frontiers | Frontiers in Animal Science

TYPE Original Research
PUBLISHED 03 January 2025
DOI 10.3389/fanim.2024.1479602

OPEN ACCESS

EDITED BY
Muhammet Rasit Ugur,
IVF Michigan Fertility Centers, United States

REVIEWED BY
Mustafa Hitit,
Kastamonu University, Türkiye
Kampon Kaeoket,
Mahidol University, Thailand

*CORRESPONDENCE
Samia Khnissi
mleilsamia@live.fr

RECEIVED 12 August 2024
ACCEPTED 11 December 2024
PUBLISHED 03 January 2025

CITATION
Khnissi S, Maaloul R, Chalouati H, Mara L, Aouadi D, Adouani B, Dattena M, Khémiri I and Fattouch S (2025) Nanotechnology-assisted cryopreservation of ovine semen: evaluation of *Thymus vulgaris* essential oil as a natural antioxidant.
Front. Anim. Sci. 5:1479602.
doi: 10.3389/fanim.2024.1479602

Nanotechnology-assisted cryopreservation of ovine semen: evaluation of *Thymus vulgaris* essential oil as a natural antioxidant

Samia Khnissi[1]*, Rania Maaloul[1], Hela Chalouati[1], Laura Mara[2], Dorra Aouadi[3], Basma Adouani[4], Maria Dattena[2], Ikram Khémiri[4] and Sami Fattouch[5]

[1]Laboratory of Animal and Forage Production, National Institute of Agronomic Research of Tunisia (INRAT), University of Carthage, Tunis, Tunisia, [2]Department of Animal Science, Agricultural Research Agency of Sardinia, Olmedo, Italy, [3]Independent Researcher, Tunisia, [4]Laboratory of Mycology, Pathology and Biomarkers, Department of Biology, Faculty of Sciences of Tunis, University of Tunis El Manar, Tunis, Tunisia, [5]EcoChemistry Laboratory, National Institute of Applied Sciences and Technology (INSAT), University of Carthage, Tunis, Tunisia

The objective of the present study was to evaluate the protective effects of nanoemulsion of thyme (*Thymus vulgaris*) essential oil (EO) during the liquid state preservation of ram semen. Storage media were prepared: one containing Thymus vulgaris essential oil (Th), another with its nanoemulsion form (NTh), and a control sample without essential oil (Dovx). The quality of the semen diluted in the prepared media after preservation at 4°C and 15°C was examined by measuring the two parameters indicative of semen quality: semen motility and progressivity refer to the ability of ram sperm cells to move actively and efficiently, which is crucial for successful fertilization. The results showed a decrease in the mobility and progressivity of the sperm in all the mediums, but the thyme EO and its nanoemulsion showed slight decreases compared to the control medium ($p<0.05$). On the other hand, the study identified a negative impact of thyme EO nanoemulsions on catalase concentrations, potentially leading to mobility inhibition ($p<0.05$). In addition, the nanoemulsion significantly ($p<0,05$) decreased the malondialdehyde concentration, increased the total protein and the glucose contents, the lactate dehydrogenase activity. A significant decrease in calcium content was observed ($p < 0.05$). The essential oil of *Thymus vulgaris*, combined with nanotechnology encapsulation as a delivery method (nanoemulsion), demonstrated a notable and effective role in enhancing the preservation of ovine semen.

KEYWORDS

semen, cryopreservation, ram, *Thymus vulgaris*, nanoemulsion

1 Introduction

The preservation of germ cells plays a pivotal role in the realm of farm animal reproduction, enabling the strategic use of spermatozoa for artificial insemination. This conservation not only has localized applications but also facilitates the global distribution of semen, allowing a single ejaculate to be divided into multiple doses for inseminating several females, thereby streamlining selective breeding (Aponte et al., 2024). Cryopreservation is a cornerstone of these efforts, allowing spermatozoa to be stored at ultralow temperatures for extended periods without significant loss of functionality (Augusto et al., 2020).

Moreover, beyond its applications in agriculture, sperm conservation plays a pivotal role in biodiversity conservation. By safeguarding the genetic heritage of endangered species, cryoconservation provides a lifeline for these populations, ensuring the preservation of their unique genetic traits (Di Iorio et al., 2023). Furthermore, sperm cryopreservation serves as a valuable tool in addressing infertility issues in animal breeding programs, offering new opportunities for assisted reproductive technologies and ensuring the continued success of breeding initiatives. However, the biology of sperm cryopreservation reveals that spermatozoa are highly susceptible to damage from environmental and chemical stressors (Khan et al., 2021). This sensitivity to conservation processes poses challenges, particularly in ovine species, where detrimental impacts on motility and viability hinder practical applications. Traditional methods like dilution, cooling, and freezing can cause irreversible damage, diminishing fertilization potential (Lv et al., 2019). Moreover, oxidative stress from reactive oxygen species (ROS) formation disrupts the oxidant/antioxidant balance. In response to these challenges, researchers worldwide are actively engaged in developing innovative conservation technologies. Nanotechnology enables the development of nanosized carriers like liposomes, polymeric nanoparticles, and nanoemulsions that protect antioxidants from degradation and enhance targeted delivery (Sova et al., 2021). Studies highlight its role in increasing antioxidant bioavailability by over 40% compared to conventional methods, as reported in recent pharmaceutical research (Mishra et al., 2022). The integration of natural substances with antioxidant properties, such as essential oils, into the composition of preservation media, presents a natural solution to enhance the quality parameters of preserved sperm. Recent studies demonstrated the antioxidant potential of nanoemulsified *Thymus vulgaris* essential oil (Ismail et al., 2020). This essential oil, rich in thymol and carvacrol, exhibits antioxidant and antimicrobial properties, enhancing sperm motility and viability during cryopreservation (Kchikich et al., 2024). In this context, the present study aims to advance sperm conservation technologies by incorporating nanotechnology of encapsulation with antioxidant-rich in *Thymus vulgaris* essential oil, into nanoemulsions. This innovative approach seeks to address cellular damage caused by oxidative stress and free radicals, ultimately improving the efficacy of sperm preservation methods. This study seeks to advance sperm preservation techniques by utilizing nanotechnology-based encapsulation to incorporate the antioxidant-rich Thymus vulgaris essential oil into nanoemulsions. This innovative strategy aims to mitigate cellular damage caused by oxidative stress and free radicals by improving the targeted delivery of active molecules to cells. The research hypothesizes that combining the antioxidant properties of natural products like Thymus vulgaris essential oil with nanoemulsion delivery systems can reduce oxidative stress and enhance the cryopreservation outcomes for ovine sperm, which is particularly susceptible to thermal shock.

