitamin C exacerbate *Cannabis sativa*-induced testicular damage when administered separately but ameliorate it when combined in rats. *J Basic Clin Physiol Pharmacol.* (2016) 27:277–87. doi: 10.1515/jbcpp-2015-0061
16. Gonzales GF, Cordova A, Vega K, Chung A, Villena A, Gonez C. Effect of *Lepidium meyenii* (Maca) a root with aphrodisiac and fertility-enhancing properties, on serum reproductive hormone levels in adult healthy men. *J Endocrinol.* (2003) 176:163–8. doi: 10.1677/joe.0.1760163
17. Valentová K, Frcek J, Ulrichová J. Yacon (*Small anthussonchifolius*) and MACA (*Lepidium meyenii*), traditional Andean crops as new functional foods on the European market. *Feedback.* (1997) 91.
18. Kilham, C. What is Maca? *Total Health.* (2000) 22:48–9. doi: 10.1080/1206212X.2000.11441599
19. Wang Y, Wang Y, McNeil B, Harvey LM. Maca: an Andean crop with multi-pharmacological functions. *Food Res Int.* (2007) 40:783–92. doi: 10.1016/j.foodres.2007.02.005
20. Del Prete C, Tafuri S, Ciani F, Pasolini MP, Ciotola F, Albarella S, et al. Influences of dietary supplementation with *Lepidium meyenii* (Maca) on stallion sperm production and on preservation of sperm quality during storage at 5°C. *Andrology.* (2018) 6:351–61. doi: 10.1111/andr.12463
21. Rubio J, Riqueros MI, Gasco M, Yucra S, Miranda S, Gonzales GF. *Lepidium meyenii* (Maca) reversed the lead acetate induced-damage on reproductive function in male rats. *Food Chem Toxicol.* (2006) 44:1114–22. doi: 10.1016/j.fct.2006.01.007
22. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* (2005) 41:1313–21. doi: 10.1002/hep.20701
23. Annunziata C, Lama A, Pirozzi C, Cavaliere G, Trinchese G, Di Guida F, et al. Palmitoylethanolamide counteracts hepatic metabolic inflexibility modulating mitochondrial function and efficiency in diet-induced obese mice. *FASEB J.* (2020) 34:350–64. doi: 10.1096/fj.201901510RR
24. Aktoz T, Durmus-Altun G, Usta U, Torun N, Ergulen A, Atakan IH. Radioiodine-induced kidney damage and protective effect of amifostine: an experimental study. *Hippokratia.* (2012) 16:40–5.
25. Coretti L, Cristiano C, Florio E, Scala G, Lama A, Keller S. Sex-related alterations of gut microbiota composition in the BTBR mouse model of autism spectrum disorder. *Sci Rep.* (2017) 7:45356. doi: 10.1038/srep45356
26. Mehraein F, Negahdar F. Morphometric evaluation of seminiferous tubules in aged mice testes after melatonin administration. *Cell J (Yakhteh).* (2011) 13:1.
27. Abarikwu SO, Wokoma AF, Mgbudom-Okah CJ, Omeodu SI, Ohanador R. Effect of Fe and Cd co-exposure on testicular steroid metabolism, morphometry, and spermatogenesis in mice. *Biol Trace Elem Res.* (2019) 190:109–23. doi: 10.1007/s12011-018-1536-2
28. Johnsen SG. Testicular biopsy score count-a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Horm Res Paediatr.* (1970) 1:2–25. doi: 10.1159/000178170
29. Teixeira TA, Pariz JR, Dutra RT, Saldiva PH, Costa E, Hallak J. Cut-off values of the Johnsen score and Copenhagen index as histopathological prognostic factors for postoperative semen quality in selected infertile patients undergoing microsurgical correction of bilateral subclinical varicocele. *Transl Androl Urol.* (2019) 8:346. doi: 10.21037/tau.2019.06.23

30. D'Angelo D, Ciani F, Zaccherini A, Tafuri S, Avallone L, D'Ingeo S, et al. Human-animal relationship dysfunction: a case study of animal hoarding in Italy. *Animals*. (2020) 10:1501. doi: 10.3390/ani10091501
31. Esposito L, Auletta L, Ciani F, Pelagalli A, Pasolini MP, Lamagna B, et al. Hair cortisol levels in captive brown hare (*Lepus europaeus*): potential effect of sex, age, and breeding technology. *Eur J Wildl Res.* (2017) 63:62. doi: 10.1007/s10344-017-1121-6
32. Ross C, Morriss A, Khairy M, Khalaf Y, Braude P, Coomarasamy A, et al. A systematic review of the effect of oral antioxidants on male infertility. *Reprod Biomed Online.* (2010) 20:711–23. doi: 10.1016/j.rbmo.2010.03.008
33. Wong WY, Thomas CM, Merkus JM, Zielhuis GA, Steegers-Theunissen RP. Male factor subfertility: possible causes and the impact of nutritional factors. *Fertil Steril.* (2000) 73:435–42. doi: 10.1016/S0015-0282(99)00551-8
34. Patil RB, Vora SR, Pillai MM. Antioxidant effect of plant extracts on phospholipids levels in oxidatively stressed male reproductive organs in mice. *Int J Reprod Biomed.* (2009) 7:35.
35. Tempest HG, Homa ST, Routledge EJ, Garner A, Zhai XP, Griffin DK. Plants used in Chinese medicine for the treatment of male infertility possess antioxidant and anti-oestrogenic activity. *Syst Biol Reprod Med.* (2008) 54:185–95. doi: 10.1080/19396360802379073
36. Zhong RZ, Zhou DW. Oxidative stress and role of natural plant derived antioxidants in animal reproduction. *J Integr Agric.* (2013) 12:1826–38. doi: 10.1016/S2095-3119(13)60412-8
37. Tafuri S, Cocchia N, Carotenuto D, Vassetti A, Staropoli A, Mastellone V, et al. Chemical analysis of *Lepidium meyenii* (Maca) and its effects on redox status and on reproductive biology in stallions. *Molecules.* (2019) 24:1981. doi: 10.3390/molecules24101981
38. Tafuri S, Cocchia N, Vassetti A, Carotenuto D, Esposito L, Maruccio L, et al. *Lepidium meyenii* (Maca) in male reproduction. *Nat Prod Res.* (2019) 35:4550–9. doi: 10.1080/14786419.2019.1698572
39. Greco A, Mancini M, Gargiulo S, Gramanzini M, Claudio PP, Brunetti A, et al. Ultrasound biomicroscopy in small animal research: applications in molecular and preclinical imaging. *J Biomed Biotechnol.* (2012) 2012:519238. doi: 10.1155/2012/519238
40. Celebi M, Paul AG. Assessment of ischaemia-reperfusion injury in the mice testis by using contrast ultrasound molecular imaging. *Andrologia.* (2016) 48:907–13. doi: 10.1111/and.12531
41. Nosarieme OA. *Cannabis sativa* (Marijuana) alters blood chemistry and the cytoarchitecture of some organs in Sprague Dawley rat models. *Food Chem Toxicol.* (2018) 116:292–7. doi: 10.1016/j.fct.2018.04.023
42. Solinas M, Massi P, Cantelmo AR, Cattaneo MG, Cammarota R, Bartolini D, et al. Cannabidiol inhibits angiogenesis by multiple mechanisms. *Br J Pharmacol.* (2012) 167:1218–31. doi: 10.1111/j.1476-5381.2012.02050.x
43. Carroll K, Pottinger AM, Wynter S, DaCosta V. Marijuana use and its influence on sperm morphology and motility: identified risk for fertility among Jamaican men. *Andrologia.* (2020) 8:136–42. doi: 10.1111/andr.12670
44. Gundersen TD, Jorgensen N, Andersson AM, Bang AK, Nordkap L, Skakkebæk NE, et al. Association between use of marijuana and male reproductive hormones and semen quality: a study among 1,215 healthy young men. *J. Epidemiol.* (2015) 182:473–81. doi: 10.1093/aje/kwv135
45. López-Cardona AP, Ibarra-Lecue I, Laguna-Barraza R, Pérez-Cerezales S, Urigüen L, Agirregoitia N, et al. Effect of chronic THC administration in the reproductive organs of male mice, spermatozoa and *in vitro* fertilization. *Biochem Pharmacol.* (2018) 157:294–303. doi: 10.1016/j.bcp.2018.07.045
46. Aktoz T, Durmus-Altun G, Usta U, Torun N, Ergulen A, Atakan IH. Radioiodine-induced kidney damage and protective effect of amifostine: an experimental study. *Hippokratia.* (2012) 16:40–5.