2 Materials and methods

The experiment took place as part of collaboration between the Laboratory of Animal and Forage Productions (INRAT), the EcoChemistry Laboratory (INSAT), and the AGRIS Laboratory (Regional Agency for Agricultural Research in Sardinia).

2.1 Plant material and essential oil (EO) extraction

T. vulgaris plants were freshly collected from Aïn Draham, Jendouba Governorate (North of Tunisia, the temperature average is 18.6°C and the precipitation average is 825.4 mm) in spring. Leaves were dried at room temperature and subjected to hydrodistillation for 3 hours with distilled water using a Clevenger-type apparatus. Distilled EO was dried over anhydrous sodium sulfate, filtered, and stored in opaque bottles at 4°C.

2.2 Analysis of phytocompounds and antioxidant activity

The procedures for analyzing total phenol content (TPC) were adopted from previous research (Singleton and Rossi, 1965). Similarly, total flavonoid content (TFC) was analyzed using the methods described in earlier studies (Kim et al., 2003). Duplicate samples of the *Thymus vulgaris* EO were tested. The antioxidant activity of *Thymus vulgaris* essential oil was evaluated using the anionic radical (DPPH) scavenging activity method, as outlined in certain earlier research reports (Marinova and Batchvarov, 2011).

The essential oil was tested at three concentrations (2, 5, and 10 mg of *Thymus vulgaris* EO/mL) within a solution composed of 70% ethanol and 30% distilled water. This approach allowed for a thorough examination of TPC, TFC, and DPPH values, providing insights into the essential oil's antioxidant potential across different concentrations within the specified dilution medium.

2.3 Animals

The experimental protocols were approved by the Animal Care and Use Committee at the University of Sassari, Italy. All procedures took place at the experimental facilities of the Department of Animal Production, AGRIS Sardegna, Bonassai,

Sassari, Italy (coordinates 40° 40′ 26″ N, 8° 22′ 1″ E), which comply with the European Union's standards for Scientific Procedure Establishments. The experiments adhered to ethical guidelines outlined in EC Directive 86/609/EEC for animal experiments. Sarda ewes and rams were housed outdoors with indoor access and provided with a maintenance ration based on live weight. All chemicals and media used were obtained from Sigma Chemical Co. (Germany), unless stated otherwise. To meet the objectives of this experiment, we used 15 adult rams (Average of age: 20 ± 3.4 months; Average of weight: 61.1 ± 2.2; Average of testes circumference: 32.57 ± 0,89) for sperm production and a single ewe for the collection process.

2.4 Nanoemulsion preparation

In view of studying the impact of nanoemulsions as a delivery system for antioxidant bioactive compounds, the optimization of nanoemulsion preparation was conducted using the design of experiment (DOE) procedure, particularly the centered mixture design method (Maiza et al., 2020). Ten mixtures were analyzed by measuring the average diameter of droplets dispersed in the nanoemulsions. Only the optimal mixture, *Thymus vulgaris* essential oil-based nanoemulsion (NTh) was selected for the rest of the study to evaluate stability over 4 days. Based on a previous study conducted at INSAT (National Institute of Applied Sciences and Technology), the mixtures had a final volume of 4000 µL while fixing the volume of the aqueous phase at 55% distilled water (2200 µL) and the volume of a certain component of the lipid phase at 15% *Thymus vulgaris* essential oil (600 µL) and 7.5% Tween 80 (300 µL), which were the most stable. The remaining volume (900 µL) was determined using the centered mixture design statistical approach by adding a combination ranging from 0% to 22.5% of Tween 80 and Tween 20 as surfactants and sorbitol as a co-surfactant as shown in Table 1. The percentages of components in each mixture were summed up to 100%.

TABLE 1 Composition of the 10 mixtures of thyme essential oil nano-emulsion (NTh).

Mixtures	Quantity of Tween80	Quantity of Tween20	Quantity of sorbitol
1	900 µl	0 µl	0 µl
2	0 µl	900 µl	0 µl
3	0 µl	0 µl	900 µl
4	450 µl	450 µl	0 µl
5	450 µl	0 µl	450 µl
6	0 µl	450 µl	450 µl
7	300 µl	300 µl	300 µl
8	600 µl	150 µl	150 µl
9	150 µl	600 µl	150 µl
10	150 µl	150 µl	600 µl

The oil phase, consisting of 600 µL of *Thymus vulgaris*, 300 µL of Tween 80, additional amounts of both surfactants, Tween 20 and Tween 80, and co-surfactant (sorbitol) mixtures determined by the centered mixture design method, were mixed in a vial for 10 minutes using a magnetic stirrer at 1500 rpm and heating at 40° C. Subsequently, 2200 µL of distilled water, as the aqueous phase, was added to the oil phase for NT. The mixture was stirred under the same conditions for an additional 30 minutes. The coarse emulsion was then covered and sonicated in a sonication bath for 10 minutes at 40°C using high-intensity ultrasound at 20 kHz. All components used in nanoemulsion preparation were preheated in a water bath set at 40°C. The resulting 10 nanoemulsions were numbered and used for the rest of the study (Donsì et al., 2012).

The stability of the 10 designed nanoemulsions was assessed over 4 days (Day 0, Day 2, and Day 4) by visually observing their macroscopic appearance. The average droplet diameter of the NTh was determined using ImageJ software (on Day 4). This parameter is crucial for both preparation and evaluation, influencing the mixture's stability, digestive processes, lipolysis, release of molecules of interest, and subsequent absorption. For diameter measurement, a drop of each sample was placed between a slide and cover slip using a micropipette, and the microscope's X100 objective was used to capture images for later analysis. ImageJ software, which distinguishes droplets in emulsions based on color contrast, was used to calculate diameters in nanometers. The diameters were calculated using the formula:

$$\text{Diameter(nm)} = 2\sqrt{(\text{Surface}/\pi)};\ \left(\text{Surface is determined in nm}^2\right)$$

2.5 Collection of semen using an artificial vagina

Semen collections were carried out using an artificial vagina. The method involved causing the ram to ejaculate into the artificial vagina during mounting. The artificial vagina provides all the conditions of the natural vagina during mating: the temperature should be approximately 38 to 39°C, pressure was maintained by insufflation of warm water through the faucet opening, and lubrication was achieved with a substance insoluble in seminal plasma and non-toxic to sperm (Vaseline).

2.6 Sperm preservation

After the analysis and approval of the semen, all ejaculates from different rams were mixed in a single tube. The collected sperm was divided into 5 aliquots and diluted using various prepared media.

2.7 Preparation of conservation media

A serial dilution was conducted to produce three conservation media with various components, as shown in Table 2.

TABLE 2 Conservation media formulations for effective preservation.

Media	Media composition
Thyme essential oil media (Th)	IMV Ovixcell = 9.9 ml 0.01% thyme essential oil = 1uL 1% DMSO = 100Ul
Thyme essential oil nano-emulsion (NTh)	IMV Ovixcell = 9.9 ml 6.66 µl thyme essential oil nano-emulsion /10 ml diluent 1% DMSO = 100uL
Control media (Dovx)	IMV Ovixcell = 10 ml 1% DMSO = 100uL

Th, *Thymus vulgaris* essential oil; NTh, Nanoemulsion of *Thymus vulgaris*.

2.8 Liquid storage

Following the assessment of initial parameters (volume, concentration, mobility), 5 tubes were taken, and in each tube, 0.5 mL of semen was combined with 0.5 mL of each preservation medium. Subsequently, the semen is stored at 15°C (in a refrigerator) for evaluation after 2 hours, 4 hours, and 6 hours, and at 4°C (in a temperature-controlled incubator) for measurements after 2 hours, 24 hours, and 48 hours of preservation.

2.9 Evaluation of parameters after preservation

2.9.1 Sperm analysis

Utilizing the Computer-Assisted Sperm Analysis (CASA) with CEROS II automated systems evaluation by Hamilton–Thorne (USA). Sperm quality assessment is done with high precision using HT CASA software, unique color-coded illumination to optimize identification of the sperm head and tail and space-saving sperm analyzer with external phase contrast microscope included. This allowed for a thorough evaluation of spermatozoa movements without manual intervention, streamlining the assessment of sperm mobility (Rijsselaere et al., 2004; Van de Hoek et al., 2022).

2.10 Oxidative stress and biochemical parameters

The oxidative stress and biochemical parameters were assessed using specific protocols and references. Oxidative stress parameters, such as Malondialdehyde (MDA), were measured using the TBARS method with absorbance at 530 nm as previously described (Gdara et al., 2018). Catalase activity was assessed through spectrophotometric measurement at 240 nm, as previously detailed (Aebi, 1984).

In terms of biochemical parameters, glucose levels were determined enzymatically at 505 nm. Total proteins were measured using a colorimetric method at 546 nm, with references to certain earlier research (Gornall et al., 1949). Calcium levels were quantified using the *o*-Cresol phthalein complexone method at 570 nm (CPC), following the procedure outlined by certain researchers (Stern and Geary, 1957). Lactate dehydrogenase (LDH) levels were determined kinetically at 340 nm following the method described in previous research (Bergmeyer and Rozalskis, 1975).

2.11 Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). GraphPad Prism 10 software (GraphPad Software Inc, San Diego, CA) was used for all statistical analyses. Data as sperm parameters, semen biochemical and oxidatif stress parameters were analyzed using one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's test, as recommended by the GraphPad software. The threshold for statistical significance was set to $p < 0.05$.

3 Results

3.1 Bioactive compounds content and antioxidant activity of *Thymus vulgaris* EO

The analysis of *Thymus vulgaris* essential oil (EO) was conducted at different concentrations (2, 5, and 10 mg of Th/mL) focusing on Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity measured through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

According to Table 3 which depicts the bioactive compounds and antioxidant capacity of *Thymus vulgaris*, the essential oil exhibited a concentration-dependent increase in TPC, with values ranging from 9.96 to 13.28 mg GAE/g ($p < 0.05$)., indicating richness in phenolic compounds and potential health benefits associated with antioxidant activity. Similarly, TFC shows an increasing, rising from 3.835 to 5.465 mg GAE/g at elevated concentrations ($p < 0.05$)., signifying a higher concentration of flavonoids. The radical trapping activity, assessed by DPPH CE50, inversely related to antioxidant activity, ranges from 2.1 to 2.2 mg/mL for *Thymus vulgaris* essential oil. The extract at 10 mg/mL displays the most powerful DPPH scavenging activity with a CE50 of 2.1 mg/mL, emphasizing its potent antioxidant properties ($p < 0.05$).

TABLE 3 Bioactive compounds and antioxidant capacity of *Thymus vulgaris* essential oil.

Concentration (mg of T. EO /ml)	TPC (mg GAE /g)	TFC (mg GAE/g)	DPPH CE50 (mg/ml)
2	10.57 ± 0,1135	4.29 ± 0,01175	2.2
5	9.96 ± 0,02548	3.835 ± 0,02958	2.2
10	13.28 ± 0,1668	5.465 ± 0,01175	2.1

Values are presented as the mean ± SEM for each group. Th, *Thymus vulgaris* essential oil; TPC, Total Phenolic Content; TFC, Total Flavonoid Content; DPPH CE50, The radical trapping activity; GAE, Gallic Acid Equivalent.

3.2 Characteristics of nanoemulsion

Following the centered mixture design approach, ten distinct mixtures of Th nanoemulsions were formulated, and the average diameter of the dispersed droplets in each mixture was analyzed. This systematic approach aimed to determine the optimal proportion of nanoemulsions leading to a minimal mean diameter and a stable structure. The results of these measurements are presented in the Table 4. According to Table 4, which details the average diameters of (NTh) observed values based on surfactant and co-surfactant content (Tween 80, Tween 20, and sorbitol): Mixtures 1, 5, and 8, consisting of pure Tween 80, Tween 80 and sorbitol only, and Tween 80, sorbitol, and Tween 20 with a dominance of Tween 80, exhibited the smallest average diameter (<50 nm). Similarly, mixtures composed of 100% Tween 20, equal amounts of Tween 20, Tween 80, and sorbitol, and only Tween 20 and Tween 80 (mixtures 2, 7, and 4, respectively) showed a reduced average diameter (50-70 nm).

The highest diameters were observed when using pure sorbitol (mixture 3) and excluding Tween 80 while using equal amounts of Tween 20 and sorbitol (mixture 6), reaching 125-150 nm and >220 nm, respectively. However, with a high sorbitol concentration (>15%), regardless of the quantity of Tween 20 and Tween 80, the diameter increased proportionally with the amount of sorbitol (>150 nm).

3.3 Analysis of sperm parameters

Figure 1 depicts the percentage of sperm mobility over time at 4°C and 15°C under various treatments ($p<0.001$). According to panels A and B, a decrease in the percentage of sperm mobility over time was observed, with improved mobility in the medium containing *Thymus vulgaris* essential oil (T. EO) and *Thymus vulgaris* nanoemulsion at 15°C even after 5 hours ($p<0.05$).

The percentage of progressive spermatozoa is illustrated in Figures 2A, B, showing better progressivity in the medium with *Thymus vulgaris* according to the findings, enhanced progressivity was noted in the medium with *Thymus vulgaris* EO nanoemulsion at 4°C after 2 until 24 hours ($p<0.05$). Our results suggest that different extenders preserve motility parameters during the initial storage hours. It is noteworthy that extenders did not behave the same at the two studied refrigeration temperatures.

TABLE 4 Mean droplet diameter of the 10 mixtures.

Mixtures	Mean Diameter (nm)
1	46,307
2	65,125
3	145,344
4	48,976
5	54,117
6	220,779
7	73,836
8	25,418
9	96,736
10	84,983

3.4 Oxidative stress parameters in semen

Malondialdehyde (MDA) is a marker of lipid peroxidation, a process where reactive oxygen species react with lipids in cell membranes, leading to the formation of MDA, which is produced through the degradation of polyunsaturated fatty acids, serves as a reliable indicator to determine the extent of peroxidation reaction and lipid damage (Asadpour et al., 2011). The results presented in Figure 3 reveal a decrease in MDA concentrations ($p<0.05$) in sperm preserved with various antioxidants compared to the control (DOvx). Among these, the inclusion of *Thymus vulgaris* essential oil (Th) yielded the most significant decrease, with levels at 49% of the control sample (DOvx). Conversely, the addition of NTh showed a result nearly equivalent to that of the control (DOvx).

Catalase (CAT) is an antioxidant enzyme crucial for defending cells against oxidative stress. It functions by breaking down hydrogen peroxide (H_2O_2) into water and oxygen, neutralizing this reactive oxygen species and preventing potential cellular damage (Nandi et al., 2019). Analysis of the results recorded in Figure 4 revealed variability in catalase content ($p<0.05$) in seminal plasma among different antioxidants. The catalase concentration notably decreases, exhibiting a reduction of approximately 87.5% compared to the control (DOvx) when using the nanoemulsion of *Thymus vulgaris* essential oil as an antioxidant, dropping from 0.8 x 10-6 UI mg-1 P to 2.9 x 10-7 UI mg-1 P.

3.5 Biochemical analysis of some parameters in semen

A comprehensive analysis of calcium, protein, glucose, and lactate dehydrogenase (LDH) content in the seminal plasma of rams subjected to diverse antioxidant treatments is shown in Table 5. The recorded results unveil significant variability in LDH content across different antioxidants. Particularly, the introduction of *Thymus vulgaris* essential oil (EO) leads to a significantly elevated LDH value, representing an increase of 55.88% compared to the control. Seminal plasma treated with *Thymus vulgaris* essential oil nanoemulsion (NTh) and *Thymus vulgaris* essential oil individually exhibited lower glucose levels, with reductions of approximately 13.30% and 15.97%, respectively, compared to the control (Dovx), which shows the highest glucose content. The observed variations in glucose levels are deemed significant, shedding light on the efficacy of added antioxidants.

Analyzing the recorded results further uncovers variability in protein content within seminal plasma treated with diverse antioxidants. *Thymus vulgaris* essential oil nanoemulsion demonstrates the highest protein content, showing an increase of approximately 54.07% compared to the control, while *Thymus*

10.3389/fanim.2024.1479602

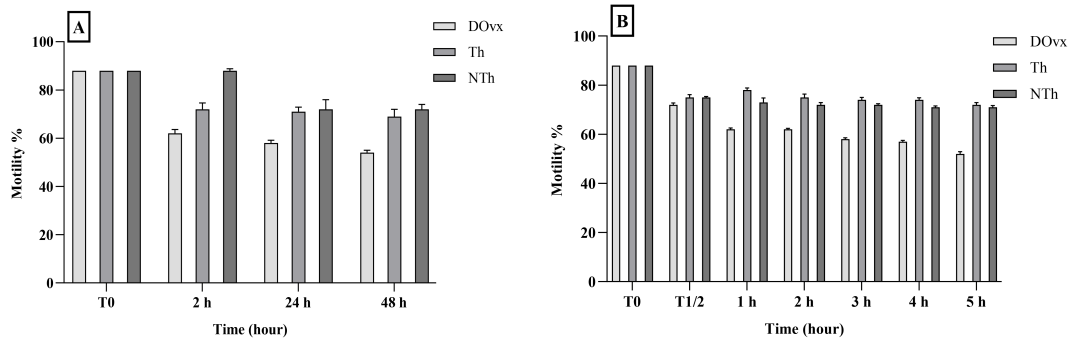

FIGURE 1
The percentage of sperm mobility over time at 4°C and 15°C under various treatments. **(A)** Variation in the sperm motility at 4°C during 48 hours. **(B)** Variation in the sperm motility at 15°C during 5 hours. Th: Thyme essential oil, NTh: Thyme essential oil nanoemulsion, Dovx: DMSO + Ovixcell (control media).

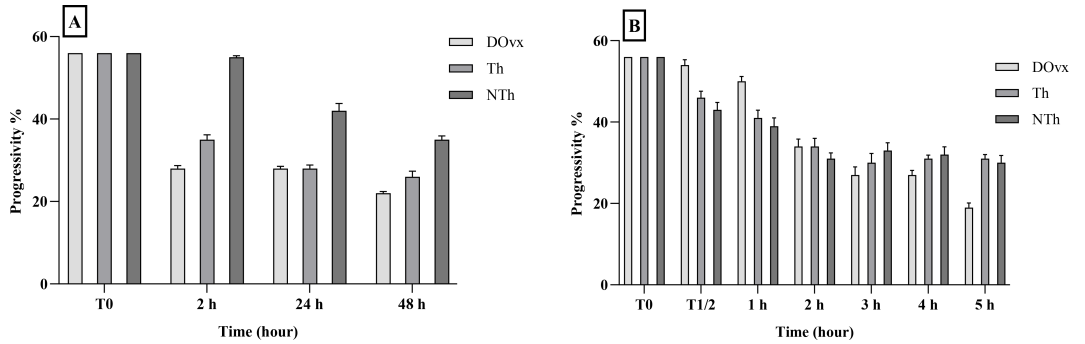

FIGURE 2
The percentage of sperm progressivity over time at 4°C and 15°C under various treatments. **(A)** Variation in the sperm progressivity at 4°C during 48 hours. **(B)** Variation in the sperm progressivity at 15°C during 5 hours. Th: Thyme essential oil, NTh: Thyme essential oil nanoemulsion, Dovx: DMSO + Ovixcell (control media).

vulgaris essential oil individually exhibits a protein content increase of about 42.35% relative to the control. The statistical analysis revealed a substantial difference ($p \leq 0.05$) in protein content attributed to the addition of antioxidants.

Moreover, an intriguing observation emerges concerning calcium content in seminal plasma treated with *Thymus vulgaris* essential oil without nanoemulsion, showcasing the highest calcium levels, with an increase of approximately 36.39% compared to the

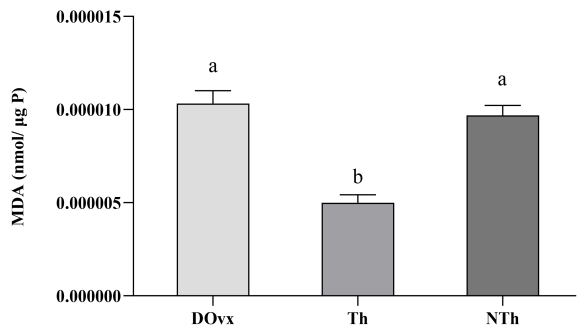

FIGURE 3
MDA concentration in the seminal plasma of rams with various treatments. Different letters (a, b) indicate the statistically significant difference ($p<0.05$) between the different concentrations of MDA in different media. Th: Thyme essential oil, NTh: Thyme essential oil nanoemulsion, Dovx: DMSO + Ovixcell (control media).

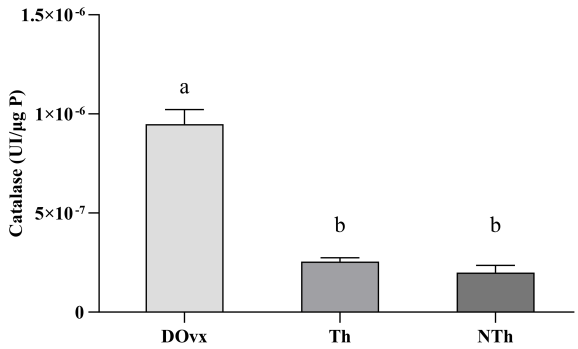

FIGURE 4
Catalase concentration in the seminal plasma with various treatments. Different letters (a, b) indicate the statistically significant difference ($p<0.05$) between the different concentrations of CAT in different media. Th: Thyme essential oil, NTh: Thyme essential oil nanoemulsion, Dovx: DMSO + Ovixcell (control media).

TABLE 5 Concentrations of biochemical components in different media.

Media	Calcium mg/l	Glucose g/l	Protein g/l	LDH U/l
Dovx	115.844 ± 1.049^{c}	1.315 ± 0.030^{a}	11.954 ± 0.314^{c}	52.615 ± 1.787^{b}
Th	158.017 ± 1.440^{a}	1.105 ± 0.060^{b}	17.016 ± 0.604^{b}	82.017 ± 2.363^{a}
NTh	134.545 ± 3.488^{b}	1.14 ± 0.076^{b}	18.412 ± 0.380^{a}	60.352 ± 2.363^{b}

Values are presented as the mean ± SEM for each group. Dovx, DMSO+ Ovixcell; Th, *Thymus vulgaris*; NTh, *Thymus vulgaris* essential oil nanoemulsion. Different letters (a,b,c) indicate the statistically significant difference ($p<0.05$) between the different concentrations of MDA in different media. Conversely, identical letters signify no statistically significant difference.

control (Dovx). Conversely, the nanoemulsion demonstrates a slight reduction in calcium content compared to *Thymus vulgaris* essential oil alone, showing a decrease of about 16.13%. The results underscore significant variations ($p<0.05$) in calcium levels among the studied rams, emphasizing the intricate influence of antioxidant treatments on this particular parameter.

4 Discussion

Antioxidant treatments aimed at preventing sperm damage during cryopreservation often yield conflicting results in studies. While some experiments have reported protective effects against cryo-induced oxidative damages (Riesco et al., 2021), others have failed to demonstrate significant effects, with some even showing impairment of sperm function (Bahmyari et al., 2020).

Free radicals can induce changes in sperm composition by activating intracellular pathways that regulate processes such as chromatin condensation, motility, capacitation, acrosome reaction, and chemotaxis (Aprioku, 2013). However, excessive levels of reactive oxygen species (ROS) can have detrimental effects on sperm, leading to reduced concentration, motility, and fertilization potential. Thus, ROS act as a double-edged sword (Dutta et al., 2019). Although semen possesses an antioxidant system, its activity is compromised during cryopreservation, leading to increased lipid peroxidation (Catalán et al., 2024). Therefore, supplementation with natural antioxidants may be insufficient to prevent lipid peroxidation during freezing and thawing. Consequently, the addition of antioxidants to the extender used in cryopreservation may have beneficial effects (Banday et al., 2017).

When considering antioxidant treatment, it is crucial to note that each type of ROS could be neutralized by a specific antioxidant system (Aprioku, 2013). Thus, a random selection of antioxidants may not effectively reduce oxidative damage. The protective role of natural antioxidants against oxidative stress in avian species has been demonstrated in various studies, suggesting their potential utility in mitigating oxidative damage in sperm (Ratchamak et al., 2023). *Thymus vulgaris* and its essential oil are highlighted for their effectiveness as supplements in rooster sperm cryopreservation extenders (Ros-Santaella and Pintus, 2021).

In our study, we investigated the potential of *Thymus vulgaris* essential oil nanoemulsion as a supplement in cryopreservation extenders to mitigate oxidative damage. Remarkably, high levels of *Thymus vulgaris* essential oil at a concentration of 10 mg/mL showed positive effects, restoring quality parameters to levels comparable to the control group. However, it is worth noting that excessive scavenging of free radicals by antioxidants may have negative effects by altering physiological levels.

The study focused on optimizing nanoemulsion preparation for delivering antioxidant bioactive compounds using a centered mixture design method. Higher levels of Tween 80 and lower levels of Tween 20 and sorbitol in different formulations, resulted in smaller droplet sizes. These findings align with previous research (Arianto and Cindy, 2019), which demonstrated stable nanoemulsions with reduced diameters using similar surfactant compositions.

Among the non-ionic surfactants studied, Tween 80 showed notable efficacy in stabilizing nanoemulsions and reducing particle sizes, depending on its concentration within the formulation. This observation is consistent with previous studies (Maccelli et al., 2019), which reported a decrease in particle size with increasing Tween 80 concentration. Furthermore, surfactant type played a significant role in droplet characteristics, with Tween 20 leading to larger droplets and broader size distribution compared to Tween 80, likely due to differences in their hydrophilic-lipophilic balance (Xue et al., 2014).

While some research reports (Ezzat Ahmed et al., 2020) suggested a potential relationship between nanoemulsion stability and *Thymus vulgaris* concentration, this study noted a consistent reduction in droplet diameter over time, potentially influenced by structural or compositional changes in the nanoemulsion components.

From a physiological perspective, our results indicated that incorporating of *Thymus vulgaris* essential oil and of *Thymus vulgaris* oil's nanoemulsion into a sperm preparation improved total and progressive sperm motility when compared to the control group. This is consistent with prior research demonstrating the positive impact of *Thymus vulgaris* essential oil on sperm characteristics like motility and viability (Lounas et al., 2020).

Thymus vulgaris essential oil has been shown to chelate iron and decrease ROS production under stressful conditions. Therefore, supplementation with *Thymus vulgaris* essential oil and its nanoemulsion in the cryopreservation medium may alleviate cooling-induced stress, leading to increased sperm progressivity and motility (Gumus et al., 2017). Our study also underlines the sperm progressive improvement in the nanoemulsion of *Thymus vulgaris* essential oil medium at 4°C after 24 hours and sustained motility at 15°C even after 5 hours, suggesting its potential as a cryoprotective agent.

Our study has adopted an approach to the study of the relation between sperm variables and MDA levels. Malondialdehyde (MDA), a marker of lipid peroxidation and oxidative stress, decreased in sperm samples preserved with various antioxidants compared to the control. Interestingly, *Thymus vulgaris* essential oil

demonstrated the lowest MDA concentration, indicating its potential to reduce oxidative damage during cryopreservation.

Catalase, a pivotal antioxidant, plays a crucial role in neutralizing reactive oxygen species (ROS) and protecting sperm from oxidative damage (Rubio-Riquelme et al., 2019). Studies have demonstrated the presence of catalase in sperm, emphasizing its significance in counteracting oxidative stress-induced sperm damage (Yang et al., 2022).

In our study, we observed a significant variability in catalase concentration in seminal plasma among different antioxidants. Notably, the addition of *Thymus vulgaris* essential oil nanoemulsion as an antioxidant led to a decrease in catalase concentration compared to the control. This data illustrates the best progressivity and motility, possibly due to the positive impact of NTh on reducing oxidative stress in seminal plasma.

However, these outcomes might be attributed to our assessments being conducted after a two-week treatment period, coinciding with the phase of antioxidant activity depletion (Shaw et al., 2022). This discovery contradicts earlier research that linked higher catalase levels with enhanced sperm motility and progressive movement, indicating intricate relationships between antioxidants and sperm function (Lounas et al., 2020). Lactate dehydrogenase (LDH), an enzyme crucial for sperm metabolism, exhibited variability in activity with different antioxidants. While previous studies (Qiu et al., 2016) have reported higher LDH levels in certain breeds of rams, our results showed lower LDH activity in seminal plasma mixed with *Thymus vulgaris* essential oil and other antioxidants. Reduced LDH activity may signify disruptions in sperm function and metabolism (Wu et al., 2021), highlighting the need for further investigation into the effects of antioxidants on sperm biochemistry. Our biochemical analysis extends to glucose levels in seminal plasma, revealing a significant increase with antioxidant supplementation. The nanoemulsion of *Thymus vulgaris* essential oil emerges as particularly effective, showcasing the highest glucose content.

These results align with studies emphasizing the role of glucose in sustaining sperm motility during liquid sperm storage (Zhang et al., 2022), shedding light on the potential benefits of specific antioxidants in supporting energy metabolism.

Variability in seminal plasma protein levels is observed with different antioxidants (Bubenickova et al., 2020), among which *Thymus vulgaris* essential oil nanoemulsion and *Thymus vulgaris* essential oil show the highest protein content, in turn motility, and progressivity. This indicates their efficacy in combating oxidative stress, maintaining protein integrity, and potentially improving sperm motility and progressivity. Moreover, elevated concentrations of specific proteins such as kallikrein and angiotensin-converting enzyme (ACE) can enhance sperm function by improving motility and capacitation, essential for successful reproduction, through interactions with sperm membranes and receptors (Moura et al., 2018).

However, some research, such as a study by (Khnissi et al., 2023), suggests a contrasting view. They found that elevated levels of stallion seminal plasma proteins are associated with decreased total and progressive sperm motility, indicating a potential negative impact.

Calcium levels in seminal plasma show significant variation with antioxidant treatment, emphasizing the complex interplay between antioxidants and sperm physiology. *Thymus vulgaris* essential oil, when used without nanoemulsion, enhance calcium levels, possibly due to its direct effects on cellular processes or calcium channels. The introduction of nanoemulsion alongside *Thymus vulgaris* essential oil may alter its bioavailability or cellular uptake, resulting in a slight reduction in calcium content compared to the non-nanoemulsified form. Comparisons with existing studies (Casao et al., 2012) underscore the need for nuanced interpretations and a deeper exploration of how antioxidants modulate calcium levels in sperm.

In conclusion: Our comprehensive study navigates the multifaceted landscape of antioxidant influence on sperm variables and oxidative stress. The intricate interplay between antioxidants, catalase, LDH, MDA, glucose, protein, and calcium underscores the need for nuanced interpretations and further research. These findings contribute to the evolving discourse on optimizing antioxidant formulations for enhancing sperm quality and fertility potential. Further researches are warranted to optimize antioxidant formulations and elucidate their mechanisms of action in protecting sperm from oxidative stress-induced damage.

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 animal study was approved by ethics committee specifically dedicated to animal experimentation in Tunisia (CEEA). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. RM: Software, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. HC: Software, Validation, Writing – original draft, Writing – review & editing. LM: Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. DA: Methodology, Software, Writing – original draft. BA: Conceptualization, Formal

analysis, Methodology, Writing – review & editing. MD: Supervision, Validation, Visualization, Writing – review & editing. IK: Conceptualization, Data curation, Methodology, Writing – review & editing. SF: Funding acquisition, Software, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was funded by financial resources of the Ministry of Higher Education and Scientific Research (MHES), Tunisia; as part of the Prima project (BIOMEnext).

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

References

Aebi, H. (1984). Catalase *in vitro*. *Methods Enzymol.* 105, 121–126. doi: 10.1016/S0076-6879(84)05016-3

Aponte, P. M., Gutierrez-Reinoso, M. A., and Garcia-Herreros, M. (2024). Bridging the gap: animal models in next-generation reproductive technologies for male fertility preservation. *Life.* 14, 17. doi: 10.3390/life14010017

Aprioku, J. S. (2013). Pharmacology of free radicals and the impact of reactive oxygen species on the testis. *J. Reprod. Infertility* 14, 158–172. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC3911811/pdf/JRI-14-158.pdf.

Arianto, A., and Cindy, C. (2019). Preparation and evaluation of sunflower oil nanoemulsion as a sunscreen. *Open Access Macedonian J. Med. Sci.* 7, 3757. doi: 10.3889/oamjms.2019.497

Asadpour, R., Jafari, R., and Tayefi, N. H. (2011). Effect of various levels of catalase antioxidant in semen extenders on lipid peroxidation and semen quality after the freeze-thawing bull semen. *Veterinary Res. Forum.* 2 (4), 218-221. Available at: https://cabidigitallibrary.org/terms-and-conditions.

Augusto, C., Kulus, D., Kaviani, B., and Vicente, E. F. (2020). Cryopreservation of agronomic plant germplasm using vitrification-based methods: an overview of selected case studies. *Int. J. Mol. Sci.* 22, 6157. doi: 10.3390/ijms22116157

Bahmyari, R., Zare, M., Sharma, R., Agarwal, A., and Halvaei, I. (2020). The efficacy of antioxidants in sperm parameters and production of reactive oxygen species levels during the freeze-thaw process: A systematic review and meta-analysis. *Andrologia* 52, e13514. doi: 10.1111/and.13514

Banday, M. N., Lone, F. A., Rasool, F., Rashid, M., and Shikari, A. (2017). Use of antioxidants reduce lipid peroxidation and improve quality of crossbred ram sperm during its cryopreservation. *Cryobiology* 74, 25–30. doi: 10.1016/j.cryobiol.2016.12.008

Bergmeyer, H. U., and Rozalskis, G. (1975). The Km of malate dehydrogenase from pig heart with oxaloacetate as substrate (author's transl). *Z. fur Klinische Chemie und Klinische Biochemie.* 13, 509–509. Available at: https://pubmed.ncbi.nlm.nih.gov/1240703/.

Bubenickova, F., Postlerova, P., Simonik, O., Sirohi, J., and Sichtar, J. (2020). Effect of seminal plasma protein fractions on stallion sperm cryopreservation. *Int. J. Mol. Sci.* 21 (17), 6415. doi: 10.3390/ijms21176415

Casao, A., Luna, C., Perez-Pe, R., Muino-Blanco, T., and Cebrian-Perez, J. A. (2012). Receptor mediated effect of melatonin on ram sperm calcium patterns. *Reprod. Domest. Animals.* 47, 1439–0531. doi: 10.1111/j.1439-0531.2012.02044.x

Catalán, J., Llavanera, M., Barranco, I., Yeste, M., and Miró, J. (2024). Impact of seminal plasma antioxidants on DNA fragmentation and lipid peroxidation of frozen–thawed horse sperm. *Antioxidants* 13, 322. doi: 10.3390/antiox13030322

Di Iorio, M., Rusco, G., Esposito, S., Roncarati, A., and Iaffaldano, N. (2023). The role of semen cryobanks for protecting endangered native salmonids: Advantages and perspectives as outlined by the LIFE Nat. Sal.Mo. Project on Mediterranean brown trout (Molise region – Italy). *Front. Mar. Sci.* 9, 1075498. doi: 10.3389/fmars.2022.1075498

Donsì, F., Annunziata, M., Vincensi, M., and Ferrari, G. (2012). Design of nanoemulsion-based delivery systems of natural antimicrobials: Effect of the emulsifier. *J. Biotechnol.* 159, 342–350. doi: 10.1016/j.jbiotec.2011.07.001

Dutta, S., Majzoub, A., and Agarwal, A. (2019). Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab J. Urol.* 17, 87–97. doi: 10.1080/2090598X.2019.1599624

Ezzat Ahmed, A., Alkahtani, M. A., and Abdel-Wareth, A. A. A. (2020). Thyme leaves as an eco-friendly feed additive improves both the productive and reproductive performance of rabbits under hot climatic conditions. *Vet. Med-Czech* 65, 553–563. doi: 10.17221/42/2020-VETMED

Gdara, I., Zrafi, I., Balducci, C., Cecinato, A., and Ghrabi, A. (2018). Seasonal occurrence, source evaluation and ecological risk assessment of polycyclic aromatic hydrocarbons in industrial and agricultural effluents discharged in Wadi El Bey (Tunisia). *Environ. Geochemistry Health.* 40, 1609–1627. doi: 10.1007/s10653-018-0075–2

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177, 751–766. doi: 10.1016/S0021-9258(18)57021-6

Gumus, R., Ercan, N., and Imik, H. (2017). The effect of thyme essential oil (Thymus vulgaris) added to quail diets on performance, some blood parameters, and the antioxidative metabolism of the serum and liver tissues. *Braz. J. Poultry Sci.* 19, 297–304. doi: 10.1590/1806-9061-2016-0403

Ismail, A. A., Abdel-Khalek, E., Khalil, W. A., Yousif, A. I., Saadeldin, I. M., Abomughaid, M. M., et al. (2020). 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.* 97, 144–152. doi: 10.1016/j.cryobiol.2020.09.002

Kchikich, A., Kirschvink, N., Raes, M., El Otmani, S., Chebli, Y., Bister, J., et al. (2024). Carvacrol and thymol enhance the quality of beni arouss buck semen stored at 4°C thanks to their antimicrobial properties. *Veterinary Sci.* 11, 406. doi: 10.3390/vetsci11090406

Khan, I. M., Cao, Z., Liu, H., Khan, A., Rahman, S. U., Khan, M. Z., et al. (2021). Impact of cryopreservation on spermatozoa freeze-thawed traits and relevance OMICS to assess sperm cryo-tolerance in farm animals. *Front. Veterinary Science.* 8, 609180. doi: 10.3389/fvets.2021.609180

Khnissi, S., Bomboi, G., Khémiri, I., Ben Salem, I., Dattena, M., Sai, S., et al. (2023). Incorporation of fresh leaves ofwormwood (Artemisia herba alba) and/or rosemary (Rosmarinusofficinalis) in the diet of rams: Effect on testicular function, sexual behavior, and blood parameters. *Food Sci. &Nutrition* 11, 3121–3130. doi: 10.1002/fsn3.3293

Kim, D. O., Jeong, S. W., and Lee, C. Y. (2003). Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chem.* 81, 321–326. doi: 10.1016/S0308-8146(02)00423-5

Lounas, M., Harichane, M., and Gasmi, S. N. (2020). Caractérisations des propriétés physico-chimiques d'un système de libération contrôlée à base de biopolymères (Algeria: Université YAHIA Fares de médéa).

Lv, C., Wu, G., Hong, Q., and Quan, G. (2019). Spermatozoa cryopreservation: state of art and future in small ruminants. *Biopreserv Biobank.* 17, 171–182. doi: 10.1089/bio.2018.0113

Maccelli, A., Vitanza, L., Imbriano, A., Fraschetti, C., Filippi, A., Goldoni, P., et al. (2019). Satureja montana L. Essential oils: chemical profiles/phytochemical screening, antimicrobial activity and O/W nanoEmulsion formulations. *Pharmaceutics* 12 (1), 7. doi: 10.3390/pharmaceutics12010007

Maiza, A., Kamgang Nzekoue, F., Ghazouani, T., Afif, M., Caprioli, G., Fiorini, D., et al. (2020). Butter oil (ghee) enrichment with aromatic plants: Chemical characterization and effects on fibroblast migration in an in-vitro wound healing mode. *Arabian J. Chem.* 13, 8909–8919. doi: 10.1016/j.arabjc.2020.10.017

Marinova, G., and Batchvarov, V. (2011). Evaluation of the methods for determination of the free radical scavenging activity by DPPH. *Bulg. J. Agric. Sci.* 17, 11–24. Available at: https://www.agrojournal.org/17/01-02-11.pdf.

Mishra, Y., Amin, H. I. M., Mishra, V., Vyas, M., Prabhakar, P. K., Gupta, M., et al. (2022). Application of nanotechnology to herbal antioxidants as improved phytomedicine: An expanding horizon. *Biomedicine Pharmacotherapy.* 153, 113413. doi: 10.1016/j.biopha.2022.113413

Moura, A. A., Memili, E., Rodrigues Portela, A. M., Viana, A. G., Cazaux Velho, A. L., Barbosa Bezerra, M. J., et al. (2018). Seminal plasma proteins and metabolites: Effects on sperm function and potential as fertility markers. *Anim. Reproduction.* 15, 691–702. doi: 10.21451/1984-3143-AR2018-0029

Nandi, A., Yan, L. J., Jana, C. K., and Das, N. (2019). Role of catalase in oxidative stress- and age-associated degenerative diseases. *Oxid. Med. Cell Longev.* 2019, 9613090. doi: 10.1155/2019/9613090

Qiu, J. H., Li, Y. W., Xie, H. L., Li, Q., Dong, H. B., Sun, M. J., et al. (2016). Effects of glucose metabolism pathways on sperm motility and oxidative status during long-term liquid storage of goat semen. *Theriogenology*. 86 (3), 839–849. doi: 10.1016/j.theriogenology.2016.03.005

Ratchamak, R., Authaida, S., Koedkanmark, T., Boonkum, W., Semaming, Y., and Chankitisakul, V. (2023). Supplementation of freezing medium with ginseng improves rooster sperm quality and fertility relative to free radicals and antioxidant enzymes. *Animals: Open Access J. MDPI* 13 (16), 2660. doi: 10.3390/ani13162660

Riesco, M. F., Alvarez, M., Anel-Lopez, L., Neila-Montero, M., Palacin-Martinez, C., Montes-Garrido, R., et al. (2021). Multiparametric study of antioxidant effect on ram sperm cryopreservation-from field trials to research bench. *Anim. (Basel).* 11, 283. doi: 10.3390/ani11020283

Rijsselaere, T., Soom, A. V., Hoflack, G., Maes, D., and De Kruif, A. (2004). Automated sperm morphometry and morphology analysis of canine semen by the Hamilton-Thorne analyser. *Theriogenology* 62, 1292–1306. doi: 10.1016/j.theriogenology.2004.01.005

Ros-Santaella, J. L., and Pintus, E. (2021). Plant extracts as alternative additives for sperm preservation. *Antioxidants* 10 (5), 772. doi: 10.3390/antiox10050772

Rubio-Riquelme, N., Huerta-Retamal, N., Gómez-Torres, M. J., and Martínez-Espinosa, R. M. (2019). Catalase as a molecular target for male infertility diagnosis and monitoring: an overview. *Antioxidants* 9 (1), 78. doi: 10.3390/antiox9010078

Shaw, P., Kumar, N., Sahun, M., Smits, E., Bogaerts, A., and Privat-Maldonado, A. (2022). Modulating the antioxidant response for better oxidative stress-inducing therapies: how to take advantage of two sides of the same medal? *Biomedicines.* 10 (4), 823. doi: 10.3390/biomedicines10040823

Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enology Viticulture.* 16, 144–158. doi: 10.5344/ajev.1965.16.3.144

Sova, M., Kristl, J., and Saso, L. (2021). Nanotechnology-based drug delivery to improve the therapeutic benefits of NRF2 modulators in cancer therapy. *Antioxidants* 10, 685. doi: 10.3390/antiox10050685

Stern, M., and Geary, A. L. (1957). Electrochemical polarization. I. A theoretical analysis of the shape of polarization curves. *J. Electrochemical Society.* 104, 56–63. doi: 10.1149/1.2428496

Van de Hoek, M., Rickard J, P., and De Graaf, S. (2022). Motility assessment of ram spermatozoa. *Biol. (Basel).* 11, 1715. doi: 10.3390/biology11121715

Wu, H., Wang, Y., Ying, M., Jin, C., Li, J., and Hu, X. (2021). Lactate dehydrogenases amplify reactive oxygen species in cancer cells in response to oxidative stimuli. *Signal Transduction Targeted Ther.* 6, 1–13. doi: 10.1038/s41392-021-00595-3

Xue, J., Schmidt, S. V., Sander, J., Draffehn, A., Krebs, W., Quester, I., et al. (2014). Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity.* 40, 274–288. doi: 10.1016/j.immuni.2014.01.006

Yang, H., Hu, E., Matthews, J. L., Varga, Z. M., and Tiersch, T. R. (2022). Is catalase an effective additive to alleviate oxidative stress during cryopreservation of zebrafish sperm at the repository level? *Cryobiology* 104, 70. doi: 10.1016/j.cryobiol.2021.10.003

Zhang, N., Tao, P., Li, S., Wang, J., Wu, X., Pan, B., et al. (2022). Ldha-dependent metabolic programs in sertoli cells regulate spermiogenesis in mouse testis. *Biology.* 11 (12), 1791. doi: 10.3390/biology11121791