47. Badawy ZS. Cannabinoids inhibit the respiration of human sperm. *Fertil Steril.* (2009) 91:2471–6. doi: 10.1016/j.fertnstert.2008.03.075
48. Morgan DJ, Muller CH, Murataeva NA, Davis BJ, Mackie K. Δ9-Tetrahydrocannabinol (Δ9-THC) attenuates mouse sperm motility and male fecundity. *Br J Pharmacol.* (2012) 165:2575–83. doi: 10.1111/j.1476-5381.2011.01506.x
49. Karasu T, Marczylo TH, Maccarrone M, KonjeIC. The role of sex steroid hormones, cytokines and the endocannabinoid system in female fertility. *Hum Reprod Update.* (2011) 17:347–61. doi: 10.1093/humupd/dmq058
50. Rajanahally S, Raheem O, Rogers M. The relationship between cannabis and male infertility, sexual health, and neoplasm: a systematic review. *Andrology.* (2019) 7:139–47. doi: 10.1111/andr.12585
51. Maccarrone M, Finazzi-Agro A. The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis. *Cell Death Differ.* (2003) 10:946–55. doi: 10.1038/sj.cdd.4401284
52. Gye MC, Kang HH, Kang HJ. Expression of cannabinoid receptor 1 in mouse testes. *Arch Androl.* (2005) 51:247–55. doi: 10.1080/014850190898845
53. Archie SR, Cucullo L. Harmful effects of smoking cannabis: a cerebrovascular and neurological perspective. *Front Pharmacol.* (2019) 10:1481. doi: 10.3389/fphar.2019.01481
54. Wolff V, Schlagowski AI, Rouyer O, Charles A-L, Singh F, Auger C, et al. Tetrahydrocannabinol induces brain mitochondrial respiratory chain dysfunction and increases oxidative stress: a potential mechanism involved in cannabis-related stroke. *Biomed Res Int.* (2015) 2015:323706. doi: 10.1155/2015/323706
55. Alagbonsi IA, Olayaki LA. Role of oxidative stress in *Cannabis sativa*-associated spermatotoxicity: evidence for ameliorative effect of combined but not separate melatonin and vitamin C. *Middle East Fertil Soc J.* (2017) 22:136–44. doi: 10.1016/j.mefs.2016.12.004
56. Mandal TK, Das NS. Testicular toxicity in cannabis extract treated mice: association with oxidative stress and role of antioxidant enzyme systems. *Toxicol Ind Health.* (2010) 26:11–23. doi: 10.1177/0748233709354553
57. Yucra S, Gasco M, Rubio J, Nieto J, Gonzales GF. Effect of different fractions from hydroalcoholic extract of Black Maca (*Lepidium meyenii*) on testicular function in adult male rats. *Fertil Steril.* (2008) 89:1461–7. doi: 10.1016/j.fertnstert.2007.04.052
58. Del Prete C, Stout T, Montagnaro S, Pagnini U, Uccello M, Florio P, et al. Combined addition of superoxide dismutase, catalase and glutathione peroxidase improves quality of cooled stored stallion semen. *Anim Reprod Sci.* (2019) 210:106195. doi: 10.1016/j.anireprosci.2019.106195
59. Del Prete C, Ciani F, Tafuri S, Pasolini MP, Della Valle G, Palumbo V, et al. Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs. *J Vet Sci.* (2018) 19:667–75. doi: 10.4142/jvs.2018.19.5.667

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership