BISPHENOLS AND MALE REPRODUCTIVE HEALTH

EDITED BY: Arcangelo Barbonetti, Nicola Bernabò, Settimio D'Andrea and David H. Volle

PUBLISHED IN: Frontiers in Endocrinology and Frontiers in Immunology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-203-6
DOI 10.3389/978-2-88966-203-6

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BISPHENOLS AND MALE REPRODUCTIVE HEALTH

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Citation: Barbonetti, A., Bernabò, N., D'Andrea, S., Volle, D. H., eds. (2020). Bisphenols and Male Reproductive Health. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-203-6

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frontiers in Endocrinology

EDITORIAL
published: 08 October 2020
doi: 10.3389/fendo.2020.597609

Editorial: Bisphenols and Male Reproductive Health

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Keywords: bisphenol, endocrine disruptors, male fertility, spermatozoa, spermatogenesis

Editorial on the Research Topic

Bisphenols and Male Reproductive Health

Bisphenols are organic industrial chemicals, widely used in the manufacture of plastic articles such as polyvinylchloride (PVC), polycarbonate plastics, and epoxy resins. Currently, bisphenol A (BPA), which represents the first-choice plasticizer due to its cross-linking properties, is produced and used in the highest volumes worldwide. Leaching of BPA monomers from inner coating of food and drink containers, especially with repeated use and following exposure to high temperature, largely accounts for the widespread human exposure to BPA by oral ingestion. However, equally important alternative non-dietary routes of absorption, including inhalation and transdermal route, have been demonstrated. Accordingly, in the National Health and Nutrition Examination Survey (NHANES), over 90% of the study population exhibited measurable urinary concentrations of BPA (1).

The ubiquitous presence and environmental persistence of BPA, along with its reputation of being an endocrine disruptor, is generating worldwide concerns about the possible links with a spectrum of human health disorders, including infertility. Due to the resultant restrictions in BPA production, the increasing use of BPA analogs is attracting interest to these new compounds, which, however, could share chemical and biological properties similar to BPA.

This special issue provides an overview of more recent clinical and basic insights about the possible impact of bisphenols on male reproductive health and expresses the opinions of experts from different areas of medicine and biology who have expanded the field with their recent discoveries.

Results from preclinical research clarified possible mechanisms by which BPA can interfere with the regulation of spermatogenesis (Castellini et al.; De Toni et al.). A polycyclic phenolic chemical structure, similar to estradiol, confers to BPA estrogenic activities exerting disrupting effects on the feedback regulation of the hypothalamic–pituitary–gonadal axis. The decreased pituitary secretion of luteinizing hormone (LH) and hypostimulation of Leydig cell steroidogenesis results in lower intratesticular levels of testosterone, which plays a pivotal role in fetal development as well as in adulthood maintenance of secondary sexual function and spermatogenesis. In addition, bisphenols can exert direct harmful effects at testicular levels. In *in vitro* studies, BPA promoted mitochondrial dysfunction, apoptosis and DNA damage of Sertoli cells with disruption of the blood-testis barrier integrity (Adegoke et al.). Detrimental reflections on spermatogenesis would be further exacerbated by intratesticular direct and indirect anti-androgenic activities, as BPA interferes with Leydig cell development and expression of steroidogenic enzymes, as well as with androgen receptor signaling (Adegoke et al.; Barbagallo et al.; Castellini et al.; Li et al.). Of note, many of these effects could be shared by several BPA analogs, which display properties of estrogen receptor agonists and androgen receptor antagonists (Li et al.).

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *21 August 2020*
Accepted: *03 September 2020*
Published: *08 October 2020*

Citation:
Barbonetti A, D'Andrea S, Bernabò N and Volle DH (2020) Editorial: Bisphenols and Male Reproductive Health. Front. Endocrinol. 11:597609. doi: 10.3389/fendo.2020.597609

Experimental studies also suggest that bisphenols could extend their biological effects on male fertility beyond the disruption of the spermatogenesis regulation. Exposure to BPA has been shown to promote epigenetic modifications in both animal and human cells, resulting in endocrine derangements, microscopic and macroscopic abnormalities of male reproductive system as well as inheritable epigenetic changes involving human reproduction (Cariati et al.). Direct effects of bisphenols on sperm functions have been also reported. In different species, including human (2), the *in vitro* exposure of spermatozoa to BPA induced pro-oxidative and apoptotic mitochondrial dysfunctions, resulting in the loss of sperm motility, viability, and DNA integrity. Furthermore, in human spermatozoa, bisphenols BPG, BPAF, BPC, BADGE, BPB, and BPBP can interfere with physiological signaling of the sperm-specific Ca^{2+} channel CatSper (Rehfeld, Andersson et al.), which is activated by the female sex steroid progesterone and plays a key role in the acquisition of sperm fertilizing ability. However, molecular mechanisms leading to activation of CatSper differ between the species, as BADGE and progesterone failed to induce Ca^{2+} signals in boar spermatozoa (Rehfeld, Mendoza et al.).

Overall, while preclinical research has provided compelling evidence that bisphenols can negatively interfere with male reproduction, clinical studies have produced quite inconclusive results. With the exception of few reports on the relationship of prenatal exposure to BPA with abnormal androgen status and pubertal timing (Hart), the claimed clinical adverse effects of bisphenols on male fertility are largely inferred from conventional semen analysis, which, however, is burdened by a number of limitations (Castellini et al.). To date, any conclusion about the cause–effect relationships is hindered by the cross-sectional design of the studies and the large spontaneous between- and within-subject variability of semen parameters (3). Furthermore, despite the adjustment for possible confounding factors in different studies, other unmeasured confounders could have influenced the associations under investigation. Obviously, the best evidence of an adverse effect of BPA on male fertility would be provided by longitudinal analyses, assessing clinically relevant endpoints, such as natural or medically assisted pregnancies among men either with different exposure degrees or with different clinical conditions (fertile/subfertile).

While this latter represents a real challenge for future research, we would like to express our sincere gratitude to all authors and referees for their contribution to this issue summarizing the multidisciplinary and collaborative efforts which in recent years have helped shed some light on a topic yet to be largely investigated.

AUTHOR CONTRIBUTIONS

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

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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 Endocrinology

ORIGINAL RESEARCH
published: 19 May 2020
doi: 10.3389/fendo.2020.00324

Bisphenol A Diglycidyl Ether (BADGE) and Bisphenol Analogs, but Not Bisphenol A (BPA), Activate the CatSper Ca^{2+} Channel in Human Sperm

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OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *05 March 2020*
Accepted: *27 April 2020*
Published: *19 May 2020*

Citation:
Rehfeld A, Andersson AM and Skakkebæk NE (2020) Bisphenol A Diglycidyl Ether (BADGE) and Bisphenol Analogs, but Not Bisphenol A (BPA), Activate the CatSper Ca^{2+} Channel in Human Sperm. Front. Endocrinol. 11:324. doi: 10.3389/fendo.2020.00324

Aim: Evidence suggests that bisphenol A diglycidyl ether (BADGE), bisphenol A (BPA), and BPA analogs can interfere with human male fertility. However, the effect directly on human sperm function is not known. The CatSper Ca^{2+} channel in human sperm controls important sperm functions and is necessary for normal male fertility. Environmental chemicals have been shown to activate CatSper and thereby affect Ca^{2+} signaling in human sperm. BPA has previously been investigated for effects on Ca^{2+} signaling human sperm, whereas the effects of other BPA analogs are currently unknown. The aim of this study is thus to characterize the effect of BADGE, BPA, and the eight analogs BPG, BPAF, BPC, BPB, BPBP, BPE, BPF, BPS on Ca^{2+} signaling, and CatSper in human sperm.

Methods: Direct effects of the bisphenols on Ca^{2+} signaling in human sperm cells were evaluated using a Ca^{2+} fluorimetric assay measuring changes in intracellular Ca^{2+}. Effects via CatSper were assessed using the specific CatSper inhibitor RU1968. Effects on human sperm function was assessed using an image cytometry-based acrosome reaction assay and the modified Kremer's sperm–mucus penetration assay.

Results: At 10 μM the bisphenols BPG, BPAF, BPC, BADGE, BPB, and BPBP induced Ca^{2+} signals in human sperm cells, whereas BPE, BPF, BPS, and BPA had no effect. The efficacy of the chemicals at 10 μM is BPG > BPAF > BPC > BADGE > BPB > BPBP. Dose-response relations of BPG, BPAF, BPC, BADGE, BPB, and BPBP yielded EC50-values in the nM-μM range. The induced Ca^{2+} signals were almost completely abolished using the CatSper inhibitor RU1968, indicating an effect of the bisphenols on CatSper. All bisphenols, except BPBP, were found to dose-dependently inhibit progesterone-induced Ca^{2+} signals, with BPG and BPAF displaying inhibition even in low μM doses. BPG and BPAF were shown to affect human sperm function in a progesterone-like manner.

Conclusion: Our results show that the bisphenols BPG, BPAF, BPC, BADGE, BPB, and BPBP can affect Ca^{2+} signaling in human sperm cells through activation of CatSper. This could potentially disrupt human sperm function by interfering with normal CatSper-signaling and thus be a contributing factor in human infertility, either alone or in mixtures with other chemicals.

Keywords: endocrine disruption, fertility, CatSper, male reproduction, bisphenol

INTRODUCTION

Humans are widely exposed to bisphenol A (BPA), a high-production-volume chemical (1), and bisphenol A diglycidyl ether (BADGE), both widely used in the production of, e.g., epoxy resins and food container linings (2). Due to concerns of the safety of BPA, it is increasingly substituted with analogous chemicals (3, 4). Although evidence suggests that BPA and its analogs can interfere with human male fertility (4–8), the effects directly on human sperm function are less well-studied.

Ca^{2+} signaling is a key regulator of human sperm function (9). The CatSper Ca^{2+} channel is the principal Ca^{2+} channel in human sperm (10, 11) and is activated by the female sex steroid progesterone, released in high amounts from the cumulus cells surrounding the oocyte (10, 12). The activation of CatSper by progesterone controls important sperm functions (13). A suboptimal progesterone-induced Ca^{2+} influx is associated with reduced male fertility (14–20) and men who lack functional CatSper are sterile (18, 21–29), illustrating the importance of CatSper and Ca^{2+} signaling for normal male fertility. Studies have shown that human CatSper can be promiscuously activated by various signaling molecules (30), steroids (31, 32), small molecules (33), and environmental chemicals (34–39). As only BPA, and none of its structural analogs, has previously been investigated for effects on Ca^{2+} signaling in human sperm cells (34, 40), we set out to screen BADGE, BPA, and its eight structural analogs BPG, BPAF, BPC, BPB, BPBP, BPE, BPF, BPS for effects on Ca^{2+} signaling, and CatSper in human sperm, as well as on human sperm cell function.

MATERIALS AND METHODS

Chemicals and Reagents

Bisphenols were purchased from Sigma-Aldrich (MO, USA) and dissolved in DMSO at a stock concentration of 10 mM. Progesterone, prostaglandin-E1 (PGE1) and ionomycin were obtained from Sigma-Aldrich (MO, USA) and dissolved in DMSO at stock concentrations of 20, 20, and 1 mM, respectively. RU1968 was obtained from Professor Timo Strünker and dissolved in DMSO at a stock concentration of 10 mM. Fluo-4, AM, and BCECF, AM were purchased from Invitrogen (CA, USA). Fluorescein isothiocyanate conjugated *Pisum sativum* agglutinin (FITC-PSA), and 4,000 cP methylcellulose were obtained from Sigma-Aldrich (MO, USA). Propidium iodide (PI), Hoechst-33342 (Hoechst), and S100 were obtained from ChemoMetec A/S (Allerød, Denmark). Human serum albumin (HSA) was obtained from Irvine Scientific (CA, USA).

Semen Samples and Ethical Approval

Healthy human volunteers donated the semen samples after their prior consent. The semen samples were produced by masturbation and ejaculated into wide-mouthed plastic containers, on the same day as the experiment and allowed to liquefy for 15–30 min at 37°C before the purification of motile sperm cells via swim-up. The volunteers were recruited from the semen donor corps, which is routinely donating samples for quality control analyses at the Department of Growth and Reproduction, Rigshospitalet. All volunteers fulfilled WHO criteria for normal sperm quality. After delivery, the samples were fully anonymized and no data on the donors fertility status, general health, or exposure to bisphenols were provided. We presumed that the donors were exposed to the same levels of bisphenols as the general population. Each donor received a fee of 500 DKK (about 75 UD dollars) per sample for their inconvenience. All samples were analyzed on the same day of delivery and destroyed immediately after the laboratory analyses. Each experimental replicate was thus based on sperm cells from a single sperm sample. Because of the full anonymization of the samples and the destruction of the samples immediately after the laboratory analyses, no ethical approval was needed for this work, according to the regional scientific ethical committee of the Capital Region of Denmark.

Purification of Motile Sperm Cells via Swim-Up

Motile spermatozoa were isolated from the semen sample by the swim-up method (41). Briefly 1 mL of semen was gently placed in the bottom of a 50 mL tube containing 4 mL of human tubular fluid (HTF^+) medium with the composition: 97.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 4 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH. After 1 h at 37°C, the upper swim-up fraction was carefully removed and after two washes, the sperm concentration was determined by image cytometry (42) and the sample adjusted to 10×10^6 sperm cells/ml in HTF^+ with human serum albumin (3 mg/ml). Hereafter the sperm cells were incubated for at least 1 h at 37°C. For the experiments with capacitated sperm cells, the semen samples were resuspended in a capacitating medium with the following composition: 72.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 25 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH. Human serum albumin (3 mg/ml) was added to the

capacitating medium and the sperm cells were incubated for >3 h at 37°C in a 5% CO_2 atmosphere.

Measurement of Changes in $[Ca^{2+}]_i$

Changes in the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in human sperm cells were measured in 384 multi-well-plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30°C as described in Rehfeld et al. (41). Briefly, sperm cells were incubated with the fluorescent Ca^{2+} indicator Fluo-4, AM (10 µM) for 45 min at 37°C. Excess dye was removed by centrifugation (700 × g, 10 min, RT) and the sperm pellet was resuspended in HTF^+ to 5 × 10^6 sperm cells/mL. Aliquots of 50 µL were loaded to the wells of a 384-well-plate using an automatic repeater pipette. Fluorescence was excited at 480 nm and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after addition of 25 µL bisphenol solutions, negative control (buffer with vehicle), positive control (progesterone, 5 µM final concentration) manually with an electronic multichannel pipette to duplicate wells. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$ (%), indicating the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before addition of bisphenols, positive control, and negative control. For the inhibition studies mean basal fluorescence (F_0) was defined as the last 5 cycles before addition of 100 nM progesterone.

Measurement of Changes in $pH_{(i)}$

Changes in $pH_{(i)}$ in human sperm cells were measured in 384-well-plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30°C as in Schiffer et al. (34). Sperm cells were loaded with the fluorescent pH indicator BCECF (10 µM) for 15 min at 37°C. Excess dye was removed by centrifugation (700 × g, 10 min, RT) and the sperm pellet was resuspended in HTF^+ to 5× 10^6 sperm/ml. Aliquots of 50 µL were loaded to the wells of the multi-well-plate. Fluorescence was excited at 440 and 480 nm (dual excitation) and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after addition of 25 µL of bisphenol solutions, negative control (buffer with vehicle), positive control (NH_4CL, 30 mM final concentration) manually with an electronic multichannel pipette to duplicate wells. Changes in the ratio of BCECF fluorescence between the 440 and 480 nm excitation are shown as $\Delta R/R_0$ (%), indicating the percentage change in the ratio of fluorescence between the two modes of excitation (ΔR) with respect to the mean basal ratio of fluorescence between the two modes of excitation (R_0) before addition of bisphenols, positive control, and negative control.

Assessment of Sperm Penetration Into a Viscous Medium

Assessment of sperm penetration was done using sperm penetration tests with 4,000 cP methylcellulose (1% w/v) as an artificial viscous medium as described in Alasmari et al. (43). The viscous methylcellulose (1% w/v) medium was prepared in HTF^+ by adding 10 mg methylcellulose per mL HTF^+ and mixing it by rotation overnight at RT. The viscous methylcellulose (1% w/v) medium was introduced into glass capillary tubes [borosilicate microslides (VitroTubes) 0.20 mm × 2.0 mm × 10 cm (VitroCom, USA)] by capillary forces, by placing the glass tubes vertically in a 1.5 mL microfuge tube with 750 µL methylcellulose (1% w/v) for 15min. Care was taken to prevent air bubbles from entering the glass tubes. The end of the glass tube that was placed in the microfuge tube was sealed with wax (Hounisens laboratorieudstyr A/S, Denmark). Hereafter the other end was cut within the part filled with methylcellulose, just before the methylcellulose-air transition, and additional wax was added to the other end to push out a small droplet of methylcellulose at the cut end. The cut end is then placed in a 1.4 mL tube (Eppendorf, Germany) with 200 µL non-capacitated sperm sample (10 × 10^6/ml in HTF^+). Just prior to the insertion of the glass tubes, either bisphenols (10 µM), 5 µM progesterone (positive control), 5 µM PGE1, or 0.1% DMSO (negative control) were added to the sperm sample. The sperm cells were allowed to migrate into the methylcellulose (1% w/v) for 60 min at 37°C. The glass tube was then removed, wiped to remove residual sperm cells from the surface of the glass, placed under a UV lamp (302 nm) in a BIO-RAD Universal Hood III (BIO-RAD, CA, US) for 3 min to paralyze the sperm cells (44) and hereafter examined using phase contrast optics on an Olympus BX45 microscope at a total magnification of ×200 (Olympus, Denmark). The number of sperm cells were counted at 2 cm distance from the opening of the tube, with two fields in each of four planes counted. Throughout the study, all samples were counted by the same observer. Only experiments with a positive increment in cell density at 2 cm for the positive control compared to the negative control and with more than 40 sperm cells counted at 2 cm for the positive control were used for the analysis.

Assessment of Acrosome Reaction

The amount of live acrosome reacted sperm cells was measured using an image cytometry-based acrosome reaction assay, as described in Rehfeld et al. (41). Briefly, capacitated sperm cells (10 × 10^6/ml) were divided into equal aliquots and mixed thoroughly with a staining solution containing 5 µg/mL FITC-PSA, 0.5 µg/mL PI, and 10 µg/mL Hoechst in HTF^+. Bisphenols (10 µM) were added to the aliquots of stained capacitated sperm cells together with the positive controls, ionomycin (10 µM), and progesterone (10 µM). As a negative control, HTF^+ with 0.2% DMSO was used. After addition of bisphenols and controls the samples were mixed well and placed on a gentle mixing heating plate at 37°C. After 30 min of incubation, the aliquots were mixed thoroughly by pipetting and a 50 µL sample was drawn and added to a 100 µL aliquot of an immobilizing solution containing 0.6 M $NaHCO_3$ and 0.37% (v/v) formaldehyde in distilled water. This solution was mixed well by pipetting, immediately loaded in an A2 slide (ChemoMetec A/S, Allerød, Denmark) and assessed in a NC-3000 image cytometer (ChemoMetec A/S, Allerød, Denmark). The following protocol was applied: 2-color flexicyte with Hoechst defining the sperm cells to be analyzed; Ex475-Em560/35: exposure time 3,000 ms, Ex530-Em675/75: exposure time 500 ms, with a minimum of 5,000 analyzed cells (positive for Hoechst). PI intensity as a function of FITC-PSA intensity was plotted on bi-exponential scales and specific quadrant gates were used to distinguish four groups:

1. PI positive and FITC-PSA positive cells: Acrosome reacted non-viable sperm cells.
2. PI negative and FITC-PSA positive cells: Acrosome reacted viable sperm cells.
3. PI positive and FITC-PSA negative cells: Acrosome intact non-viable sperm cells.
4. PI negative and FITC-PSA negative cells: Acrosome intact viable sperm cells.

Only experiments with an increase of live acrosome reacted sperm cells for both positive controls compared to the negative control at ≥100% were included in the analysis.

Statistical Analysis

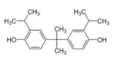
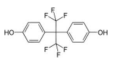
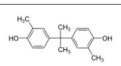
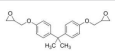
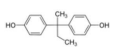
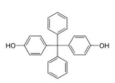
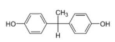
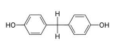
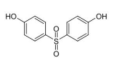
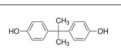
Data from sperm penetration tests and the acrosome reaction assay were analyzed using a mixed effects model with Geissner-Greenhouse correction. Normality was assumed based on a QQ-plot of residuals. *P*-values were corrected for multiple comparison type I error inflation by Dunnett's method. Statistical analyses were performed using GraphPad Prism 8.3.1 (GraphPad Software Inc., USA).

RESULTS

Bisphenols Induce Ca^{2+} Signals in Human Sperm Cells

We investigated the 10 bisphenols BADGE, BPA, BPG, BPAF, BPC, BPB, BPBP, BPE, BPF, and BPS for their ability to induce Ca^{2+} signals in human sperm cells (**Table 1**), using a Ca^{2+} fluorimetric assay (34). The bisphenols were screened at a concentration of 10 μM, along a positive control (progesterone, 5 μM), and negative control (HTF^{+} with vehicle). Changes in $[Ca^{2+}]_i$ were recorded for 4 min after addition of the chemicals and controls. We calculated the relative peak Ca^{2+} signal in % induced by the bisphenols, by dividing the peak Ca^{2+} signal with that of the paired positive control, in order to compare

TABLE 1 | Bisphenols ranked according to the mean relative peak Ca^{2+} signal induced at 10 μM, i.e., the peak Ca^{2+} signal induced by the bisphenol at 10 μM divided by the peak Ca^{2+} signal induced by progesterone at 5 μM in the same experiment.

Rank		Name	CAS number	Abbrevation	Mean relative peak Ca^{2+} signal at 10 μM (in %) ($n = 3$)	Chemical structure
Positive hits	1	Bisphenol G	127-54-8	BPG	109.02	
	2	Bisphenol AF	1478-61-1	BPAF	57.95	
	3	Bisphenol C	79-97-0	BPC	21.67	
	4	Bisphenol A diglycidyl ether	1675-54-3	BADGE	14.79	
	5	Bisphenol B	77-40-7	BPB	11.75	
	6	Bisphenol BP	1844-01-5	BPBP	9.09	
Negative hits	7	Bisphenol E	2081-08-5	BPE	5.37	
	8	Bisphenol F	620-92-8	BPF	5.09	
	9	Bisphenol S	80-09-1	BPS	4.41	
	10	Bisphenol A	80-05-7	BPA	1.44	

Based on their ability to induce Ca^{2+} signals, the bisphenols are categorized into "positive hits," which induced mean relative peak Ca^{2+} signals above that of the negative controls (HTF^{+} with vehicle) ± 3 × SD (0.0 ± 3 × 2.3 = 6.9%) and "negative hits." CAS number, abbreviation, and chemical structure are also listed in the table.

data from the different experiments. Six of the ten bisphenols tested induced a mean relative peak Ca^{2+} signal larger than that of negative controls (HTF^{+} with vehicle) ± 3 × SD (0.0 ± 3 × 2.3%, giving a maximal value of 6.9%, **Table 1**). These six bisphenols were categorized as "positive hits" and investigated in further detail.

Dose Response Relationship for the "Positive Hit" Bisphenols

Dose response relations were assessed for the "positive hit" bisphenols to examine whether they induced Ca^{2+} signals in human sperm cells at physiologically relevant levels. Saturating dose response relations could be made for all six bisphenols, with mean EC_{50}-values within the concentration range 0.79–15.87 μM and mean EC_{05}-values within the concentration range 0.18–2.37 μM (**Table 2**, **Figure 1**).

TABLE 2 | Left and middle columns: EC_{50} and EC_{05} for the dose response curves (mean and SD, n = 3–7) of all "positive hit" bisphenols.

	EC_{50}, μM		EC_{05}, μM		IC_{50}, μM	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
BPG	1.27	0.61	0.18	0.16	1.86	0.80
BPAF	2.40	0.93	0.36	0.09	12.3	4.12
BPC	10.26	1.83	0.70	0.27	45.2	4.25
BADGE	8.18	3.88	1.82	1.01	–	–
BPB	14.87	4.42	2.37	1.75	39.9	8.05
BPBP	0.79	0.06	0.38	0.26	–	–

Right column: IC_{50} (mean and SD, n = 3–5) for the dose response curves of Ca^{2+} signals induced by 100 nM of progesterone after pre-incubation of human sperm cells with various concentrations of the 6 "positive hit" bisphenols. Note that the dose response data generated from the preincubation experiments with BADGE and BPBP could not be used to estimate IC_{50}-values.

Bisphenols Induce Ca^{2+} Signals Through an Activation of CatSper

To test if the six "positive hit" bisphenols induced Ca^{2+} signals through CatSper, we used the novel and specific CatSper inhibitor RU1968 (13). We compared the Ca^{2+} signals induced by the bisphenols at doses inducing peak Ca^{2+} signals (5–50 μM) and progesterone at 5 μM, in the presence or absence of 30 μM of RU1968 (**Figure 2**). We found that the Ca^{2+} signals induced by all six bisphenols, like that of progesterone, were highly inhibited by RU1968. This strongly indicates that the bisphenols induce Ca^{2+} signals via a specific activation of CatSper in human sperm cells. Furthermore, the shape of the Ca^{2+} signals induced by the bisphenols at these doses, except BPBP, which only induce a small Ca^{2+} signal, resembles that induced by progesterone (**Figure 2**). This further indicates an action of the bisphenols on CatSper. As CatSper can be activated both by the endogenous ligands progesterone and prostaglandins, as well as by intracellular alkalization, we examined if the bisphenols induced changes in $pH_{(i)}$. At bisphenol doses inducing peak Ca^{2+} signals (5–50 μM) no increase $pH_{(i)}$ was observed (n = 3, **Figure 3**). This suggests that the bisphenols most likely act on the ligand-dependent pathway of either progesterone or prostaglandins leading to activation of CatSper.

Bisphenols Dose-Dependently Inhibit Progesterone-Induced Ca^{2+} Signals

As the bisphenols were found to induce Ca^{2+} signals through CatSper we examined whether pre-incubating the human sperm cells with the bisphenols could inhibit progesterone-induced Ca^{2+} signals. We compared the amplitude of the

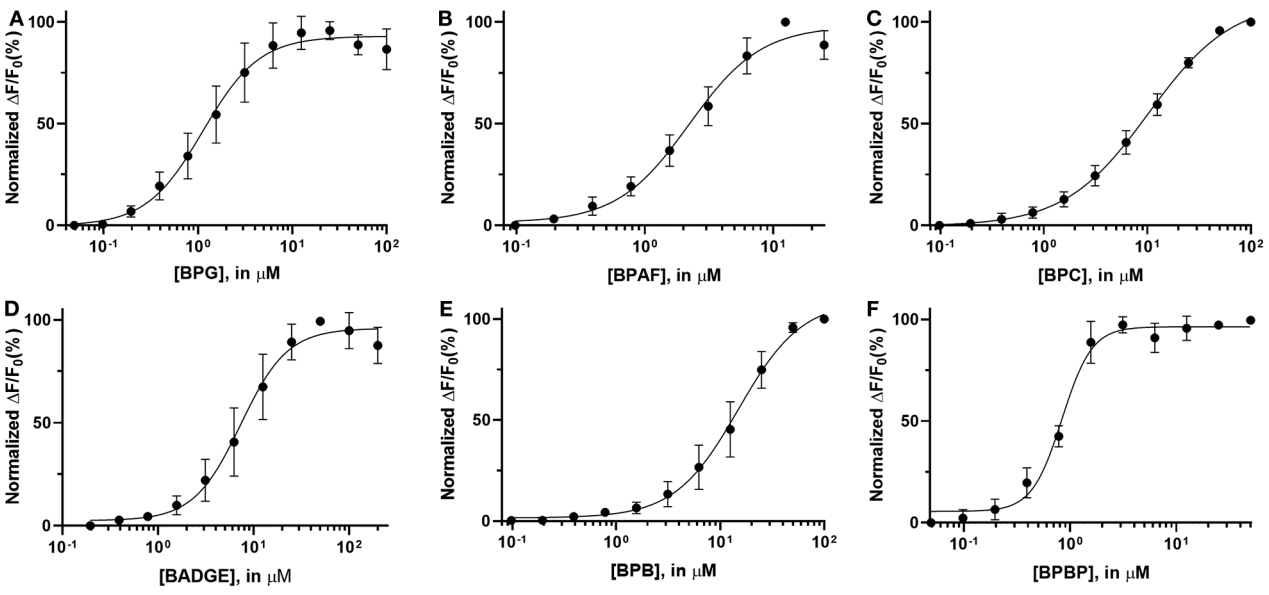


FIGURE 1 | Normalized dose response curves (mean ± SD) for "positive hit" bisphenols. **(A)** BPG, **(B)** BPAF, **(C)** BPC, **(D)** BADGE, **(E)** BPB, and **(F)** BPBP, n = 3–7.

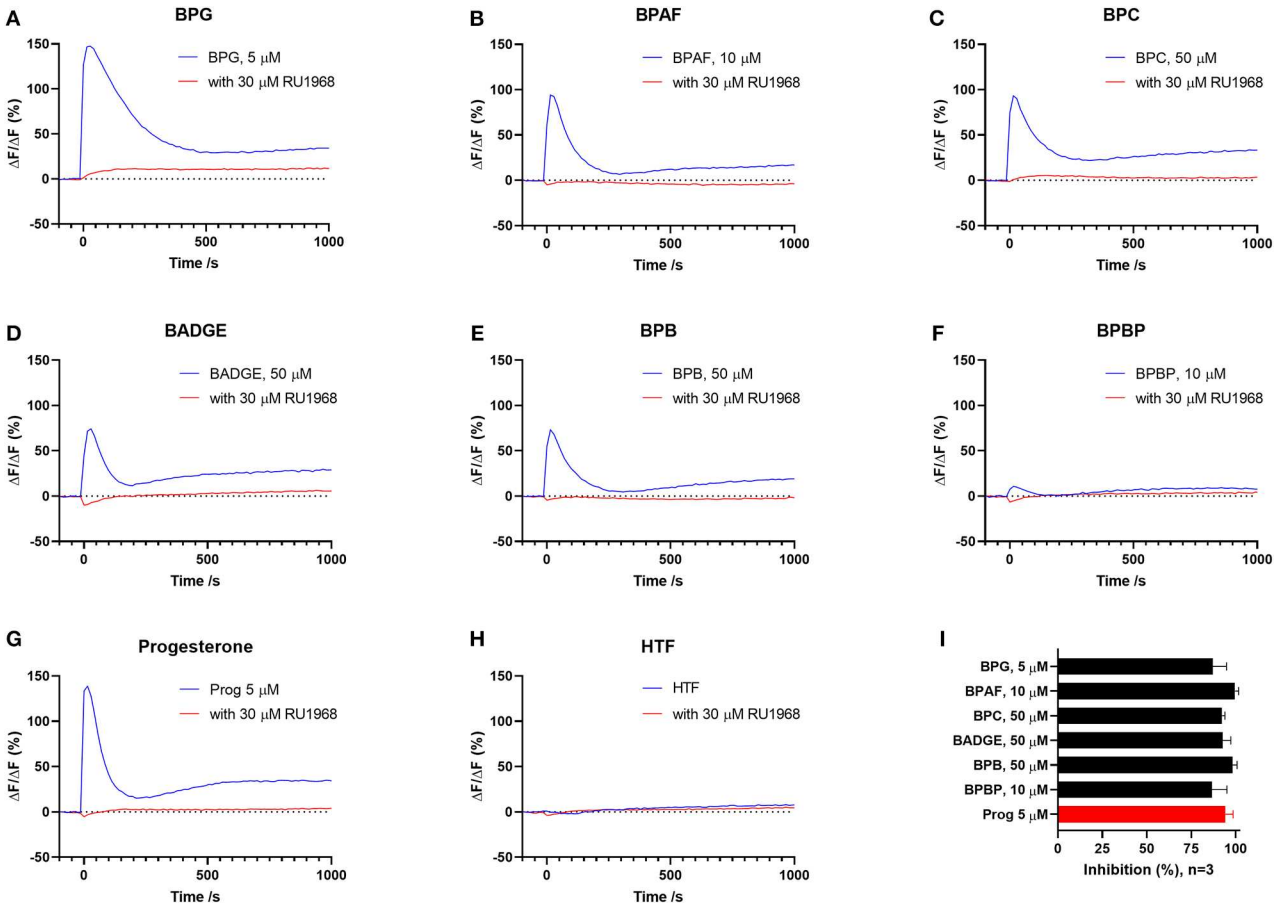


FIGURE 2 | Ca^{2+} signals induced by **(A)** BPG, **(B)** BPAF, **(C)** BPC, **(D)** BADGE, **(E)** BPB, **(F)** BPBP, **(G)** the endogenous CatSper ligand progesterone, and **(H)** HTF^{+} buffer in the absence and presence of CatSper inhibitor RU1968, 30 μM. **(I)** Mean inhibition (in %) of the induced Ca^{2+} signal ±SD in the presence of RU1968, 30 μM ($n = 3$).

Ca^{2+} signals induced by 100 nM of progesterone in human sperm cells after 5 min of pre-incubation with serially diluted doses of the bisphenols or a negative buffer control. Our results showed that all bisphenols, except BPBP, were able to dose dependently inhibit the progesterone-induced Ca^{2+} signals (**Figure 4**). The mean IC_{50}-values estimated from the fitted dose response curves were within the concentration range 1.86–45.2 μM (**Table 2**).

Effects of Bisphenols on CatSper-Mediated Human Sperm Responses

To examine whether the bisphenols could affect CatSper-mediated human sperm responses, we examined the effect of the two most efficacious bisphenols, BPG and BPAF at 10 μM, on sperm penetration into a viscous medium, as well as on acrosome reaction. BPG and BPAF were found to induce a significant increase in the numbers of human sperm cells penetrating into a viscous medium (**Figure 5**), similar to the effect of the endogenous CatSper ligands, progesterone, and prostaglandin E1 at 5 μM. Furthermore, BPG and BPAF were found to induce a significant increase in live acrosome reacted sperm cells (**Figure 6**), similar to the effect of the endogenous CatSper ligand progesterone at 10 μM, in capacitated human sperm cells.

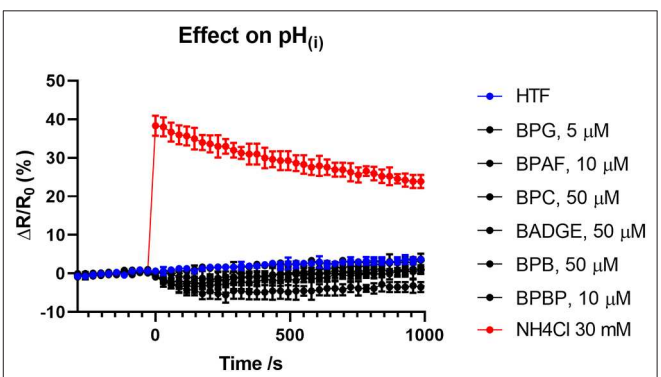


FIGURE 3 | Changes (mean ± SD) in $pH_{(i)}$ induced by the bisphenols (5–50 μM), HTF^{+} buffer and positive control NH_4Cl at 30 mM ($n = 3$).

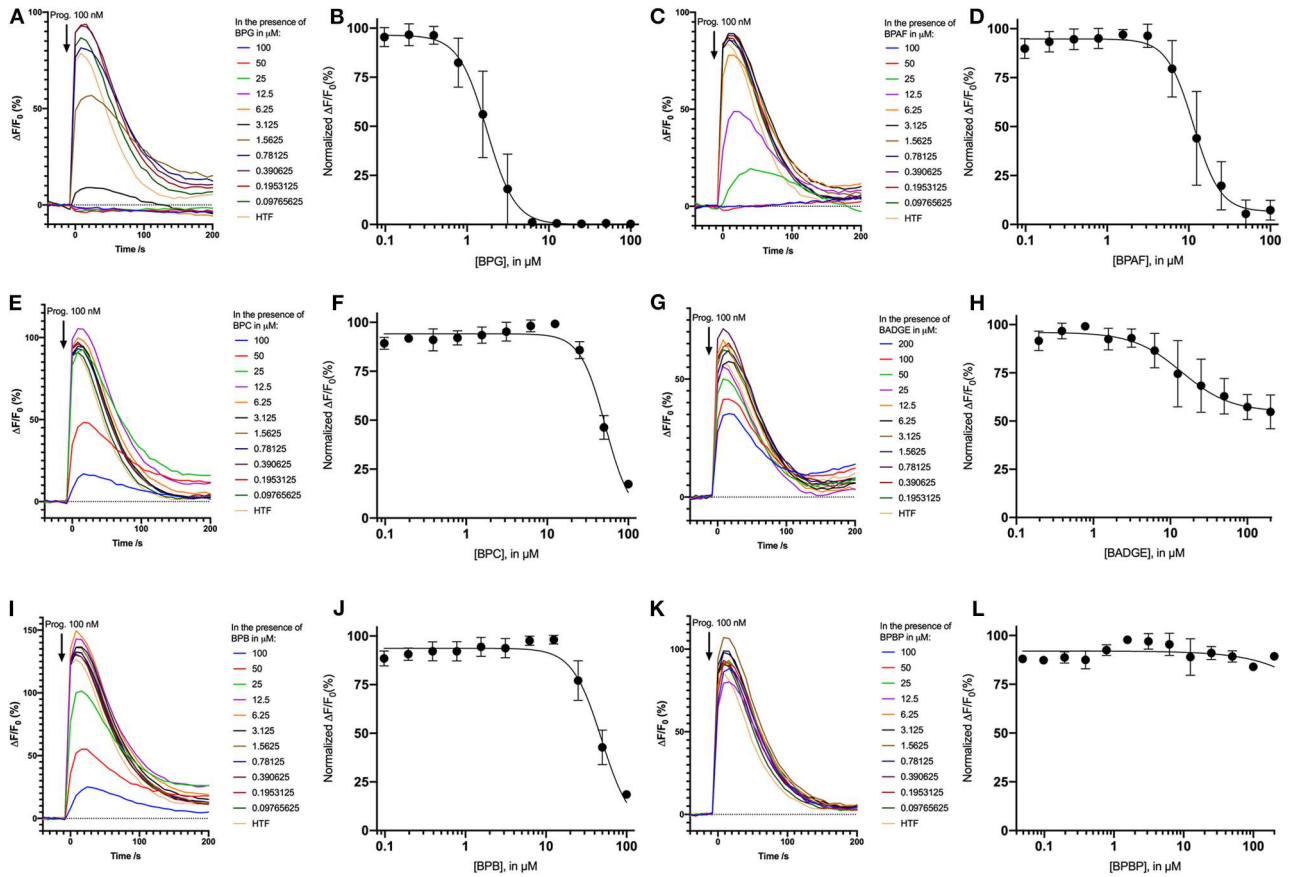


FIGURE 4 | Inhibition of Ca^{2+} signals induced by 100 nM progesterone after 5 min of pre-incubation with the negative control HTF and different concentrations the bisphenols: **(A)** BPG, **(C)** BPAF, **(E)** BPC, **(G)** BADGE, **(I)** BPB, and **(K)** BPBP. Normalized dose response relations ($n = 3–5$) of Ca^{2+} signals induced by 100 nM progesterone after 5 min of pre-incubation with the different concentrations the bisphenols: **(B)** BPG, **(D)** BPAF, **(F)** BPC, **(H)** BADGE, **(J)** BPB, and **(L)** BPBP.

DISCUSSION

Our study showed that BADGE and the five bisphenol analogs BPG, BPAF, BPC, BPB, and BPBP can induce Ca^{2+} signals in human sperm cells at 10 µM, whereas BPA and three other bisphenols BPE, BPF, and BPS induced no Ca^{2+} signals in human sperm cells at this concentration (**Table 1**). The efficacy of the chemicals at 10 µM was BPG > BPAF > BPC > BADGE > BPB. It seems that the bisphenols with larger/bulkier side chains are more efficacious and that relatively small molecular differences between the bisphenols can alter their effects significantly (**Table 1**). This is in line with a previous study showing that the read-across approach was non-applicable for otherwise structurally comparable bisphenols (45). Interestingly, low doses of BPAF, BPB, BPF, BPS, and BPA have all been shown to induce Ca^{2+} signals in SKBR3 cells via the G protein-coupled estrogen receptor (GPER) (46), with BPAF and BPB being more efficacious than BPF, BPS, and BPA. This is somewhat similar to our findings, although we in our assay see no effect for BPF, BPS, and BPA. Furthermore, even though BPA showed no effect in our assay, it has been shown both to activate (47) and inhibit other voltage-activated Ca^{2+} channels (48).

The induced Ca^{2+} signals could be used to form saturating dose response curves for all six "positive hit" bisphenols (**Figure 1**). The EC_{50}-values estimated from these curves ranged from 0.79 to 14.87 µM and the lowest effective dose values (EC_{05}) ranged from 0.18 to 2.37 µM (**Table 2**). In the literature, we could only identify human plasma or serum levels for BPAF, BADGE, and BPB out of the six "positive hit" bisphenols (49–53). A reported maximal human serum concentration of BADGE (3.45 µM) (50) is above the EC_{05} estimated in our study (1.82 µM), whereas the reported maximal human serum levels of BPAF (0.05 µM) (50) and BPB (0.59 µM) (50) are below the estimated EC_{05}-values of 0.36 µM for BPAF and 2.37 µM for BPB.

We found that the induced Ca^{2+} signals were almost completely inhibited by the specific CatSper inhibitor RU1968 (**Figure 2**), like the Ca^{2+} signal induced by the endogenous CatSper ligand progesterone. This indicates that the six bisphenols induce Ca^{2+} signals in human sperm cells via CatSper. Furthermore, the shape of the Ca^{2+} signals induced by all bisphenols, except BPBP, which only induced a small peak Ca^{2+} signal, resembled that of the Ca^{2+} signal induced by progesterone (**Figure 2**), similarly suggesting an effect of

these bisphenols on CatSper. Human CatSper can be activated by a ligand-dependent pathway, by the endogenous CatSper ligands progesterone and prostaglandins (10, 12), as well as by a ligand-independent pathway through intracellular alkalization (10, 12). Our data showed that the induction of Ca^{2+} signals by the bisphenols is not due to an increase in $pH_{(i)}$ (**Figure 3**), suggesting that the bisphenols act on the ligand-dependent pathways of either progesterone or prostaglandins leading to activation of CatSper in human sperm cells (10, 12). Interestingly, progesterone has been suggested to activate CatSper through an activation of the enzyme ABHD2, whereas the molecular target of prostaglandins leading to CatSper activation remains unknown (54). The direct action of the bisphenols on the ligand-dependent pathway leading to activation of CatSper in human sperm cells, is similar to what has been shown for multiple other environmental chemicals previously (34, 35, 55).

Pre-incubation of the human sperm cells with the bisphenols BPG, BPAF, BPC, BADGE, and BPB was found to dose-dependently inhibit progesterone-induced Ca^{2+} signals (**Figure 4**). BPG and BPAF were found to be much more potent inhibitors of progesterone-induced Ca^{2+} signals than the other bisphenols, which only inhibited progesterone-induced Ca^{2+} signals at high μM doses (**Table 2**). Exposure of human sperm cells to these bisphenols may thereby inhibit the action of progesterone on CatSper, as has been shown for other environmental chemicals acting on the ligand-dependent pathway (34–36, 38).

In addition, our results showed that the two most efficacious bisphenols at 10 μM, BPG and BPAF, could both increase sperm penetration into a viscous medium, like the response induced by the endogenous CatSper ligands progesterone and PGE1 (**Figure 5**), and induce acrosome reaction in capacitated human sperm cells, similar to the response induced by progesterone (**Figure 6**). Again, this is in line with previous studies where other environmental chemicals activating CatSper were found to exert progesterone-like effects on human sperm function (34, 36–38, 56).

Only few studies have examined the effect of bisphenols on human sperm cell function. One study showed that very high doses of BPA (≥300 μM) induced mitochondrial dysfunction in human sperm (57), another study showed that BPA at 0.1 nM−1 μM could affect human sperm motility parameters and that BPA at 1 μM could induce a rapid, transient increase in $[Ca^{2+}]_i$ in a whole population of observed single human sperm cells (40), whereas BPA at 0.1, 1, and 10 μM did not affect $[Ca^{2+}]_i$ in human sperm cells in a large screening of environmental chemicals by Schiffer et al. (34). Our results here support the findings by Schiffer et al. (34) that BPA at concentrations up to 10 μM do not induce Ca^{2+} signals in human sperm cells.

Our findings add BADGE and the five bisphenol analogs BPG, BPAF, BPC, BPB, and BPBP to the growing list of environmental chemicals that can induce Ca^{2+} signals in human sperm cells through CatSper (34–39). Studies have shown that chemicals acting on CatSper can cooperate in low dose mixtures to activate CatSper both additively (34, 35) and synergistically (55). As humans in the industrialized part of the world are suggested to be exposed to thousands of environmental chemicals (58), such a low dose mixture exposure scenario is quite realistic.

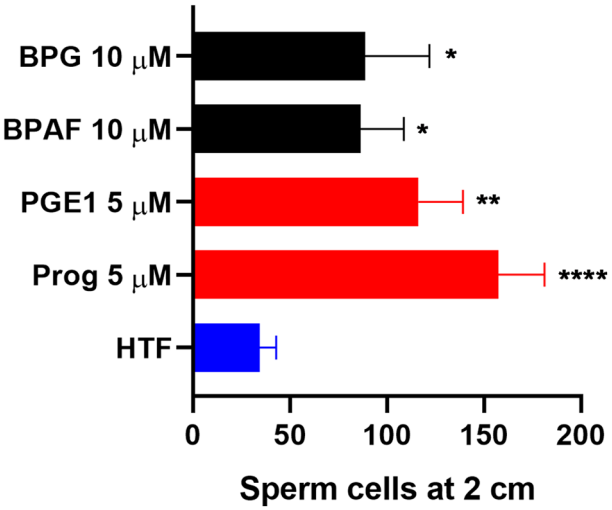


FIGURE 5 | Human sperm cells at 2 cm into a viscous medium (mean ± SEM) after treatment with negative control (HTF^+ with 0.1% DMSO "HTF"), positive controls (5 μM progesterone "Prog" and prostaglandin E1 "PGE1"), 10 μM BPG, and 10 μM BPAF ($n \geq 5$). Statistics from multiple comparison between negative control and treatments: ****adjusted $P \leq 0.0001$; **adjusted $P = 0.0029$; *adjusted $P \leq 0.0295$.

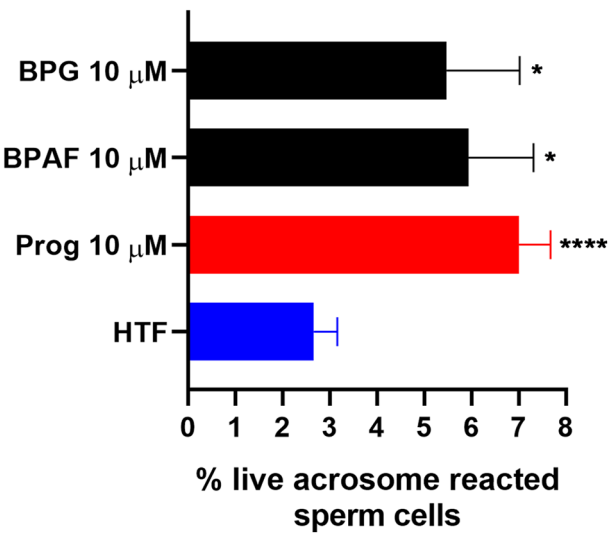


FIGURE 6 | Percentage live acrosome reacted sperm cells (mean ± SEM) after 30 min treatment of capacitated human sperm cells with negative control (HTF^+ with 0.2% DMSO "HTF"), positive control (10 μM progesterone "Prog"), 10 μM BPG, and 10 μM BPAF ($n \geq 8$). Statistics from multiple comparison between negative control and treatments: ****adjusted $P \leq 0.0001$; *adjusted $P \leq 0.0249$.

This indicates that the bisphenols could affect Ca^{2+} signaling in human sperm cells even at doses well below the EC_{05}, when present in mixtures with other chemicals acting on CatSper. This is important as only BADGE has been found with a maximal serum concentration (3.45 μM) (50) above the EC_{05} estimated in our study (1.82 μM).

Whether exposure of the human sperm cells, either within the male or female reproductive tract, to environmental chemicals acting on CatSper can interfere with the fertilization process remains to be shown. However, the fact that impaired progesterone-signaling is associated with reduced male fertility (14–20) and that men who lack functional CatSper are completely infertile (18, 21–29) hints that environmental chemicals interfering with this signaling pathway could make it more difficult for the human sperm cells to successfully locate and fertilize the oocyte. As our experiments have been performed on sperm cells *in vitro* future studies would be needed to validate our results and to examine the effects of exposure to bisphenols on fertility *in vivo*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher upon request.

ETHICS STATEMENT

Because of the full anonymization of the samples and the destruction of the samples immediately after the laboratory analyses, no ethical approval was needed for this work, according to the regional scientific ethical committee of the Capital Region of Denmark. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AR, AA, and NS conceived the study and drafted the manuscript. AR designed, planned, and performed the experiments.

FUNDING

This study was supported by a EDMaRC research grant from the Kirsten and Freddy Johansen's Foundation and the Danish Center on Endocrine Disrupters.

ACKNOWLEDGMENTS

The authors would like to acknowledge Sissel Marie Bredesen for her assistance in performing the laboratory experiments and Brian Vendelbo Hansen for his assistance with obtaining reagents and chemicals from the various providers.

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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 Endocrinology

MINI REVIEW
published: 29 May 2020
doi: 10.3389/fendo.2020.00320

The Impact of Prenatal Exposure to Bisphenol A on Male Reproductive Function

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Bisphenol A (BPA) is a recognized xenoestrogen, in that it possesses oestrogenic and anti-androgenic properties. These endocrine-disrupting effects of BPA at the estrogen receptor (ER) occur despite the very low affinity of BPA for the ERβ, which is 10,000 times lower than that of 17-β estradiol, and despite the European regulatory authorities stating that BPA is safe, at usual exposure concentrations, the use of BPA in baby drink bottles was banned in 2011. There exists conflicting evidence from human epidemiological studies as to its influence on adult male reproductive function, although animal data is more convincing. This mini-review will report on the limited epidemiological data from human studies relating early life exposure to BPA on adult male reproductive function. A long term follow-up study from Western Australia using a birth cohort, the Raine Study, demonstrated no adverse associations of antenatal exposure to BPA, and potentially a positive association with antenatal BPA exposure with sperm concentration and motility at 20 years of age, although recent scientific reports suggest traditional measures of BPA exposure may underestimate exposure levels, which makes data interpretation potentially flawed.

Keywords: BPA, sperm count, testosterone, male reproduction, raine study, endocr disrupting chemicals, early life exposures, *in-utero*

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *16 November 2019*
Accepted: *27 April 2020*
Published: *29 May 2020*

Citation:
Hart RJ (2020) The Impact of Prenatal Exposure to Bisphenol A on Male Reproductive Function. Front. Endocrinol. 11:320. doi: 10.3389/fendo.2020.00320

INTRODUCTION

Bisphenol A (BPA) is a widely used chemical which is ubiquitous within the environment, being present within plastics and epoxy resin. In the United States the Centre for Disease Control and Prevention reported that more than 90% of individuals, in the early years of the twenty-first century, had measurable concentrations of BPA present within their body (1). The production of BPA has increased substantially over the last 15 years and the projection for 2020 is 9,600 kilo tons http://www.digitaljournal.com/pr/2009287 (2). Exposure to BPA can be through the diet, drinking, inhalation or dermal contact, although inhalation exposure appears to be negligible in comparison to the dietary route (3). Furthermore, measurable levels of BPA have been detected in breast milk, amniotic fluid, and cord blood. Furthermore, the fetus is at risk of BPA exposure as it freely crosses the placenta. In the circulation BPA is present in the free form at about 8% of the total BPA in the blood (4). Subsequent to eating, after gastric absorption, peak serum BPA concentrations are reached within 90 min (5), and BPA is rapidly eliminated, after gut absorption (6), dermal and sub-lingual absorption have different pharmacokinetics. After undergoing rapid conjugation, forming inactive glucuronides and sulfates by the liver of the mother and fetus, and

by the placenta, BPA is excreted in the urine (7), although some work suggests that the glucuronide metabolite may be active (8). Consequently, it is theoretically possible for the fetus to be exposed to a greater concentration of BPA than the mother, as the placenta can de-conjugate BPA by placental sulphatase and beta-glucuronidase enzymes (9), and furthermore, the immaturity of fetal liver would make the BPA conjugation poorly effective in the fetus (9). However, the significance of this placental metabolism is believed to be low (7), although studies suggest almost universal exposure of pregnant women to BPA, and a substantial variation in its metabolic clearance, which will lead to substantial variability of fetal exposure (10).

Despite reassurances of the safety of BPA by the European Food Safety Authority (EFSA) as recently as 2015, the EFSA reduced the tolerable daily intake of BPA from 50 μg/kg body weight per day (bw/day) to 4 μg/kg bw/day, and stated that the average daily exposure was below this "safe" level (11). With estimated BPA dietary intake in infants and toddlers (up to 0.875 μg/kg bw/day), with reproductive aged women having dietary exposures comparable to men of the same age (up to 0.388 μg/kg bw/day), and adolescent exposure of upto 1.449 μg/kg bw/day, in 2011 the European Union banned the use of BPA within baby bottles. Interestingly, due to the lipophilic properties of BPA, BPA could concentrate in the breast milk, and levels of infant exposure to BPA decrease with the introduction of solid foods (11).

Due to its prevalence within the environment, and its known endocrine disrupting effects, it has been suggested that BPA may have a negative impact on male fertility acting as a xenoestrogen. Unconjugated BPA binds as a weak agonist to estrogen receptors α and β (12, 13), as well as the androgen receptor (14). Hence, it may be expected to potentially impact the reproductive development of the male, particularly if exposure was to occur during a vulnerable period of development of the male fetus during pregnancy. It has been demonstrated in rodent models that a "masculinization programming window" exists in pregnancy, and would be expected to correlate with 8–14 weeks gestation in humans (15). Features of lack of male androgenisation are a shorter anogenital distance, impairment of sperm production, hypospadias and cryptorchidism (15), which have been grouped together as part of a "testicular dysgenesis syndrome" (TDS) (16, 17). Consequently, it is during this period of time that the male fetus would, theoretically, be at greatest vulnerability to chemicals that either interfere with the secretion, transport, action, metabolism, and excretion of testosterone; the hormone primarily responsible for fetal masculinisation. This is particularly of relevance as BPA freely diffuses across the placenta (7), and the placenta's ability to conjugate, and hence potentially de-activate BPA is limited. Hence, BPA at maternal serum concentrations may freely pass to the fetus across the placenta, leading to near-equivalent levels in fetal and maternal blood (7), therefore measuring maternal circulating concentrations is a reasonable proxy for fetal exposure.

It has been assumed by many experts that sperm counts may have been diminishing over the last 30 years, although this is hotly debated (18, 19), however it is not disputed that the incidence of undescended testis, hypospadias and testicular cancer is increasing in some countries (20–23). The TDS hypothesis proposes that, as a result of abnormal testicular development, a secondary abnormality in Leydig and/or Sertoli cells results during male sexual differentiation, leads to reproductive disorder in later life (24, 25), again, this assertion has been disputed (26). However, with the increasing prevalence of oestrogenic endocrine disrupting chemicals within the environment it is plausible, but unproven, that human fetal Sertoli cell proliferation may be altered by an excessive oestrogenic environment in early life. Consequently, researchers have attempted to study potential associations of early life exposures to oestrogens (27), and endocrine disrupting chemicals (28, 29), with the incidence of cryptorchidism (30), anogenital distance (a reliable marker of prenatal androgenisation) (31), pubertal timing (32), sperm counts (27), and adult markers of testicular function (27). This mini-review will review the epidemiological studies of prenatal BPA exposure on human male reproductive function.

BACKGROUND ANIMAL STUDIES OF EXPOSURE TO BPA

Data from animal studies provide potential mechanistic insights to the human data and are briefly reviewed for context. Animal studies suggest that exposing mice early in the neonatal period to BPA, at concentrations that humans encounter daily, may reduce sperm number, motility, and maturation, without influencing testicular histology (33). Perinatal BPA administration to female rats has been reported to reduce the fertility of the mature male offspring (34). Furthermore, negative influences on plasma testosterone and estradiol concentrations have been reported subsequently, after maturity, when pre-pubertal rats were exposed to low doses of BPA, inducing some degree of androgen deficiency features in adulthood (35, 36).

Male mice exposed *in utero* to BPA have been demonstrated to have reductions in concentrations of serum and intra-testicular testosterone (37), impairments of testicular development (37) and spermatogenesis (37), with reduced sperm counts (38). Indeed, studies suggest that BPA may be a testicular toxicant in animal models (39, 40). Furthermore, adverse effects of BPA exposure on rodents' developing testis and prostate stem cells have been also reported (41, 42). Other animal studies suggest that BPA may exert it effects through central influences from *in-utero* maternal BPA exposure causing alterations in gonadotrophin releasing hormone and kisspeptin secretion, and consequently influence anterior pituitary function (43). From **Figure 1** (44), it can be seen that the influence of BPA exposure at different stages of development in the animal model appears to produce similar effects on reproductive function in adulthood. Due to the concerns of the potential health effects of BPA on human health analogs to BPA have been introduced into commercial production. However, this approach may not be entirely beneficial, as one study that administered BPA, and its analogs bisphenol B, bisphenol F, and bisphenol S, at various low concentrations to pregnant rats, demonstrated in the male offspring a decrease in sperm production, testosterone secretion,

Bisphenol- A and Male Reproduction in animal models			
Exposure effects	**Fetal life**	**Pubertal life**	**Adult life**
Anatomic structure	Retardation of testicular development	Enlarged ventral prostate gland Disruption of blood-testis barrier	Reduced weight of testes and prostate Thinner seminiferous epithelium Loss of structural integration in the gonadal compartment
Hormone profile	↓Testicular and serum T	↓Plasma T ↑Insulin growth factor	↓FSH, LH,GnRH, T
Semen quality	Impaired spermatogenesis ↓Sperm count ↓Sperm motility	↓Epidiymal sperm count	↓Type A spermatogonia, spermatocytes and spematids Inhibition of spermiation ↓ Sperm count and motility ↑Oxidative stress in epididymal sperm ↑Sperm DNA fragmentation

FIGURE 1 | Reproduced with permission Cariati et al. (44). FSH, Foliular stimulating hormone; LH, Luteinising hormone; GnRH, Gonadotrophin releasing hormone; T, Testosterone.

and histological changes in the reproductive tissues with these analogs (45).

HUMAN STUDIES OF PRENATAL EXPOSURE TO BPA

Due to the difficulty of completing human studies, there are understandably less studies that have addressed human prenatal exposure to BPA on subsequent male reproductive development. This is in part due to the duration of follow-up required to study potential exposure effects, the potential multiple confounders inherent in any human exposure study, and consequently the cost of such long-term studies. As the measured anogenital distance (AGD) is now a recognized marker of prenatal androgenisation (46), with a longer AGD being a marker of greater prenatal androgen exposure, this offers a potential reference point to assess prenatal androgenisation. Researchers from Shanghai measured the AGD of male infants, and related this distance to the maternal urine BPA concentration at 12–16 weeks of gestation (47). This early stage of pregnancy is considered a critical time for prenatal androgenisation, as described the masculinization programming window (48), where perturbations in the androgenic environment, may have long term consequences. This study demonstrated that those boys, whose mothers had detectable levels BPA in their urine, at 12–16 weeks of gestation, were more likely to have shorter AGD at birth, than boys with undetectable levels of maternal BPA (47). These findings were consistent when measured again at both 6 and 12 months of age, and was irrespective of ascertainment of AGD by measuring from the anus to the base of the penis, or the scrotum (47). A further study was performed using cord blood measurements of BPA in relation to the AGD among 72 boys, which demonstrated an inverse relationship between cord blood BPA concentrations and male newborn ano-scrotal distance (49).

With respect to pubertal timing, a recent study demonstrated an association of greater prenatal exposure to BPA, assessed by maternal urine measurement, with later puberty in girls and earlier puberty in boys (50). Nevertheless, when data were adjusted for overweight/obesity status, all associations for boys were attenuated, suggesting a contribution of body fat in mediating the associations (50). An earlier study, possibly the first reported study, of BPA exposure as assessed by a 3rd trimester urine sample relating exposure to pubertal timing, did not demonstrate any association with hormone levels or pubertal staging in adolescence (51). However, this may relate to the sampling timing in this study not being performed at a vulnerable time in pregnancy.

With respect to deriving associations of *in-utero* exposures to BPA with adult reproductive assessment only one study has been undertaken (28). This study, led by the author of this mini-review, studied early life influences on adult testicular function. This was a birth cohort study where men from the birth cohort, who had been followed very closely through childhood, were recruited at 20 years of age to undergo a thorough testicular assessment (serum sex steroids and gonadotrophins were measured, semen assessment undertaken, and a testicular ultrasound examination performed). The mothers of 149 of the men had blood drawn at 18 and 34 weeks of gestation in 1990–1991, and their paired samples were mixed to provide an "average" of antenatal exposure. The total BPA concentrations in the maternal samples were measured by mass spectrometry and correlated with their sons' adult testicular function. There was a substantial range in serum concentrations measured in maternal serum, with the 10, 25, and 95th percentile concentrations recorded as $\leq$0.005, 0.08, and 2.15 μg/l, respectively, reflecting a large variation in exposures. The result of the analysis after adjustment for time since last ejaculation, maternal smoking, sexual abstinence, and presence of a varicocele, demonstrated that maternal BPA exposure was positively associated with their sons' sperm concentration and motility in adulthood. In addition, no associations of maternal serum BPA concentrations were detected with their sons' testicular volume or hormonal measures of male reproductive function in adulthood; serum testosterone, LH, FSH, or inhibin B concentrations (28). The positive association of maternal BPA exposure with sperm concentration and motility may be chance findings (**Table 1**), in view of the lack of other associations being identified. However, the association may be real, but the study is limited by numbers of participants (maternal BPA measures were available for 284 men, however only 149 of them underwent testicular assessment at 20 years of age). It is important to state that

TABLE 1 | Correlation between adult testicular volume and semen parameters with BPA exposure.

Ranked phthalates		Testis volume (mls)	Semen sample parameter					
			Volume (mls)	Sperm output (million)	Concentration (million/ml)	SCSA (%)	Normal morphology morphology	Motility (a + b grade)
BPA	Correlation	−0.05	−0.05	0.13	**0.18**	0.05	0.00	**0.18**
	P-value	NS	NS	NS	**0.037**	NS	NS	**0.036**

All correlations were adjusted for abstinence period, presence of a varicocele and maternal smoking and in addition, testicular volume was also adjusted for adult height (z-scores). NS, not statistically significant; BPA, bisphenol A; SCSA, sperm chromatin structural assay.
Values in bold purely highlighting the statistically significant results.

contemporary BPA exposure was not measured, which may have influenced the results, as recent xenoestrogen exposure has the potential to influence the testicular assessment. Furthermore, any potential associations may be lost by the long duration of follow-up, due to the multiple exposures and life events that will have occurred over 20 years. Within our study the median total serum BPA concentration within the maternal blood was 0.25 μg/l, which is similar to reported by the EFSA (11), and other authors (52, 53). However, it must be stated that the assessment of BPA exposure was via serum sampling, whereas the standard method of assessment is urine, hence the serum concentration documented may not reflect a more sustained exposure as recorded in urine measurement. As the concern with serum measures is that urine provides significantly less variability than serum for a compound with a relatively short half-life, although even urinary total BPA concentrations vary across different times in pregnancy (54), and individuals have a diurnal variation, with the exposure levels generally being lower in the morning than the evenings (55). Consequently variability of the concentrations recorded understandably reduces the power of any statistical analysis. Furthermore, as recently proposed, if the method of analysis of BPA concentrations was flawed, then the exposure levels may have been greater than reported, and subtle associations may have been missed (56), although the recognized measurement of serum BPA is well-established and reliable, as BPA contamination can be controlled during sample collection and inadvertent hydrolysis of BPA conjugates can be avoided during sample handling (57, 58).

CONCLUSIONS

The focus of this mini-review has been to determine if there is any association between prenatal BPA exposure and human male reproductive function. There have been many cross-sectional studies looking at linking assessment of reproductive function with current BPA exposure, such as timing of puberty and sperm counts, however the purpose of this review was to determine if the exposures to BPA at a critical stage of development, the "masculinization programming window" may lead to a permanent perturbation in the hypothalamic-pituitary-gonadal axis. Furthermore, from animal studies it may be suspected that BPA exposure may also have a permanent gonadotoxic effect. The benefit of animal studies are numerous, in that they are comparatively cheap, have the ability to control for multiple confounders and exposures within an environment, and due to their short gestation, and pubertal maturation period, provide an ability to review a life-span in a relatively short period of time. However, a major problem with animal studies of endocrine disrupting chemicals is that these chemicals are known to have potential different effects at different concentrations leading to difficulty in extrapolating animal effects to the human situation. Furthermore, whilst it appears form the animal studies that BPA has an endocrine disrupting influence when administered in the prenatal, and perinatal period, it is important to determine whether human exposures are at, above or below, safe levels of exposure in the perinatal period. Controversially, the EFSA stated in 2015 that current levels of exposure are below the tolerable daily intake (<4 μg/kg bw/day) and as such current BPA exposure does not pose a threat to the fetus (11). However, work performed by independent researchers cast some doubt on these claims, and raise concerns that very low doses of exposure may pose a risk during development (59, 60). Furthermore, there is evidence to suggest that the previous methods used to measure BPA exposure, using enzyme de-conjugation, may substantially underestimate human exposure, and hence fail to detect any subtle associations (56). The explanation for this is that an assay that reduces the variance in BPA concentrations, underestimates the exposures of those most highly exposed, tends to lead to an increase in the likelihood of false negative findings. Furthermore, it is proposed that the current safe levels are flawed, as evidence suggests that low-dose BPA exposure induces marked adverse effects below the considered safe levels (40). Indeed, the greatest number of effects were observed, in one study, at doses substantially lower than the current "safe" dose of BPA for humans (59). As this CLARITY study found that there were evidence of detrimental effects detected at doses of 2.5 μg/kg per day (59). With respect to human serum levels of free BPA, the serum concentrations have been reported to be below the limits of detection (<0.2 μg/L) in several cohorts (11, 52), which has led to doubts around the potential for environmental BPA exposure to exert endocrine disrupting effects (52).

The limited human data presented suggest that prenatal exposure to BPA may have a potential negative association with early life anogenital distance for boys, but the evidence for an influence on pubertal timing is less clear. Furthermore, it is unclear whether prenatal exposure to BPA *in-utero* has an influence on later life mature male reproductive health,

with the data suggesting a potential positive association with sperm concentration and motility at 20 years of age. Certainly there is a need for further long-term studies of early life exposure to endocrine disrupting chemicals, such as BPA, to assist individuals and regulatory authorities in their decision making for the use of chemicals in the environment.

AUTHOR CONTRIBUTIONS

RH conceived the mini-review and wrote the manuscript.

FUNDING

This study was supported by Australian NHMRC Grant Number 634457.

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Conflict of Interest: The author has equity interests in Western IVF.

frontiers in Endocrinology

REVIEW
published: 04 June 2020
doi: 10.3389/fendo.2020.00301

Bisphenols and Male Reproductive Health: From Toxicological Models to Therapeutic Hypotheses

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Bisphenols, and in particular bisphenol A (BPA), have been widely used for the production of plastic manufacts in the last 50 years. Currently, BPA is present in a variety of daily use polycarbonate plastics and epoxy resins, and dietary ingestion is considered the main route of human exposure. Accordingly, BPA is the chemical pollutant with the widest exposure in humans, involving nearly 90% of general population, according to recent studies. Concerns about BPA effects on human health date back to 1930s, when severe impact on male sexual development was suggested. Now, the acknowledged biological effects of BPA are various. In regard to human fertility, BPA has been shown to disrupt hormone signaling even at low concentrations. Results from human epidemiological studies have reported BPA interference with follicle stimulating hormone, inhibin B, estradiol, testosterone levels, and sexual function in male subjects. Moreover, recent studies have reported an association between BPA levels and reduced sperm concentration, motility, normal morphology, sperm DNA damage, and altered epigenetic pattern, resulting in trans-generational legacy of BPA effects. In this review, the recognized effects of BPA on male reproductive health are described, from the most recent issues on experimental models to epidemiological data. In addition, the very recent interest about the use of nutraceutical remedies to counteract BPA effects are discussed.

Keywords: endocrine discruptors, semen parameters, endocrine axes, drug metabolism, exposure markers

OPEN ACCESS

Edited by:
David H. Volle, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Laura Maria Mongioì, University of Catania, Italy
Giulia Rastrelli, University of Florence, Italy

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *04 March 2020*
Accepted: *20 April 2020*
Published: *04 June 2020*

Citation:
De Toni L, De Rocco Ponce M, Petre GC, Rtibi K, Di Nisio A and Foresta C (2020) Bisphenols and Male Reproductive Health: From Toxicological Models to Therapeutic Hypotheses. Front. Endocrinol. 11:301. doi: 10.3389/fendo.2020.00301

INTRODUCTION

Bisphenols, and in particular the phenol compound 2,2 Bis (4-hydroxylphenyl)–propane, universally known as Bisphenol-A (BPA), are widely used as additives for the production of plastic materials, such as polycarbonate, phenol and epoxy resins, and polyesters and polyacrylates, as well as an antioxidant in foodstuffs and cosmetics (1, 2). Specifically, nearly 75% of the industrial production of BPA is intended for the manufacture of polycarbonate-based products, which find wide application in food industry, such as in containers for food and beverages, in plastic dishes, in kitchen utensils, in containers for microwave cooking, and until 2011, in bottles (3). Of note, BPA is also used in epoxy resin films used as binary patina: the internal coatings in the cans for canned food (4).

BPA is a solid at 25°C with a melting temperature of 156°C, insoluble in water but soluble in alcohol, ethers, and fats. Accordingly, BPA can migrate for continuity in food and drinks by direct contact with plastic container under certain conditions. Prolonged storage times, exposure to high temperatures (e.g., >70°C), and the presence of foods with a significant lipid component

represent some of these conditions. Consequently, BPA enters the food chain due to the massive use of plastics, as containers or technological packaging, and as a function of increasing the shelf life of foods (5). A recent report from the European Food Safety Agency (EFSA) showed that the highest concentrations of BPA were found in packaged products (on average 18.68 μg/kg) compared to unpackaged foods (on average 1.50 μg/kg) (6). To this regard, the most relevant concentrations of BPA (>30 μg/kg) were observed in packaged food, such as cereals, meat and fish, ready-to-use foods, snacks, and sweets. As for other bulk foods, the presence of the contaminant is most likely due to the production processes. Among unpackaged foods, the highest concentrations were found in fish, with average values of 9.40 μg/kg. These data strongly suggest a major role of massive plastic pollution in waterways (7).

As a result, there is a significant risk of human exposure to BPA through ingestion, skin contact, or inhalation (8, 9). Once accessed into the body, nearly the 12% of BPA is metabolized in the liver by glucuronidation, providing more water-solubility and quicker excretion in urine, even if the concentrations in plasma and urine are very low and difficult to detect (10, 11). In addition, the conjugated form of BPA is equally accounted in the pool of the active forms (12). For this reason, total urinary BPA, including both conjugated and unconjugated BPA, is generally used as a biomarker of exposure to BPA (13). Epidemiological data from the United States have reported detectable levels of BPA in urine samples from more than 90% of general population, resulting a major problem of exposure to chemical substance (14).

Concerns about BPA issues on the human health date back to 1930s, when severe impact on male sexual development had been suggested. From a mechanistic point of view, the most relevant risks associated with the exposure to BPA are mainly due to its action as an endocrine disruptor (ED), being able to interfere with the balance of the hormonal system and thus causing harmful effects on the whole body (15). Available reports in late 1990s firstly documented a stimulating activity of BPA on estrogen receptor α that differed, however, from the classical pattern observed in weak estrogens, partial agonists, and pure antagonists (16, 17). This evidence was confirmed by subsequent investigations, reporting that BPA binds several nuclear receptors, mimicking the action of endogenous steroids, maintaining the target molecule in active conformations or blocking the access of endogenous 17β-estradiol to receptor's binding site by competition (18–20). In addition, unconjugated BPA showed a binding activity to other two receptors: the G protein-coupled estrogen receptor 30 (GPR30), also known as membrane estrogen receptor alpha (mERα) (21, 22) and the orphan nuclear estrogen-related receptor gamma (ERR-gamma) (23, 24). Finally, experimental animal studies demonstrated that BPA binds also to the androgen receptor (AR), to the peroxisome proliferator-activated receptor gamma (PPAR-gamma), and the thyroid hormone receptor (19).

On these bases, the exposure to BPA is increasingly suspected to exert major reproductive issues, such as the impairment of semen production in men as well as alteration of the hormonal cycle and oocyte maturation in women (25). This narrative review will cover available evidence regarding the male reproductive outcomes associated with the exposure to BPA. In addition, possible remedies to counteract BPA effects are discussed.

METHODS

PubMed, Scopus and Web of Science databases were used to perform a literature search on the time interval 2000–2019. The following terms were included: "bisphenol male fertility," "bisphenol testis," "bisphenol reproductive outcome," "bisphenol semen parameters," "bisphenol spermatozoa," "bisphenol nutraceuticals," "bisphenol dietary supplements," "bisphenol antioxidant," "bisphenol medicinal plants." We included studies on cell models, studies on murine models, and observational studies in humans.

The overall 6,865 records were then screened for relevance to the topics, for a total of 77 studies finally considered for the review. Data from eligible studies were considered separately, according to the different following topics: "data from animal studies," "data from human studies," and "nutritional remedies to BPA related disorders."

Disrupting Effects of BPA on Male Fertility: Data From Animal Studies

A wide amount of data from animal studies shows a clear effect of BPA on male reproductive system, even at very low doses. One of the first investigations on this topic relied of the fact that BPA is massively used in sealant made of resin-based composite materials for dental use, with the consequent oral ingestion of BPA. Al-Hiyasat et al. were among the first to investigate the reproductive outcome in male mice exposed to BPA by oral ingestion, suggesting possible issues for infertility, genital tract malformations and increased cancer rates in estrogen sensitive target tissues (26). BPA doses >25 ng/kg were associated with reduced sperm count, both at epididymal and ejaculated level, and with significant reductions of the absolute weights of the testes and seminal vesicles. These early results were confirmed by more recent studies reporting decreased sperm count associated with the exposure of BPA in rodent models, suggesting major impairment of the spermatogenetic process. (27–33). In addition, lower levels of exposure were equally associated with reduced semen quality, particularly with regard to motility parameters and markers of adequate cell-redox balance (27, 29, 31, 33, 34). Furthermore, the exposure to BPA has been associated with the alteration of other non-conventional markers of sperm quality such as the index of DNA fragmentation, suggesting a possible role as mutagen (29, 31, 34–42). Also, in a recent study from Wisniewski et al. acrosomal integrity, an overall marker of the fertilization potential, was significantly reduced by PBA exposure in murine models (27).

As outlined by the aforementioned studies, BPA showed major abilities to interfere with spermatogenesis and germ cell maturation, a process largely regulated by the synthesis of testosterone (T) from the Leydig cell population of the testis under the direct control of pituitary luteinizing hormone

(LH) (43). Several studies have been performed to disclose the possible disruption of the hypothalamus/hypophysis/testis axis (HHTA) associated with BPA exposure in animal models, with the result of a fairly complex picture that invariably leads to the impaired production of T (28, 44). In this regard, both direct effects on Leydig cells and indirect effects on HHTA were recognized. Among the direct effects, a study conducted in the classical murine Leydig MA-10 cell model, Lan et al. showed that BPA forces a detour of the normal steroidogenic activity by stimulating, on one hand, the production of 17-hydroxy-pregnenolone and T from cholesterol, but on the other hand, the expression of CYP19A1, the aromatase activity that converts T into 17-β estradiol, resulting in a overproduction of this latter (45). Other studies suggested that BPA triggers multi-level dysfunction in Leydig cells, altering either insulin signaling and glucose transport or the mitochondrial activity, with a resulting downstream redox imbalance and altered steroidogenesis (46, 47). As anticipated, BPA was also suggested to indirectly suppress the pituitary LH release through the massive aromatase upregulation in the testes; the consequent increase of serum estrogens would then exert a negative hormonal feedback at central level (48). Importantly, because of its high lipid solubility, BPA undergoes to trans-placental transfer in animal models with a consequent detection in cord blood, an evidence reported also in humans (49–52). Accordingly, BPA exposure during the prenatal period was associated with the impairment of both fetal development and the endocrine function of the testis, with reduced Leydig cell proliferation and fetal testosterone production (53–55). Additional data from animal models suggests that the endocrine disruption associated with BPA exposure in male fetuses negatively affects fertility in adult life. To this regard, in a study by Salian et al., maternal exposure to BPA was associated with reduced sperm count and motility in male offspring and, in turn, with post implantation loss and decreased litter size (56). However, the mechanisms by which BPA interferes with testis development and function, whether in fetal or in adult life, seem to be wider than the exclusive endocrine disruption of the HHTA. In fact, exposure to BPA alters the glucose homeostasis in germ cells through the decreased expression of GLUT-8 glucose transporter, particularly in spermatocytes and spermatids (39). In addition, an increased oxidative stress in the testis was claimed as the responsible for the impaired seminal quality associated with exposure to BPA (35, 57). For example, excessive production of reactive oxygen species (ROS) and consequent mitochondrial dysfunction induced by BPA, was associated with Sertoli cells apoptosis (58). BPA was also suggested to directly interfere with apoptotic signaling and to induce the morphological changes in Sertoli cell mitochondria, the triggering of Pten/Akt signaling pathway, or the activation of the JNKs/p38 MPAK pathway, with the consequent nuclear translocation of NF-kB and Fas/FasL system (59–61). Despite this severe interference with Sertoli cell cycle, a morphological alteration of testicular histologic architecture was not observed frequently, largely depending on the protocol of administration. In fact, Aikawa et al. showed that the experimental exposure of male mice to 50 μg BPA for 5 days after birth caused a decrease in normal morphology and sperm motility with no significant histologic changes of testes (62). Jiang et al. observed ultrastructural lesions in Sertoli and Leydig cells after the administration of 5 mg/kg/day of BPA to rats for 8 weeks (63).

Of note, very recent studies disclosed some transgenerational effects associated with BPA exposure. Manikkam et al. showed that the early exposure of female gestating rats to a cocktail of plastic additives, including BPA, was associated with a significant increase of the prevalence of diseases and abnormalities in F1 and F3 generation males, particularly pubertal abnormalities, testis disease and obesity (64). Likewise, similar effects were exerted by replacement bisphenols, namely compounds structurally similar to BPA used in "BPA-free" products (65). Subsequent studies were able to detect major genetic abnormalities associated with exposure to BPA. Firstly, BPS showed a mutagen effect on male germ cells, resulting in blocked meiotic progression of germ cells (31, 66). Furthermore, Shi et al. showed that both BPA and replacement bisphenols are able to modify the expression of DNA methyltransferases and the pattern of histone methylation in the neonatal and adult testes (67).

However, earlier studies by Hass et al. on this topic (68) reported that male offspring from pregnant Wistar rats, gavaged with bisphenol A from gestation day 7 to pup day 22, showed a significant reduction of the sperm count only at the lowest bisphenol A dose (25 μg/kg/day). Higher doses had no effect on either sperm parameters or the weight and histology of the reproductive organs. These results suggest a likely transgenerational toxicity of bisphenols, with a possible mechanistic involvement of epigenetics on the impairment of male reproductive functions. However, a more complex scenario should be hypothesized given the observed non-monotonic dose–response relationship.

DISRUPTING EFFECTS OF BPA ON MALE FERTILITY: DATA FROM HUMAN STUDIES

Despite the large availability of data in animal models, fewer studies assessed the possible relationship between BPA exposure and semen quality in humans. The first reports on this topic dealt with occupational medicine; particularly, Li et al. found a negative association between urinary BPA and sperm concentration, total sperm count, viability, and motility in 215 factory workers, further distinguished into occupationally exposed to high or low levels of BPA. However, in the subgroup with lower creatinine-adjusted urinary BPA, the only significant association was with reduced sperm concentration. Notably, urinary BPA levels were not associated to altered morphology in this study (69). As for data in animal models, other studies investigated the possible association of BPA exposure with alterations of sperm DNA. Meeker et al. explored the possible correlation between urinary BPA concentration and sperm DNA damage, evaluated by neutral comet assay, in a cohort of 190 subfertile male patients (70). Urinary BPA concentration was associated with reduced sperm concentration, motility, and morphology, whereas a positive association with sperm DNA damage was observed. However, two independent studies on

male partners from infertile couples attending infertility clinics were not able to retrieve any significant association between BPA urinary concentration and altered semen parameters. Importantly, a relatively high variability of exposure markers was observed, since the mean urinary BPA concentration in these two studies were, respectively, 1.5 and 0.6 ng/mL (71, 72).

Another field of investigation pursued was the possible correlation between exposure to BPA and alteration of the endocrine pattern, but widely varying scenarios can be observed. Hanaoka et al. conducted a study on 42 workers occupationally exposed to BPA through the handling of epoxy resin spray containing BPA (73). Interestingly, authors have found lower serum levels of follicle-stimulating hormone (FSH) in exposed workers compared to those non-exposed, although non-obvious differences in plasma LH and free T levels were observed. Also, Galloway et al. investigated the relationship between urinary BPA and male reproductive hormones in a cohort of 715 healthy adults aged 20–74 years from the general population (74). Surprisingly, urinary BPA levels were positively and significantly associated with serum T levels, but no associations with either 17-β estradiol, sex hormone-binding globulin (SHBG), or free T were observed. On the other hand, Lassen et al., in a study on 308 healthy males from the general population, found increased serum T, free T, LH, and estradiol in subjects pertaining to higher urinary BPA concentrations quartile, compared with the lowest quartile. Subjects in the highest urinary BPA quartile also showed reduced progressive sperm motility compared with the lowest quartile (75). Also, Mendiola et al. performed a similar study on 375 fertile men recruited from prenatal clinics, finding that urinary BPA concentrations were positively associated with serum SHBG levels and inversely correlated with free androgen index (FAI), calculated as total T × 100/SHBG and the FAI/LH ratio. However, serum FSH, LH, total T, inhibin B, and free T levels showed no obvious correlation with urinary BPA concentration (72). In addition, Meeker et al. found a negative association between urinary BPA levels and both serum inhibin B levels and 17-β estradiol/T ratio in male partners of subfertile couples attending a fertility clinic; however, BPA was positively associated with both FSH and FSH/inhibin B ratio (76).

Finally, few studies aimed to assess the possible impact of BPA exposure on the overall fertility potential in males through the overall evaluation of the relationship between BPA levels and the reproductive outcome in the setting of assisted reproduction facilities. In a study enrolling 215 infertile couples undergoing assisted reproduction techniques, with roughly equal distribution between *in vitro* fertilization and intrauterine insemination, Dodge et al. (77) investigated the possible correlation between urinary concentrations of parabens and BPA with the live-birth rate. Authors found minimal association between paternal urinary propyl paraben levels and reduced live-birth rate in a correlation model corrected by possible confounders. However, no significant association emerged between paternal urinary BPA and reproductive outcomes after fertility treatments (77). On the other hand, Buck-Louis et al. in the Longitudinal Investigation of Fertility and the Environment (LIFE) Study, a multicenter investigation involving 501 infertile couples from 16 targeted counties in the middle-east of the United States (78), evaluated the possible relationship between time to pregnancy (TTP) and urine levels of more than 15 environmental pollutants, including BPA, in both males and females. Urinary BPA concentration in either males or females was not associated with increased TTP, which was instead correlated with male urinary concentration of monomethyl, mono-n-butyl, and monobenzyl phthalates.

Overall, available data are supportive of detrimental role of BPA on semen parameters, but this is not accompanied by clear data on sex hormones and on fertility outcomes. As suggested by other authors (79), within the limits of the availability of data in humans, a possible reconciling explanation could rely on a greater direct toxicity of BPA on germ line cells, rather than in an albeit important endocrine disruption of the HHTA. This hypothesis is somewhat supported by very few studies reporting the interference of BPA on germ cell development in human fetal testis and on mitochondrial activity and energy metabolism in ejaculated human sperms (57, 80, 81).

NUTRACEUTICAL APPROACHES TO OVERCOME BPA EFFECTS

Given the large availability of evidence reporting detrimental effects of BPA on testis function, especially in animal models, this chemical has progressively gained a role as a reference substance, able to induce endocrine disruption in several experimental models, from laboratory animals to *in vitro* cell cultures (82). On this basis, some recent studies have focused on possible approaches to treat or prevent BPA-induced derangements and testicular toxicity. Since the direct toxicodynamics of PBA on both Leydig and germ cells of the testis were largely related to the impairment of cell redox system, most of the treatment approaches relied on the use of natural sources of antioxidants.

Based on the fact that the expression of the enzymes glutathione peroxidase and glutathione reductase are regulated by melatonin, a study from Anjum et al. aimed to disclose the possible effect of melatonin on mitochondrial lipid peroxidation observed in mouse testis after BPA exposure (41). Interestingly, the treatment with melatonin reduced mitochondrial lipid peroxidation, restored the overall mitochondrial enzyme machinery and improved the mitochondrial antioxidant pool compromised by BPA. However, major limitations of the study were represented by the high dosage of melatonin administered intraperitoneally. In 2012, El-Beshbishy et al. demonstrated some mitigation of the mitochondrial toxicity exerted by BPA exposure in rats, by the co-administration of lipoic acid (44). Also, Khalaf et al. (83) recently reported a protective effect of selenium (Se) against BPA-induced testis impairment in albino male rats. In particular, co-administration of Se attenuated the reproductive issues induced by BPA toxicity through the restoration of testicular antioxidant activity and the amelioration of sperm genetic abnormalities observed in BPA exposed animals (83). Similar results were obtained by Kaur et al. who reported decreased lipid peroxidation in mouse testis associated with the co-administration of Se and BPA, compared with sole exposure to BPA (84).

TABLE 1 | Summary of the references supporting the possible effects of bisphenols on male reproductive health.

	Animal Model		Human Model	
Outcome	***In vitro***	***In vivo***	***In vitro***	***In vivo***
Testis Histology		(62) ↔ (63) ↓		
Effect on sperm count		(26) ↓ (29) ↓ (28) ↓ (61) ↓ (↓Sertoli cell function) (32) ↓ (33) ↓ (30) ↓ (31) ↓ (59) ↓ (↓Sertoli cell function) (60) ↓ (↓Sertoli cell function) (27) ↓		(71) ↔ (69) ↓↔ (95) ↓ (72) ↔
Effect on sperm motility/mitochondrial function		(39) ↓ (↓ Germ/Sertoli cells metabolism) (29) ↓ (34) ↓ (33) ↓ (31) ↓ (27) ↓	(57) ↓ (35) ↓ (80) ↓ (81) ↓	(71) ↔ (75) ↓ (95) ↓ (72) ↔
Sperm DNA Fragmentation		(41) ↑ (35) ↑ (39) ↑ (29) ↑ (42) ↑ (40) ↑ (36) ↑ (34) ↑ (37) ↑ (31) ↑ (38) ↑		(70) ↑
Testosterone Production	(46) ↓ (↓ redox balance) (47) ↓ (↓ redox balance) (45) ↓ (↑ CYP19)	(53) ↓ (↓Fetal testis development) (44) ↓ (28) ↓ (55) ↓ (↓Fetal testis development) (54) ↓ (↓Fetal testis development) (48) ↓ (↓ LH by estrogens)		(74) ↑ (73) ↔ (75) ↑ (76) ↓ (72) ↓↔
Fertility Outcome				(78) ↔ (77) ↔
Fertility in Offspring		(66) ↓ (68) ↓↔ (64) ↓ (56) ↓ (↓ Fetal testis development) (67) ↓ (31) ↓		

For each outcome considered, the respective references are listed according to the model used, animal or human, the in vitro or in vivo evidence and the observed effect (↓, decrease; ↑, increase; ↔↓, mild decrease or no effect). When available, mechanistic details are provided.

Another key vitamin supplementation, namely vitamin D, showed a partial restore of testicular fibrosis in a complex rat model of diabetes, obtained by streptozotocin treatment, associated with BPA-induced hypogonadism (85). Interestingly, this effect appeared as the result of a direct downregulation of nuclear factor kappa B exerted by vitamin D, rather than the indirect involvement of the central pituitary/testis axis.

Of note, the composition of the antioxidant mixture seems to have major relevance on the efficacy of the treatment. In fact, the classical vitamin C administration failed to produce any amelioration on the testicular oxidative damage induced by BPA in rats, or even exerted worsening effects (86). On the contrary, Rahman et al. (87), in an *in vitro* experimental model on isolated mouse spermatozoa, showed that the combination of glutatione, vitamin C, and vitamin E effectively prevented the oxidative stress and the respective downstream tyrosine phosphorylation-signaling pathway, avoiding the premature acrosome reaction and possibly improving the fertilization capacity of sperm cells exposed to BPA. (87).

Interestingly, a wide variety of phytochemicals and plant extracts showed ameliorating effects of testis function in rodent

models exposed to BPA (88). *Cordyceps militaris*, a medical fungus largely employed in Chinese traditional medicine, restored the histological architecture of seminiferous tubules and epididymis in male rats exposed to BPA, with a significant recovery of the sperm count, through the likely reduction of the oxidative stress damage (89). Also, lycopene showed a detoxifying activity toward testicular damages associated with BPA exposure, as evidenced by the protection from the loss of germ cell population, the reduction of testis and epididymis weight, as well as the impairment of sperm motility, exerted by the treatment of male rats with the sole BPA (90). Furthermore, co-administration of quercetin, an antioxidant phytochemical member of the polyphenolic flavonoid family, amended the toxic effects on testis and epididymis exerted by BPA (91).

Our group recently showed that the metabolic/mitochondrial disruption, induced by the *in vitro* exposure of human spermatozoa to BPA, was effectively compensated by low dose treatment with aqueous extract from leaves of *Eruca sativa*, a plant of the Brassicaceae family widely represented in the Mediterranean region. Importantly, the characterization of the extract showed to be extremely rich in natural antioxidants, such as polyphenols and flavonoids. The treatment with high concentration of the aqueous extract was unexpectedly associated with severe disruption of both mitochondrial and cell membrane redox balance, resulting in a significant loss of sperm motility (81). Importantly, these preliminary results have been confirmed by a subsequent study performed on Wistar rats (92). Consistent with *in vitro* data, the overall hormonal and semen disruption associated with BPA exposure was significantly ameliorated by the low-dose administration of *Eruca sativa* aqueous extract, while it was worsened by high dose treatment.

Despite these encouraging results, exogenous antioxidants may exert a double-edged effect. In particular, the SELECT study found that the supplementation of vitamin E significantly increased the risk of prostate cancer among healthy men (93). Furthermore, more recently, it has been shown that vitamin E can act as pro-oxidant agent promoting DNA damage and cell transformation (94). Thus, the use of antioxidants-based dietary supplements for the prevention of disease states in general, and in particular for the compensation from altered states associated with exposure to environmental factors, should be considered with caution.

CONCLUSIONS AND FUTURE PERSPECTIVES

Bisphenol A represents one of the most controversial chemical pollutants, with the typical features of an endocrine disruptor. Early toxicological evidence on BPA date back to nearly 30 years ago, when major interference with estrogen signaling pathway was claimed. Since that time, a wide range of cell mechanisms of both endocrine and metabolic disruption have been claimed by the use of experimental models. In particular, major impairment of the male hypothalamus/hypophysis/testis axis has been recognized as associated with the exposure to BPA during both the fetal and the adult life, resulting in altered testis development, impaired endocrine function and infertility. In this regard, direct disruption of sperm characteristics, such as reduced motility performances and development genetic abnormalities have been identified. On the other hand, data obtained in humans are actually limited and poorly conclusive to identify a strict causal role of BPA in reduced male fertility potential. A summary of references supporting each singular effects of bisphenols on male reproductive health is reported in **Table 1**.

Methodological differences and different study populations are factors that can explain some discrepancies. Moreover, available clinical outcomes, such as semen parameters and time to pregnancy, are likely susceptible of variation related to many different confounding factors. It should be noted that, as for most of chemical pollutants, the identification of a reliable marker of exposure remains a major issue. Specifically, for BPA, urinary concentrations are surely reliable data from an analytical point of view, but may not be representative of the real exposure to BPA due to its short half-life. To this regard, Vitku et al. reported that BPA levels in blood plasma were positively correlated with BPA levels in semen, but only seminal BPA was negatively associated with seminal quality (96). Finally, the cross-sectional design of the available studies surely provides proof of association, but limited evidence of causality.

One of the main problems associated with exposure to endocrine disruptors in general, and to BPA in particular, is represented by the potential activity at low concentrations. This represents a critical issue during the development phases, such as embryo/fetal life and newborn or peri-pubertal age, since the effects in these time windows may be irreversible and are generally detected only at adulthood (15). Accordingly, populations at higher risk includes pregnant women, infants, and adolescents. On these bases, the current European law restricted the use of BPA in the production of packaging and materials in direct contact with food by limiting migration rate to 0.05 mg/kg of food and prescribing the total absence in products for newborns, from food to food containers and clothes (6). In addition, based on new toxicological data and methodologies, the European Authorities adjusted the tolerable daily intake from 50 to 4 μg/kg body weight/day with an overall lowering rate of 12 times, highlighting the increasing level of attention for these health concerns.

In conclusion, reproductive issues associated with bisphenol A exposure still remains an intense field of investigation, particularly dealing with health consequence reported in males. Current challenges for the future are represented by the identification of efficient markers of exposure in order to address the extent of health consequences in different age ranges.

AUTHOR CONTRIBUTIONS

CF supervised manuscript drafting. MD reviewed clinical data. GP and KR reviewed data on nutraceuticals. AD reviewed experimental data. LD drafted the manuscript.

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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.

The reviewer GR declared a past co-authorship with one of the authors MD to the handling editor.

frontiers in Endocrinology

REVIEW
published: 12 June 2020
doi: 10.3389/fendo.2020.00353

Bisphenol A and Male Fertility: Myths and Realities

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Bisphenol A (BPA) represents the main chemical monomer of epoxy resins and polycarbonate plastics. The environmental presence of BPA is widespread, and it can easily be absorbed by the human body through dietary and transdermal routes, so that more than 90% of the population in western countries display detectable BPA levels in the urine. As BPA is qualified as an endocrine disruptor, growing concern is rising for possible harmful effects on human health. This review critically discusses the available literature dealing with the possible impact of BPA on male fertility. In rodent models, the *in vivo* exposure to BPA negatively interfered with the regulation of spermatogenesis throughout the hypothalamic–pituitary–gonadal axis. Furthermore, in *in vitro* studies, BPA promoted mitochondrial dysfunction and oxidative/apoptotic damages in spermatozoa from different species, including humans. To date, the claimed clinical adverse effects on male fertility are largely based on the results from studies assessing the relationship between urinary BPA concentration and conventional semen parameters. These studies, however, produced controversial evidence due to heterogeneity in the extent of BPA exposure, type of population, and enrollment setting. Moreover, the cause–effect relationship cannot be established due to the cross-sectional design of the studies as well as the large spontaneous between- and within-subject variability of semen parameters. The best evidence of an adverse effect of BPA on male fertility would be provided by prospective studies on clinically relevant endpoints, including natural or medically assisted pregnancies among men either with different exposure degrees (occupational/environmental) or with different clinical conditions (fertile/subfertile).

Keywords: endocrine disruptors, environmental pollution, oxidative stress, spermatozoa, sperm DNA damage

OPEN ACCESS

Edited by:
Ravinder Anand-Ivell, University of Nottingham, United Kingdom

Reviewed by:
Nafis Hasan, Tufts University, United States
Richard Graham Lea, University of Nottingham, United Kingdom

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *05 March 2020*
Accepted: *05 May 2020*
Published: *12 June 2020*

Citation:
Castellini C, Totaro M, Parisi A, D'Andrea S, Lucente L, Cordeschi G, Francavilla S, Francavilla F and Barbonetti A (2020) Bisphenol A and Male Fertility: Myths and Realities. Front. Endocrinol. 11:353. doi: 10.3389/fendo.2020.00353

INTRODUCTION

Bisphenol A (BPA), 4,40-isopropylidenodi-phenol, 2,2-bis(4-hydroxyphenylo)propane, is a crystalline chemical compound widely used as key monomer of epoxy resins and polycarbonate (PC) plastics for more than 50 years (1). The industrial use of BPA is impressive with ∼9 million tons per year produced worldwide (2–4). Resiliency, flexibility, and durability have decreed the large-scale success of BPA-based PC plastics, leading to their use in many and various fields, ranging from the arms industry, for components of safety equipment (helmets), to the manufacture of medical devices, including dental sealants and fillers. In the food industry, synthetic materials containing BPA are widely employed for manufacturing long-term food and drink containers and represent key components of protective coatings, including those covering the internal surface of cans (2–4). A wide variety of other commonly used articles also contain BPA and its derivatives:

fridges, baby bottles, dishes, lenses, sunglasses, hair dryers, CD and DVD, cell phones, computers, and thermal paper (1, 5, 6). Owing to its ubiquitous presence, environmental persistence, and the reputation of being an endocrine disruptor, BPA is now regarded as a potential threat to human health, and concerns arise from its possible link with cardiovascular diseases, metabolic disorders, cancer, and infertility (7–10).

The aim of this review is to critically outline and discuss the available literature dealing with the possible impact of BPA on male fertility.

TOXICOKINETICS OF BPA

BPA is an ideal plasticizer because of its cross-linking characteristics; nevertheless, free monomers can be released into food content after polymerization, especially on exposure to high temperature and with re-use of the containers: this makes possible BPA entering the organism (11–14). As early as 1994, Brotons et al. (15) reported that vegetables preserved in lacquer-coated cans acquire estrogenic activity due to contamination by BPA, which is leached from the lacquer coating. More recently, Kubwabo et al. (16) reported that BPA also migrated from the wall of PC baby bottles: high temperatures and prolonged incubation times resulted in increased leaching of BPA, especially when fatty foods were used, whereas BPA leaching from non-PC baby bottles appeared to be negligible under the same experimental conditions (16). Hence, as a precautionary measure, the European Union banned BPA in the production of baby bottles in 2011(2011/8/EU). Resin-based dental filling materials have been feared as another possible source of oral exposure (17). According with two recent systematic reviews, BPA can leach from some resin-based dental materials into the saliva (18, 19), reaching detectable urinary concentrations that peak 24 h after treatment (19). However, the extent to which such an increase may affect the health of patients remains an open question (18).

According to the main regulatory agencies, dietary route represents the primary source of human exposures (2, 20–26) and a tolerable daily intake (TDI) of 50 μg/kg body weight/day has been established, based on studies from rodent models, where clear harmful effects at much higher doses were registered. Based on an analysis of consumer exposure to BPA, the European Food Safety Authority (EFSA) stated that the current levels of exposure are below the TDI (27). Therefore, they would not represent a threat for consumers at any age, taking also into account the short half-life of orally ingested BPA (27).

After ingestion, most of BPA is quickly bound to glucuronic acid by the liver enzyme uridine diphosphonate glucuronosyl transferase (UGT) to produce BPA glucuronide (BPA-G) (25, 28). This rapid first-pass liver metabolism makes BPA more soluble in water, with a half-life of elimination in urine of 5.4–6.4 h (28, 29). Therefore, at oral doses ranging from 50 to 100 μg/kg body weight (far above the TDI), in humans, BPA elimination is essentially complete within 24 h, with free BPA accounting for < 1% of total PBA (28, 29). Of note, toxicokinetic processes can be influenced by physiological changes related to pregnancy, as the placenta exhibits beta-glucuronidase enzymatic activity that deconjugates BPA-G (26, 30, 31). Once having crossed the placental barrier, the BPA conversion to BPA-G in the fetus would be poorly effective, due to the immature liver functions (30).

The highly effective detoxifying system of the human body could counteract possible consequences of a large-scale exposure to BPA. In line with the ubiquitous use of this substance, the reports by the Center for Disease Control and Prevention revealed that more than 90% of the US population displays detectable BPA levels in urine (32, 33). In the largest population study by Calafat et al. (34), detectable levels of total (free plus conjugated) BPA were found in 92.6% of urine samples from 2,517 participants aged ≥6 years in the 2003–2004 National Health and Nutrition Examination Survey (NHANES). According to a recent study by Gerona et al. (35), these epidemiological data could even be underestimated. Indeed, the results of the largest population studies were produced by using indirect methods requiring the enzymatic hydrolysis of conjugated BPA to free BPA, before its quantification in urine by liquid chromatography–mass spectrometry (LC-MS). Unfortunately, the deconjugation process would be largely incomplete in many cases (35).

Interestingly, when NHANES data were adjusted for the fasting hours preceding the collection of the urinary samples, no clear inverse relationship between fasting hours and urinary BPA levels was found (36). This finding seems to be at the variance with the assumption that BPA is rapidly eliminated after ingestion and that the digestive tract represents the main source of exposure. On the contrary, NHANES data could suggest that either the half-life of BPA is longer than we think or this substance can, to some extent, remain stored in the body or it can even be assimilated through alternative non-dietary routes. Indeed, BPA is also detectable in indoor/outdoor air and floor dust and is widely used in products that come into contact with skin, including not only cosmetics but also thermal paper, where BPA is used as a heat-activated developer (2, 27, 37). This makes possible its absorption by alternative non-dietary means, such as inhalation and transdermal route. While the estimated inhalation exposure would be negligible when compared to dietary route (38, 39), with the exception of some factory workers with high occupational exposure (40, 41), transdermal absorption should deserve special attention. Unlike plastics and can linings, where BPA is largely in a polymerized form (PC or PVC), the printing surface of many thermal papers contains milligrams of unbound (free) BPA per gram of paper (42–45), thus explaining the quick transdermal absorption of BPA from this source after handling (26, 45–47): this raises special concerns in individuals who work as cashiers (48). The absorption degree is further enhanced by chemicals which are present in hand sanitizers that can cause a breakdown of the dermal barrier (45). According to EFSA (49), apart from oral exposure, the skin contact with thermal paper represents a major source of exposure to BPA. Of note, while almost all of bloodstream circulating BPA following oral ingestion is in the conjugated form (50–52), after entering the body via a transdermal route, BPA bypasses the liver metabolism, resulting in significantly higher concentrations of unconjugated form in the bloodstream (50, 53–56). This is relevant to toxicodynamics because only unconjugated BPA can activate estrogen receptors and is regarded as the biologically active one.

EFFECTS OF BPA ON MALE FERTILITY

Effects on Spermatogenetic Function: Evidence From Preclinical Studies

In the last decades, results of preclinical research revealed endocrine-disrupting effects of BPA on male reproductive functions, clarifying possible mechanisms by which BPA can interfere with the regulation of spermatogenesis mainly throughout the hypothalamic–pituitary–gonadal axis.

In rodent models, with some exceptions (57–59), the *in vivo* exposure to BPA at different doses (largely ranging from 2 μg/kg/day to 960 mg BPA/kg body weight/day) and time intervals (from 5 to 84 days) resulted in a significant decrease in sperm counts (60–70), sperm motility (61, 62, 67), normal sperm morphology (62), increase in sperm DNA damage (63, 67), and poor spermatogenesis (64–66, 70–72). A large between-studies heterogeneity in both cumulative effective doses and tolerable daily intakes was observed. It might partially be due to differences in susceptibility to BPA effects across rodent species and strains. Genetic factors of animal models can modulate the metabolic rate of a chemical substance, accounting for the variability of its toxicokinetics among the species (73, 74). This could determine a variable extent of sensitivity of different species and strains to the same chemical under the same experimental conditions (75).

An interference at hypothalamic–pituitary level of the gonadal axis has been clearly demonstrated in the rat, where, with a few exceptions (65, 76), the administration of BPA significantly lowered both the expression of the GnRH gene in cells of preoptic area (64) and circulating levels of gonadotropins and/or testosterone (64, 69, 70, 77–81). Interestingly, the perinatal phase would represent a sensitive exposure window (3), as the treatment of pregnant and nursing dams with BPA decreased intratesticular (77) and circulating (82) testosterone levels of male offspring in adulthood.

BPA is qualified as a xenoestrogen because it mimics estrogen effects due to its characteristic polycyclic phenolic chemical structure, similar to estradiol (77). In a study by Matthews et al. (83), BPA, but not the soluble product of its glucuronidation, was able to displace tritiated 17-β estradiol from the estrogenic alpha and beta receptors (ERα and ERβ, respectively). The authors observed a more evident dose dependence for ERβ, to which BPA exhibited higher affinity than to ERα (83). The affinity for ERα is 10,000 times lower than that of 17-β estradiol, more than 20,000 times lower than that of diethylstilbestrol, a synthetic molecule with a powerful estrogenic activity, and 3–700 times lower than that of various polychlorinated biphenyls, which represent ubiquitous organic polluting compounds in the environment (84). Despite the low affinity, the binding of BPA to ERs is biologically functional in terms of ER-dependent transcription of target genes, as demonstrated by the luciferase reporter gene assay (83). Noteworthy, although BPA acts as a weak estrogen on ERs, it exhibits a very higher affinity (similar to estradiol) for the membrane G protein-coupled estrogen receptor (GPER) of the non-classical estrogenic pathways, mediating rapid non-genomic effects of BPA even at low doses (85). In males, such an estrogen-like endocrine disruption is expected to interfere with the feedback mechanisms of the hypothalamic–pituitary–gonadal axis, leading to a reduced pituitary secretion of gonadotropins and consequent hypostimulation of spermatogenesis and Leydig cell steroidogenesis.

Indeed, the decrease in testosterone levels in animals exposed to BPA could reflect a combination of central (hypothalamic–pituitary) and peripheral (testicular) effects. The *in vitro* treatment of Leydig cells from adult rat with BPA decreased testosterone biosynthesis as a result of decreased expression of steroidogenic enzymes (77, 86).

Further possible mechanisms leading to an androgen deficiency status could be sought in the endocrine perturbation exerted by BPA on the differentiation and functions of the adipose tissue. BPA promotes both adipogenesis (87) and lipid storage in adipocytes (88); furthermore, animals treated with low doses of BPA exhibited obesity-related metabolic dysfunctions (89). In this view, BPA is now regarded as a possible environmental obesogen (90). In the complex and bidirectional relationship between obesity and low testosterone, it is well-demonstrated that adipocytes express aromatase activity which is responsible for testosterone conversion into estradiol (91), which can exert a synergistic inhibitory effect on pituitary secretion of luteinizing hormone (LH) (92, 93). An excess of fat mass is also associated with increased levels of circulating leptin which exerts a direct inhibition of Leydig cell steroidogenesis (94, 95). Noteworthily, the chemical structure of BPA is lipophilic; therefore, the effects on adipocytes could be amplified and maintained by its retention in fat mass, establishing a possible vicious circle (96).

BPA can also exert anti-androgenic activity by interfering with the signaling of the androgen receptor (AR) at several levels (9, 69, 97). BPA acts as a competitive (98) and non-competitive (99) antagonist of AR and decreases the expression of AR in the testis (66). Other mechanisms of the anti-androgenic interference include the disruption of the nuclear AR translocation (99) and the enhancement of the interaction of AR with its corepressors, such as the silencing mediator of retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor co-repressor (N-CoR) (100). As spermatogenesis requires both high intratesticular levels of testosterone and an adequate functionality of the AR (101, 102), it is not surprising that the effects of BPA on testosterone biosynthesis and activity could affect spermatogenic function.

Independently of its hormonal disrupting effects, BPA could interfere with spermatogenesis processes even through other mechanisms. After *in vivo* exposure to BPA, an impaired testicular glucose homeostasis has been reported in the rat (103), and an increased testicular oxidative stress has been revealed both in the rat (70, 103) and in the mouse (104, 105). BPA can also induce apoptosis in cultured Sertoli cells from rodents (106–109) by inducing dysfunction of mitochondria and generation of reactive oxygen species (ROS) (110). Moreover, an impaired expression of junctional proteins of Sertoli cell has been found in rats that were exposed to BPA neonatally (61), while a downregulated expression of genes involved in Sertoli cell functions has been found in mice that were exposed to BPA prenatally (62).

Effects on Semen Quality and Reproductive Outcomes: Evidence From Clinical Studies

Due to the obvious lack of controlled clinical trials investigating the effects of BPA on human male fertility, information is largely inferred from findings of observational epidemiological studies that, with a few exceptions, used semen quality as a surrogate endpoint, producing divergent results likely due to heterogeneity in the extent of BPA exposure, sample sizes, type of population, and enrollment setting (**Table 1**). Some studies included men from the general population (112–115), others included men attending fertility clinics with (116–120) or without known subfertility (121); one study was restricted to men with proven fertility (122). Only in two studies were men with occupational exposure to BPA included (111, 112).

Inconclusive results arise from studies on semen quality in the general population. In a study by Lassen et al. (114) on 308 young men enrolled during physical examinations for military service in Denmark, those in the highest quartile of BPA urinary excretion exhibited significantly lower percentages of progressive sperm motility when compared to the lowest quartile group. Adoamnei et al. (115) reported a significant negative association of urinary BPA concentrations with sperm concentration and total sperm count, but not with motility, in 215 healthy young university students. On the contrary, no significant associations were found between urinary BPA concentrations and any standard semen parameter in the Longitudinal Investigation of Fertility and the Environment (LIFE) study, which recruited 418 men from 16 counties in Michigan and Texas (113).

Also inconclusive are the findings on semen quality arising from studies that enrolled men attending fertility clinics. In a study by Meeker et al. (116), where 190 male partners of couple seeking treatment for infertility were dichotomized as either equal/above or below the reference range for total sperm number, sperm concentration, and sperm motility, according to the WHO 1999 criteria (123), urinary BPA concentration was not associated with a significant odd for having semen parameters below the reference levels. Nevertheless, when variables were modeled continuously in multivariable linear regression models, an increase in urinary BPA levels was associated with a slight, albeit just significant, decrease in the percentage of normal sperm morphology ($p = 0.049$), curvilinear velocity at the computer-aided semen analysis ($p = 0.04$) and increased sperm DNA damage ($p = 0.048$) at the comet assay. In a large case–control study by Chen et al. (120), no significant differences were found in urinary BPA concentrations between 877 men with idiopathic infertility and 713 fertile control men. In the same study, crude and multivariable adjusted models did not show significant associations between BPA levels and standard semen parameters (120). In a subsequent study on 149 couples undergoing their first or second *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) procedure, an increased urinary BPA concentration in male partners was associated with lower sperm count, sperm concentration, and sperm vitality (117). However, parameters of embryo development (from the fertilization of oocyte to the stage of blastocyst) were not related to the exposure to BPA (117). In another study on 191 Czech men with infertile marriages, seminal BPA, but not plasma BPA, levels were negatively associated with sperm concentration, sperm count, and, to a lesser extent, normal sperm morphology (118). More recently, Radwan et al. (119) reported that urinary concentration of BPA in 315 men with normal sperm concentration according to the WHO 2010 criteria (124) was negatively associated with sperm motility and positively associated with the percentage of sperm sex chromosome disomy. Finally, in a recent report on a preconception cohort of 161 men without known subfertility, higher urinary BPA concentrations were found in the group of men with abnormal sperm tail morphology, whereas no association was found with sperm count, and no information was provided about other semen parameters (121).

In the Study for Future Families (SFF), the only one enrolling men with proven fertility (315 male partners of pregnant women), regression models revealed no relationship between urinary BPA levels and semen parameters (122).

Noteworthy, Li et al. (112) assessed the relationship between urinary BPA levels and semen parameters among 218 factory Chinese workers with or without occupational exposure to BPA. Men with occupational exposure to BPA, who exhibited much higher urinary BPA concentrations, also displayed a significant negative association of BPA with sperm count, viability, and motility. A significant association with lower sperm concentration remained when analysis was restricted to non-occupationally exposed workers. In another study on factory Chinese workers (111), BPA-exposed men ($n = 75$) had significant lower sperm concentration when compared to unexposed group ($n = 65$). Interestingly, authors also found a negative independent association between urinary BPA levels and global methylation degree of sperm DNA, pointing to possible epigenetic consequences of BPA exposure, as already suggested by *in vitro* studies (125, 126).

Actually, two studies assessing more clinically relevant endpoints seem to weaken concerns about the possible adverse impact of BPA exposure on male fertility. In a series from the population-based LIFE study, where Buck-Louis et al. (127) assessed the time to pregnancy in 501 men who were actively trying to conceive, no significant association was found between higher BPA concentrations in the urine and longer duration of pregnancy attempts, after controlling for a number of possible confounders that included partner age. In the cohort of the Environment and Reproductive Health (EARTH) Study, including 218 couples who underwent assisted reproductive technologies (ART) procedures (intrauterine inseminations or IVF), no association was found between paternal urinary BPA concentrations and ART outcomes (128).

Effects on Sperm Functions: Evidence From *in vitro* Studies

Experimental studies suggest that BPA could extend its biological effects on male fertility beyond the disrupting effect on the regulation of spermatogenesis, by directly affecting sperm functions. In animal models, the *in vitro* treatment with different doses of BPA adversely affected sperm motility in fish (129),

TABLE 1 | Epidemiological studies on the relationship of urinary BPA concentration with semen quality and/or other reproductive outcomes.

Study	Population	BPA urinary concentration: mean ± SD or median (range)	Results	Adjustments
Mendiola et al. (107)	Fertile men ($n = 375$)	1.5 (0.80–3.0) μg/l*	No significant associations between urinary BPA and semen parameters.	Age, BMI, smoking status, ethnicity, center, urinary creatinine concentration, and time to motility analysis.
Meeker et al. (101)	Male partners of subfertile couples ($n = 190$)	1.3 (1.8–2.5) ng/ml	Urinary BPA concentrations were linearly associated with lower percentages of sperm with normal morphology (β regression coefficient: −0.90, 95% CI: −1.79, −0.004, $p = 0.049$), lower VCL values (β regression coefficient: −3.97, 95% CI: −7.65, −0.27, $p = 0.04$) and increased DNA damage (β regression coefficient: 3.88, 95% CI: 0.01, 7.74, $p = 0.048$).	Age, BMI, abstinence period, and smoking.
Li et al. (97)	Factory workers with and without occupational BPA exposure ($n = 218$)	38.7 (6.3–354.3) μg/grCr in exposed and 1.4 (0.0–17.9) μg/grCr in non-exposed	Urinary BPA was associated with lower sperm concentration (β regression coefficient: −15.6; $p < 0.001$), total sperm count (β regression coefficient: −42.1; $p = 0.004$), sperm vitality (β regression coefficient: −4.6; $p < 0.001$), and motility (β regression coefficient: −3.1; $p < 0.001$).	Age, education, history of chronic disease, previous exposure to other chemicals and heavy metals, employment history, marital status, age at first intercourse, smoking, alcohol consumption, and center.
Chen et al. (105)	Infertile men ($n = 877$) and fertile controls ($n = 713$)	Geometric means: 0.612 ng/ml in cases and 0.621 ng/ml in controls	No significant associations between urinary BPA levels and standard semen parameters	Age, BMI, and urinary creatinine concentration.
Buck Luis et al. (109)	Couples recruited upon discontinuing contraception to become pregnant ($n = 501$)	1.04 (0.91–1.18) ng/ml*	BPA concentration was not associated with time to pregnancy.	Partner age, BMI and urinary creatinine concentration, female urinary BPA concentration, smoking, and center.
Knez et al. (102)	Male partners of couples seeking infertility treatment ($n = 149$)	1.55 (0.81–3.27) ng/ml*	Urinary BPA was associated with lower total sperm count (β regression coefficient: −0.241, 95% CI: −0.47, −0.012), sperm concentration (β regression coefficient: −0.219, 95% CI: −0.436, −0.003), and viability (β regression coefficient: −2.66, 95% CI: −4.991, −0.392). No association between urinary BPA concentration and embryo development parameters at IVF/ICSI.	Male age, BMI, current smoking status, alcohol consumption, abstinence period, and urinary creatinine concentration.
Lassen et al. (99)	General population ($n = 308$)	3.25 (0.59–14.89) ng/ml	BPA urine concentration was significantly associated with lower progressive motility (−6.7%; 95% CI: −11.76, −1.63).	Smoking, varicocele, cryptorchidism, genital conditions, and time to motility analysis.
Miao et al. (111)	Factory workers with and without occupational BPA exposure ($n = 140$)	36.23 ± 7.69 μg/grCr in exposed and 1.38 ± 6.89) μg/grCr in non-exposed	Exposed men ($n = 75$) exhibited a lower sperm concentration when compared to unexposed group ($n = 65$): 94.93 ± 58.58 × 10^6/ml vs. 126.42 ± 82.26 × 10^6/ml ($p = 0.03$). Higher urinary BPA levels were independently associated with lower global methylation degree of sperm DNA.	Age, education, history of disease, smoking, and alcohol consumption
Dodge et al. (110)	Couples seeking infertility treatments ($n = 218$)	1.6 (0.8–2.8) ng/ml	Lower male BPA concentrations were associated with a greater proportion of high-quality embryos in IVF cycles (RR = 1.92; 95% CI: 1.13, 3.25).	Maternal age, paternal normal weight, maternal normal weight, and maternal smoking.
Goldstone et al. (98)	Male partners of couples who discontinued contraception to become pregnant ($n = 418$)	0.51 (0.46–0.58) μg/grCr*	No significant association was found between urinary BPA levels and any standard semen parameter.	Age, abstinence time, alcohol consumption, BMI, smoking, previously fathered pregnancy, center, and ethnicity.
Vitku et al. (103)	Male partners in couples seeking infertility treatment ($n = 191$)	0.075 (0.055–0.100) ng/ml#	Seminal BPA but not plasma BPA was negatively associated with sperm count ($r^{§} = -0.178$; $p = 0.018$), concentration ($r^{§} = -0.198$; $p = 0.009$), and morphology ($r^{§} = -0.160$; $p = 0.044$).	Age, BMI, and abstinence time.

(Continued)

TABLE 1 | Continued

Study	Population	BPA urinary concentration: mean ± SD or median (range)	Results	Adjustments
Adoamnei et al. (100)	Healthy young university students ($n = 215$)	1.8 (0.14–11.9) μg/grCr*	Urinary BPA concentration was negatively associated with sperm concentration (β regression coefficient = −0.04, 95% CI: −0.07; −0.02) and total sperm count (β regression coefficient = −0.05, 95% CI: −0.08; −0.02).	BMI, smoking, varicocele, abstinence time, and time to motility analysis.
Radwan et al. (104)	Male partners of couples seeking infertility treatment ($n = 315$)	1.64 ± 2.32 μg/grCr	Higher urinary BPA concentration was related to lower sperm motility ($p = 0.03$), increased percentage of immature sperm ($p = 0.018$), and sperm sex chromosome disomy ($p = 0.01$).	Abstinence time, age, smoking, alcohol consumption, and past diseases.
Pollard et al. (106)	Male partners in couples seeking to become pregnant without history of infertility ($n = 161$)	2.5 ng/ml[†]	Higher urinary BPA concentrations were associated with increased percentage of sperm with abnormal tail morphology ($p = 0.032$).	Age, ethnicity, income, smoking, and BMI.

*BMI, body mass index; BPA, bisphenol A; CI, confidence intervals; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; RR, rate ratio; VCL, curvilinear velocity. *values are mean (25th−75th percentiles); [#]seminal BPA levels; [§]r = correlation coefficient of partial correlation; [†] geometric mean.*

bovine (130), mouse (131), and chicken (132) and also impaired sperm fertilizing ability in mouse (131) and chicken (132). Consistent with data from *in vitro* studies on several types of cells (106–108, 133–137), these effects could be mediated by oxidative–apoptotic mechanisms: it has been reported that the exposure to BPA reduces mitochondrial membrane potential ($\Delta\Psi$m) in chicken spermatozoa (132) and promotes ROS generation in bovine spermatozoa (130); oxidative stress and high DNA fragmentation have been also reported in fish spermatozoa exposed to BPA (129). To date, only two studies have assessed the direct *in vitro* effects of BPA on human spermatozoa. In the first study, which was carried out from our group (138), the exposure of motile spermatozoa to scalar concentrations of BPA (10–800 μM) for 4 h produced a decrease in $\Delta\Psi$m, starting from 300 μM, which was accompanied by mitochondrial superoxide anion generation, activation of caspase-9 and caspase-3, and motility decrease. As a late consequence of oxidative stress, a 20-h exposure to 300 μM BPA (but not to lower doses) also produced a significant decrease in sperm viability, complete sperm immobilization, and oxidative damage of DNA, as revealed by the generation of the oxidized base adduct 8-hydroxy-2′-deoxyguanosine (138). An inhibitory effect on $\Delta\Psi$m in human spermatozoa exposed to BPA has been also reported, even at very lower doses, in a subsequent study by Grami et al. (139).

DISCUSSION

Due to the widespread presence of BPA, environmental exposure to this chemical spares no one: large epidemiological studies revealed that more than 90% of the population in western countries displays detectable BPA in the urine (32–34) and toxicokinetic analyses pointed to dietary and transdermal routes as the primary sources of human exposure. As BPA is qualified as a xenoestrogen endocrine disruptor, growing concern is rising for possible harmful effects on human health, including fertility. Indeed, except for some factory workers with high occupational exposure, measured BPA levels in biological fluids are usually low and the hazards to fertility for the general population remain a matter for debate.

Overall, while preclinical studies have clearly shown that BPA can negatively interfere with the regulation of spermatogenesis, as well as with sperm functions, the claimed clinical adverse effects on male fertility are largely based on the results from conventional semen analysis, that, however, produced controversial evidence (**Table 1**), being strongly weakened by a number of limitations. Firstly, the cross-sectional design of the studies and the large spontaneous between- and within-subject variability of semen parameters (140) hinder any conclusion about the cause–effect relationships. Although analyses were adjusted for a number of possible confounding factors, it cannot be excluded that other unmeasured confounders have not influenced the examined associations. Other endocrine-disrupting substances are ubiquitous in the environment and may coexist in the human body, leading to possible synergic effects on semen quality with BPA not necessarily playing the major role. Secondly, heterogeneity arises from the inclusion of different study populations with variable degrees of exposure to BPA and, probably, from the variable susceptibility to its effects: in fertile men with low unintentional environmental BPA exposure, any detectable effect on reproductive functions is likely to be small, with uncertain clinical significance. Whether or not low unintentional environmental BPA exposure can worsen the fertility potential in subfertile men would represent a more relevant issue, but it is difficult to be ascertained. On the other hand, when men with or without occupational exposure to BPA were compared, those with

occupational exposure, who exhibited much higher urinary BPA concentrations, also displayed a significant negative association of BPA with sperm count (111, 112), viability, and motility (112). Further studies on occupationally exposed workers are warranted.

The best evidence of an adverse effect of BPA on male fertility would be provided by prospective studies on clinically relevant endpoints, including natural or medically assisted pregnancies among men either with different exposure degrees (occupational/environmental) or with different clinical conditions (fertile/subfertile). However, this is a hard challenge.

AUTHOR CONTRIBUTIONS

AB and FF contributed conception and design of the study. CC, MT, and AP performed the literature search and wrote sections of the manuscript. SD'A, LL, and GC contributed literature search. AB wrote the first draft of the manuscript. SF, FF, and AB critically revised the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

Supported by a grant to SF: PRIN 2017, MIUR, Italy.

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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 Endocrinology

REVIEW
published: 30 June 2020
doi: 10.3389/fendo.2020.00373

Effects of Bisphenols on Testicular Steroidogenesis

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Over the last decades, the adverse effects of human exposure to the so-called "endocrine disruptors" have been a matter of scientific debate and public attention. Bisphenols are synthetic chemicals, widely used in the manufacture of hard plastic products. Bisphenol A (BPA) is one of the best-known environmental toxicants proven to alter the reproductive function in men and to cause other health problems. Consumer concern resulted in "BPA free" products and in the development of bisphenol analogs (BPA-A) to replace BPA in many applications. However, these other bisphenol derivatives seem to have effects similar to those of BPA. Although a number of reviews have summarized the effects of BPA on human reproduction, the purpose of this article is to review the effects of bisphenols on testicular steroidogenesis and to explore their mechanisms of action. Testicular steroidogenesis is a fine-regulated process, and its main product, testosterone (T), has a crucial role in fetal development and maturation and in adulthood for the maintenance of secondary sexual function and spermatogenesis. Contradictory outcomes of both human and animal studies on the effects of BPA on steroid hormone levels may be related to various factors that include study design, dosage of BPA used in *in vitro* studies, timing and route of exposure, and other confounding factors. We described the main possible molecular target of bisphenols on this complex pathway. We report that Leydig cells (LCs), the steroidogenic testicular component, are highly sensitive to BPA and several mechanisms concur to the functional impairment of these cells.

Keywords: bisphenols, BPA, endocrine disruptors, testicular steroidogenesis, spermatogenesis

OPEN ACCESS

Edited by:
David H. Volle, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Ren-Shan Ge, Wenzhou Medical University, China
Settimio D'Andrea, University of L'Aquila, Italy

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *01 January 2020*
Accepted: *11 May 2020*
Published: *30 June 2020*

Citation:
Barbagallo F, Condorelli RA, Mongioì LM, Cannarella R, Aversa A, Calogero AE and La Vignera S (2020) Effects of Bisphenols on Testicular Steroidogenesis. Front. Endocrinol. 11:373. doi: 10.3389/fendo.2020.00373

INTRODUCTION

Over the last decades, the adverse effects of human exposure to the so-called "endocrine disruptors" have been a matter of deep debate by the scientific community and the layman. Particular attention has been paid to their toxicity on the reproductive function. Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA) is among the most well-known endocrine disruptors proven capable of impairing the male reproductive function and to cause other health problems. BPA is an organic synthetic compound, including the group of dyphenylmenthane derivatives and bisphenols, widely used in the manufacture of hard plastic products. BPA has been used since the 1950s, in food packaging, industrial materials, dental sealants, personal hygiene products, and thermal receipts (1, 2). A significant exposure to BPA for children is given by toys, books, and feeding bottles (3, 4). BPA penetrates the body through the skin, inhalation, and the digestive system (5). Once adsorbed, BPA is then metabolized by the liver and excreted with the urine in 24 h

(2). Despite the rapid metabolism, BPA can accumulate in different tissues (6). Consumer concern for BPA effects on health resulted in "BPA free" products and in the development of bisphenol analogs to replace BPA in many applications. However, these compounds seem to have endocrine disrupting capabilities similar to BPA and their impact on reproduction has been little investigated (7–9).

BPA seems to influence fetal testis development and predispose to the testicular dysgenesis syndrome (TDS). This syndrome may manifest itself not only at birth with cryptorchidism and hypospadias, but also in adulthood when it shows up with testicular tumors, hypogonadism, and/or infertility (10). Current evidence suggests that BPA can cause testicular histological abnormalities, which encompass dysregulated proliferation and apoptosis of Leydig cells (LCs) and alteration of steroidogenesis (11). In mice, pubertal exposure to high doses of BPA causes LC and germ cells apoptosis, resulting in underdeveloped testis with histopathological changes including atrophied seminiferous tubules, decreased number of late spermatids, and increased karyopyknotic cells (12). The reduction of testicular weight and the alteration of spermatogenesis persist till adulthood, long after the period of BPA exposure (12). The gestational period is a sensitive window of exposure to BPA. Male rats maternally exposed to BPA from gestation to the postnatal period have low testicular weight and testosterone (T) levels in the testicular interstitial fluid in adulthood (13). These effects may involve different molecular pathways discussed in section Bisphenol A Molecular Mechanisms of Action on Testicular Steroidogenesis.

Many studies have investigated the effects of BPA on human reproduction and extensive reviews have addressed the strength of the evidence on BPA toxicity (9, 10, 14, 15). Contradictory outcomes may depend on several factors including study design, BPA dose, timing, and route of exposure and other confounding factors (15). Several mechanisms of action have been described. First of all, BPA exhibits weak estrogenic and antiandrogenic proprieties. It binds to both estrogen receptors (ERs), ERα and ERβ (1, 10), and at high concentrations, BPA binds to the androgen receptor (AR) on which it acts as an antagonist (16). In addition to binding to the ARs, it disturbs the hypothalamic–pituitary–testicular axis and modulates gene expression and the enzymatic activity of testicular steroidogenesis (16). Furthermore, exposure to BPA is also associated with a decrease in the activity of the antioxidant system, resulting in increased oxidative stress, the most common cause of sperm damage (17, 18). Although several studies have supported the harmful effects of BPA on testicular function, its mechanism remains not fully understood.

The purpose of this article is to review the evidence on the relationship between bisphenols and testicular steroidogenesis, focusing on their mechanism(s) of action on LCs function.

TESTICULAR STEROIDOGENESIS

The testis is a complex endocrine organ regulated by intra- and extra-testicular pathways that interact synergistically (19). LCs have a crucial role in the regulation of steroidogenesis and spermatogenesis. LCs produce testosterone (T), which has a main role in fetal development and maturation. During the masculinization programming window, the fetal testes begin to produce T, which allows male gonadal differentiation and development (20). Hence, T is necessary for the maintenance of secondary sexual function and spermatogenesis (21). Intratesticular T levels are approximately 100 times higher than the levels found in systemic circulation (22). The high local production rate of T implies the need for its intratesticular transport from LCs to Sertoli cells which nourish and support the development of the germinal cells during the various stages of spermatogenesis (23). LCs derive from mesenchymal cells located in the interstitial compartment of the testis. Their development occurs through three different stages during which they are called progenitor, immature, and adult LCs. Apoptosis seems to have a main role in maintaining a constant population of LCs, although other mechanisms may be involved (9).

LCs produce T in response to the luteinizing hormone (LH). LH binding to the LH receptors (LHR) on LCs activates Gs protein and adenylyl cyclase, increasing cAMP levels. cAMP acts as a key second messenger and upregulates the expression of genes related to the steroidogenesis (24). The steroidogenesis consists in a complex multi-enzyme process by which precursor cholesterol is converted to biologically active steroid hormones in a tissue-specific manner (**Figure 1**). Cholesterol can be synthesized in the endoplasmic reticulum but the first source of this precursor for steroidogenesis is via uptake of cholesteryl esters from high-density lipoprotein by the scavenger receptor SR-B1 (25). Therefore, SR-B1 has a key role for the maintenance of cholesterol balance. The first step in steroidogenesis takes place within mitochondria. The steroidogenic acute regulatory protein (StAR) mediates the transport of cholesterol from the outer to the inner mitochondrial membrane (26). The StAR-mediated transport of cholesterol is a crucial step for steroidogenesis (27, 28) and appropriate concentrations of cAMP are necessary for the regulation of StAR expression (29). However, cAMP/PKA is not the only pathway that regulates StAR expression. Other factors such as steroidogenic factor, activator protein, and cAMP-response element-binding protein are also associated with StAR regulation (30). Then, cholesterol is metabolized to pregnenolone into the smooth endoplasmic reticulum through a cascade of reactions that are catalyzed by the cytochrome P-450 proteins. Pregnenolone is then converted to T by 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase/17,20 lyase (CYP17A1), and 17β-hydroxysteroid dehydrogenase (17β-HSD). This complex process of steroidogenesis itself can be responsible for the increase of reactive oxygen species (ROS) (31). Thus, the normal products of steroidogenesis can act as pseudosubstrates and interact with P-450 enzymes, resulting in a pseudosubstrate–P-450–O_2 complex, which is a source of dangerous free radicals (32).

BISPHENOLS AND TESTICULAR STEROIDOGENESIS

Effects of BPA on Steroid Hormone Levels

Experimental studies in male animals have shown that exposure to BPA is associated with altered hormone levels suggesting

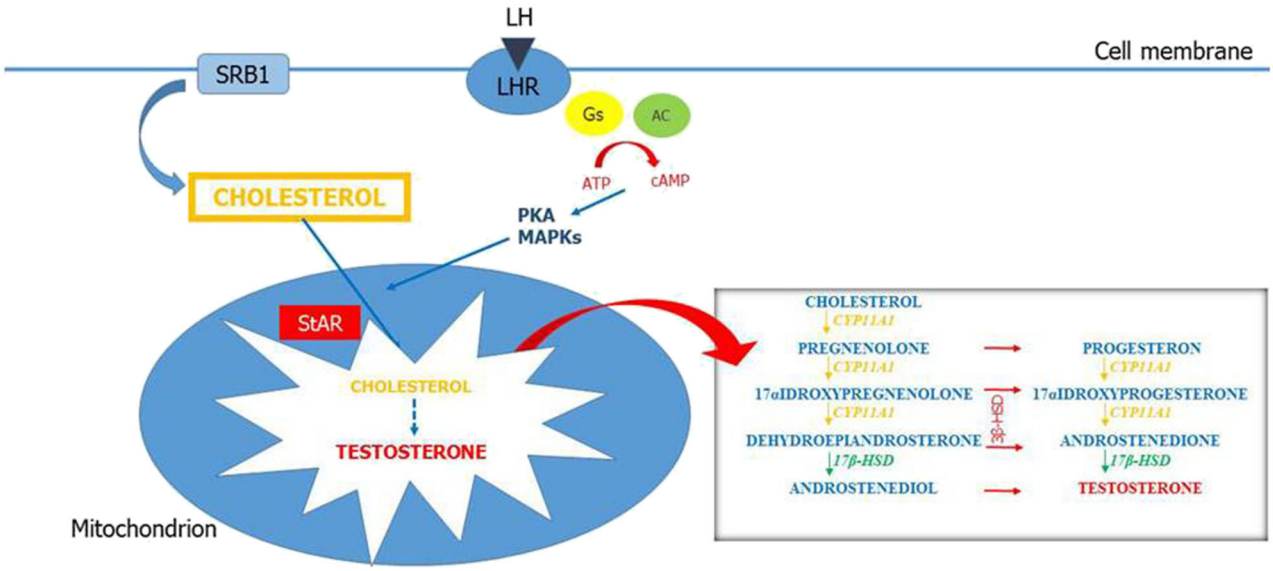

FIGURE 1 | Leydig cell steroidogenesis. LH binds to its receptors (LHR) on the Leydig cell (LC) membrane. This results in activation of Gs protein and adenylyl cyclase and increased concentration of intracellular cAMP. cAMP stimulates the mobilization and transport of cholesterol within the mitochondria in part by activating PKA and MAPK signaling. The first source of cholesterol for steroidogenesis is via uptake of cholesteryl esters from high-density lipoprotein (HDL) by the scavenger receptor SR-B1. Steroidogenic acute regulatory enzymes (StARs) regulate cholesterol transport from the outer to the inner mitochondrial membrane. At the inner mitochondrial membrane, cholesterol is converted into pregnenolone by CYP11A1 and pregnenolone is converted into testosterone by enzymes in the smooth endoplasmic reticulum (3β-HSD, CYP17A1, and 17β-HSD).

direct effects of BPA on LCs. However, these data are discordant. Low-dose BPA decreased T levels in CD-1 mice exposed during perinatal and postnatal periods (33), but not in adult C57BL/6 mice exposed *in utero* (34). In addition, low-dose BPA lowered T levels in Holtzman rats exposed during gestation or in the neonatal age (35, 36) and albino (37) and Wistar (38) rats exposed in adulthood. In contrast, by examining the gestational and neonatal exposure of low-dose BPA in Long–Evans (39) or Sprague–Dawley (SD) rats (40, 41), the levels of T did not change. Treatment with increasing concentrations of BPA (1 to 1,000 nM) did not significantly lower basal or hCG-stimulated T secretion by primary culture of LCs of young adult male rats (42). However, although Sánchez et al. reported that low-dose BPA did not decrease T levels in Wistar rats, dihydrotestosterone levels decreased (43). Gamez et al. reported that exposure to low-dose BPA led to an increase in serum LH and FSH levels in young Wistar rats (44). Nevertheless, another study in adult Wistar rats showed that exposure to BPA decreased serum T, LH, and FSH levels, but increased the levels of 17β-estradiol (E_2) (45). In two studies in SD rats, postnatal exposure to low-dose BPA decreased serum T and E_2 levels (46). BPA exposure lowered T levels in Swiss albino and C57BL/6 mice, but at variable dosage between 0.5 μg/kg and 100 mg/kg (47, 48). Sadowski et al. described a decrease in FSH concentrations in Long–Evans rats at weaning, after exposure to BPA at both 4 and 400 μg/kg/day (49). An *in vitro* study conducted on fetal testes explanted from mice, rats, and humans demonstrated that exposure to 10 nM of BPA was enough to decrease basal T secretion in human fetal testes, but higher concentrations were required in rats and mice (10 and 1 μM, respectively) (50).

The epidemiological studies evaluating the effects of BPA exposure on serum hormone levels in men have also shown conflicting results. In the INChianti adult population study, Galloway et al. found a correlation between higher urinary BPA concentrations and higher serum T, but not E_2 levels in 307 Italian men living in Chianti, Italy (51). Another study, conducted on 308 young men from Denmark's general population, reported that higher urinary BPA concentration was associated with a significant increase of LH, T, and E_2 levels (52). In contrast, in a cross-sectional study of 290 men, Zhou et al. found that increased serum BPA concentrations were statistically significantly associated with the reduction of androstenedione, free T and free androgen index (FAI) levels, and with the increase of sex hormone-binding globulin (SHBG) levels (53). Two cross-sectional studies, respectively, of 167 and 302 men, did not report any associations between BPA and T concentrations (54, 55). According to Meeker and colleagues, men with elevated urinary BPA concentrations had higher FSH and lower inhibin B levels with a higher FSH/inhibin B ratio and a lower E_2/T ratio (54). Mendiola et al. found that higher urinary BPA levels were associated with lower FAI and FAI/LH and free T/LH ratios in fertile men (55). Two cross-sectional studies reported that urinary BPA levels were associated with higher SHBG in men occupationally exposed to BPA (56, 57). The NHANES 2011-2012 study showed an inverse correlation between urinary BPA levels and serum T concentrations in male adolescents (58). However, a retrospective

cohort study did not find any effects on hormone levels in boys aged 8 to 14 years after prenatal or childhood exposure to BPA (59).

Although these results are controversial, they suggest that BPA alters steroid hormones pathways in men.

BPA Molecular Mechanisms of Action on Testicular Steroidogenesis

Although both animal and human studies support the harmful effects of BPA on steroid hormones, the mechanism of action of BPA in negatively interfering with testicular steroidogenesis remains unclear. Since LCs are the site of testicular steroidogenesis, several studies have been conducted on these cells to investigate the effects of BPA. In Wistar/ST pubertal rats, continuous exposure to BPA at high doses reduced the number of LCs and the expression of steroidogenic enzymes in these cells (60). In contrast, Long–Evans rats exposed to a low dose of BPA during gestation and at birth had an increase in the number of LCs in adulthood through the upregulation of mitogen factors. However, although a low dose of BPA increased LC proliferation, the expression of steroidogenic enzymes and T biosynthesis decreased (61). Chen et al. reported that BPA did not stimulate staminal LC proliferation, but it induced the differentiation of stem LCs into more mature LCs. They used an *in vivo* ethane dimethane sulfonate (EDS)-induced LC regeneration model to mimic the pubertal development of LCs. They treated rats with EDS to eliminate LCs and then they injected BPA within the testis. The intratesticular injection of BPA avoided possible interference of hypothalamus and pituitary. The results of this study showed that BPA significantly increased the number of 11β-HSD1-positive cells, which is a biomarker for LCs at an advanced stage. Thus, BPA promoted the differentiation of staminal LCs, increasing T production and upregulating LC-specific genes (LHCGR, StAR, CYP11A1, HSD3B1, CYP17A1, HSD17B3, and HSD11B1). These findings suggest a possible role of BPA in sexual precocious puberty in males (62). Exposure to high doses of BPA (480 and 960 mg/kg/day at postnatal days 31–44) has been reported to induce apoptosis in Leydig and germ cells *via* the upregulation of Fas, FasL, and caspase-3 (12). The apoptosis of LCs was associated with a decreased testicular testis weight and histopathological changes, which persisted into adulthood (12). In another study, Thuillier et al. reported that SD rats exposed *in utero* to BPA had an increase number of LCs but did not present significant change in serum T levels (63). Moreover, BPA can also induce *Nur77* gene expression, an orphan nuclear receptor that plays an important role in the regulation of LH-mediated steroidogenesis, altering LC steroidogenesis (64). BPA induced Nur77 gene expression via PKA and MAPK signaling pathways in a time- and dose-dependent manner. BPA-mediated Nur77 expression resulted in the upregulation of steroidogenesis both *in vitro* and *in vivo*, with a significant increase of T synthesis (two-fold) (64).

The inhibition of testicular steroidogenesis by BPA can also be associated with a decreased LH secretion. Akingbemi et al. reported that Long–Evans rats exposed to low doses of BPA (2.4 μg/kg/day) from postnatal days 21–35, decreased both serum LH and T levels, downregulating pituitary LHβ expression but increasing ERβ pituitary mRNA levels (13).

The expression of LH and FSH receptors may also be altered by BPA. Li et al. showed that treatment of adult male zebrafish (Danio rerio) by 500 ng/L BPA for 7 weeks downregulated the expressions of FSHr and LHCGr (65). For the first time, Roelofs et al. demonstrated that BPA, BPF, and TBBPA showed clear glucocorticoid receptor antagonism, other than AR antagonism. They also found that bisphenol analogs upregulated the 5αRed1 gene expression, suggesting a redirection of steroidogenesis, which may have significant consequences for fetal testis development and function (7).

Within the steroid hormone biosynthetic pathway, steroidogenic enzymes are recognized as important targets for the actions of endocrine-disrupting chemicals. Several studies showed that BPA decreases the expression of steroidogenic enzymes (33, 41, 60, 61, 66, 67). Moreover, some compounds, including BPA, seem to disturb steroidogenesis by inhibiting the cAMP pathway. Nikula et al. analyzed the effects of BPA at micromolar concentration in cultured mouse Leydig tumor cells (mLTC-1). BPA did not have any effects on hCG binding to LH receptors, but it inhibited LH-receptor-mediated signal transduction by decreasing hCG-stimulated cAMP. Specifically, they found that after preincubation of mLTC-1 cells for 48 h with different doses of BPA, hCG-stimulated cAMP and progesterone production was inhibited. Whereas, preincubation with 17β-estradiol inhibited progesterone production but had no effect on cAMP. Thus, the effects of BPA did not seem to be estrogen-related (68). Moreover, the inhibitory effect of BPA could not be seen when cAMP formation was directly stimulated by forskolin (Fk) or through Gs protein by cholera toxin (CT), and when steroidogenesis was directly activated by 8-Br-cAMP, which can penetrate the plasma membranes and directly activate the protein kinase A. These results suggested that the negative effect of BPA is exerted between the LH receptor and the adenylate cyclase. Accordingly, Feng et al. found that BPA exposure inhibited progesterone secretion in hCG-stimulated mouse Leydig tumor cell line (mLTC-1) by decreasing SR-B1 and P450scc expression due to the adverse effects on cAMP. Moreover, lower SR-B1 levels cause a reduction in cholesterol levels within LCs that alters steroidogenesis (69). The role of StAR is instead controversial. According to Feng et al. (69), StAR seems not be the molecular target of BPA. Similarly, male rats exposed to BPA showed decreased T levels but did not exhibit significant changes in StAR expression (61). However, other previous studies have reported that BPA decreased StAR expression in cell culture *in vitro* (15, 33, 47), but, in contrast, other studies have shown that StAR expression is upregulated (41, 65). Takamiya et al. reported that StAR gene expression increased in the presence of both hCG (10 μg/L) plus BPA (10^{-5} M) or by hCG alone, but was not influenced by BPA alone. They found that BPA had only a weak modulating effect on gene expression of hCG-stimulated mLTC-1 cells (70). Li et al. showed that the exposure of adult male zebrafish to low doses (0.22–2.2 nM) of BPA for 7 weeks resulted in abnormal expression of genes involved in testicular steroidogenesis, specifically of 3β-HSD1, CYP17A1, and CYP11C1 (65). Samova et al. found that BPA significantly

and dose-dependently affected the functions of 3β-HSD and 17β-HSD in the testis of inbred Swiss strain male albino mice (67). Ye et al. reported that BPA significantly inhibited 3β-HSD, CYP17A1, and 17β-HSD3 activities in both human and rat testis. However, the inhibition of 17β-HSD3 activity was much weaker compared with that on the other two enzymes. They also found that human enzymes were more sensitive to BPA (71). Specifically, their results suggested that BPA did not exert a competitive inhibition of 3β-HSD against its substrate (pregnenolone), but it competed with the cofactor NAD+ in the cofactor binding site of the enzyme, whereas BPA inhibition of CYP17A1 was mixed type for enzyme substrate progesterone, indicating a combination of two different types of reversible enzyme inhibition, both competitive and uncompetitive (71). Additionally, not only BPA, but also bisphenol S (BPS) and bisphenol F (BPF) exposure decreased T production in fetal mouse testis by inhibiting mRNA expression of StAR, 3β-HSD, and cytochrome P45017A1 (CYP17A1), but not of P450scc (72). Moreover, Dankers et al. suggested that the changes in T secretion after BPA or TBBPA exposure were only partly due to alterations of steroidogenic enzyme expression. These authors hypothesized that the inhibition of ATP-binding cassette (ABC) transporters, expressed in the blood–testis barrier (BTB), may play a role in this process. The BTB divides the seminiferous epithelium into a basal and an apical compartment and provides structural and protective support for the differentiation of spermatogonia into spermatocytes. It consists of tight junctions, testis-specific atypical adherent junctions, desmosomes, and gap junctions. In the active part of BTB, ABC transporters are present to allow the passage of endogenous molecules involved in cellular signaling and to block the passage of dangerous compounds within the testes and to protect germ cells. The cellular membranes of LCs, Sertoli cells, and capillary endothelial cells are provided of these transporters. For this reason, the association between endocrine disruptors and ABC transporters has a strong toxicological impact (23). The breast cancer resistance protein (BCRP/*ABCG2*), the P-glycoprotein (P-gp/*ABCB1*), and the multidrug resistance proteins 1 and 4 (MRP1, 4/*ABCC1,4*) are the major efflux transporters in the BTB with a differential expression in the various parts of the BTB (23). LCs express P-gp, MRP1, and MRP4, but not BCRP in adult human testis (73, 74). Dankers et al. investigated the effects of BPA and of TBBPA (tetrabromobisphenol A) on BCRP, MRP1, MRP4, and P-gp. They found that TBBPA inhibited all these transporters; thus, it is considered a non-competitive transporter inhibitor, whereas BPA inhibited only BCRP activity. They also showed that BPA, but not TBBPA, is transported by BCRP (23). Interestingly, they found that, although exposure to BPA and TBBPA significantly increased T level in MA-10 cells, the effects on steroidogenic genes were not so significant. Thus, these authors hypothesized that the changes in T levels upon BPA or TBBPA exposure were associated with the inhibition of efflux of T precursors. Increased availability of these precursors, such as androstenedione or DHEA, could be responsible for the increased T levels found.

Moreover, many compounds increase the levels of ROS in the testis, altering steroidogenesis. Oxidative stress has also been found to induce apoptosis in LCs and germ cells (64). Recent

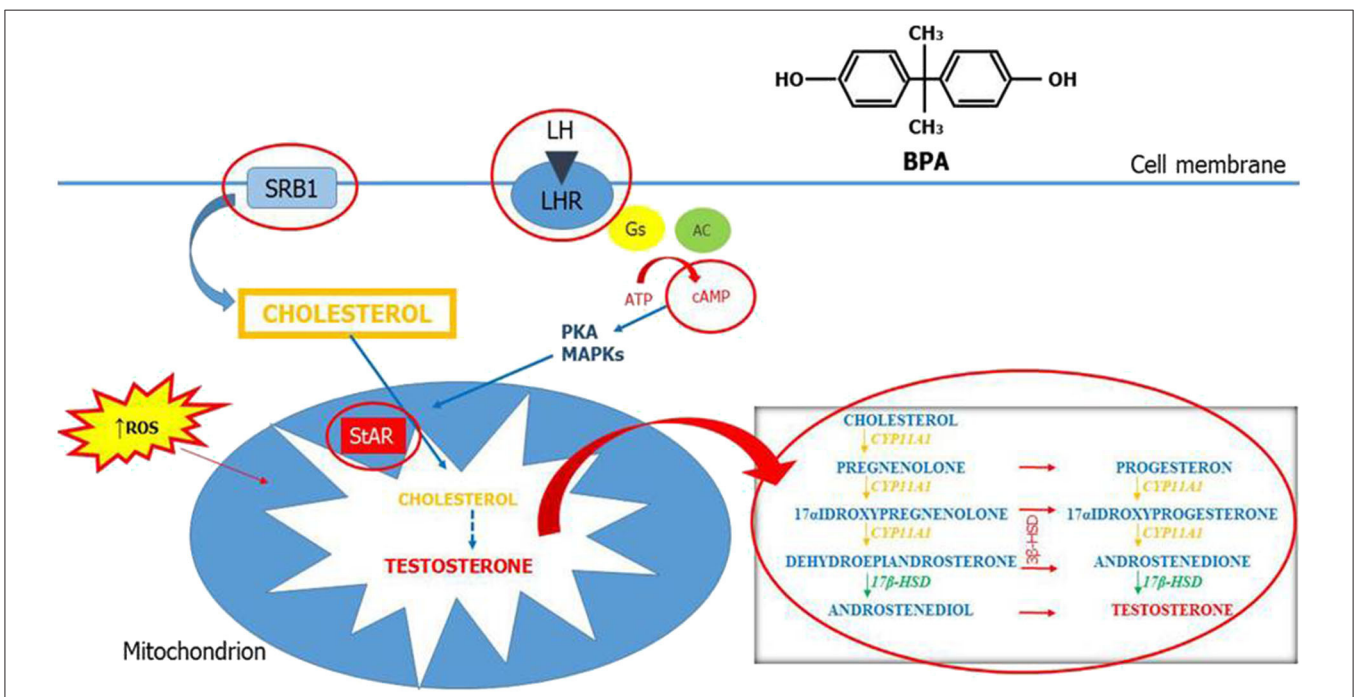

FIGURE 2 | Mechanisms of action of bisphenol A on testicular steroidogenesis. Testicular steroidogenesis is a complex and fine-regulated process that bisphenol A (BPA) can perturb by acting with several mechanisms represented in this figure (circled in red).

studies have reported an inverse relationship between NOS activity and StAR expression (47). Chouhan et al. exposed Swiss albino mice to BPA at concentrations of 0.5, 50, and 100 μg/kg body weight/day intraperitoneally for 60 days. They showed that BPA upregulated the expression of iNOS, downregulating the expression of StAR in mouse testis (47). It was also supposed that BPA impaired steroidogenesis by decreasing testicular glucose levels (38). Glucose homeostasis is crucial for testicular spermatogenesis and steroidogenesis. D'Cruz et al. reported that low-dose BPA exposure impaired insulin signaling interacting with GLUT-2 and GLUT-8 and inhibiting the uptake in the testis (38).

Recently, a number of studies suggest epigenetic effects of BPA, including DNA methylation, histone modifications, and non-coding RNAs. Epigenetic mechanisms can have long-term effects and may be transmitted across several generations (75). Specifically, Gao et al. (76) have recently investigated the epigenetic effects of BPA on the expression of non-coding RNAs (e.g., microRNAs) in the regulation of testicular steroidogenesis. They used both cell culture and *in vivo* mouse models and showed that miR-146a-5p was expressed only in LCs, and this expression was significantly induced by BPA. Consequently, the high miR-146a-5p expression intensifies the negative effects of BPA on testicular steroidogenesis by directly targeting the 3′ UTR of Mta3 gene (76). Mta3 is a subunit of the Mi-2/nucleosome remodeling and deacetylase (NuRD) protein complex that is exclusively expressed in LCs (77). Specifically, Mta3 role in the control of testicular steroidogenic function is proven by its negative regulation by the high levels of circulated insulin (77). He et al. showed that a deficiency of Mta3 in LCs of diabetic mice was associated with low serum T level, indicating that Mta3 expression in LCs may be associated with androgen deficiency (77). Thus, the downregulation of mir-146a-5p/Mta3 cascade seems to be involved in steroidogenic alterations caused by BPA (76).

DNA methylation is one of the best characterized epigenetic mechanisms. Liu et al. investigated the effects of BPA on DNA methylation in rare minnow *Gobiocypris rarus*. DNA hypermethylation consists of an addition of a methyl group to the cytosine bases of DNA to form 5-methylcytosine and it may be associated with changes in gene expression. In their study, Liu et al. found that the global DNA methylation level was significantly increased in testis of male *G. rarus* exposed to BPA for 7 days. Then, they specifically analyzed the change in DNA methylation in the 5′ flanking region of the cytochrome P450 aromatase (CYP19A1A) gene. After 35-day exposure, the DNA methylation levels of CYP19A1A did not have significant change in the testis, whereas they significantly increased in the ovary (78).

CONCLUSIONS

This review summarizes the current evidences on the association between BPA and testicular steroidogenesis. Altogether, these results show that LCs are very sensitive to BPA and that several mechanisms concur to the functional impairment of these cells. Testicular steroidogenesis is a complex and fine-regulated process and each component of this pathway may be the molecular target of BPA. The main possible sites of BPA action are summarized in **Figure 2**. The conflicting results of both human and animal studies may be related to various factors that include study design, dose of BPA, timing and route of exposure, and other confounding factors. This review confirms that the widespread use of bisphenols is certainly dangerous for testicular development and function and that a reduction of its use is necessary to preserve male sexual and reproductive health.

AUTHOR CONTRIBUTIONS

FB, SL, and AC: concept and design. RAC, LM, and RC: articles research. FB: writing of the original draft. SL, AC, and AA: final approval. All authors contributed to the article and approved the submitted version.

FUNDING

The publication costs of this article are partially supported by the 2020–2022 grant Linea Open Access, University of Catania, Catania, Italy.

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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 Physiology

ORIGINAL RESEARCH
published: 07 July 2020
doi: 10.3389/fphys.2020.00785

Bisphenol A Diglycidyl Ether (BADGE) and Progesterone Do Not Induce Ca^{2+} Signals in Boar Sperm Cells

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Aim: Exposure of boar sperm cells to Bisphenol A diglycidyl ether (BADGE) has been shown to lead to reproductive failure in sows, however, the mode of action is unknown. As we have recently shown that BADGE can interfere with Ca^{2+} signaling in human sperm cells through an action on CatSper, and as CatSper has been shown to be expressed in boar sperm cells, we hypothesized that a similar mechanism in the boar sperm cells could be responsible for the reproductive failure.

Methods: Direct effects of BADGE and the endogenous ligand of human CatSper, progesterone, on Ca^{2+} signaling in human and boar sperm cells were evaluated side-by-side using a Ca^{2+} fluorimetric assay measuring changes in intracellular Ca^{2+}. Effects of BADGE on Ca^{2+} signaling in boar sperm were furthermore assessed by flow cytometry by an independent laboratory.

Results: The exact same solutions of BADGE and progesterone induced transient biphasic Ca^{2+} signals in human sperm cells, but failed to do so in both non-capacitated and capacitated boar sperm cells. BADGE also failed to induce transient biphasic Ca^{2+} signals in boar sperm cells in the flow cytometric assay.

Conclusion: BADGE and progesterone failed to induce Ca^{2+} signals in boar sperm cells. This indicates that the signaling mechanisms leading to activation of CatSper differs between human and boar sperm cells, and suggests that the mode of action by which exposure of boar sperm cells to BADGE can lead to reproductive failure in sows does not involve effects on Ca^{2+} signaling.

Keywords: Endocrine disruption, fertility, CatSper, boar sperm, bisphenol

OPEN ACCESS

Edited by:
Arcangelo Barbonetti, University of L'Aquila, Italy

Reviewed by:
Donatella Paoli, Sapienza University of Rome, Italy
Rosita Angela Condorelli, University of Catania, Italy

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *08 April 2020*
Accepted: *15 June 2020*
Published: *07 July 2020*

Citation:
Rehfeld A, Mendoza N, Ausejo R and Skakkebæk NE (2020) Bisphenol A Diglycidyl Ether (BADGE) and Progesterone Do Not Induce Ca^{2+} Signals in Boar Sperm Cells. Front. Physiol. 11:785. doi: 10.3389/fphys.2020.00785

INTRODUCTION

The CatSper Ca^{2+} channel is a sperm specific Ca^{2+} channel highly conserved in mammals (Cai and Clapham, 2008), but also present in a wide range of other species (Romero and Nishigaki, 2019). Ca^{2+} signaling is a key regulator of sperm function and CatSper thus controls important sperm functions (Lishko et al., 2012). In human (Lishko et al., 2011; Strünker et al., 2011) and macaque sperm cells (Sumigama et al., 2015) CatSper has been shown to be activated by the female

sex steroid progesterone, released in high amounts from the cumulus cells surrounding the oocyte (Lishko et al., 2011; Strünker et al., 2011). However, in mouse sperm cells progesterone fails to activate CatSper (Lishko et al., 2011; Schiffer et al., 2014), hinting that signaling pathways leading to CatSper activation may be more different than similar, even between mammalian species (Kaupp and Strünker, 2017). CatSper has been shown to be expressed in boar sperm cells (Song et al., 2011; Vicente-Carrillo et al., 2017), and has been suggested to be functional through the use of CatSper-inhibitors (Vicente-Carrillo et al., 2017; Machado et al., 2019), but the exact role of CatSper in boar sperm cells remains unclear.

Bisphenol A diglycidyl ether (BADGE) is synthesized through O-alkylation of bisphenol A (BPA) with epichlorohydrin and is a widely used constituent of, e.g., epoxy resins, paints, and food container linings (Chamorro-García et al., 2012). Recently, a study found that BADGE could leach from plastic bags used for storage of boar semen and that exposure of boar sperm cells to BADGE led to reproductive failure in sows (Nerin et al., 2014), however, without any clear mode of action identified. As we have shown that BADGE in μM concentrations can induce transient biphasic Ca^{2+} signals via an activation of CatSper in human sperm cells (Rehfeld et al., 2020), we hypothesized that a similar mechanism in the boar sperm cells could be responsible for the reproductive failure in sows. Here we set out to test this hypothesis, by investigating whether BADGE could interfere with Ca^{2+} signaling in boar sperm cells through an examination of the effect of both BADGE and the endogenous ligand of human CatSper, progesterone, on human and boar sperm cells side-by-side using a Ca^{2+} fluorimetric assay.

MATERIALS AND METHODS

Chemicals and Reagents

Bisphenol A diglycidyl ether (BADGE) was purchased from Sigma-Aldrich (St. Louis, MO, United States) and dissolved in DMSO at a stock concentration of 10 mM. Progesterone, A23187 and ionomycin were obtained from Sigma-Aldrich (St. Louis, MO, United States) and dissolved in DMSO at stock concentrations of 20 mM, 100 mM and 1 mM, respectively. Fluo-4, AM, was purchased from Invitrogen (Carlsbad, CA, United States). Fluo-3, AM, and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO, United States). Human serum albumin was obtained from Irvine Scientific (Santa Ana, CA, United States). Dulbecco's Phosphate Buffered Saline with calcium chloride and magnesium chloride (DPBS+) (Item # D8662) and Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (DPBS-) (Item # D8537) were obtained from Sigma-Aldrich (St. Louis, MO, United States).

Semen Samples

Human semen samples from volunteer donors were produced by masturbation and ejaculated into wide-mouthed plastic containers, on the same day as the experiment and allowed to liquefy for 15–30 min at 37°C. The volunteer donors were recruited from the semen donor corps, which is routinely donating samples for quality control analyses at the Department of Growth and Reproduction, Rigshospitalet. All volunteers fulfilled WHO criteria for normal semen quality. Each experimental replicate was based on sperm cells from a single sperm sample.

Boar semen samples for the Ca^{2+} fluorimetric assay were produced the day before the experiment and obtained as raw semen samples from Ringsted Forsøgslaboratorium, Denmark, a part of Hatting A/S. For the flow cytometry experiments eight ejaculates were collected on the same day as the experiment from eight different animals in different Spanish boar studs, diluted 1:10 in commercial boar semen extender Duragen® and then immediately sent to Magapor SL laboratories. The viability was evaluated by flow cytometry using propidium iodide staining and motility was evaluated using a commercial computer assisted sperm analysis system (CASA) (ISAS Proiser, Spain) as in (Nerin et al., 2014).

Purification of Motile Sperm Cells via Swim-Up

For the Ca^{2+} fluorimetric assay, motile sperm cells were isolated from human and boar semen samples by the swim-up method (Rehfeld et al., 2019). Briefly 1 mL of semen was gently placed in the bottom of 50 mL tube containing 4 mL of human tubular fluid (HTF^+) medium with the composition: 97.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 4 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH. After 1 h at 37°C, the upper swim-up fraction was carefully removed and after two washes, the sperm concentration was determined by image cytometry (Egeberg et al., 2013) using an NC-3000 (ChemoMetec AS, Denmark) and samples were adjusted to 10×10^6 sperm cells/mL in HTF^+ with human serum albumin (3 mg/mL). Hereafter the sperm cells were incubated for at least 1 h at 37°C. For the experiments with capacitated boar sperm cells, the samples were treated similar to in a previous study on boar sperm (Bernecic et al., 2019) and resuspended in a capacitating medium with the following composition: 72.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 25 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH. Human serum albumin (3 mg/mL) was added to the capacitating medium and the sperm cells were incubated for >3 h at 37°C in a 5% CO_2 atmosphere.

For the flow cytometry experiments the boar semen samples diluted 1:10 in extender, were simply diluted further to a final concentration of 4×10^7 cells per mL in DPBS+.

Measurement of Changes in $[Ca^{2+}]_i$ in Ca^{2+} Fluorimetric Assay

Changes in the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in human and boar sperm cells were measured in 384 multi-well plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30°C as described in Rehfeld et al. (2019). Briefly, sperm cells were incubated with the fluorescent Ca^{2+}

indicator Fluo-4, AM (10 μM) for 45 min at 37°C. Excess dye was removed by centrifugation (700 × *g*, 10 min, RT) and the sperm pellet was resuspended in HTF^+ to 5 × 10^6 sperm cells/mL. Just before loading the sperm cells to the 384-well plates, sperm motility was evaluated manually using phase contrast optics on an Olympus BX45 microscope at a total magnification of ×200 (Olympus, Denmark) to make sure that the sperm cells used for the experiments were motile and thus viable. Aliquots of 50 μL were loaded to the wells of a 384-well plate using an automatic repeater pipette. Fluorescence was excited at 480 nm and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after addition of 25 μL bisphenol solutions, negative control (buffer with vehicle), and positive controls (progesterone, 5 μM final concentration, and ionomycin, 10 μM final concentration) manually with an electronic multichannel pipette to duplicate wells. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$ (%), indicating the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before addition of BADGE, positive control and negative control.

Measurement of Changes in $[Ca^{2+}]_i$ Using Flow Cytometry

Changes in $[Ca^{2+}]_i$ in boar sperm cells were additionally measured using Fluo-3 and flow cytometry, similar to what other have previously used in boar sperm cells (Schmid et al., 2013; Yeste et al., 2015). The measurements were performed on a BD Accuri™ C6 (Becton Dickinson, Madrid, Spain) with BD software. At least 40,000 events were counted in every measurement. 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. To stain the boar sperm cells 3.5 μL of Fluo-3, AM stock solution (2 mM in DMSO) was added to 400 μL of sperm samples (4 × 10^7 cells per mL), giving a final Fluo-3, AM, concentration of 17.5 μM and incubated for 45 min at 37°C protected from light. After the incubation, small aliquots of the BADGE stock solution were added to each sample, respectively, yielding final BADGE concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 1.562 μM. As a positive control the calcium ionophore A23187 was added at 1 mM final concentration to one of the samples and as a negative control DPBS- buffer was added. Just after adding BADGE, the positive control or the negative control, samples were measured in the flow cytometer at different times. Changes in Fluo-3 fluorescence are shown as $\Delta F/F_0$ (%), indicating the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before addition of BADGE, positive control and negative control.

Statistical Analyses

Comparison of peak Ca^{2+} signal amplitudes were done using one-way ANOVA. *P*-values were corrected for multiple comparison type I error inflation by Dunnett's method. Statistical analyses were performed using GraphPad Prism 8.3.1 (GraphPad Software Inc., United States).

Ethical Approval

Healthy human volunteers donated the semen samples after their prior consent. The volunteers were recruited from the semen donor corps, which is routinely donating samples for quality control analyses at the Department of Growth and Reproduction, Rigshospitalet. After delivery, the samples were fully anonymized and no data on the fertility status or general health of donors is provided. Each donor received a fee of 500 DKK (about 75 UD dollars) per sample for their inconvenience. All samples were analyzed on the same day of delivery and destroyed immediately after the laboratory analyses. Because of the full anonymization of the samples, and the destruction of the samples immediately after the laboratory analyses, no ethical approval was needed for this work, according to the regional scientific ethical committee of the Capital Region of Denmark.

RESULTS

Effects of BADGE and Progesterone on Ca^{2+} Signaling in Boar Sperm Cells

We investigated BADGE for its ability to induce Ca^{2+} signals in human and boar sperm cells, using a Ca^{2+} fluorimetric assay (Schiffer et al., 2014). BADGE was tested at decreasing serially diluted concentrations from a starting concentration of 50 μM, along with the endogenous ligand of human CatSper, progesterone, at 5 μM, ionomycin at 10 μM, and a negative buffer control (HTF^+). Changes in $[Ca^{2+}]_i$ were recorded for 250 s after addition of the compounds. Our results showed that addition of the exact same solutions of BADGE and progesterone to the sperm cells, induced transient biphasic Ca^{2+} signals in the human sperm cells, but failed to do so both in non-capacitated and capacitated boar sperm cells ($n \geq 3$) (**Figures 1A–C**), whereas addition of 10 μM ionomycin induced rapid and saturating Ca^{2+} signals in both human and boar sperm cells. In boar sperm, only a small, slowly rising Ca^{2+} signal was induced by 50 and 25 μM of BADGE, but these Ca^{2+} signals did not resemble the transient biphasic Ca^{2+} signal induced by BADGE in human sperm cells. When comparing the amplitude of the induced Ca^{2+} signals 30 s after addition of compounds, a time point where both the progesterone- and BADGE-induced Ca^{2+} signals peak in human sperm cells, we found that BADGE at concentrations ≥3,125 μM induced Ca^{2+} signals significantly larger than those induced by HTF buffer alone in human sperm cells (**Figure 1D**). 5 μM progesterone and 10 μM ionomycin, similarly induced significantly larger Ca^{2+} signals in human sperm cells (**Figure 1D**). In contrast to this, only 10 μM ionomycin induced Ca^{2+} signals significantly larger than those induced by HTF buffer alone in both non-capacitated and capacitated boar sperm cells (**Figures 1E,F**).

Effects of BADGE on Ca^{2+} Signaling in Boar Sperm Cells in an Independent Laboratory

To scrutinize our negative results for BADGE above, we contacted an independent laboratory to get them to repeat

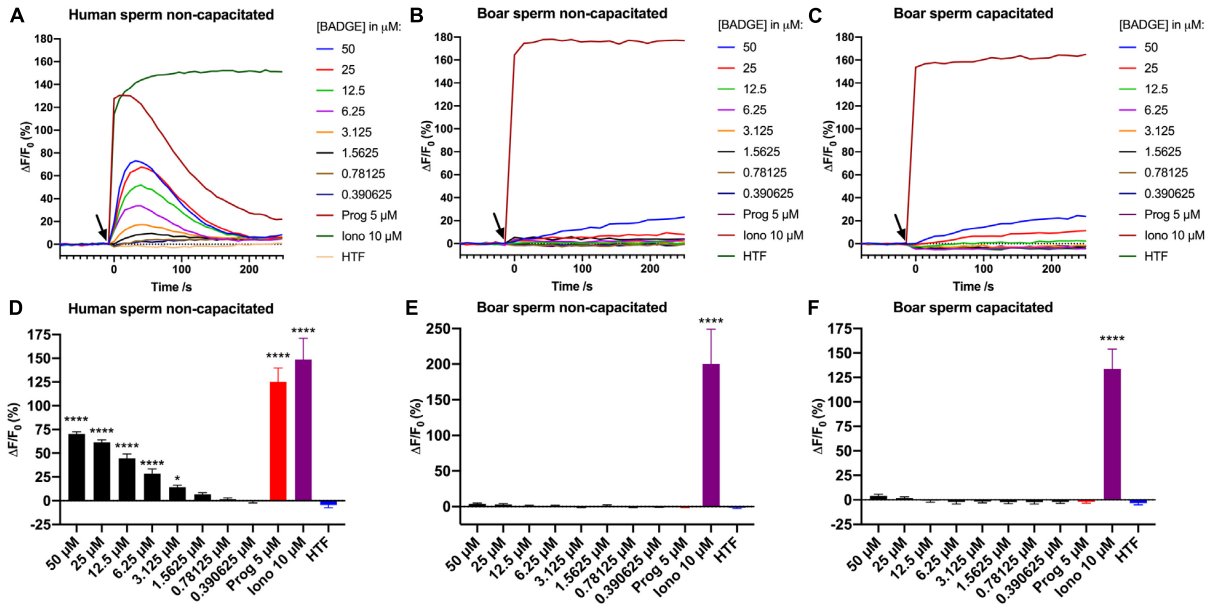

FIGURE 1 | Ca^{2+} signals induced by addition of serially diluted doses of BADGE, 5 µM progesterone, 10 µM ionomycin, and a negative buffer control "HTF" to **(A)** non-capacitated human sperm cells, **(B)** non-capacitated boar sperm cells, and **(C)** capacitated boar sperm cells. The black arrow depicts the time of addition of solutions to the sperm cells. Graphs **(A–C)** are from single representative experiments. Mean amplitude of the induced Ca^{2+} signals 30 s after addition of compounds and controls are shown for **(D)** non-capacitated human sperm cells (n = 4), **(E)** non-capacitated boar sperm cells (n = 6), and **(F)** capacitated boar sperm cells (n = 3). Statistics are from one-way ANOVA analyses comparing the mean amplitude of the induced Ca^{2+} signals 30 s with the mean amplitude of the Ca^{2+} signal induced by HTF buffer alone. **** depicts an adjusted p-value of <0.0001, and * depicts an adjusted p-value of 0.0286.

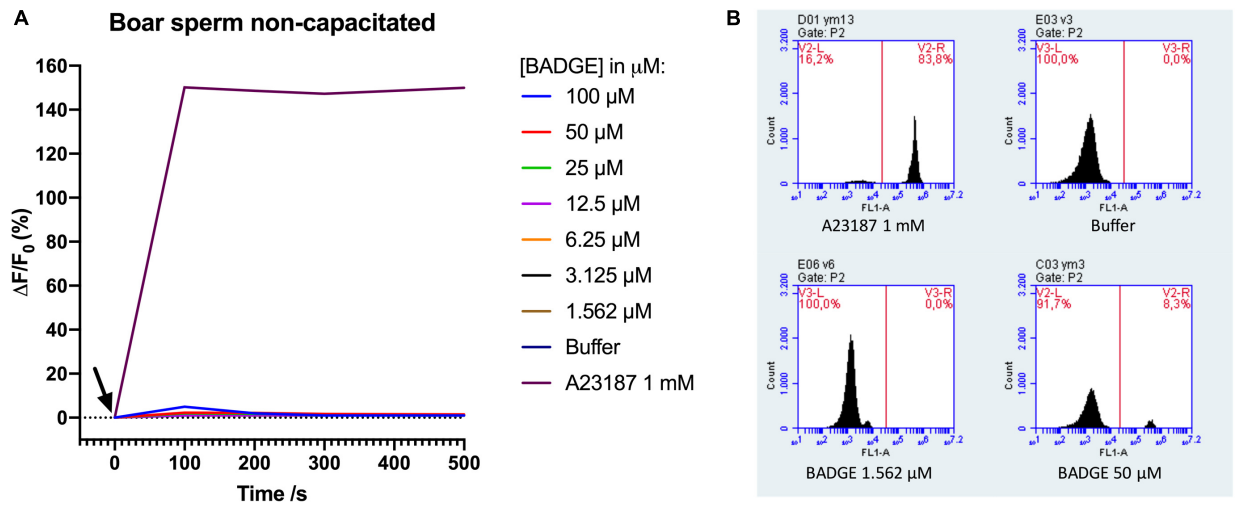

FIGURE 2 | (A) Ca^{2+} signals measured by flow cytometry after addition of serially diluted doses of BADGE, 1 mM of ionophore A23187, and a negative buffer control (DPBS-) to non-capacitated boar sperm cells. The black arrow depicts the time of addition of solutions to the sperm cells. Representative data from a single experiment. **(B)** representative flow cytometric Fluo-3 fluorescence images from a single experiment.

the experiment. They similarly tested BADGE for effects on Ca^{2+} signaling in boar sperm cells using a slightly different experimental setup. Using boar sperm of a diluted, raw boar semen sample instead of swim-up purified sperm, a flow cytometer instead of a plate reader, and the Ca^{2+}-fluorophore Fluo-3 instead of Fluo-4, BADGE was again tested at decreasing serially diluted concentrations from a starting concentration of 100 µM, along with a positive control, ionophore A23187 at 1 mM, and a negative buffer (DPBS-) control. The data from these experiments were almost identical to our initial results from the Ca^{2+} fluorimetric plate reader based assay. BADGE at any concentration failed to induce Ca^{2+} signal in non-capacitated boar sperm cells (n = 8) (**Figure 2**), whereas addition of 1 mM A23187

induced rapid and saturating Ca^{2+} signal in the boar sperm cells.

DISCUSSION

A recent study showed that exposure of boar sperm cells to BADGE could lead to reproductive failure in sows (Nerin et al., 2014), although the mode of action remained unknown. Intriguingly, no effects were found on routine boar sperm parameters, including motility and viability, or on in vitro fertilization tests, but only on in vivo fertility rates in sows (Nerin et al., 2014). We speculated that the reproductive failure could be due to effects on Ca^{2+} signaling through an action on CatSper in the boar sperm cells, as CatSper has been shown to be expressed in boar sperm cells (Song et al., 2011; Vicente-Carrillo et al., 2017), has been suggested to be functional through the use of CatSper-inhibitors (Vicente-Carrillo et al., 2017; Machado et al., 2019), and as we have recently found that BADGE in μM concentrations can interfere with Ca^{2+} signaling through an action on CatSper in human sperm cells (Rehfeld et al., 2020). Our results here, however, showed that addition of the exact same solutions of BADGE to the sperm cells, induced transient biphasic Ca^{2+} signals in the human sperm cells, but failed to do so both in non-capacitated and capacitated boar sperm cells ($n \geq 3$) (**Figure 1**), similar to after addition of 5 μM progesterone. Furthermore, a similar experiment in an independent laboratory, using a slightly different experimental setup, confirmed the lacking ability of BADGE to induce transient biphasic Ca^{2+} signals in boar sperm cells (**Figure 2**). As the sperm cells were motile and thus viable just prior to running the Ca^{2+} fluorimetric assay experiments, the difference in the Ca^{2+} responses between human and boar sperm cells are unlikely to be caused by a lack of viable boar sperm cells. In line with this, the very large Ca^{2+} signal induced by the Ca^{2+}-ionophores ionomycin (**Figure 1**) and A23187 (**Figure 2**) indicates that, at the moment of adding the ionophones to the sperm cells, a large proportion of the sperm cells must have been viable, as unviable sperm cells cannot maintain their Ca^{2+} gradient across the cell membrane. Our findings therefore do not support our hypothesis that exposure of boar sperm cells to BADGE leads to reproductive failure in sows (Nerin et al., 2014) through an effect on Ca^{2+} signaling in boar sperm cells, similar to the effect that we have recently shown for BADGE on human sperm cells (Rehfeld et al., 2020).

Interestingly, the structurally similar compound BPA, has been shown to inhibit mouse CatSper transiently in low pM-nM concentrations and to cause a significant reduction in motility and acrosome reaction in mouse sperm cells (Wang et al., 2016). A similar inhibitory action of BADGE on boar CatSper could take place, although we did not observe a large decrease in Fluo-4 or Fluo-3 fluorescence [$\Delta F/F_0$ (%)] (**Figures 1**, **2**) after application of BADGE, similar to what has been shown after addition of the potent CatSper inhibitor RU1968 to human sperm cells (Rennhack et al., 2018). Future studies will have to examine this using electrophysiological measurements on boar sperm cells.

Furthermore, our finding that the same solution of progesterone (5 μM) induced a large biphasic Ca^{2+} signals in the human sperm cells, but failed to do so both in non-capacitated and capacitated boar sperm cells ($n \geq 3$) (**Figure 1**) also do not support the hypothesis that boar CatSper should be activated by progesterone as seen for human CatSper, as has been suggested by others (Machado et al., 2019). The fact that 17-OH-progesterone (Strünker et al., 2011) and pregnenolone sulfate (Mannowetz et al., 2017; Brenker et al., 2018) are potent ligands of human CatSper, but that both 17-OH-progesterone and pregnenolone did not mimic the action of progesterone in boar sperm cells (Machado et al., 2019), further fails to support a similar mechanism of activation between human and boar CatSper. It is possible that boar sperm cells need to undergo some form of maturation process, other than capacitation, before boar CatSper can be activated by progesterone. Future studies will have to examine this. However, as human CatSper can be activated by progesterone even in testicular and epididymal human sperm cells (Smith et al., 2013), without the sperm cells having to go through any form of maturation process first, this would again suggest large differences in the events leading to activation of CatSper activation between human and boar sperm cells. Even though CatSper has been shown to be expressed in boar sperm cells (Song et al., 2011; Vicente-Carrillo et al., 2017), and has been suggested to be functional through the use of CatSper-inhibitors (Vicente-Carrillo et al., 2017; Machado et al., 2019), electrophysiological evidence of CatSper-conductance in boar sperm cells need to be obtained to confirm a possible functional role in this species. To our knowledge, such data are yet to be published.

We are unaware of any studies showing induction of transient biphasic Ca^{2+} signal by progesterone in physiological (low μM) concentrations in boar sperm cells, although, studies have found an induction of transient Ca^{2+} signals by progesterone at very high concentrations (100 μM) (Kim et al., 2008) and (10 μg/mL or 31.8 μM) (Yeste et al., 2015), similar to what has been seen in mouse sperm cells after addition of progesterone at very high concentrations (40 and 100 μM) (Romarowski et al., 2016). As mouse CatSper is not activated by progesterone (Lishko et al., 2011; Schiffer et al., 2014), these Ca^{2+} signals must be induced by some other mode of action, which is supported by the finding that progesterone at 1 mM can even induce Ca^{2+} signals in CatSper$^{-/-}$ mouse sperm cells (Ren et al., 2001). However, progesterone at a much lower nM concentrations has been shown to affect boar sperm penetration through a cell separation media (100 nM) (Campbell, 2013), to affect the acrosome reaction in capacitated boar sperm cells (100 nM) (Campbell, 2013), to affect the release of boar sperm cells from oviductal cells (80 nM) (Machado et al., 2019), and to induce a slow increase in intracellular Ca^{2+} evident after 30 min of incubation (80 nM) (Machado et al., 2019), probably associated with an induction of capacitation. Furthermore, a progesterone gradient from a starting concentration of 1 μM has been shown to act chemotactically on boar sperm cells (Berendsen et al., 2020). It is likely that progesterone exerts these effects in boar sperm cells through a mechanism unrelated to Ca^{2+} signaling and CatSper. If this is the case, BADGE could possibly have

caused the reproductive failure through this same unknown pathway. Future studies are needed to clarify this. Importantly, our findings are in line with other studies showing that the activation of CatSper by progesterone is unique to human (Lishko et al., 2011; Strünker et al., 2011) and primate sperm cells (Sumigama et al., 2015), whereas mouse CatSper is insensitive to both the chemicals affecting human CatSper (Schiffer et al., 2014) and to progesterone (Lishko et al., 2011). This means that observations on reproductive toxicology in non-primate animal models cannot simply be translated to humans in terms of effects on CatSper-mediated sperm function and consequently in terms of effects on fertility.

CONCLUSION

In conclusion, our study fails to support our hypothesis that exposure of boar sperm cells to BADGE leads to reproductive failure in sows (Nerin et al., 2014) through an effect on Ca^{2+} signaling in boar sperm cells. Furthermore, our data do not support the hypothesis by others (Machado et al., 2019) that boar CatSper can be activated by progesterone as seen for human CatSper. Future studies will have to validate our results and further explore the mode of action by which exposure of boar sperm cells to BADGE can lead to reproductive failure in sows (Nerin et al., 2014), as this could be of high interest given the widespread human exposure to BADGE (Chamorro-García et al., 2012).

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 study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Ethical review and approval was not required for the animal study because we only used anonymous boar sperm samples from a commercial provider.

AUTHOR CONTRIBUTIONS

AR and NS conceived the study and drafted the manuscript. AR designed, planned, and performed the experiments on the plate reader. NM and RA designed, planned, and performed the experiments on the flow cytometer. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a EDMaRC research grant from the Kirsten and Freddy Johansen's Foundation and the Danish Centre on Endocrine Disrupters.

ACKNOWLEDGMENTS

The authors would like to acknowledge Sissel Marie Bredesen for her assistance in performing the laboratory experiments and Brian Vendelbo Hansen for his assistance with obtaining reagents and chemicals from the various providers.

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Conflict of Interest: RA and NM were employed by the company Magapor SL.

The remaining 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 Endocrinology

REVIEW
published: 30 July 2020
doi: 10.3389/fendo.2020.00453

Bisphenol A-Induced Epigenetic Changes and Its Effects on the Male Reproductive System

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Bisphenol A (BPA) is a widespread chemical agent which can exert detrimental effects on the male reproductive system. Exposure to BPA has been shown to induce several epigenetic modifications in both animal and human cells. Specifically, BPA could not only modify the methylation pattern of multiple genes encoding proteins related to reproductive physiology but also directly influence the genes responsible for DNA methylation. BPA effects include hormonal alterations, microscopic and macroscopic alteration of male reproductive organs, and inheritable epigenetic changes involving human reproduction. BPA exposure was also linked to prostate cancer. This review aims to show the current scenario of BPA-induced epigenetic changes and its effects on the male reproductive system. Possible strategies to counter the toxic effect of BPA were also addressed.

Keywords: bisphenol A, male reproduction, infertility, epigenetic, oxidative stress, DNA methylation, spermatogenesis, prostate cancer

OPEN ACCESS

Edited by:
Nicola Bernabò, University of Teramo, Italy

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Laura Maria Mongioì, University of Catania, Italy

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *21 March 2020*
Accepted: *09 June 2020*
Published: *30 July 2020*

Citation:
Cariati F, Carbone L, Conforti A, Bagnulo F, Peluso SR, Carotenuto C, Buonfantino C, Alviggi E, Alviggi C and Strina I (2020) Bisphenol A-Induced Epigenetic Changes and Its Effects on the Male Reproductive System. Front. Endocrinol. 11:453. doi: 10.3389/fendo.2020.00453

INTRODUCTION

Epigenetics is the science that studies the environmental influence over the genetic heritage without modifying the DNA sequence. Epigenetic modifications include chromatin remodeling, histone modifications, and non-coding RNA mechanisms, which in turn could affect the phenotype of different types of cells. Epigenetics acts in the regulation of the expression of silencing genes in response to a variety of environmental exposures, allowing cells to answer and adapt to environmental stressors (1). Moreover, epigenetic modifications in parents can determine long-lasting changes, evolving in pathologies for the offspring (1).

It is now acknowledged that widespread chemical agents can exert detrimental effects on human physiology. Some substances identified as "endocrine-disrupting chemicals" (EDC) could also interfere with the endocrine system (2–4).

Bisphenol A (BPA) is an organic synthetic compound largely used for the production of polycarbonate plastics and epoxy resins. Due to the widespread industrial use of polycarbonate plastics (e.g., food/drink packaging, production of compact discs, impact-resistant safety equipment, and medical devices), the presence of BPA is ubiquitous (5). BPA can be detected in various body fluids like urine, saliva, blood, breast milk, and amniotic fluid, as well as on the skin

(6). The most harmful effect of BPA is due to both its estrogenic and anti-estrogenic properties (7, 8). BPA is able to bind to multiple targets both inside and outside the nucleus, inducing alterations in various endocrine-related pathways (7). The effects of BPA on the hypothalamic–pituitary–gonadal axis, determining pathologic consequences on the reproductive system, have been elucidated in animal and human studies (9). In addition to short-term effects, it has been demonstrated that BPA can alter epigenetic mechanisms producing also long-term effects (1).

The ability of BPA to alter normal epigenetic patterns has been recently demonstrated. Some studies revealed a role in the differentiation of spermatogenic cells, through the functional modification of some genes (10, 11). This review intends to summarize the epigenetic mechanisms by which BPA acts on both animal and human male reproductive systems. Furthermore, the possible strategies to counteract BPA effects were also disclosed and discussed.

MATERIALS AND METHODS

The search was conducted using Medline, Embase, Web of Science, Scopus, ClinicalTrials.gov, Ovid, and Cochrane Library as electronic databases. Studies were identified using the combination of the following search terms: "bisphenol A" AND "epigenetic" OR "epigenetic changes" OR "male" OR "male reproduction" OR "reproduction" OR "male reproductive system" OR "sperm" OR "sperm function" OR "sperm changes" OR "spermatogenesis" OR "prostate cancer" OR "oxidative stress" OR "offspring" OR "transgenerational" OR "transgenerational effects" OR "transgenerational changes" OR "DNA methylation," from inception of each database to May 2020. Therefore, all data from both animal and human studies on the relationship among BPA and the different aspects of the male reproductive system were considered for inclusion. However, only data regarding epigenetic changes were included in the review. All discrepancies were resolved by discussion among authors. No restrictions for language were applied. Unpublished studies were not included. Data were presented and categorized in relation to the different level at which BPA may induce impairment of male reproductive system physiology. The list of the genes mentioned throughout the whole text is presented with their proper nomenclature and role in **Table 1**.

Reproductive Endocrine System

BPA is able to bind hormonal receptors, stimulating, or inhibiting the physiologic pathway. Consequently, the ability to interfere with the hormonal axis has been observed, thereby influencing steroid signaling (12). BPA affects testis competence, varying the gene expression of steroid hormone receptors and influencing the enzymes that catalyze DNA methylation, as demonstrated by *in vivo* and *in vitro* animal studies (13–21).

In fishes (adult males of rare minnow *Gobiocypris rarus*), BPA has been demonstrated to affect the gene expression of steroid hormone biosynthesis, blood–testis barrier, proteolysis, lipid transport, and metabolism (13).

In rats, similar data were obtained, showing how BPA exposure influences the hypothalamic–pituitary–gonadal axis, finally modifying the levels of steroid hormone receptors in testes, with important consequences on sperm parameters as motility and count (14). Again, when neonatal male rats were exposed to BPA for the first 5 days of life, a change in gene expression of estrogen receptors (ERα and ERβ) in adult testis and an increase in both transcript and protein levels of DNA methyltransferases (DNMT3A and DNMT3B) were revealed (15). Interestingly, El Henafy et al. (16) obtained very similar results, analyzing the methylation pattern of DNMT3A and ERα, showing hypermethylation for both genes, in male rat pups exposed to BPA by transplacental and trans-lactational routes. In addition, their findings indicated that if the period of exposure was longer (pregnancy plus lactation), the effects were higher, suggesting a dose–response effect (16).

The interference of BPA with hormones is also suggested from an *in vivo* study in mice, where a flavonoid-based diet was administered to counteract the epigenetic effects induced by BPA. DNA methyltransferase expression was inhibited, with a decrease in epigenetic methylation of ERα and H19/IGF2 genes (the H19 imprinting is associated with IGF2 since they have the same gene locus and common enhancers) and of reproductive hormone levels, thus contrasting BPA's effect (17).

Another mechanism by which epigenetic changes are induced may be the increase in oxidative stress caused by BPA exposure (18, 19); in this sense, an *in vitro* study from Zhang et al. (20) showed that after exposing mouse testicular cells to BPA, the mRNA levels of proteins involved in sexual hormone steroidogenesis as StAR, P450scc, Cyp17a1, and 3β-HSD were reduced but were also normalized after exposure to melatonin. Finally, the exposure to BPA in mouse preimplantation embryo produces a disruption of testicular synthesis of testosterone and reduction of StAR promoter histone acetylation, thereby inducing a retard of testis development (21).

Teratogenesis and Gonadal Morphology

The majority of the studies principally focus on molecular mechanisms of pathophysiological changes and not on proper structural abnormalities. However, some evidence from animal studies showed how the BPA exposure promotes teratogenesis and affects testis morphology.

In zebrafish embryo–larvae, BPA shows teratogenic properties, provoking different anomalies going from cardiac edema to craniofacial abnormalities, spinal malformations, cranial hemorrhage, and yolk sac deformity, depending on dose of exposure (22).

In mice, BPA administration appears to compromise the testis morphology; especially the size of seminiferous tubules and the epithelium were significantly reduced with impairment of spermatogenesis at various stages (21).

Moreover, El Henafy et al. (16) evidenced that BPA could significantly impair anogenital distance, which represents an important measure of genital development, as well as testis and epididymis weight.

Another study showed the involvement of Sertoli cells, essential for physical and nutritional support of developing germ cells, as a target of epigenetic and transcriptome alterations from

TABLE 1 | Genes studied in relation to BPA exposure and male reproductive system alterations.

Acronym	Gene	Role	Reference paragraph
DNMT3A	DNA methyltransferase 3 alpha	*De novo* methylation	Reproductive endocrine system Spermatogenesis impairment Risk of prostatic cancer
DNMT3B	DNA methyltransferase 3 beta	*De novo* methylation	Reproductive endocrine system Spermatogenesis impairment Risk of prostatic cancer
ERα	Estrogen receptor α	Sexual development and reproductive function	Reproductive endocrine system
H19	Imprinted maternally expressed transcript 19	• Imprinted gene only expressed from the maternally inherited chromosome • Epigenetic changes in this gene have been associated with Beckwith–Wiedemann syndrome • Epigenetic deregulations at H19 imprinted gene in sperm have been observed associated with male infertility	Reproductive endocrine system Spermatogenesis impairment Transgenerational effects
IGF2	Insulin-like growth factor 2	• Imprinted gene only expressed from the paternally inherited chromosome • Epigenetic changes at this locus are associated with Wilms tumor, Beckwith–Wiedemann syndrome, rhabdomyosarcoma, and Silver–Russell syndrom	Reproductive endocrine system Spermatogenesis impairment Transgenerational effects
StAR	Steroidogenic acute regulation protein	Regulation of steroid hormone synthesis by enhancing the conversion of cholesterol into pregnenolone	Reproductive endocrine system
P450scc	Cytochrome P450 family	Drug metabolism and synthesis of cholesterol, steroids, and other lipids	Reproductive endocrine system
CYP17A1	Cytochrome P450 family	Drug metabolism and synthesis of cholesterol, steroids, and other lipids	Reproductive endocrine system
3β-HSD	3β-Hydroxysteroid dehydrogenase	Catalyzation of the oxidative conversion of delta (5)-ene-3-beta-hydroxy steroids and the oxidative conversion of ketosteroids	Reproductive endocrine system
EXPO5	Exportin 5	Transport of small RNAs and double-stranded RNA-binding proteins from the nucleus to the cytoplasm	Teratogenesis and gonadal morphology
DICER	Ribonuclease type III	Production of small RNA component that represses gene expression	Teratogenesis and gonadal morphology
DROSHA	Ribonuclease type III	MicroRNA (miRNA) synthesis	Teratogenesis and gonadal morphology
AGO2	Argonaute RISC catalytic component 2	Short-interfering-RNA-mediated gene silencing	Teratogenesis and gonadal morphology
DNMT3L	DNA methyltransferase 3 like	• *De novo* methylation • Transcriptional repression	Spermatogenesis impairment
H3K9	Histone H3-lysine 9	• Involvement in acetylation of genes for activation, methylation of genes for silencing • Marker of heterochromatin	Spermatogenesis impairment Transgenerational effects
H3K4	Histone H3-lysine 4	Involvement in acetylation of genes for activation, methylation of genes for silencing	Spermatogenesis impairment
DNMT1	DNA methyltransferase 1	*De novo* methylation	Spermatogenesis impairment Risk of prostatic cancer
H3K9Me3	Histone H3-lysine 9	• Trimethylation at the 9th lysine residue of the histone H3 protein • Binding heterochromatin protein 1 (HP1) to constitute heterochromatin	Spermatogenesis impairment
H3K27Me3	Histone H3-lysine 27	• Trimethylation at the 27th lysine residue of the histone H3 protein • Involvement in the peroxisome-associated pathway and induction of peroxisome loss to ameliorate oxidative stress	Spermatogenesis impairment
H3K9Me1	Histone H3-lysine 9	• Monomethylation at the 9th lysine residue of the histone H3 protein	Spermatogenesis impairment
H3K9Me2	Histone H3-lysine 27	• Dimethylation at the 9th lysine residue of the histone H3 protein • Mark of the inactivated X chromosome (Xi)	Spermatogenesis impairment
MYBPH	Histone H3-lysine 9	Biased expression in prostate	Spermatogenesis impairment
PRKCD	Protein kinase C δ	Tumor suppressor and cell cycle progression	Spermatogenesis impairment
IGF2R	Insulin-like growth factor 2 receptor	• Intracellular trafficking of lysosomal enzymes • Activation of transforming growth factor beta • Degradation of insulin-like growth factor 2	Spermatogenesis impairment
G9a	Lysine methyltransferase	Key histone methyltransferase for H3K9me1 and H3K9me2	Spermatogenesis impairment
GNMT	Glycine N-methyltransferase	Catalyzation of the conversion of S-adenosyl-L-methionine (along with glycine) to S-adenosyl-L-homocysteine and sarcosine	Spermatogenesis impairment

(Continued)

TABLE 1 | Continued

Acronym	Gene	Role	Reference paragraph
TET	Ten–eleven translocation protein	Regulation of DNA demethylation, gene transcription, embryonic development, and oncogenesis	Spermatogenesis impairment
LINE-1	Long interspersed nucleotide elements 1	• Gene regulation by the 5′ UTR methylation level • Active in germ cells and silent in most of the somatic cells	Spermatogenesis impairment
ACHE	Acetylcholinesterase	Hydrolyzation of the neurotransmitter acetylcholine in choline and acetic acid	Spermatogenesis impairment
H3K27	Histone H3-lysine 27	• Epigenetic mark • Regulation of chromatin structure and gene expression	Transgenerational effects
H4K12	Histone H4-lysine 12	• Epigenetic mark • Regulation of chromatin structure and gene expression	Transgenerational effects
SIRT1	Sirtuin 1	Regulation of epigenetic gene silencing and suppression of rDNA recombination	Transgenerational effects
ERβ	Estrogen receptor β	• Transcription activation • Inhibition of the activity of other estrogen receptor family members	Transgenerational effects
CAV-1	Caveolin 1	Involvement in the Ras-ERK pathway and promotion of cell cycle progression	Transgenerational effects
IGF2R	Insulin-like growth factor 2 receptor	• Intracellular trafficking of lysosomal enzymes • Activation of transforming growth factor beta • Degradation of insulin-like growth factor 2	Transgenerational effects
PEG3	Paternally expressed 3 gene	• Paternally expressed • Involvement in cell proliferation and p53-mediated apoptosis	Transgenerational effects
SLC12A2	Na-K-Cl cotransporter	Mediation of sodium and chloride transport and reabsorption	Risk of prostatic cancer
PDE4D4	Phosphodiesterase 4D4	3′,5′-Cyclic-AMP phosphodiesterase activity and cAMP degradation	Risk of prostatic cancer
HPCAL1	Hippocalcin-like 1	Calcium-dependent regulation of rhodopsin phosphorylation with implication in neuronal signaling in the central nervous system	Risk of prostatic cancer
MBD2	Methyl-CpG-binding domain protein 2	• Binding specifically to methylated DNA sequences • Transcription repression from methylated gene promoters • Mediation of the biological consequences of the methylation signal	Risk of prostatic cancer
GPCR14	Putative G-protein coupled receptor	Mediation of signaling processes to the interior of the cell via activation of heterotrimeric G proteins	Risk of prostatic cancer
PDGFRα	Platelet-derived growth factor receptor alpha	Mitogenesis for cells of mesenchymal origin	Risk of prostatic cancer
PLCβ3	Phospholipase C beta 3	Catalyzation of the diacylglycerol and inositol 1,4,5-triphosphate from phosphatidylinositol in G-protein-linked receptor-mediated signal transduction	Risk of prostatic cancer
NSBP1	Nucleosomal binding protein 1	Nucleosomal binding and transcriptional activating protein	Risk of prostatic cancer
HMGN5	High-mobility group nucleosome-binding domain 5	Nucleosomal binding and transcriptional activating protein	Risk of prostatic cancer
PITX3	Paired-like homeodomain 3	Lens formation during eye development	Risk of prostatic cancer
WNT10B	Wnt family member 10B	• Oncogenesis • Regulation of cell fate and patterning during embryogenesis	Risk of prostatic cancer
PAQR4	Progestin and adipoQ receptor family member 4	Tumor suppression by inhibition of the Raf/MEK/ERK signaling cascade	Risk of prostatic cancer
SOX2	SRY-box transcription factor 2	Regulation of embryonic development and determination of cell viability	Risk of prostatic cancer
CHST14	Carbohydrate sulfotransferase 14	Catalyzation of sulfate transfer to the C-4 hydroxyl of N-acetylgalactosamine residues in dermatan sulfate	Risk of prostatic cancer
TPD52	Tumor protein D52	Tumor progression	Risk of prostatic cancer
CREB3L4	CAMP-responsive element-binding protein 3 like 4	Adiposity and male germ cell development	Risk of prostatic cancer
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit	Maintaining of the transcriptional repressive state of genes over following cellular generations	Risk of prostatic cancer
UHRF1	Ubiquitin-like with PHD and ring finger domains 1	Regulation of gene expression	Risk of prostatic cancer
BCR	Breakpoint cancer region	• Serine/threonine kinase activity • GTPase activation of protein for p21rac and other kinases	

(Continued)

TABLE 1 | Continued

Acronym	Gene	Role	Reference paragraph
PTGS2	Prostaglandin-endoperoxide synthase 2	Involvement in prostaglandin biosynthesis	Risk of prostatic cancer
TIMP3	Tissue inhibitor of metalloproteinase 3	Inhibition of the matrix metalloproteinases with a role in tumor suppression	Risk of prostatic cancer
ZMYDN10	Loss of zinc finger MYND-type containing 10	Tumor suppressor	Risk of prostatic cancer
GSTP1	Glutathione S-transferase Pi 1	Detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione	Risk of prostatic cancer
LOX	Lysyl oxidase	Tumor suppression	Risk of prostatic cancer
MGMT	O-6-Methylguanine-DNA methyltransferase	Cellular defense against mutagenesis and toxicity from alkylating agents	Risk of prostatic cancer
NEUROG	Neurogenin 1	Transcriptional regulator	Risk of prostatic cancer
TSC2	TSC complex subunit 2	Tumor suppression	Risk of prostatic cancer
PDLIM4	PDZ and LIM domain 4	Bone development	Risk of prostatic cancer
PYCARD	PYD and CARD domain containing	Mediation of signaling complex assembly in the inflammatory and apoptotic signaling pathways via the activation of caspase	Risk of prostatic cancer
KDM5B	Lysine demethylase 5B	Transcriptional repression	Risk of prostatic cancer
NSD1	Nuclear receptor-binding SET domain protein 1	Androgen receptor transactivation	Risk of prostatic cancer

environmental toxicant exposures. These epigenetic alterations are related to testis abnormalities (23).

Cho et al. studied the influence of BPA on micro-RNA (miRNA): in mouse Sertoli cell lines, the BPA was shown to alter miRNA expression, with subsequent gene expression modification, and related changes in reproductive patterns (24).

An *in vitro* study on testicular fragments culture from 7-day-old male pigs exposed to BPA demonstrated a downregulation of EXPO5 and Dicer genes and an upregulation of Drosha and AGO2 genes, involved in miRNA pathways. Also, Leydig cells' morphology was not altered but interstitial tissue collagen was increased (25).

Spermatogenesis Impairment

Epigenetic modifications can occur at different steps during spermatogenesis. Firstly, primordial germ cells are subjected to genomic imprinting through a process of DNA/histone demethylation and deacetylation of H4 (Histone 4). DNA methyltransferases already expressed at this stage are DNMT3A, DNMT3B, and DNMT3L. Then, *de novo* DNA methylation occurs in spermatogonia and remains stable until fertilization and zygote development (26). This mechanism appears necessary to complete spermatic meiosis, as suggested in a study in which DNA methyltransferase knockout mice resulted to be sterile because they were unable to sustain meiosis (26). Furthermore, H3K9 and H3K4 methylation takes place in spermatocytes; DNMT1 is expressed in round spermatids, where hyperacetylated H4 is found and replacement of histone variants by transition proteins is starting. Elongated spermatids show establishment of a DNA methylation pattern associated with histone H3K9 demethylation. The histone-to-protamine transition is completed at this stage of spermatogenesis. Finally, the genomic imprinting is saved in spermatozoa (27). The histone-to-protamine transition permits a packaging of spermatic DNA, with condensation of sperm heads and protection of DNA from damage and mutagenesis. However, a low percentage of histones can remain in sperm, at undefined genes or gene promoter levels, causing possible post-translational modifications, resulting in severely altered reproductive phenotypes (11, 28, 29). It has been demonstrated that the alteration of the histone–protamine ratio affects male fertility (27).

In mice, *in vitro* studies on testis germ cells exposed to high doses of BPA demonstrated a decrease in the global DNA methylation levels, due to a reduction in DNMT1 protein and mRNA. At the same time, histone hypomethylation of H3K9Me3, H3K27Me, H3K9Me1, and H3K9Me2 was revealed. These changes seem to be mediated by a reduction in G9a proteins, which are essential methyltransferases for the meiotic process and hence for the whole spermatogenesis (20, 30).

The toxic effect of BPA on mouse semen quality was demonstrated from Zhang et al., who observed an increased number of morphologically altered and headless spermatozoa; in addition, sperm motility was reduced, after subcutaneous injection or feeding with BPA (31). Yin et al. demonstrated the alteration of DNA methylation of MYBPH and PRKCD, eliciting a change in spermatocyte proliferation and motility in a murine model (32).

In fishes, several studies showed that BPA exposure causes an impairment of global DNA methylation in the testes and consequently reduced rate of fertilization (33–36).

In details, in *Gobiocypris rarus*, BPA-induced DNA hypermethylation was demonstrated and explained by several mechanisms, including *de novo* synthesis of glutathione and oxidative stress, in addition to a significant decrease of the TET protein levels, responsible for demethylation (33, 36, 37).

It was also observed that administration of antioxidants as N-acetylcysteine may reverse such damages, protecting DNA integrity and sperm motility (36).

On the contrary, in zebrafish gonads, a global DNA demethylation due to a transcriptional miss-regulation of the DNA methylation/demethylation-associated genes (DNMTs, GNMT, and TETs) was noticed (35, 38). Moreover, a compromised spermatogenesis in male zebrafish exposed to a high dose of BPA was demonstrated. As a matter of fact, a significant decrease in sperm count was seen together with an increase in apoptosis; in addition, a miss-regulation of transcription of enzymes responsible for epigenetic remodeling was proven, leading to an increase in histone acetyltransferase activity and causing alterations in embryo development (34, 39).

In *Danio rerio* zebrafish, Lombó et al. observed sperm DNA fragmentation dependent on dose and time of BPA exposure (6).

In humans, the dimethylation of histone H3 on lysine K4 has been demonstrated to be negatively correlated with sperm concentration, motility, and mitochondrial function (40). In particular, a genome-wide study on semen samples from workers exposed to BPA and unexposed controls showed the ability of that compound to interfere with gene expression during spermatogenesis, with DNA hydroxymethylation due to H3 trimethylation, clinically ending in reduced sperm concentration motility (41). More recent data confirm previous findings, especially demonstrating a LINE-1 hydroxymethylation (42). Since LINE-1 activation has already been studied in relation to male infertility, its epigenetic modifications induced by BPA exposure may be one of the mechanisms for this EDC's toxicity. In another study, blood and semen samples collected from BPA exposed vs. non-exposed men were analyzed, in order to evaluate the toxic effect on a marker of genome-wide methylation status as LINE-1. Results showed a significantly lower methylation level of sperm LINE-1 in workers exposed to BPA. In addition, the BPA urinary levels were associated with low semen quality, even though they were inversely correlated with LINE-1 methylation (43).

Men exposed to BPA showed an increase in the rate of 5-hydroxymethylcytosine (5-hmc, which is a marker of DNA demethylation processes and demonstrates active gene transcription) of the sperm ACHE gene. Therefore, the accumulation of 5-hmc is associated with demethylation status. Taking into consideration that this type of alteration is correlated with sperm concentration and motility, the authors suggested that male infertility could be a consequence of BPA exposure (10, 44). Indeed, the effects of BPA on spermatogenesis are widely discussed in literature, whereas the majority of the studies do not explicitly mention if the underlying pathogenetic mechanisms are epigenetic (5, 14).

Moreover, since the effects of BPA exposure are also unfolded by DNA damage and epigenetic modifications, information on the influence of BPA on spermatogenesis and related male infertility is derived not only by studies directly analyzing sperm parameters but also through evidence of embryo and offspring abnormalities, as for transgenerational effects, thus described accordingly.

Transgenerational Effects

The process of DNA methylation is closely linked to the well-known phenomenon of genomic imprinting, wherein a gene is differentially expressed depending on whether it has been inherited from the mother or from the father. Examples of imprinting-derived diseases are Angelman syndrome and Prader-Willi syndrome. These, although caused by the epigenetic modification of the same gene, elicit different consequences depending on which parent it has been inherited from (45).

When a "safe" dose of bisphenol A was administered for a long time in rats, a decrease in histone acetylation of H3K9, H3K27, and H4K12, an increase in deacetylase Sirt1 expression with reduced binding, and finally an increase in estrogen receptor β (ERβ) to caveolin-1 (Cav-1) binding were observed. These processes and the related findings provided clues about the underlying mechanisms for epigenetic inheritance induced by BPA exposure (46).

An indirect proof of alteration of the sperm epigenome came from the study of Doshi et al., who evaluated the percentage of post-implantation loss and expression of DNMTs in embryos of pregnant female rats coupled with BPA-exposed males. They pointed out that post-implantation loss rate appeared to be higher and resorbed embryos had lower expression of DNMTs when sired by BPA-exposed males, compared to viable embryos from both BPA-exposed and control males (47). In addition, in their following work on the imprinting control region (ICR) of two genes implicated in embryonic growth and cellular proliferation, H19 and IGF2, the methylation pattern was analyzed. The authors showed hypomethylation at the H19-ICR in both spermatozoa and resorbed embryos from neonatally BPA-exposed rats, demonstrating that epigenetic mechanisms regulate both infertility, and transmission to offspring (48).

Oppositely, Zhang et al. noticed no changes in methylation of IGF2, IGF2R, Peg3, and H19, which are imprinted genes. However, they acknowledged that the offspring of BPA-exposed mice had smaller size and worse pelage quality, thus admitting a certain effect of this compound (31).

Shi et al. demonstrated how BPA modifies the mRNA expression of DNA and histone methyltransferases and their associated factors in the testis of a generation of mouse neonates prenatally exposed to that compound and how these effects were transmitted to the third generation of offspring (49).

In *Danio rerio* zebrafish, treatment with BPA during embryogenesis did not impact the methylation profile of sperm, although a decrease in H3K9ac, involved in sperm development, was observed (50).

An *in vivo* study on adult zebrafish males exposed to BPA during spermatogenesis and mated with non-exposed females revealed a disruption of cardiogenesis in forthcoming generations (51).

Akhter et al. studied the appearance of different malformations in various generations of zebrafish, after that the parental generation was exposed to BPA, finding abnormalities in the testes of the second-generation males and explaining this as a trans-generational effect most probably due to epigenetic mechanisms (52).

Other lines of evidence from animal studies showed that sperm motility was associated with methylation variation affecting genes involved in chromatin organization. The result of this alteration could affect embryo development (53, 54).

In a study on perinatal exposure of pregnant rats to BPA, the authors observed male fertility impairments in the three subsequent generations (13).

Hong et al. observed a reduction in the population of all sperm cells at different stages of development (spermatogonia, spermatocytes, and spermatids) in adult mouse testes, after exposure of preimplantation embryos to low-dose BPA, suggesting it as a consequence of epigenetic mechanisms (21).

Moreover, male rats subjected to neonatal BPA exposure showed downregulation of DNMT gene expression and related transcription factors, with impact on sperm epigenome and therefore influence on embryo development and implantation process (47).

In addition, after fetal exposure to BPA *in utero*, male rats were mated with unexposed female rats: the results showed an epigenetic alteration of IGF2 methylation in the male germline and subsequently promotion of glucose intolerance and β-cell dysfunction in the offspring, proving therefore the inheritance of epigenetic pattern changes, leading to dysregulation and disease (55, 56).

Furthermore, a study on pregnant rats exposed to environmental compounds including BPA during embryonic gonadal sex determination showed pubertal abnormalities, testis disease, obesity, and ovarian disease in the third generation. Apoptosis of spermatogenic cells resulted to be impaired through different generations of offspring (57). Moreover, 197 differential DNA methylation regions (DMR) in the gene promoter were shown in the sperm epigenome in the third generation after exposure. Authors stated that the sperm DMRs could represent epigenetic biomarkers for transgenerational disease and/or ancestral environmental exposures (58).

Risk of Prostatic Cancer

Ho et al. identified 28 genes as possible markers of epigenetic modifications, looking in particular to DNA methylation, leading to increased predisposition to adult-onset prostate cancer in rats, after neonatal estrogenic or BPA exposure. The majority of such genes were implicated in signal transduction pathways: Na–K–Cl cotransporter (SLC12A2), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (GPCR14 and PDGFRα), phosphokinase C pathway (PLCβ3), and cAMP pathways (PDE4D4 and HPCAL1). In particular, the prostatic PDE4D4 gene remains expressed in all rats early exposed to a low dose of BPA, before adult-onset prostatic lesions; in addition, HPCAL1 showed a specific methylation and expression alteration with aging. Therefore, the authors concluded that early exposure to BPA could provoke permanent impairment of the prostate epigenome, determining a predisposition to prostate cancer (59–62). Later, Tang et al. showed that few genes, such as DNMT3A, DNMT3B, and MBD2,

TABLE 2 | Characteristics of the studies which analyzed hormonal axis disturbances.

Epigenetic modifications	Effects	Study type	Species	References
Genes expression	• Induction of gene expression in the renin–angiotensin system pathway • Inhibition of tRNA processing-related gene expression • Decreases in hemostasis and blood coagulation-related gene expression	*In vivo*	Fish	(13)
DNA methylation	• Hypermethylation of ERα/ERβ promoter regions • Increase in DNMT3A and DNMT3B expression	*In vivo*	Rat	(15)
DNA methylation	Hypermethylation within DNMT3A and ER∞	*In vivo*	Rat	(16)
DNA methylation	• Hypermethylation of the ERα promoter and H19/Igf2 imprinting control region in the testis • Increase of DNMT expression	*In vivo*	Mouse	(17)
DNA methylation	• Decrease in G9a-dependent H3K9 di-methylation • Impairment of spermatogenesis	*In vitro*	Mouse	(20)
Histone acetylation	• Decrease in H3 and H3K14 acetylation in the StAR and P450 in the testes • Decrease in the expressions of testicular StAR and P450scc	*In vivo*	Mouse	(21)

TABLE 3 | Characteristics of the studies which analyzed morphological alterations.

Epigenetic modifications	Effects	Study type	Species	References
Histone acetylation	Reduction in diameter and epithelium height of seminiferous tubules and spermatogenic cells at different stages	*In vivo*	Mouse	(21)
Gene expression	Upregulation or downregulation of 37 miRNA related to overexpression of genes implicated in metabolism and reproduction	*In vitro*	Mouse	(24)
miRNA biogenesis and function	• No changes in Leydig cell morphology • No changes in lipid droplet content and distribution • Changes in lipid and autophagy protein abundance • Downregulation of EXPO5 and Dicer genes and an upregulation of Drosha and AGO2 genes	*In vitro*	Boar	(25)

responsible for epigenetic mechanisms, were overexpressed in rats after early exposure to BPA. Moreover, this study defined three patterns of epigenetic changes, characterizing genes like NSBP1 and HMGN5, persistently present epigenetic markers of early-life exposure; a second group represented by PDE4D4, which appear only at genital maturation but persist throughout life; and the last group, including genes such as HPCAL1 considered modifiable epigenetic markers, whose later appearance depends on early-exposure features and subsequent events during adult life (63). Moreover, Cheong et al. analyzed the prostatic tissue of BPA early-exposed rats for the methylation pattern of 7 genes (PITX3, WNT10B, PAQR4, SOX2, CHST14, TPD52, and CREB3L4), at the promoter region, showing that 4 of them (PITX3, WNT10B, PAQR4, and TPD52) were differently methylated when comparing prostatic cancer cells with normal adjacent tissues. They also noticed a connection with recurrence-free survival of prostatic cancer patients (64). Interestingly, Prins et al., discovered that different prostatic regions and lobes in rats have variable sensitivities to different doses of early-administered BPA in later-developing cancerous lesions, with different dose-dependent methylation patterns: CREB3L4, TPD52, and PITX3 showed a noteworthy hypomethylation at lower doses of BPA, with a normalization toward higher doses; PAQR4 showed significant hypomethylation for all BPA doses; and SOX2 showed an inverse correlation between hypomethylation and BPA doses (65). In a study on healthy primary human prostate epithelial cells (PrECs) exposed to high concentrations of BPA and analyzed using a whole-genome microarray, the authors noticed that BPA can modify the expression of epigenetic factors as EZH2, DNMT1, DNMT3B, and UHRF1, producing transcriptional perturbations with epigenetic consequences and even raising cancer risks (66). In addition, Karaman et al., studying prostatic carcinoma cells, observed hypermethylation in the p16 promoter region as well as for BCR, PTGS2, TIMP3, and ZMYDN10, with different changes seen in GSTP1, LOX, MGMT, NEUROG, and TSC2 methylation pattern. Also, a low dose of BPA could determine hypomethylation of PDLIM4 and PYCARD. Moreover, exposure to BPA induces downregulation

TABLE 4 | Characteristics of the studies which analyzed the epigenetic impairment of spermatogenesis.

Epigenetic modifications	Effects	Study type	Species	References
Histone methylation	• Decrease of DNMT • Reduction in the global DNA methylation levels in spermatogonia	*In vitro*	Mouse	(30)
DNA methylation	• No effect on DNA methylation of imprinted genes (IGF2, IGF2R, PEG3. and H19) in germ cells • Increase in ERα expression • Impairment of meiotic progression of germ cells • Decrease in quality and quantity of spermatozoa	*In vivo*	Mice	(31)
DNA methylation	• Reduction in DNA replication capacity • Alteration of the genome-wide DNA methylation level in GC-2 cells • Alteration of DNMT expression levels • Regulation of MYBPH and PRKCD methylation	*In vivo*	Mouse	(32)
DNA methylation	Promotion of the DNA methylation process in the testes by novo synthesis of glutathione and oxidative stress	*In vivo*	Fish	(33)
DNA methylation	Alteration of the global DNA methylation level of gonads	*In vivo*	Fish	(34)
DNA methylation	• Alteration of the global DNA methylation level of gonads • Transcriptional change of genes (DNMTS, GNMT, and TEST)	*In vivo*	Fish	(35)
DNA methylation	Variation in DNA methylation levels	*In vivo*	Fish	(36)
DNA methylation	Hypermethylation of global DNA in the testes	*In vivo*	Fish	(37)
DNA methylation	Global DNA demethylation	*In vivo*	Fish	(38)
DNA methylation	• Decrease of spermatocytes • Increase in apoptosis • Downregulation of CCNB1 and SYCP3 • Upregulation of GPER1 and ESRRGA receptors • Miss-regulation of epigenetic remodeling enzyme transcripts • DNA hypermethylation • H3K27me3 demethylation • Increase in histone acetyltransferase activity	*In vitro*	Fish	(39)
DNA methylation	• Di-methylation of lysine K4 on histones H3 • Impairment of motility, concentration, and mitochondrial activity in sperm	*In vivo*	Human	(40)
DNA methylation	Trimethylation of histone 3 (H3K27me3, H3K4me2, or H3K4me3) in sperm	*In vivo*	Human	(41)
DNA methylation	Hypomethylation of LINE-1	*In vivo*	Human	(42)
DNA methylation	• Decrease in sperm LINE-1 methylation status • Association between BPA urinary levels and low semen quality	*In vivo*	Human	(43)
DNA methylation	• Correlation between 5hmC rates of AChE and low sperm motility • Correlation between HoxC4 promoters and sperm concentration	*In vivo*	Human	(44)

TABLE 5 | Characteristics of the studies which analyzed the transgenerational effects of BPA exposure.

Epigenetic modification	Effects	Study type	Species	References
Histone acetylation DNA methylation	Apoptosis and impairment of the meiotic process	*In vitro*	Fish	(39)
DNA methylation	• Decrease in histone acetylation of H3K9, H3K27, and H4K12 • Increase in protein expression of deacetylase Sirt1 • Reduction in binding of Sirt1 and ERβ to caveolin-1	*In vivo*	Mouse	(46)
DNA methylation	Downregulation of the gene expression of DNMTS and related transcription factors	*In vivo*	Rat	(47)
DNA methylation	• Hypomethylation of the H19 imprinting control region • Downregulation in the transcript expression of IGF2 and H19	*In vivo*	Rat	(48)
DNA methylation	• Expression of DNMT3A in Sertoli cells • Strengthening of DNMT3B and weakening H3K9me2 and H3K9me3 in germ cells of the neonatal testis	*In vivo*	Mouse	(49)
DNA methylation	• Impairment of primordial germ cell (PGC) migration to the genital ridge • Dysregulation of genes involved in PGC migration (CXCR4B and SDF1A) • No alteration of DNA methylation	*In vivo*	Fish	(50)
Gene expression	• Increase in the rate of heart failure of progeny up to the second generation deriving from females that mated with males exposed to BPA • Downregulation of 5 genes involved in cardiac development in first-generation embryos • Decrease in parents and first-generation sperm remnant mRNAs related to early development	*In vivo*	Fish	(51)
DNA methylation	Maintenance of chromosome structure through epigenetic regulation correlated with sperm functionality	*In vivo*	Bull	(53)
DNA methylation	Hypermethylation of IGF2, glucose intolerance, and β-cell dysfunction in islets in offspring	*In vivo*	Rat	(55)
DNA methylation	Global DNA methylation decreased in the first-generation sperm	*In vivo*	Rat	(56)
DNA methylation	Sperm DMR correlation with several adult-onset pathologies (e.g., mammary tumors, prostate disease, kidney disease, testis abnormalities, immune abnormalities) in offspring	*In vivo*	Rat	(57)

TABLE 6 | Characteristics of the studies which analyzed the risk of prostate cancer induced by BPA exposure.

Epigenetic modification	Effects	Study type	Species	References
DNA methylation	Hypomethylation of the prostate cancer gene (PDE4D4)	*In vivo*/*In vitro*	Human	(59)
DNA methylation	• Aberrant NSBP1 promoter demethylation and transcriptional overexpression persisting in adult life • Aberrant HPCAL1 promoter hypermethylation and transcriptional suppression with a little degree of gene expression in adult life • High expression of DNMT3A and DNMT3B in early life, diminishing with aging • Involvement in early-life reprogramming of DNA methylation patterns in target genes such as NSBP1 or HPCAL1	*In vitro*	Rat	(63)
DNA methylation	DNA methylation-mediated gene expression of 6 genes linked to embryonic stem cell pluripotency	*In vivo*	Rat	(64)
DNA methylation	DNA hypomethylation of genes that confer carcinogenic risk	*In vivo*	Rat	(65)
DNA methylation	Deregulation of EZH2, DNMT1, DNMT3B and UHRF1	*In vitro*	Human	(66)
DNA methylation	• Expression levels of p16 gene decreased significantly after promoter hypermethylation • p16-related histone modifications • Dose-dependent promoter hypermethylation of tumor suppressor genes as BCR, PTGS2, TIMP3, and ZMYDN10 • Hypomethylation of PDLIM4 and PYCARD • Demethylation of GSTP1, LOX, MGMT, NEUROG, and TSC2 • Significant decrease of gene expression levels and downregulation of KDM5B and NSD1 measured in RT-PCR (real-time polymerase chain reaction)	*In vitro*	Human	(67)

of chromatin-modifying enzymes like KDM5B and NSD1 (67).

DISCUSSION

Our review intended to highlight the mechanisms by which BPA modifies at various levels the reproductive system. In particular, we looked into literature and summarized the studies that analyzed the epigenetic changes leading to impairment of the different aspects of male reproduction, both in animals and in humans.

Epigenetics is responsible for the control of many genes implicated in hormonal production, sperm parameters, and inheritable abnormalities (**Tables 2–6**).

The interesting point that comes out from our analysis is that BPA acts on two levels of epigenetic changes. In fact, on the one hand, it is responsible for the widely altered DNA methylation, the most commonly studied epigenetic mechanism; on the other hand, studies showed that the main effect of bisphenol A is on genes related to methylation proteins. In other words, BPA might be considered as an example of a proper epigenetic controller.

In this paper, we have also illustrated the possible strategies to counteract the epigenetic effect of BPA. Indeed, several antioxidants can ameliorate reproductive function by inhibiting BPA's effect on oxidative stress (68–70).

Since the increase in ROS (reactive oxygen species) is one of the recognized effects of BPA in male spermatogenesis, causing reduction in sperm viability and motility, due also to mitochondrial dysfunction, a study explored the efficacy of taurine in reversing such events, although not properly epigenetic changes, observing good results in a dose-dependent fashion (71). N-Acetylcysteine also has been evaluated to reduce ROS after BPA exposure, showing amelioration of sperm motility (36).

As abovementioned, flavonoids can defend from the epigenetic modifications induced by bisphenol A, due to their antioxidant and similar estrogenic properties (17). In addition, thanks to its antioxidant and free radical scavenger properties, melatonin has been demonstrated to pass the blood–testis barrier and protect steroidogenesis and spermatogenesis, acting principally on H3K9me2 and DNA methylation (20, 72).

Folates are methyl donors, essential for the DNA methylation process and for stabilization of the methylation status of the epigenome. Mao et al. (73) studied the efficacy of folate supplementation during pregnancy in restoring pancreatic function after BPA administration in rats, obtaining a reversal of its epigenetic changes. Moreover, Dolinoy et al. (74) demonstrated that supplementation with folate or phytoestrogen as genistein during pregnancy could counteract the effects of BPA exposure in *Agouti* mice, showing reduction in the hypomethylation pattern and hence pelage modification. These studies support that the transgenerational effects of BPA could be reduced by folate administration.

In 2011, Hardy and Tollefsbol coined the term "epigenetic diet" to refer to the dietary intake of all the compounds with protective properties against epigenetic modifications, including folates, isothiocyanates, isoflavones, resveratrol, curcumin, and tea polyphenols, among others (75). However, data on therapeutic options to reduce the impact of BPA are still quite scarce.

To the best of our knowledge, this is the first comprehensive narrative review on BPA-induced epigenetic changes and its consequence on male reproductive health. Indeed, we explored the effect of BPA in any aspect of reproductive system anomalies, considering different species. Furthermore, various epigenetic targets of BPA in reproductive disorders were also analyzed. On the other hand, we recognize that this led us also to a limitation, since we did not apply a systematic approach.

Given the relevant epigenetic effect of BPA and other EDCs, it could be useful in future to define specific epigenetic markers associated with male reproductive dysfunction during preconceptional analysis (8, 76, 77). In addition, since epigenetic changes can be potentially treated, target therapies could represent a very interesting topic of study in order to preserve fertility in subsequent generations.

CONCLUSION

Exposure to BPA has the potential to induce epigenetic modifications in both animal and human cells. Such modifications could in turn play a role in male reproductive disorders and cancer development. An epigenetic transmission to offspring was also demonstrated.

Further research is needed to define the mechanisms underlying BPA-related epigenetic changes in paternal sperm and offspring phenotype and to find appropriate therapies to reduce the impact of BPA-induced dysfunctions.

AUTHOR CONTRIBUTIONS

FC, IS, and AC contributed to the conception and design of the study. AC, FB, and CC organized the database. FC and LC wrote the first draft of the manuscript. CB, IS, EA, SP, and CA wrote the sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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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 Endocrinology

REVIEW
published: 31 July 2020
doi: 10.3389/fendo.2020.00447

Bisphenols and Leydig Cell Development and Function

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Bisphenol A (BPA) is a ubiquitous environmental pollutant, mainly from the production and use of plastics and the degradation of wastes related to industrial plastics. Evidence from laboratory animal and human studies supports the view that BPA has an endocrine disrupting effect on Leydig cell development and function. To better understand the adverse effects of BPA, we reviewed its role and mechanism by analyzing rodent data *in vivo* and *in vitro* and human epidemiological evidence. BPA has estrogen and anti-androgen effects, thereby destroying the development and function of Leydig cells and causing related reproductive diseases such as testicular dysgenesis syndrome, delayed puberty, and subfertility/infertility. Due to the limitation of BPA production, the increased use of BPA analogs has also attracted attention to these new chemicals. They may share actions and mechanisms similar to or different from BPA.

Keywords: bisphenol, bisphenol analogs, Leydig cells, steroids, reproductive function

OPEN ACCESS

Edited by:
David H. Volle, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Nicolas Chevalier, Centre Hospitalier Universitaire de Nice, France
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *04 January 2020*
Accepted: *08 June 2020*
Published: *31 July 2020*

Citation:
Li X, Wen Z, Wang Y, Mo J, Zhong Y and Ge R-S (2020) Bisphenols and Leydig Cell Development and Function. Front. Endocrinol. 11:447. doi: 10.3389/fendo.2020.00447

INTRODUCTION

Leydig cells (LCs) are a group of cells specifically located in the interstitium of the testis [see review (1)]. They secrete two important hormones: testosterone (T, androgen), which is an androgen, and insulin-like 3 (INSL3) [see review (2)]. There are at least two generations of LCs, namely fetal LCs (FLCs) and adult LCs (ALCs) (2). These two generations of LCs have different development processes and different functions (2). In fetuses, T and metabolically activated dihydrotestosterone (DHT) from T by 5α-reductase is essential for the development of the male reproductive tract (3). Failure to synthesize T may cause abnormalities in the male reproductive tract, such as hypospadias and small penis [see review (4)]. Androgens are also essential for testis descent (4). INSL3 binds to its receptor in the gubernaculum and pulls the testis from the kidney position to the lower part of abdomen (5). *Insl3* knockout in mice leads to cryptorchidism, indicating that it is important for testis descent (6, 7). Therefore, defects in FLCs may cause the fetal part of Testicular Dysgenesis Syndrome (TDS) (8). TDS was coined to refer to diseases such as cryptorchidism and hypospadias in neonates and testicular cancer, as well as decreased fertility in men with common fetal causes (9). Although the exact cause is unclear, the high incidence of male reproductive tract defects in male neonates has brought significant attention to children's health (10, 11). In adults, T is essential for the onset of puberty, the maintenance of secondary sexual characteristics, the promotion of spermatogenesis, and the promotion of muscle health (4). INSL3 is essential for regulating bone metabolism in adult males (12) and acts as an anti-apoptotic factor against germ cell apoptosis (13).

There is increasing evidence that environmental pollutants can cause TDS, androgen deficiency, and infertility. A group of highly studied environmental chemicals comprises bisphenol A [2,2-bis (4-hydroxyphenyl) propane, BPA, **Figure 1**] and related compounds, such as bisphenol AF, AP, B, C, F, H, S, Z, and other similar chemicals (**Table 1**).

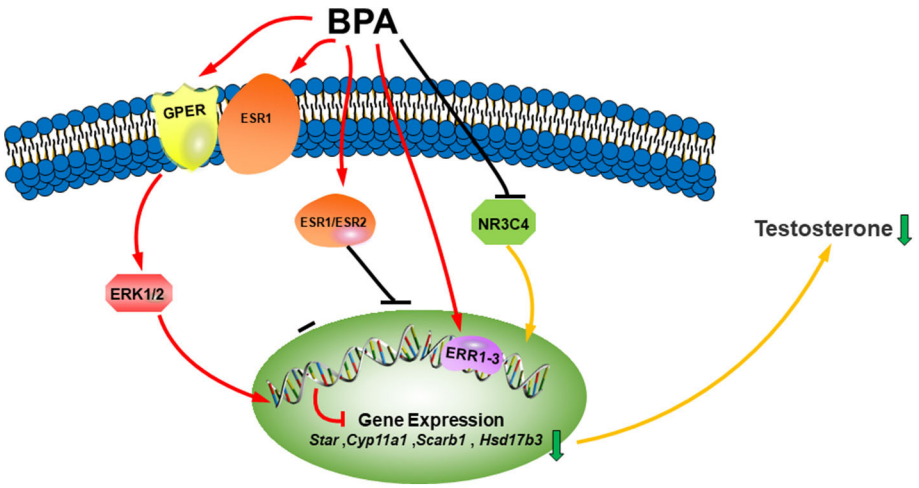


FIGURE 1 | Illustration of BPA on LC development. Bisphenol A (BPA) or its analogs can bind both estrogen receptors (ESR1 and ESR2) and estrogen-related receptors (ERR1–3), which blocks LC gene expression, binds to androgen receptor (NR3C4) as an antagonist to block the activation of LC genes. BPA can also bind membrane G-coupled receptor (GPER) or ESR1, which activates ERK1/2 pathway to inhibit the differentiation of Leydig cells. The combined consequence of BPA action leads to lower testosterone synthesis.

BPA is widely used in our industrial and consumer products and seriously pollutes our environment. BPA was first synthesized in 1891. Since then, BPA has been widely used in various products and applications as a common ingredient in plastic manufacturing. Plastics containing BPA are used to make children's toys, food containers, water bottles, medical equipment, and other durable materials (14–16). Many countries and regions are synthesizing BPA, including the United States, China, and European countries (16–19). Plastics are widely used in our consumer products and have changed our lifestyles, including the environment (20, 21). The widespread use of BPA-containing plastics has prompted BPA to spread in the environment. Therefore, BPA is ubiquitous in the environment, including air, drinking water, water systems, sewage sludge, soil, house dust, and food (16, 22). Humans are exposed to BPA mainly through food intake, dust, and skin contact (14, 15). BPA exposure through water and food is considered to be the main source (16, 22). Surveys indicate that 90% of urine samples in the general population of the United States can detect BPA levels (14, 17). The average urine BPA concentration in American people is about 2.5–10.95 ng/ml (14, 17). BPA can also penetrate the placenta and enter the fetal circulation. The average level of BPA in pregnant women's plasma is 0.3–18.9 ng/ml, and the average level of BPA in fetal plasma is 0.2–9.2 ng/ml (23, 24), and the level of BPA in placental tissue is 1.0–104.9 ng/g. BPA can enter breast milk, and the BPA level in breast milk is 0.28–0.97 ng/ml (23, 24). After ingestion through the oral route, BPA rapidly combines with blood proteins, and the concentration of free BPA in the blood is about 1 ng/ml (15).

There is increasing evidence that BPA is associated with the occurrence of reproductive toxicity (25, 26) and other health problems such as diabetes (27), neurotoxicity (28–30), immunotoxicity (31), and cancer (32–34).

BPA is classified as an endocrine disruptor that mainly mimics the effects of estrogen and disrupts the synthesis of male androgens (35–37). BPA is one of the most studied endocrine disrupting compounds. The toxicological effects of BPA may cause TDS (38) and other reproductive toxicities. The relationship between BPA and TDS and other reproductive effects has been well-studied in human epidemiology (18, 19, 39, 40). Due to the reproductive toxicity of BPA, some new BPA analogs, such as bisphenol AF, AP, B, C, F, H, S, and Z, were introduced into the market (**Table 1**) (41–43). These new compounds have received little attention. Many data on BPA reproductive toxicity have been collected from mice and rats. In this review, we mainly discuss the effects of BPA and its analogs on the development and function of LCs.

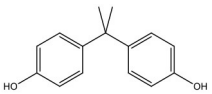
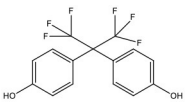
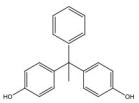
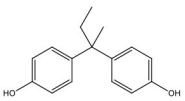
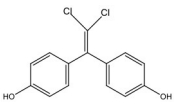
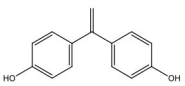
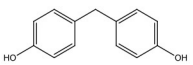
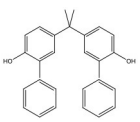
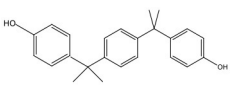
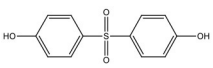
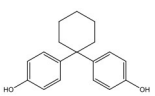
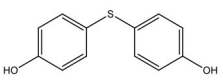
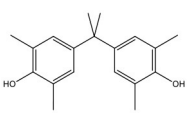
ACTION OF BPA

Estrogen Receptors

The classic mechanism of estrogens requires them to bind to estrogen receptor (ESR), a type of nuclear receptor (44). There are two subtypes of ESR, namely ESR1 and ESR2 (45, 46). Estrogen binds to ESR to form a nuclear ESR dimer that binds to the ligand. This dimer binds to the DNA sequence (GGTCACAGTGACC) and is called an estrogen response element (ERE) in the target gene promoter to induce ESR transactivation (44). ESRs bind to the same sets of ERE in the target genes (47). When different isoforms exist in the same cell, the ESR bound to the ligand can form homodimers or heterodimers.

In addition to the genomic pathway of ESR, cytoplasmic/membrane-bound ESR interacts with many other proteins to mediate the activation of several kinase pathways that are hormone-dependent (48).

TABLE 1 | Bisphenol analogs and their structures.

Bisphenols	Abbreviation	CAS No.	MW	Structure
Bisphenol A	BPA	80-05-7	228.28	
Bisphenol AF	BPAF	1478-61-1	336.23	
Bisphenol AP	BPAP	1571-75-1	290.36	
Bisphenol B	BPB	77-40-7	242.31	
Bisphenol C	BPC	14868-03-2	281.13	
Bisphenol E	BPE	2081-08-5	12.24	
Bisphenol F	BPF	620-92-8	200.24	
Bisphenol FL	BPFL	3236-71-3	350.41	
Bisphenol H	BPH	24038-68-4	380.48	
Bisphenol P	BPP	2167-51-3	346.50	
Bisphenol S	BPS	201-250-5	250.27	
Bisphenol Z	BPZ	843-55-0	268.35	
4,4′-Thiodiphenol	TDP	2664-63-3	218.27	
Tetramethyl bisphenol A	TMBPA	5613-46-7	284.39	

Estrogen-related receptors α, β, and γ (ERRα, ERRβ, and ERRγ, also known as ERR1–3) are another subfamily of orphan nuclear receptors with sequence similarity to ESR1 (49). However, 17β-estradiol (E2) is not its natural ligand, and ERR has constitutive activity (50). ERRs contain a DNA binding domain with two highly conserved zinc finger motifs in a specific DNA binding element (TCAAGGTCA, called ERRE). ERR and ERRE are combined into monomers or homodimers or heterodimers with coactivators (51). In addition to ERRE, ERR can also be bound to ERE. ESR1, but not ESR2, can also be combined with ERRE (52), so ESR1 and ERRs will affect each other.

In addition, estrogen can bind to G protein-coupled membrane estrogen receptor (GPER, also known as GPR30), which is a member of the G protein receptor superfamily. This receptor mediates the rapid signaling of estrogen. After activation, estrogen can induce ERK1/2 activation by releasing HB-EGF through transactivation of EGFR (53). GPER works through a pertussis toxin-sensitive pathway that depends on Gβγ (53). Then, GPER activation through Gαs protein activation (54) stimulates adenylate cyclase, increases cAMP, and weakens the EGFR-MAPK signaling axis (55). The activation of cAMP further leads to the activation of PKA-CREB signal (56, 57) and the transcriptional activation of CREB. GPER also activates other signaling, including PI3K (58), PKC (59), and calcium (60).

Estrogen Receptors in LCs

In rodents, there are two generations of LCs: namely, FLCs and ALCs (2). The two generations of LCs have different development trajectories and functions (2, 61). The first generation of FLCs was found in fetal age (GD) 12 of mice, GD14 in rats, and fetal testes of human around gestational age (GW) 6 (62, 63). After birth, FLCs involute, and a few FLCs persist in the adult testes (64, 65).

The second-generation ALCs begin to develop around the 9th day after birth (PND) in rats, transit to progenitor LCs in PND21 (pre-pubertal period), develop into immature LCs during PND28-35, and finally mature to ALCs around PND56 (66).

ESRs, ERRs, and GER are differently expressed in LCs during the development, depending on two generations of LCs and species. ESR1 has been detected in mouse (67) and rat (68) FLCs, as well as mouse (69) and rat (70) ALCs. ESR2 was also found in mouse and rat (71) FLCs as well as mouse and rat (72) ALCs. It has been shown that the GPER level of rat LC is higher (73). In human fetal testes, ESR1 and ESR2 are located in FLCs (74, 75). Human LCs also have low levels of ESR1 and ESR2 and high levels of GPER (76–78). All three ERRs are found in mouse tumor LCs (79). In ESR1 knockout mice, ALCs are hypertrophic and serum T levels are elevated (80, 81). However, the ESR2 knockout mice did not change, but the average cell volume of ALC decreased (81).

The Action of BPA and Its Analogs via Estrogen and Estrogen-Related Receptors in LCs

Both FLCs and ALCs mainly synthesize T from steroid cholesterol. High-density lipoprotein transport through scavenger receptor class B member 1 (SCARB1) contributes to the formation of most cholesterol in LCs (82, 83). Under the stimulation of luteinizing hormone (LH) or human chorionic gonadotropin (hCG) by binding to LH receptor (LHCGR) on the surface of LCs, adenylate cyclase is activated to increase intracellular adenosine 3′,5′cyclic monophosphate (cAMP) levels, triggering protein kinase A signaling (84). Then, the expression and phosphorylation of steroidogenic acute regulatory protein (StAR) is activated (85, 86) and, together with the translocation protein (TSPO) (87), they transport cholesterol to the mitochondrial inner membrane. In this organelle, there is a complex of P450 cholesterol side chain cleavage enzyme (CYP11A1), which catalyzes the production of pregnenolone by cholesterol (88). Pregnenolone diffuses from the mitochondria to the smooth endoplasmic reticulum, where 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase/17,20-lyase (CYP17A1), and 17β- hydroxysteroid dehydrogenase 3 (HSD17B3) catalyzes a chain-reaction to generate T (89).

INSL3 is encoded by *Insl3* in LCs and is secreted into the circulatory system (6, 90). *Insl3* is also only expressed by two types of LCs. *Insl3* encodes a G protein, a leucine-rich repeat sequence GPCR 8 (also known as relaxin family peptide receptor 2, RXFP2). Knockdown of INSL3 or RXFP2 resulted in failure of testis descent (90–92), indicating that INSL3 is critical for testis descent. INSL3 constitutive expression depends only on LC number, but also on differentiation status. INSL3 is different from T. When T synthesis is low, it is restored to normal levels by supplementing LH levels (93). Because INSL3 is specifically expressed by LCs, INSL3 is a powerful sensitive biomarker that is affected by environmental endocrine disruptors even when exposed to them during pregnancy (94).

The detailed mechanisms of BPA and its analogs to interfere with LC functions has been reviewed (95). When ESR1 was used to compare the estrogen potency of BPA with E2 through the endogenous estrogen regulatory gene in human MCF7 cells, the potency of BPA was found to be four to six orders of magnitude lower than E2 (96). Some of these studies have shown that the BPA analog BPS has lower estrogen potency than E2 when measured in nuclear receptor models. However, BPS has the same or higher estrogen potency as E2 by binding membrane ESR [see review (95)].

In mouse MLTC-1 tumor LCs, BPA, and E2 have similar potency and can inhibit LH-stimulated cAMP production with <0.7 nM after 1 h, which may be caused by GPER (97).

Mouse tumor LCs express ERRs (79). BPA significantly binds to human ERR3, with an IC_{50} value of 13.1 nM, and binds to the ERR3 receptor cavity, and its two OH groups form a hydrogen bond; one forms a hydrogen bond with Glu275 and Arg316, and the other binds to Asn346 (98). BPA and ERR3 effectively bind as antagonists (28), and may inhibit cAMP-induced *Star* promoter activation by inhibiting the transcriptional activity of Nur77 (99). These results indicate that BPA works by binding different receptors, depending on the concentration. High concentrations of BPA mainly target

ESR1, while low concentrations of BPA mainly target GPER and ERR3.

Direct Inhibition on LC Steroidogenic Enzymes

Besides the receptor-mediated actions, BPA also directly interferes with androgen synthesis. The direct effects of BPA on rat and human T synthetic enzymes, including CYP17A1, HSD3B, and HSD17B3, were evaluated using testis microsomes. BPA directly inhibited rat and human CYP17A1, HSD3B, and HSD17B3 enzyme activities. IC_{50} values of BPA for rat and human testicular HSD3B were about 27 and 8 μM; IC_{50} values for rat and human CYP17A1 were about 65 and 19 μM, and BPA inhibited both rat and human HSD17B3 around 100 μM (100). Adult rat LCs also express both HSD11B1 and HSD11B2 (101), behaving in oxidative inactivation of cortisol or corticosterone, which can suppress androgen synthesis (102). BPA inhibited human HSD11B1, with an IC_{50} of about 15 μM and rat enzyme with IC_{50} of about 19 μM. BPA also weakly inhibited both human and rat HSD11B2 with IC_{50} values about 100 or over 100 μM (103). These results indicate that BPA directly inhibits steroidogenic enzyme activities at the higher concentrations.

Other Mechanisms of BPA

Studies using *Nr3c4* (androgen receptor) knockout mice (104, 105) and Tfm mice (106, 107) showed that androgen is very critical for LC development. Knockout of *Nr3c4* in Sertoli cells, LCs, and peritubular myoid cells (104, 105) also caused the delay of ALC development. BPA might act as antiandrogen via blocking the activation of NR3C4. Lee et al. used a yeast detection system for the antiandrogenic effects of BPA and found that BPA antagonized DHT binding at 50 nM (108).

BPA-induced reactive oxygen species (ROS) generation has also been proposed for BPA-mediated suppression of T synthesis in LCs. ROS has been shown to disrupt LC steroidogenesis (109, 110). BPA was orally administered to adult male rats at 0.005, 0.5, 50, and 500 μg/kg/day for 45 days, and it significantly increased testicular ROS levels, suggesting that BPA-induced ROS might also be involved in its inhibition of T synthesis in LCs (111). Rats were administered BPA via gavage at 10 mg/kg/day BPA for 14 days, and it lowered T levels and decreased testis weight and inhibited antioxidants (such as SOD2 and catalase) and co-treatment with an antioxidant (lipoic acid) was able to reverse it (112). Male adult rats were administrated via gavage of 200 mg/kg BPA for 4 weeks, and it inhibited serum LH and T levels after decreasing SOD, GPx, and GSH and increasing ROS generation and antioxidants can attenuate BPA-induced inhibition (113). Adult male Wistar albino rats (aged 3 months) were gavaged with 50, 500, and 1,000 μg/kg BPA and/or vitamin E (40 mg/kg) for 3 months, and BPA significantly lowered T levels, testis weights, and sperm count, and vitamin E could attenuate it (114). These results indicate that BPA at high or very high doses also increases ROS levels.

ANIMAL STUDIES

Effect of *in utero* BPA Exposure on Male Reproductive Tract Development

Reports on the effects of *in utero* BPA exposure on T production and male reproductive tract development are conflicting. This difference may be due to the dosage, developmental period, and species. Pregnant CD mice orally exposed to 50 μg/kg BW/day BPA from GD16 to 18 and F2 male pups had an increase in AGD on PND3 (115) (**Table 2**).

However, other studies have shown that BPA inhibits T synthesis in fetal testes. Oral administration of BPA from GD1 to GD22 to pregnant rats inhibited T production in neonates (129). Pregnant Sprague Dawley rats were administered 4, 40, and 400 mg/kg BW BPA via gavage daily from GD12 to 21, and BPA dose-dependently reduced serum T levels and down-regulated the expression of *Insl3* and *Hsd17b3* and their proteins at 40 and 400 mg/kg and that of *Lhcgr*, *Cyp11a1*, and *Cyp17a1* and their proteins at 400 mg/kg (26). BPA inhibited FLC proliferation at 400 mg/kg (26). Pregnant Sprague Dawley rats were administered 0.002, 0.02, 0.5, 50, or 400 mg/kg BW or 0.001, 0.01, 0.1, 1, or 10 μg/kg BW 17α-ethynyl estradiol (EE, as the positive control of ESR1 agonist) daily s.c. from GD11 to GD20. Gene microarray analysis in GD20 fetal testes revealed that BPA at 400 mg/kg and EE at 10 μg/kg significantly down-regulated the expression of FLC genes, including *Scarb1*, *Star*, *Cyp11a1*, *Cyp17a1*, and *Svs5* (116), and they had similar down-regulation patterns, suggesting that BPA exerts ESR1-mediated inhibition of FLC function (116). High doses of BPA exert similar effects to E_2. Horstman et al. also exposed pregnant Sprague Dawley rats to 0.001 or 0.1 μg/kg BW/day EE or 0.02, 0.5, and 400 mg/kg/day BPA via s.c. from GD11 to GD20 and found that the highest concentration of EE and BPA down-regulated the expression of *Star* gene and proteins (117) (**Table 2**). These studies indicate that BPA may show different actions at low and high doses and it may mainly bind to ESR1 to take action at the high doses.

Effects of *in utero* BPA Exposure on Postnatal Male Reproduction

There are also conflicting reports about the effects of *in utero* BPA exposure on the production of T after birth. This difference may also be due to dose, duration of treatment, and species. Pregnant rats were orally administered with 4 or 40 mg/kg BW/day BPA from GD6 to PND20, and BPA did not affect AGD in PND21 male offspring. This study cannot conclude the inhibitory effect of BPA on T secretion (118). From GD7 to PND18, pregnant Long Evans rats were administered doses of 2, 20, and 200 μg/kg BW/day, which had no effect on AGD at PND2 and nipple retention at PND14 in male offspring, suggesting that low doses of BPA cannot cause TDS (119). Pregnant mice were exposed to 50 μg/kg BW/day BPA from GD16 to GD18, which increased AGD and prostate size and decreased epididymal weight without affecting testicular weight at PND3, 21, and 60 (115) (**Table 2**).

Pregnant CD-1 mice who were administered low doses of BPA (2 and 20 μg/kg/day) via gavage of from GD11 to GD17 had significantly lower relative testicular weight compared to 8 and 12-week-old male mice without affecting serum T levels (120).

TABLE 2 | Bisphenol A (BPA) and animal studies.

Species	Regimen	Outcome	References
***In utero* exposure on FLC development and function**			
CD mouse	po 50 μg/kg/day from GD16 to 18	Increase of AGD and decrease of epididymal weight without affecting testicular weights at PND3	(115)
SD rat	po 4–400 mg/kg/day from GD12 to 21	Reduction of serum T levels and expression of *Lhcgr*, *Insl3*, and *Hsd17b3* and FLC proliferation at 40 or 400 mg/kg	(26)
SD rat	s.c. 0.002–400 mg/kg/day from GD11 to 20	Reduction of expression of *Scarb1*, *Star*, *Cyp11a1*, *Cyp17a1*, and *Svs5* at 400 mg/kg	(116)
SD rat	s.c. 02–400 mg/kg/day from GD11 to 20	Reduction of expression of *Star* at 400 mg/kg	(117)
***In utero* exposure on postnatal LC development and function**			
SD rat	po 4 or 40 mg/kg/day form GD6 to PND20	Effect on at PND21 and inconclusive effect on T synthesis	(118)
LE rat	po 2–200 μg/kg/day from GD7 to PND18	No effect on AGD examined at PND2 and nipple retention at PND14	(119)
CD mouse	po 50 μg/kg/day from GD16 to 18	Decrease in epididymal weight without affecting testicular weights at PND21 and 60	(115)
CD mouse	po 2, 20 μg/kg/day from GD11 to 17	Reduction of relative testicular weights at PND56 and 84 without affecting serum T levels	(120)
SD rat	po 0.0025–250 mg/kg/day from GD6 to PND90	Reduction of testis/epididymis weights only at 250 mg/kg	(121)
LE Rat	po 2.5–25 μg/kg/day from GD12 to PND21	Increase of LC number and reduction of LHCGR and HSD17B3 and T secretion at PND90	(122)
Neonatal exposure on postnatal LC development and function			
SD rat	s.c. 0.002–97 mg/kg/day from PND0-9	No effect of preputial separation, T levels, and fertility rate on PND10 and PND150	(123)
LE rat	po 2.4 μg/kg/day from PND21 to 35	Reduction of serum LH and T levels	(124)
Adult exposure on postnatal LC development and function			
Swiss mouse	po 5–100 μ/kg BW/day from PND21 to 35	Reduction of absolute testis weights, seminal vesicle weight and sperm counts and fertility rate	(125)
SD rat	po from PND21 for 56 days	Reduction of free T levels without affecting LH levels	(126)
Wistar rat	s.c. 20–200 mg/kg BW BPA from PND21 for 42 days	Inhibition of plasma T and LH levels and down-regulation of *Cyp11a1* and *Scarb1*	(127)
SD rat	s.c. 1 mg/kg BW BPA at adulthood for 14 days	Decrease in plasma T level and increase in LH level	(128)
SD rat	po 10 mg/kg BW BPA at adulthood for 14 days	Reduction of serum T levels and testis weight	(112)
SD rat	po 0.005–500 μg/kg BW BPA at adulthood for 45 days	the testis as well as HSD3B1, HSD17B3, and StAR protein levels and T levels	(111)

AGD, anogenital distance; GS, gestational day; LH, luteinizing hormone; PND, postnatal day; po, gavage; s.c., subcutaneous; T, testosterone.

Sprague Dawley pregnant rats were given 0.006, 0.025, 0.25, 2.5, 25, and 250 mg/kg BW/day by oral administration from GD6 to GD21, and their male pups were directly administered via gavage of the same doses of BPA from PND1 to PND90. BPA only suppressed the weight of testes and epididymis at a dose of 250 mg/kg (121) (**Table 2**).

However, when pregnant CD-1 mice were given 0.1, 1, or 10 mg/kg BPA BW/day by gavage and another plasticizer bis (2-ethylhexyl)-phthalate (DEHP) from GD1 to GD21, and further, in the weaning period (PND1-21), the mixture down-regulated the *Star* expression and reduced sperm count in epididymis at PND42 (130). This effect may be confused by the addition of DEHP. In pregnant Long-Evans rats gavaged with 2.5–25 μg/kg BW/day from GD12 to PND21, BPA stimulated LC proliferation during prepuberty and increased the number of LCs at PND90, but down-regulated LHCGR and HSD17B3 and decreased T secretion by LCs (122) (**Table 2**). These different actions of BPA might be due to the doses of BPA.

Effects of Neonatal and Prepubertal BPA Exposure on Postnatal Male Reproduction

There are also conflicting reports on the effects of BPA exposure on postnatal T production and reproduction. This difference may also be due to dose, duration of treatment, and species. Male Sprague Dawley rats were daily s.c. administered 0.002–97 mg/kg BW BPA or 0.9 mg/kg BW E_2 from PND0 to PND9, and BPA did not affect preputial separation (an androgen-dependent process), T levels, and fertility rate on PND10 and PND150, while E_2 inhibited these parameters (123). However, Long Even rats were orally exposed to 2.4 μg/kg BW/day BPA from PND21 to PND35, and BPA inhibited serum LH and T levels (124). Rats were exposed to 2.4 μg/kg BW/day BPA from GD12 to PND21, and BPA inhibited T levels in adulthood (124). Prepubertal mice were administered BPA via gavage for 56 days, and they had significantly lower free T levels without a change in LH levels (126). Prepubertal Wistar male rats (28 days old) were injected subcutaneously with 20, 100, and 200 mg/kg BW/day

BPA, and BPA inhibited plasma T and LH for 6 weeks but did not affect FSH levels. BPA down-regulated steroidogenic enzymes and cholesterol carrier proteins in LCs and decreased LC number (127) (**Table 2**).

Effects of Adult BPA Exposure on Male Reproduction

Adult male Swiss mice were given BPA by gavage of 5, 25, and 100 μg/kg BW for 28 days. BPA significantly lowered absolute testis weights, seminal vesicle weight, and sperm count and fertility rate (125). Adult male rats were exposed subcutaneously to 1 mg/rat BPA for 14 days. BPA decreased plasma T level and increased LH levels, suggesting that BPA directly inhibits LC function (128). Adult rats were administered via gavage of 10 mg/kg BW/day BPA for 14 days. BPA lowered T levels, decreased testis weight, and inhibited antioxidants, and co-treatment with an antioxidant (lipoic acid) could reverse it (112). Adult male rats were administered by gavage of 0.005, 0.5, 50, and 500 μg/kg BW/day BPA for 45 days. BPA significantly decreased insulin, insulin receptor, insulin receptor substrate-1, phosphoinositide 3-kinase (PI3K), and GLUT-2 in the testis as well as HSD3B1, HSD17B3, and StAR protein levels and T levels (131). Adult male rats were gavaged with 400 or 800 μmol/kg BW/day BPA for 14 days. BPA significantly decreased CYP17A1, POR, CYP1B1, and CYP2A1 protein levels without affecting HSD3B1 protein levels (132) and this potency of BPA was similar to 4 μmol/kg BW/day E_2 (132). Treatment of ALCs with 0.01 μM BPA decreased T synthesis by down-regulating expression of *Cyp17a1* (124) (**Table 2**). This further demonstrates that BPA has different effects depending on doses.

HUMAN STUDIES

Human Epidemiological Study

Some epidemiological studies have explored the relationship between human exposure to BPA during pregnancy and male reproductive diseases. The results are contradictory. Fénichel et al. measured unconjugated BPA levels in cord blood in 152 boys born after GW34 with cryptorchidism and 106 controls and did not find any association between BPA and cryptorchidism (133). Cord blood BPA levels were measured in 52 neonates with cryptorchidism and 128 controls in France. No correlation was found between BPA and T or cryptorchidism, but a significant negative correlation was found between BPA and INSL3 (18). Because INSL3 and T are important for testis descent, no relationship of BPA with cryptorchidism might be involved in more confounding factors. Serum BPA levels were detected in 98 (1–4 years old) unilateral cryptorchidism boys and 57 controls. No association between free BPA levels and cryptorchidism was found. However, they did find a significant association between total BPA levels and cryptorchidism (134). Fernandez et al. measured free BPA levels in term placenta in 28 boys of cryptorchidism/hypospadias and 51 controls, finding an association between BPA levels and cryptorchidism/hypospadias in the third tertile of cases (135). Miao et al. investigated maternal occupational exposure to BPA and AGD in 56 BPA-exposed male offspring and 97 unexposed controls and found that BPA was significantly negatively correlated with AGD (136). Liu et al. investigated the effect of BPA on sex hormone levels in 100 mother–infant pairs in two hospitals in China and found that maternal urinary BPA levels were negatively correlated with male fetal cord blood T levels and T/E2 ratios in male fetal cord blood without association with AGD (137). Therefore, more human studies are needed to clarify the effect of BPA on FLC functions of male fetuses and newborns.

For BPA-mediated effects on adult reproduction, Adoamnei et al. measured urinary BPA levels, serum LH levels, and sperm counts in 215 healthy young men (ages 18–23 years) in southern Spain, and found that urinary BPA was positively associated with serum LH levels and negatively with sperm concentrations, suggesting that BPA disrupts LC function and spermatogenesis (138). Den Hond et al. measured the urinary BPA levels and serum sex hormones in 163 subfertile men in four fertility clinics and found that there was a negative association between urinary BPA concentrations and serum T levels (139). Meeker et al. measured urinary BPA levels and serum reproductive hormone levels in 167 infertile men and found an inverse relationship between urinary BPA levels and free T (T/SBBG) (140). Mendiola et al. reported on 375 men with partners of pregnant women in four cities of the United States and found that urinary BPA level was not associated with semen quality, but was negatively related to free T index and positively related to SHBG (141).

In vitro Studies Using Human Testis

The effect of BPA on FLC function was evaluated in human fetal testes. Exposure of BPA to human GW6-11 fetal testis explants for 3 days did not affect T secretion at 1 nM, but significantly lowered T secretion at 10 and 10 μM (142). Ben Maamar et al. found that BPA exposure to human GW7-12 human fetal testis explants for 3 days significantly inhibited T synthesis under the basal and LH or hCG-stimulated conditions at 10 μM (143, 144). BPA exposure also inhibited T secretion under a basal condition at 10 nM, but not under a LH-stimulated condition at this low concentration (143). Similar data were observed on the basis of BPA exposure to GW6-11 human testis and LH-stimulated T synthesis (41). Interestingly, Eladak et al. performed the first and second trimester human fetal testis xenograft to explore effect of BPA on T secretion and found that exposure of host mice to 10 μM BPA in water or 0.5 or 50 μg/kg BPA via gavage for 35 days did not influence T secretion from xenografts (41, 145).

BPA ANALOGS

Exposure of BPA Analogs

Due to strict restrictions on the production and use of BPA, several BPA analogs are gradually replacing BPA. Recent studies have reported that there was widespread exposure to a variety of chemicals with structural or functional similarity to BPA, referred to as BPA analogs (**Table 1**). BPA and its analogs were reported to exist in food stuffs (16, 146) and indoor dust (147) in both China and the United States. BPS and BPF are highly detectable in many water supply systems (148) and paper (149). BPA analogs can enter human tissues, circulation, and urine. In a survey for 190 women in Hangzhou, China, showed that,

besides BPA (average level of 2.5 ng/mL), BPS (0.19 ng/mL) and BPAF (0.092 ng/mL) were also detectable in breast milk (150). In the serum samples of 181 Chinese pregnant women, BPS, BPF, BPAF, BPB, BPP, BPZ, BPAP, TBBPA, tetrabromobisphenol S (TBBPS), and tetrachlorobisphenol A (TCBPA) were detected, and TBBPS was 0.593 ng/mL and BPS was 0.113 ng/mL (151). BPB was detected in the urine of Portuguese volunteers, and its level was similar to BPA (152) (**Table 1**).

In vitro Studies of BPA Analogs

Despite extensive research on the effects and toxicity of BPA on the male reproductive endocrine system in mammals, including humans, little is known about the activity of most BPA analogs. Several studies have been conducted on the toxicological effects of certain BPA analogs on Leydig cell function.

As mentioned above, LCs contain NR3C4 and androgen agonists, and antagonists can affect their development and function. The effects of BPA, BPF, BPS, and tetrabromobisphenol (TBBPA) on the activation of human NR3C4 were studied *in vitro*. BPA, BPF, and TBBPA antagonized NR3C4 activation with IC_{50} values of 39, 20, and 0.982 μM, while BPS did not affect it (153) (**Table 3**). Using a human recombinant androgen receptor (NR3C4) competitive binding test, it was found that BPB binds NR3C4 at a potency similar to BPA (157, 158). However, BPS bound NR3C4 weakly (157). BPA and its analogs were compared using *in vitro* and *in vivo* reporter assays for androgen agonism and antagonism. BPA significantly antagonized DHT androgenic activity in mouse fibroblast cell line NIH3T3 with TMBPA> BPAF >BPAD >BPB >BPA, whereas TBBPA and TCBPA were inactive (159). In another assay, like BPA, the following BPA analogs, TBBPA, BPAF, BPB, BPZ, BPE, 4,4-BPF, 2,2-BPF, BPC, TGSA, and TMBPA were NR3C4 antagonists between 3 and 100 μM, where BPS and TCBPA were inactive (160).

A series of estrogen receptor luciferase assays of BPA analogs in all 127 test compounds showed that BPC bound ESR1 with the highest affinity, with IC_{50} of 2.81 nM, and other BPA analogs such as BPAF (53.4 nM), BPM (56.8 nM), BPZ (56.9 nM), BPP (176 nM), BPB (195 nM), BPAP (259 nM), and BPA (1,780 nM) (155) (**Table 3**). Estrogen receptor binding experiments have shown similar effects of these BPA analogs (154, 156) (**Table 3**). Comparing the estrogen activity of BPA and its analogs in human breast cancer cell line MCF-7, the results showed that the estrogen activity was TCBPA> BPB> BPA> TMBPA (159). Using an *in vivo* uterotrophic assay in ovariectomized mice, anti-estrogenic activity against E_2 was observed with TMBPA and TBBPA (159).

Compared with ESR1, BPAF also binds to ESR2 more effectively. The IC_{50} value of BPAF for ESR2 as an antagonist is 18.9 nM. Reporter gene assay showed that BPAF is a full agonist of ESR1, inactive to ESR2, and has very weak binding to ERR3 (161).

In vitro studies showed that after 24 h of treatment, BPAF was found to dose-dependently inhibit the production of P4 in mLTC-1 tumor LCs after 24 h of treatment with an IC_{50} value of 70.2 μM. BPAF also lowered intracellular cAMP levels and down-regulated *Scarb1* and *Cyp11a1* expression without affecting *Star* expression (162). This indicates that at high concentrations, BPAF has similar effect to BPA.

When MA-10 tumor LCs were treated with BPA analogs, TBBPA induced T synthesis, while BPF and BPS increased P4 levels (153). Fetal human testis was exposed to BPA, BPF, and

TABLE 3 | Bisphenols as estrogen receptor agonists and androgen receptor (NR3C4) antagonists.

	ESR1 agonist						ESR2 agonist				NR3C4 antagonist	
Chemical	**EC_{50} (nM)[a]**	**Rel to BPA**	**EC_{50} (nM)[b]**	**Rel to BPA**	**EC_{50} (nM)[c]**	**Rel to BPA**	**EC_{50} (nM)[a]**		**EC_{50} (nM)[c]**	**Rel to BPA**	**EC_{50} (nM)[c]**	**Rel to BPA**
BPA	1200	1	1780	1	180	1	350	1	250	1	17500	1
T	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	2.8	6250
E2	0.042	28571	0.88	2225	0.9	200	1.1	318	0.3	833	30	583
BPAF	130	9	53.4	33.3	ND	ND	46	7.6	ND	ND	ND	ND
BPAP	-	-	259	6.9	2600	0.07	-	-	-	-	5400	3.2
BPB	320	4	195	9.1	ND	ND	-	-	ND	ND	ND	ND
BPC	780	2	2.81	633	3900	0.05	3200	9.1	-	-	1800	9.7
BPE	1400	1	ND	ND	ND	-	460	1.3	ND	ND	ND	ND
BPF	1600	1	ND	ND	1800	0.1	1300	3.7	3800	0.07	5800	3.0
BPFL	ND	ND	2230	0.8	-	-	ND		IA	IA	30	583
BPH	ND	ND	ND	ND	-	-	ND		-	-	1100	15.9
BPP	5600	0.2	176	10.1	ND	ND	-	-	ND	ND	ND	ND
BPS	1300	1	ND	ND	ND	ND	2100	6	ND	ND	ND	ND
BPZ	400	3	56.9	31.3	80	2.3	500	1.4	1100	0.23	1600	10.9
TDP	ND	ND	ND	ND	480	0.4	ND	ND	1100	0.23	5800	3.0
TMBPA	1100	1.1	1630	1.1	-	-	-	-	-	-	1100	15.9

[a]Potency of bisphenols in estrogen receptor (ESR) and androgen receptor (NR3C4) luciferase reporter gene assays (154); [b]Ligand binding assay (155); [c]Ligand binding assay (156); IA, inactive; ND, not detected; -, no active activity; REL, potency relative to BPA.

BPS *in vitro*. These compounds inhibited T secretion at 10 nM (41). Fetal mouse testis was exposed to BPA and its analogs; these chemicals inhibited T secretion at higher concentrations, and the minimum effective concentrations were 1 μM for BPA and BPF as well as 100 nM for BPS (41). These data indicate that there is species-dependent difference for the inhibition of T synthesis between humans and mice, and human is more sensitive to BPA analogs than mouse. These chemicals also lowered *Insl3* transcription level at 10 μM in fetal mouse testis (41).

In vivo Studies of BPA Analogs

Only some reproductive and developmental toxicity studies have been conducted on BPA analogs. BPAF did not change fetal T secretion from male fetuses on GD18 when exposed to BPAF by GD14 to 18 at a dose of 200–500 mg/kg/day (163). Exposure of rats to 5, 25, and 50 μg/L BPA and its analogs BPB, BPF, and BPS from GD1 to GD21 in drinking water caused significantly low antioxidant enzyme, plasma testosterone, and estrogen concentrations and altered morphological changes of testis and epididymis in male offspring after birth (164). *In vivo* studies of 5 mg/kg/day of BPA, BPB, BPF, and BPS exposed to adult male rats for 28 days showed that they led to decreased T levels and increased ROS levels (165). Male prepubertal rats exposed to 5, 25, and 50 μg/L BPA, BPB, BPF, and BPS in drinking water for 48 weeks also showed a decrease in T levels in the highest dose group (166). These results indicate that BPA analogs BPB, BPF, and BPS have similar effects on the development of the male reproductive system to BPA.

CONCLUSION

BPA is a ubiquitous environmental pollutant, mainly from the manufacture and use of plastics and its degradation of waste related to industrial plastics. More and more animal experiments have shown that BPA has endocrine disruption to the development and function of LCs. Studies on laboratory animals have shown that the effect of BPA is usually more harmful in the uterus, which is a critical stage of embryonic development. BPA has been found to cause defects in the embryo, such as feminization of the male fetus, atrophy of the testes and epididymis, as well as shortened AGD and changes in adult sperm parameters. BPA also disrupts the development of LCs after birth and the function of LCs in adulthood. BPA may have several molecular mechanisms: (1) binding to different ESR (ERS1 and ERS2) and ERR (1-3) as agonists, and NR3C4 as antagonist (**Figure 1**); (2) binding to the membrane receptor (GPER) (**Figure 1**); (3) direct inhibition of steroidogenic enzyme activity; and (4) stimulation of ROS production. Epidemiological studies provide some data indicating that BPA can change male reproductive function in men. There are dose-dependent effects, including low-dose and high-dose effects and species-dependent effects. Human testes may be more sensitive to the T inhibition of BPA analogs.

AUTHOR CONTRIBUTIONS

XL and ZW wrote the paper. R-SG and YZ edited the paper. YW prepared the tables. JM draw the figure. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NSFC (81730042 to R-SG, and 81901467 to YW), Science and Technology Department of Zhejiang Province (2019C03035 to R-SG).

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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 Endocrinology

REVIEW
published: 11 September 2020
doi: 10.3389/fendo.2020.00624

Bisphenols Threaten Male Reproductive Health via Testicular Cells

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Male reproductive function and health are largely dependent on the testes, which are strictly regulated by their major cell components, i. e., Sertoli, Leydig, and germ cells. Sertoli cells perform a crucial phagocytic function in addition to supporting the development of germ cells. Leydig cells produce hormones essential for male reproductive function, and germ cell quality is a key parameter for male fertility assessment. However, these cells have been identified as primary targets of endocrine disruptors, including bisphenols. Bisphenols are a category of man-made organic chemicals used to manufacture plastics, epoxy resins, and personal care products such as lipsticks, face makeup, and nail lacquers. Despite long-term uncertainty regarding their safety, bisphenols are still being used worldwide, especially bisphenol A. While considerable attention has been paid to the effects of bisphenols on health, current bisphenol-related reproductive health cases indicate that greater attention should be given to these chemicals. Bisphenols, especially bisphenol A, F, and S, have been reported to elicit various effects on testicular cells, including apoptosis, DNA damage, disruption of intercommunication among cells, mitochondrial damage, disruption of tight junctions, and arrest of proliferation, which threaten male reproductive health. In addition, bisphenols are xenoestrogens, which alter organs and cells functions via agonistic or antagonistic interplay with hormone receptors. In this review, we provide *in utero*, *in vivo*, and *in vitro* evidence that currently available brands of bisphenols impair male reproductive health through their action on testicular cells.

Keywords: bisphenol, Sertoli cell, Leydig cell, germ cell, apoptosis, tight junction

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *30 January 2020*
Accepted: *30 July 2020*
Published: *11 September 2020*

Citation:
Adegoke EO, Rahman MS and Pang M-G (2020) Bisphenols Threaten Male Reproductive Health via Testicular Cells. Front. Endocrinol. 11:624. doi: 10.3389/fendo.2020.00624

INTRODUCTION

In recent years, the declining trend in male reproductive health has generated public concern, and industrialized countries are the most affected (1, 2). A recent study indicated that the fertility rate has drastically declined in the United States of America, European countries, Japan, South Korea, and Singapore (2). About half of these infertility occurrences are linked to male factors (3–5). Studies relating to infertility in both human and animals have identified endocrine disruptors, including bisphenols, among etiologies (2, 5, 6). Bisphenols are man-made organic chemicals used to manufacture plastics, epoxy resins, and other products. The most common and widely used analog of bisphenol, bisphenol A (BPA), was designed by Diani in 1891 and synthesized by Zincke in 1905 (7). However, there are growing concerns about BPA which constitutes a major component of food packaging, plastics and other household products becoming a threat owing to its tendency

to leach into the surroundings. The perceived harmful tendency of BPA has led to a complete ban, regulatory policies, and search for safer substitutes in many countries (8–10). Consequently, there is variation in its usage in different countries (8, 9).

As BPA is being removed from consumer products, there is a progressive move to its derivatives: bisphenols F, E, B, and S as materials for polycarbonate resin (11). Other industrial application of BPS include wash fastening and electroplating (12), BPF epoxy resins are used in lacquers, liners, adhesives, dental sealants, and food packaging (13), while other analogs of BPA such as 2,2-bis-(3,5- dibromo-4-hydroxyphenyl)propane (TBBPA), are commonly used as fire retardant in several materials (11). An ideal substitute for replacing BPA, whose safety is of public concern, should be inert or less toxic. Unfortunately, these analogs have been implicated in male reproductive health problems and found in several household commodities, for instance; body cream, shampoo, meat, and milk (14, 15), making them imperfect substitutes for BPA. Bisphenols are ubiquitous contaminants in humans, livestock, wildlife, and the environment (16). Humans get exposed to bisphenols through food, skin, and inhalation (17, 18). Protective coatings of drinks and food cans, and household water containers are made of phenolic epoxy resins that contain BPA (8).

Once BPA and analogs are absorbed into the body, their major targets include testicular cells (19, 20). Functional cells of the testes include Leydig, Sertoli, and germ cells (21). Although the testis houses other important cells such as peritubular myoid, nerve, blood, and lymphatic endothelial cells, information on impact of bisphenols on these cells in relation to male reproductive health is few (22). Leydig cells produce testosterone, which perform a crucial function in differentiation of the germ cells and maintenance of testicular functions (21). Additionally, testosterone produced by Leydig cells perform important functions in the maintenance of the prostate gland (23). Sertoli cells phagocytize apoptotic germ cells to maintain testicular homeostasis for normal spermatogenesis and regulate germ cell proliferation and differentiation (24). These cells control male somatic sex determination during embryogenesis and spermatogenesis in adulthood (25). In addition, Sertoli cells secrete lactate and pyruvate, which are sources of energy for germ cells (26–29). Their number in the testes is, therefore, closely related to testicular volume and sperm yield (21). The production of viable spermatozoa involves a sequence of gradual differentiation of germ cells via mitosis and meiosis, and final transformation into mature sperm (30, 31).

Previous studies have revealed estrogen signaling as important signaling involved in endocrine disrupting activities of bisphenols, especially BPA (11, 17, 32). Estrogen signaling occurs through multiple pathways in which estrogen receptors (ERα and ERβ) regulate transcription of target genes directly or indirectly (33). BPA binds with cytoplasm estrogen receptors (cERs) or ERs located in the nucleus (nERs) in the genomic pathway. The binding to these receptors affects nuclear chromatin function and regulates the transcription/translation of genes and protein. Consequently, the cell proliferation, differentiation, and survival are altered (17, 34). In non-genomic signaling pathway, BPA could bind to G-protein coupled receptor (GPR30) on the membrane of testicular cells especially sperm cells (17). The activation of these receptors by BPA in sperm cells results in rapid phosphorylation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), protein kinase A (PKA), and alteration in levels of cyclic adenosine monophosphate, protein kinase C, and intracellular calcium which result in serious cellular effect (11). BPA can also elicit its effect on testicular cells via reactive oxygen species-mediated damage and apoptosis through activation of pro-apoptotic signaling (MAPK, Fas/FasL, Caspase 3 and 9, Bax) (35).

Although, a study indicated that BPA probably incapable to elicit observable effects at low concentration through estrogen receptors (ERα and ERβ) by demonstrating a low affinity between BPA and these receptors (32), recent studies reported that BPA possesses a strong affinity with membrane-bound estrogen receptors and G protein–coupled receptor 30 (GPR30) and evokes cellular effects at low doses (picomolar and nanomolar concentrations), which are lower compared to concentrations needed to stimulate nuclear ERs (36, 37). This review suggests that extremely low doses of BPA that are incapable of producing detrimental effects in tissues and organs via ERα and ERβ will produce negative effects in testicular cells through GPR30 and ERR-γ which are more abundant in the cells (**Figure 1**). Therefore, the objective of this review is to clarify conflicting studies surrounding the effect of bisphenols on male reproductive health, and produce evidence that BPA and currently available analogs threaten male health and fertility through their action on testicular cells, which results in alterations to testicular functions and culminates in impairment of male reproductive health.

FUNCTION OF LEYDIG CELLS IN MALE REPRODUCTIVE HEALTH

Leydig cells are found in the interstitial spaces of the testis (38). They are vital parts of male reproductive organ development and reproduction (39). Androgens produced by Leydig cells are essential for the differentiation of male genitalia and masculinization in response to luteinizing hormones from the pituitary (39). Sexual differentiation in males is a complex sequence of processes that involves activities of hormones produced by somatic cells in the gonads, including Leydig cells (38). There are two populations of Leydig cells in eutherian mammals: fetal and adult Leydig cells (38–40). The post-natal surge in androgens has led to suggestion that there is neonatal or infantile population of Leydig cells in certain species such as humans and primates (38, 40). The fetal Leydig cells are found in the embryonic testes following formation of the testis until parturition (40, 41). In mouse, the fetal Leydig cell secrete androstenedione which is consequently converted to testosterone by hydroxysteroid 17-beta dehydrogenase 3(HSD17B3) produced by Sertoli cells, whereas fetal Leydig cells of rat produce testosterone commencing from gestational day 15.5 (39–41). The adult Leydig cells synthesize testosterone needed for the development of male reproductive organ and the commencement of spermatogenesis (38). Although both fetal and adult Leydig cells possess some distinctions in

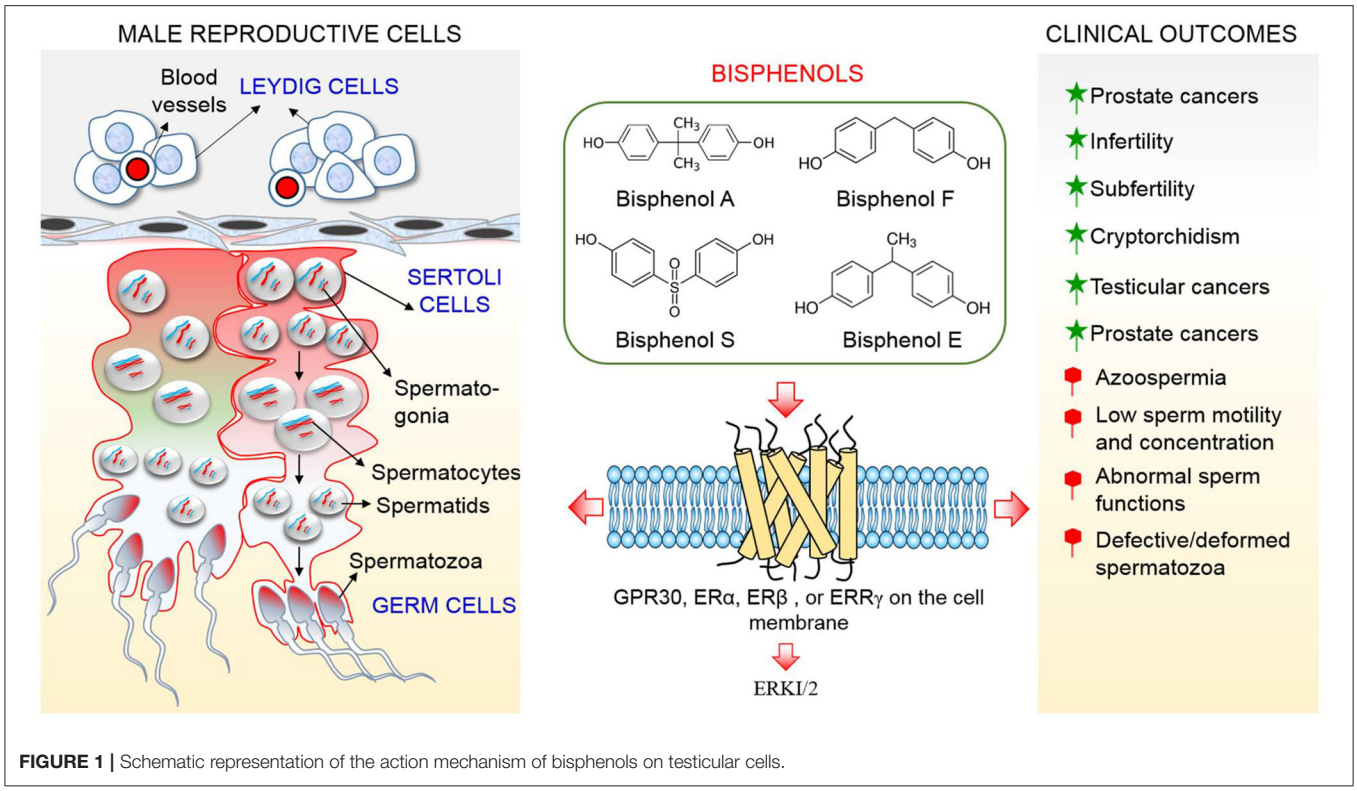

FIGURE 1 | Schematic representation of the action mechanism of bisphenols on testicular cells.

morphological characteristics, they perform same function of androgen production (38). Androgens and other hormones produced by Leydig cells are indispensable to male reproductive system development and health (38). The absence or dysfunction of these cells give rise to maldevelopment of reproductive organs and disorders associated with incomplete masculinization of the male fetus (41, 42). Additionally, the alteration of Leydig cell function can adversely impair fertility in men (43). Normal male sex differentiation procedure involves movement of the testes from their initial location close to the kidneys into the extracorporeal position inside the scrotum. There are two separate and successive phases of testicular descent: the intraabdominal phase, where the testes migrate to the abdominal base; and the inguinoscrotal phase, which involves the movement of the testes through the inguinal canal into the scrotum (43). Each of these phases is controlled by specific hormones; insulin-like 3 (INSL3) and testosterone (40), produced by Leydig cells. The intraabdominal phase which occurs in man between 8 and 10 weeks of gestation is controlled by INSL3, while testosterone regulates the inguinoscrotal phase which occurs between 20 and 26 weeks of gestation (44, 45). Therefore, Leydig cells perform a critical role in male reproductive development and health.

THE EFFECTS OF BISPHENOLS ON LEYDIG CELLS AND THE DEVELOPMENT OF MALE REPRODUCTIVE ORGANS

Leydig cells are the main producers of testosterone in the male reproductive system, and harm to them can lead to infertility (6). A previous study identified a high occurrence of undescended testes in several developed nations (43); one third of male born prematurely have unilateral cryptorchidism, while 2–8% cases are found in full-term males, indicating cryptorchidism as prevalent male reproductive abnormality (46). Leydig cells have been identified among the target cells of bisphenols and other environmental contaminants (47–49). There is increasing evidence of the detrimental implication of BPA and derivatives on health and function of male reproductive system acting via Leydig cells in a dose-dependent manner (48, 49). A previous study examined the effect of BPA on testicular testosterone production using human (6th−11th gestational weeks) and rodent [(Wistar rat:14.5 dpc), (C57BL/6 mice 12.5 dpc)] testicular explants using Fetal Testis Assay (FeTA) method and found that testosterone production was unaffected when exposed to 10^{-12} M BPA for 3 days. However, a reduction in testosterone secretion was noticed with exposure to 10^{-8} M BPA (48). By implication, the effect of BPA on Leydig cells is dose dependent. In the same study, 10^{-8} M BPA decreased testosterone production in human testicular explant, while a higher concentration of 10^{-5} M was required to produce same effect in mice and rats. These results indicate that the effect of BPA is species dependent. Similar to the results obtained from testosterone secretion, BPA decreased mRNA levels of INSL3 in both human and mouse testicular explants in species-dependent manner (48). Meanwhile, a new experiment investigating the effect of BPA on Leydig cells using human and two strains of rodent testicular explants (49) reported that the administration of BPA doses of 10^{-8} M and 10^{-5} M (the concentration that decreased testosterone and INSL3 in humans in an earlier study) for 72 h suppressed testosterone

secretion in Sprague-Dawley fetal rat testicular explants, while a higher concentration of 10^{-5} M repressed testosterone in Wistar strain. This report implies that higher concentration of BPA is required to reduce testosterone production in Wistar rat, while a lower concentration similar to that of human is required to produce same effect in Sprague–Dawley strain. Therefore, whether data regarding the effect of bisphenols on Leydig cells from all strains of rodents under the same conditions can be used to make inferences for human risk assessments requires further investigation. Another experiment conducted using the TM3 Leydig cell from mice indicated that BPA decreased testosterone production, cell viability, growth, metabolic active mitochondria, and induced cell death and alteration of mitochondria membrane potential (50). Exposure of adult Leydig cells to 0.01 nm BPA altered testosterone production by 25%, while 2.4 μg/kg/day decreased testosterone production, androgen biosynthesis, and *CYP17* gene expression in rats in the same study (6). Another study conducted to investigate the involvement of BPA in the maldevelopment of male reproductive organs and heath demonstrated that BPA increased aromatase mRNA levels but suppressed testosterone production in R2C cell line (from rats) *in vitro* (51).

Furthermore, studies on the effect of bisphenols on development of male reproductive organs revealed that 10,000 nmol/L of BPA and its analogs; BPS and BPF, reduced the mRNA level of fetal Leydig cell-related genes [Steroidogenic acute regulatory protein (*Star*), 3 beta-hydroxysteroid dehydrogenase/Delta 5–>4-isomerase type 1 (*Hsd3b1*), cytochrome P450 family 17 subfamily A member 1 (*Cyp17a1*), *INSL3*] in 12.5 dpc fetal mouse testicular explants cultured for 3 days (52). In the same study, the author expanded their earlier finding that used (FeTA) method (48) by adopting basal condition to investigate the differential effect of BPA on rat (14.5 dpc), mouse (12.5 dpc), and human (6.3–11.1 gestational weeks) testicular explants. It was confirmed that 1,000 nmol/L (10^{-6} M) of BPA significantly reduced basal testosterone secretion of human and mouse fetal testes (52). The authors concluded that the minimum observed adverse effect level is 100-fold higher in mouse than in human testes and 100 or 1,000-fold higher in rat than in human testes in basal conditions (52). Moreover, higher occurrences of undescended testes may stem from impairment of the functions of Leydig cells during embryonic stage. In another study, BPA concentration in the blood was negatively correlated with INSL3 expression level (53). Several other studies have indicated that BPA and its analogs reduced both Leydig cell number and testosterone production (54–59). Interestingly, a clinical study conducted on 160 neonate males (Control:80, hypospadias patients: 80) suffering from hypospadias had seven folds BPA concentration in their blood compared to normal newborns (60). Testosterone controls the masculinization of male genitourinary system and a decrease or alteration in its production between days 15 and 18 post-coitus results in developmental defects in male rat fetuses (52). Despite the variation in the mode of study (*in vitro* or *in vivo*), route (subcutaneous or gavage), duration (short or long), and species, all researchers confirmed the effect of bisphenols on Leydig cells. Variations observed in reports are linked with dosage, species, age, duration, and solvent used for dissolving bisphenols. Meanwhile, the interspecies discrepancies present critical concern since animal studies are commonly employed in risk assessment of bisphenols. Human risk assessment data extrapolation from *in vivo* animal studies has generated a concern because metabolic process of BPA in man and rodent is different (61–63). The sensitivity to BPA in rodent fetal and adult-type Leydig cells cannot be comparatively assessed because most studies involving mature animals were conducted *in vivo*, moreover, hypothalamus-pituitary-testicular axis can be affected by bisphenols at varying developmental stage differently (62). Furthermore, the effect of BPA and analogs could be cell specific, e.g., BPA, BPF, BPS, BPE, BPB, and bisphenol A diglycidyl ether (BADGE) inhibit testosterone production in Leydig cells from human testicular explants, whereas Sertoli and germ cells were not affected by the same concentration in the study (54). This suggests variation in the action mechanism of bisphenols on different cells of the testes. A summary of effects of bisphenols on Leydig cells is shown in **Table 1**.

EFFECT OF BISPHENOLS ON LEYDIG CELLS AND TESTICULAR DYSGENESIS SYNDROME

Hypospadias, cryptorchidism, impaired spermatogenesis, and testicular cancer are categorized as testicular dysgenesis syndrome (TDS). They also represent indices of impaired prenatal testicular development (64, 65). This syndrome (TDS) is associated with embryonic maldevelopment of the testis, which impair differentiation of somatic cells (66). Cryptorchid testes mostly harbor twisted tubules and undifferentiated Sertoli cells (66, 67). Although many factors have been hypothesized as causes of TDS, some studies have linked TDS to the effect of endocrine disruptors on Leydig cells (68–70). Bisphenols are endocrine disruptors that can cause malfunctioning of Leydig cells (47–49). Cases of TDS are characterized by the failure of gonads to fully develop and emergence of intersex genitalia (64). The influence of BPA on human fertility and its involvement in several reproductive complications, including TDS, germ cell cancers, and Sertoli cell only syndrome have been reported (10, 71–73). In a study conducted to evaluate the BPA in the blood samples of 98 cryptorchid males admitted for surgery, serum BPA levels ranged from 4.1 to 89.8 ng/mL (74). The study concluded that serum BPA was positively correlated with cryptorchidism. Effect of BPA on Leydig cells resulting to hypoplasia of the cell can be complete or incomplete. Complete hypoplasia is characterized by presence of both male and female copulatory organs. However, atrophy or hypertrophy of testicles characterized incomplete forms (48). These findings prove beyond doubt the involvement of bisphenols, especially BPA in the maldevelopment of the male reproductive organs and system. The aftermath of decreases in the expression of INSL3 and testosterone synthesis are masculinization defects. Low

TABLE 1 | Effects of Bisphenols on Leydig cells.

Chemical name	Dosage	Species	Effects	References
BPA	2.4 μg/kg/day	Long-Evans rat	Decreased testosterone production (1.62 ± 0.16 ng/ml; vs. control, 2.52 ± 0.21)	(6)
			Decreased androgen biosynthesis	
			Suppression of CYP17 gene expression	
			Inhibition of testicular steroidogenesis	
BPA	0.01	90-day old Rat adult Leydig cell	Decreased testosterone biosynthesis by 25%	(6)
BPA	10, 25, and 50 μg/ml	TM3 cell line	Decreased testosterone secretion by 30.4, 69.2, 79.5 % for 10, 25, and 50 μg/ml, respectively	(47)
			Decreased viability	
BPB	10, 25, and 50 μg/ml	TM3 cell line	Decreased testosterone secretion by 41, 76.1, and 91% for 10, 25, and 50 μg/ml, respectively	(47)
BPS	10, 25, and 50 μg/ml	TM3 cell line	Decreased testosterone secretion by 8.8, 7, and 19.4% for 10, 25, and 50 μg/ml, respectively	(47)
BPF	25 and 50 μg/ml	TM3 cell line	Decreased testosterone secretion by 3.8 and 13.8% for 25 and 50 μg/ml, respectively	(47)
BPA	10^{-8} M	Human (6.5–10.5 gestational weeks) testicular explant	Decreased testosterone by 20% compared to control	(48)
			Reduced expression of INSL3 by 20% compared to control	
BPA	10^{-5} M	Wistar rat (14.5 dpc) testicular explant	Decreased testosterone by approximately 50 % on 3rd day of culture	(48)
			Reduced expression of INSL3 by approximately 20%	
BPA	10^{-5} M	Sprague-Dawley Rat (14.5 dpc) testicular explants	Inhibition of testosterone by 10^{-5M} BPA diluted in DMSO at all periods 24 h: 53%; 48 h: 40%; 72 h: 39%,	(49)
			Suppressed INSL3 by 76%	
BPA	10^{-5} M	Man (7–12 gestational week) testicular explants	Inhibition of testosterone by 10^{-5} BPA diluted in DMSO by 28%	(49)
BPA	1, 10, and 100 μM	TM3 cell line	Decreased testosterone production by 22%, 28%, and 39%, for 1, 10, and 100 μM, respectively, when compared to the negative control	(50)
			Decreased cell viability	
			Decreased cell growth	
			Decreased metabolically active mitochondria	
			Alteration of mitochondrial membrane potential	
BPA, BPS, and BPF	10,000 nmol/L	Mice (12.5 dpc)	Reduced INSL3 expression	(52)
			Reduced expression of testosterone biosynthesis related genes (*Star, Hsd3b1, Cyp17a1*) and *Lhcgr*	
BPA, BPS, and BPF	10 nmol/L	Human (6.3–11.1 gestational weeks)	Decreased basal testosterone secretion	(52)
BPA and BPB	10^{-9}-10^{-5} M	Human (46.7 ± 4.65) testicular explant	Inhibition of testosterone production (BPA, 28.7 and 39.2 % at 24 and 48 h, respectively) (BPB, 17 and 47% at 24 and 48 h, respectively.	(54)
BPAF	200 mg/kg/day	7 weeks Sprague–Dawley rat	Reduction of testosterone production by 90.6% compared to control	(55)
			Altered testosterone biosynthesis	
BPA	100 and 200 mg/kg/day	Wistar/ST rat	Reduced plasma and testicular testosterone production	(56)
			Reduced number of Leydig cell	
BPA	4, 40, and 400 mg/kg	Sprague–Dawley rats (Gestational day 21)	Disruption of fetal Leydig cell number, proliferation and distribution	(57)
			Downregulation of Leydig cell genes	
			Decreased expression of INSL3	

fertility and germ cell cancer are major complications linked with testicular dysgenesis (43). The association between the actions of bisphenols on Leydig cells and testicular dysgenesis is as shown in **Figure 2**.

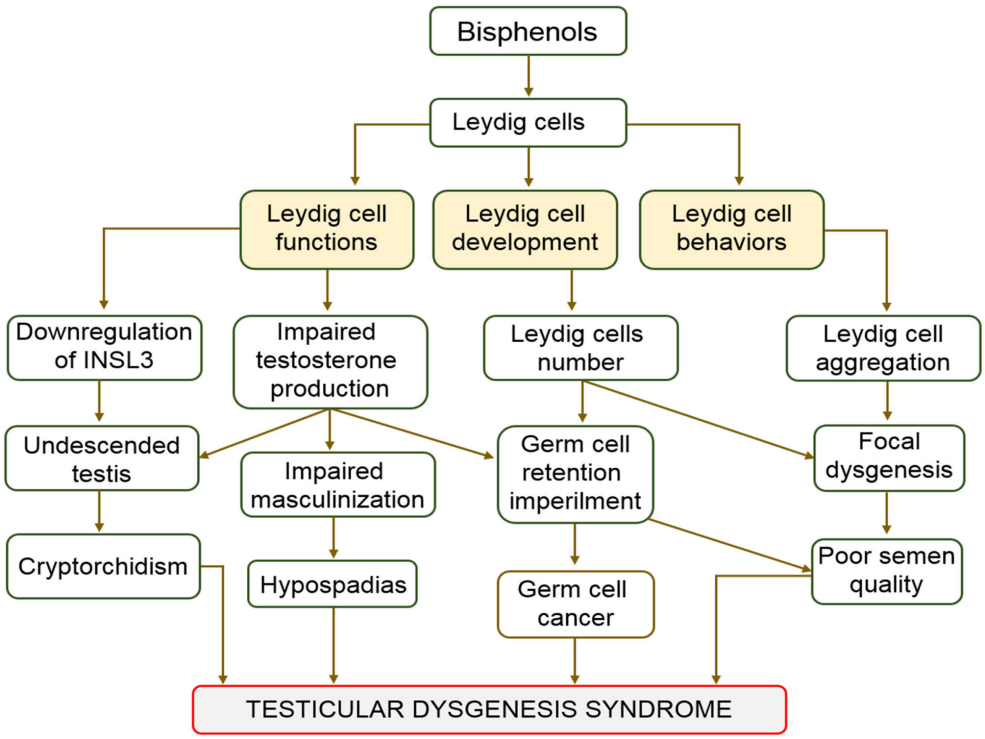

FIGURE 2 | Schematic diagram of the effect of Bisphenols on Leydig cells.

THE ACTION OF BISPHENOLS ON LEYDIG CELLS AND PROSTATE HEALTH

Testosterone performs a very important function in male reproductive health. Reduction or alteration of its synthesis is connected to complications of hypogonadism and impairment of male reproductive health (52). Estrogen and testosterone execute crucial roles in the onset, advancement, and growth of prostate cancer (75). The growth and maintenance of prostate gland is regulated by testosterone and prolactin (23). Citrate production by prostate glands is performed by the highly specialized citrate-producing acini epithelial cells (23). The capability of the acini epithelial cells to produce substantial citrate depends on their zinc accumulating ability and inhibition of citrate oxidation. Meanwhile, the zinc and citrate levels are controlled by testosterone and prolactin (76, 77). There is increasing concern that exposure to xenoestrogen, including bisphenols, during critical developmental window may multiply vulnerability to prostate cancer. Leydig cells are involved in testosterone production and are major targets of bisphenols (47–49). Earlier research findings revealed that BPA decreases testosterone synthesis via Leydig cells, indicating that it is also a threat to prostate health. As shown in previous studies, BPA contamination is related to development of prostate cancer and elevates centrosome amplification *in vitro* (78). In another study, BPA caused enlargement of prostate and increased EGFR mRNA level in mature Sprague–Dawley rats when administered orally (79). Another study demonstrated that BPA enhanced human prostate stem cell proliferation (80). However, a recent study conducted by the same author using a rat model and human prostate epithelial cells indicated that BPA alone did not drive prostate pathology but low doses of BPA augment vulnerability to prostate cancer and induced homeostatic imbalance in adult prostate stem cell (81). At present, these studies indicate bisphenols, especially BPA, as predisposing factors of prostate cancer via decreased testosterone production. However, further investigation into the severity of the involvement of bisphenols as a single chemical and in a mixture with other xenoestrogen in the development of prostate cancer is required.

ACTION MECHANISMS OF BISPHENOLS ON LEYDIG CELLS

As bisphenols are estrogenic in nature, estrogen receptors (ERs) are expressed in Leydig cells and are controlled by estrogen activities (82). Information regarding the localization of ERs in Leydig cells has been inconsistent, they were reported absent (83) and present (84) in the mouse Leydig cell. It was found to be localized in fetal rat Leydig cells (85), but not in adult rat Leydig cells (86). Similarly, ERs were not detected in mouse Leydig cells (6). It was further demonstrated that ERα expression is non-detectable in human fetal testes (87), suggesting that there is no involvement of ERα in impact of BPA on human testes. This result was considered applicable in mice because BPA induced decrease in testosterone production was maintained following

ERα withdrawal (48). Meanwhile, studies regarding the presence or absence of ERβ in human and mouse Leydig cells need to be confirmed in future research. Low concentrations of BPA have reportedly elicited effects via GPR30 or estrogen-related receptor gamma (ERR-γ) (88–90). Both GPR30 and ERR-gamma were expressed in human and mouse fetal testes (48) and ERR-γ has a high affinity for BPA (91). Therefore, GPR30 and ERR-γ represent means by which bisphenols act on testicular cells.

BISPHENOLS, SERTOLI CELLS, AND MALE REPRODUCTIVE HEALTH

Function of Sertoli Cells in Spermatogenesis

Spermatogenesis is a successive development of male germ cells to mature spermatozoa. It involves mitotic and meiotic divisions of germ cells. Sertoli cells play a key role in every stage of spermatogenesis, by clinching tightly to developing germ cells in the seminiferous tubules thereby providing a suitable milieu necessary for their development. Sertoli cells produce pyruvate for nourishment of germ cell. Lactate and pyruvate produced by Sertoli cells are needed by germ cells for energy and survival (26–29), thereby providing nutrition for their development (92). About thirty to fifty germ cells at various developmental phases can be nourished in the seminiferous epithelium by every Sertoli cell (93). The cumulative number of Sertoli cell is positively correlated with testicular size and sperm count (94). Tissue transformation that takes place during spermiation is achieved through the activities of proteases produced by Sertoli cells (21). Similarly, plasminogen activator which facilitates the migration of preleptotene spermatocytes are produced by Sertoli cells (21). Importantly, Sertoli cells expansive junctional networks and communication provide structural support for developing germ cells (20).

Effect of Bisphenols on the Blood-Testis Barrier and Its Implication on Spermatogenesis

Several studies have provided strong evidence that BPA derivatives (BPE and BPS) affect Sertoli cell functions (19, 20, 95). An experimental study on the effect of BPA on Sertoli cells at >150 μM concentration time and dose dependently reduced cell viability, while those exposed to 200 μM BPA reduced to approximately two-thirds of the control (96). The study further revealed that Sertoli cells treated with BPA *in vitro* at a concentration of 200 μM induced morphological distortions such as collapse of cytoskeleton, chromatin impairment, and DNA damage in the cells. Immunocytochemistry studies of the cells showed the expression of caspase-3, colocalization of active caspase-3, and fragmentation of actin filaments (96). The authors concluded that stimulation of apoptotic pathways within the cells rather than necrosis was responsible for their death (96). In another study involving Sertoli cells, BPA induced cellular damage and apoptosis; the BPA-induced damage was attributed to its ability to block endoplasmic reticulum-Ca^{2+} homeostasis (97). BPA was also reported to affect anchoring junction which

TABLE 2 | Effects of bisphenols on Sertoli cells.

Chemical name	Dosage	Species	Effects	References
BPA	50 mg/kg (Rats)	Sprague–Dawley rats and Wistar rats (20 day old);	Disruption of the blood-testis barrier integrity	(19)
BPA	200 μM (Sertoli cell)	Sertoli cells (20-day-old Sprague-Dawley rats)	Perturbation of Sertoli cell tight junction permeability barrier	(19)
			Activation of ERK1/2 in the cell	
			Downregulation of basal ectoplasmic specialization and gap junction at the blood-testis barrier	
BPA	150–200 μM	18-day-old Wistar rats Sertoli cell	Decreased cell viability	(96)
			Induction of membrane blebs, cell rounding, cytoskeletal collapse, chromatin condensation, and DNA fragmentation	
			Expression of caspase-3	
			Disorganization of the actin cytoskeleton	
			Decreased hormone (transferrin) secretion	
BPA	200 μM	Mouse Sertoli TTE3 cells	Induction of cellular damage and apoptosis	(97)
			Induction of endoplasmic reticulum stress	
			Endoplasmic reticulum Ca^{2+} homeostasis blockage	
BPA	20 and 200 μg/kg	ICR mice (3 months old)	Impairment of ectoplasmic specialization between the Sertoli cell and spermatids	(98)
		Wistar rat (4 months)	Incomplete, redundant ectopic specialization	
BPA	200 μM	Rat and SerW3 Sertoli cell line	Perturbation of the Sertoli cell tight junction permeability barrier function	(99)
			Downregulation of blood-testis barrier proteins	
			Redistribution of blood-testis barrier-associated proteins	
			Alteration of the distribution of integral membrane proteins and their peripheral adaptors	
BPA	45 μM	SerW3 Sertoli cells	Alterations of Sertoli cell functions	(101)

(Continued)

TABLE 2 | Continued

Chemical name	Dosage	Species	Effects	References
			Metabolic, endocrine and/or paracrine dysfunctions	
BPA	50 mg/kg	ICR mice (6 weeks)	Downregulation of Sertoli cell-related genes (*Msi1h, Ncoa1, Nid1, Hspb2*, and *Gata6*)	(102)
BPA	2.4 μg/kg/day	Neonatal Holtzman rat	Impairment of fertility	(105)
			Perturbations tight junctions and decreased expression of junctional proteins	
BPA	40 and 200 μM	Human Sertoli cell (12, 23, and 36-year old)	Truncation and depolymerization of actin	(106)
			Microfilaments	
			Disorganization of F-actin	
			Changes in the localization and distribution of F-actin regulatory proteins in Sertoli cell epithelia	
			Retraction of actin microfilaments	
BPA	4, 40, and 400 mg/kg	Sprague–Dawley rat	Downregulation of Sertoli cell genes	(57)

attached spermatids to Sertoli cells (98). This effect of BPA characterized the human Sertoli cell only (SCO) testes, in which the testes were void of blood-testis barrier and constituent proteins, especially connexin 26, which mediates adhesion or communication at the site of attachment of Sertoli cells and spermatogonial are downregulated (99). Several other studies (19, 20, 42) showed that bisphenols, especially BPA, impair male reproductive health. BPA reportedly perturbed Sertoli cell tight junction, downregulated the level of blood-testis barrier constituent proteins (JAM-A, ZO-1, N-cadherin, connexin 43), activated ERK1/2, and redistributed cell-cell interface proteins (19, 20). Although these effects were reported to be non-significant in adult rats, significant effects were observed in immature (20-day-old) rats at the same concentration, indicating a higher susceptibility of immature rats and infants to BPA (19). Information regarding the effect of bisphenols on Sertoli cells isolated from adult rats is not available. Therefore, it remains uncertain whether the effect will be significant if Sertoli cells isolated from mature rats are exposed to BPA *in vitro*. When cultured *in vitro*, Sertoli cells form a blood-testis barrier and intercellular junctions that mimic *in vivo* conditions between 48 and 72 h after culture (100, 101). Using *in vitro* methods, another study (101) investigated the mechanism of BPA action on Sertoli cells and confirmed that BPA interferes with junctional proteins of the cells, for example; occludin, connexin 43, and E-cadherin. In addition to *in vitro* evidence, *in vivo* studies involving 6-week-old male mice revealed that BPA downregulated a wide range of genes connected to Sertoli cell function [Musashi RNA Binding Protein 1 (*Msi1h*), Nuclear Receptor Coactivator 1(*Ncoa1*), nidogen 1 (*Nid1*), Heat shock protein beta-2 (*Hspb2*), and GATA-binding factor 6 (*Gata6*)] following prenatal exposure (102). Downregulation of the junctional and functional proteins of Sertoli cells is potentially capable of disrupting the blood-testis barrier, thereby impairing spermatogenesis (103). Clinical and laboratory reports indicate that BPA exerts higher effects on male reproduction and fertility after prenatal and neonatal exposure than adults because they are resistant to BPA (19, 104, 105). For example, oral administration of 0.02–50 mg/kgbw doses of BPA to adult rats did not alter normal sperm production; meanwhile, it disrupted the blood-testis barrier integrity when neonatal rats were treated with 50 mg/kgbw/day of BPA. Similar result was obtained during *in vitro* BPA treatment of rat Sertoli cells at 40–200 μM (76). Most effects of bisphenols on testicular cells become visible after many years of accumulated effects at a cellular level. A summary of the effect of bisphenols on Sertoli cells is shown in **Table 2**.

Bisphenol Effects on Sertoli Cells and Testicular Homeostasis

During spermatogenesis, few developing germ cells experience programmed cell death while some exfoliate some cytoplasmic materials following completion of differentiation procedures (107). Phagocytosis activities of Sertoli cells rid the testes of dead germ cells and exfoliated materials from full developed germ cells. Sertoli cells ensure elimination of noxious materials originating from dead cells and the removal of autoantigens that may induce an autoimmune response in the testes. Alteration of Sertoli cell phagocytosis is responsible for disease development and testicular dysfunction, consequently, infertility. In addition, disruption of phagocytic function of Sertoli cells interferes with spermatogenic cycle (108). Critical cellular functions like phagocytosis, structural support, and movement are achieved via actin cytoskeleton. Meanwhile, research findings on the effects of BPA on the actin cytoskeleton of the Sertoli cells revealed that BPA induced changes in the organization and location of F-actin proteins in Sertoli cell epithelia (106). The distribution of F-actin system was dose dependently disorganized when human Sertoli cells were cultured in the presence of BPA *in vitro* (106). The same study found that 0.4 μM BPA caused reduction of Sertoli cells actin microfilament. However, high doses, ranging from 40 to 200 μM, of BPA retracted actin microfilaments close to the nucleus (106). BPA also caused disorganization of the actin cytoskeleton in rat Sertoli cells (96). These changes were attributed to the mislocalization of two actin regulatory proteins leading to its failure to aid Sertoli cell blood-testis barrier function (17, 106). In addition, another recent study revealed that disengagement of spermatozoa from Sertoli cells at spermiation is controlled by modifications in arrangement of actin cytoskeletons at the apical ectoplasmic specialization (109). The disruption and mislocalization of actin proteins did not only interfere with testicular homeostasis but

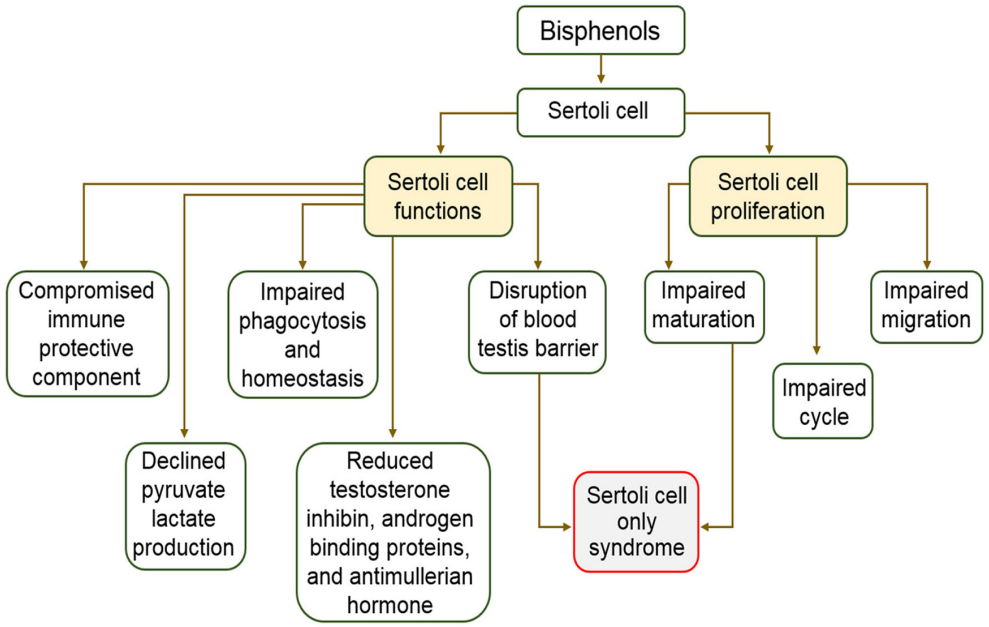

FIGURE 3 | Schematic description of the effects of bisphenols on Sertoli cells and male reproductive health.

lead to untimely release of spermatids into the seminiferous epithelium and are consequently trapped within seminiferous epithelium. These studies confirm that through the action of BPA on actin cytoskeleton, Sertoli cell phagocytic function and testicular homeostasis are impaired. The description of the effects of bisphenols on Sertoli cells and male reproductive health is represented in **Figure 3**.

The Action of Bisphenols on Sertoli Cells and the Defense of Testicular Immunity

Developing germ cells within the seminiferous epithelium are shielded by Sertoli cells being immune privileged cells (110). They perform this critical role by secreting chemicals that can suppress stimulation of pro-inflammatory cytokines and growth of B and T lymphocytes (111). Previous studies show that they synthesize complement inhibitors (112), and endured exposure to antigen (113). In addition, they secrete several protective factors that perform a critical function in immunomodulation and protection of spermatogonia and spermatids (111, 112, 114). Moreover, galectin-1, a highly conserved β-galactosidase-binding protein, capable of inhibiting pro-inflammatory cytokine activation was also discovered in Sertoli cells (115). Sertoli cells are a primary target of BPA and its analogs BPE and BPS impair its functions (19, 20, 95). The impairment of Sertoli cells defensive and immune functions is highly critical to the testes and male reproductive health because it predisposes developing germ cells to external attack. Despite varying dosages and strains of animals used in investigating the effects of bisphenols on Sertoli cells, all studies confirm the negative effect of bisphenols on Sertoli cells, especially during the neonatal window of development.

Action Mechanism of the Effects of Bisphenol on Sertoli Cells

Bisphenols are endocrine disruptors that elicit their impact via affinity with estrogen, androgen, or thyroid hormone receptors (88). These steroid receptors are expressed in Sertoli cells (116, 117). The exposure of fetal rats to BPA reportedly activated Raf1 and p-ERK1/2 in the testes while further evaluation indicated increased level of Raf1 and ERK1/2 proteins in Sertoli cells in response to BPA exposure (118). A study also established that disruption of Sertoli cell tight junction barrier by BPA was accompanied by upregulation of p-ERK in Sertoli cells (19). Another study demonstrated that upregulation of pERK1/2 in cultured Sertoli cells with established junctional barrier declined to lowest level following BPA withdrawal, indicating the involvement of ERK1/2 in BPA induced disruption of Sertoli cell tight junction barrier (119). Although PD98059, an inhibitor of ERK, suppresses the BPA-induced ERK1/2 upregulation in Sertoli cells (120), whether the same inhibitor can repress other effects of BPA on rat and human Sertoli cells remains unknown. Information regarding the mechanism of BPA analogs on Sertoli cells is lacking. Therefore, studies regarding whether BPA analogs have the same or different mechanisms of action on Sertoli cells is necessary. Nonetheless, these findings indicated that BPA elicits its effects on Sertoli cells via the estrogen-ERK signaling pathway.

Bisphenol Effects on Germ Cells and Spermatogenesis

Differentiation process by which spermatogonial stem cells become full developed spermatozoa is called Spermatogenesis (121) Spermatogonial stem cells experience mitotic and meiotic cell divisions to become functional spermatozoa (121). The

quality of germ cells largely determines the fertility of males. Meanwhile, research findings have shown the negative impacts of bisphenols on different growth stages of male germ cells (121–124). BPA concentrations of 10 and 100 μM reportedly induced apoptosis, meiotic abnormalities, and altered stemness properties of ICR (CD-1) and C57BL/6-TG-EGFP mice spermatogonial stem cells cultured for 1 week (121). In the same study, BPA inhibited proliferation and induced alterations in testicular germ cells. Similar findings were reported in another study wherein BPA decreased the density and survival rate of rat spermatozoa (122). Contrary to earlier reports (19) that bisphenols only elicit effects on neonatal males, it was demonstrated in a more recent finding that BPA caused DNA damage, reduced sperm count, and motility in adult rats (123). This study was also supported by a recent finding that BPA through IFNβ-XAF1-XIAP signaling pathway caused germ cell apoptosis in adult mice (125). The incidence of hypomethylation was discovered in the spermatozoa of neonatal males exposed to 2.4 μg of BPA/ pup. Another recent study found that BPA, BPE, and BPS at concentrations of 0.5, 20, and 50 μg/kg/day, respectively, induced oxidative stress and apoptosis, altered the transition of germ cell stages (1–VI, VII, and VIII), caused spermatogenic defects, and decreased sperm motility (126). The study further used *in utero* exposure to confirm that lower concentration of BPE and BPS (between calculated human exposure and no adverse effect doses of BPA) are sufficient to interrupt germ cell differentiation in males. By implication, BPE and BPS are not safe alternatives to BPA in terms of the threat they pose to male reproductive health. The same authors also reported in their earlier study that BPA (10 mg/kgbw), BPE (50 μg/kgbw), and BPS (10 mg/kgbw) caused developmental distortion during spermatogenesis, disrupted male germ cell differentiation, induced germ cell apoptosis and DNA breaks in pachytene spermatocytes in mice (127). BPA, BPB, BPF, and BPS at dosages of 50 μg/L abated the number of germ cells (spermatocytes and spermatids), reduced sperm motility, and daily sperm production (128). This condition represents a critical situation in male reproductive health. In addition, 2 and 20 μg/kgbw of BPA induced oxidative stress in epididymal spermatozoa, caused abnormalities in sperm morphology, and decreased epididymal sperm counts and motility (129). When mice were administered 50 mg/kg/day of BPA, the seminiferous tubule contained a lower number of germ cells and undifferentiated germ cells (130). BPS at a dosage of 50 μg/L induced the generation of reactive oxygen species (ROS) and the apoptosis in germ cells (131). Another *in vivo* study that investigated the effect of 1, 5, and 100 mg/kg body weight of BPA in rats observed absence of germ cells within seminiferous tubules. In addition, the seminiferous epithelia appeared to disintegrate and germ cells were disengaged from Sertoli cells. There were no germ cells in the epididymis but filled with cellular debris (132). In another study that bordered on the impact of BPA on male germ cells using chickens, the results showed that male chickens orally administered BPA dosage of 2 mg/kg body weight every 2 days for 23 weeks had smaller seminiferous tubules exhibiting constrained spermatogenesis (133). The induction of ROS, undifferentiated germ cells, and empty epididymal tubules are indicators of impaired spermatogenesis. These alterations attributed to BPA and its analogs are not only impairments of spermatogenesis but also represent threats to male reproductive health. The effects of bisphenols on germ cells are summarized in **Table 3**.

The Effects of Bisphenols on Sperm Functions

Clinical data consistently revealed an adverse association between BPA exposure and sperm function. Decreased sperm counts and motility observed in occupationally exposed men (138) and infertile patients (139) positively correlated with their urinary BPA concentration. Additionally, an investigation involving middle aged men in Denmark showed lower sperm motility in persons in the upper percentile of urinary BPA concentration compared to those in lower percentile (140). Animal studies on impact of prenatal or neonatal exposure to BPA on spermatozoa showed deleterious aftermath on sperm production in adulthood. For example, the seminiferous tubule of ICR mice and Holtzman rats exposed to low concentration of BPA *in utero* contained reduced number of elongated spermatids and reduced sperm counts (134). The time taken for copulation in F1, F2, and F3 generations of the male offspring of rats exposed to 1.2 and 2.4 μg of BPA was significantly higher compared to that of their control counterparts in respective generation (135). Sperm motility, viability, mitochondrial functions, and intracellular ATP levels have been reported to be negatively affected by BPA through activation of the mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase-A pathways (136). A study which investigated the effect of BPA on sperm function revealed that *in utero* exposure of male vesper mice to BPA at 40, 80, and 200 μg/kg/day altered sperm membrane integrity and motility (141). In addition, pubertal exposure of C57BL/6J male mice to BPA at 50 mg/kg/day concentration caused deformity in ∼9% of sperm population compared to the control group (142). The sperm acrosome integrity of postnatal day 50 male Wistar rats exposed to 5 and 25 mg/kg/day of BPA decreased by 8 and 16%, respectively (72). Similarly, same concentrations reduced sperm plasma membrane integrity by 2% (72). Capacitation-associated proteins in spermatozoa relates to male fertility (143, 144), unfortunately, these proteins are downregulated by BPA (145). Maternal transfer of BPA during nursing was reported caused impairment of spermatozoa in male offspring (146). BPA concentrations of 5 and 50 mg/kg/day reduced sperm motility and intracellular ATP levels of ICR mice (147). In another experiment in which the effect of BPA on sperm function was investigated, BPA concentrations of 50 and 250 μg/kg/day significantly decreased the acrosome reaction which is an indicator of fertilizing ability of the sperm in mice (148). These studies aimed at evaluating the impacts of BPA on male reproductive health and consistently indicated that exposure to low doses of BPA across all developmental stages affected sperm production and fertilizing quality. Although there were differences in exposure windows, period of time, and species investigated, effects unanimously noticed included reduced sperm number, stimulation of sperm apoptosis and

TABLE 3 | Effect of bisphenols on germ cells.

Chemical name	Dosage	Species	Effects	References
BPA	100 μM	ICR mice	Alteration of motility characteristics, acrosome reaction, fertilization, and early embryonic development	(71)
			Downregulation of fertility-related proteins	
			Altered capacitation status	
BPA	10 and 100 μM	Germ cell (ICR mice)	Induction of apoptosis in cultured spermatogonial stem cells	(121)
			Inhibition of testicular germ cell proliferation	
			Alteration of stemness properties of spermatogonial stem cells	
			Induction of meiotic abnormalities in spermatogonial stem cells	
			Induction of proteome alterations in germ cells	
BPA	50, 100, and 200 mg/kg/day	Wistar male rats (aged 28 days)	Sperm abnormality	(122)
			Decreased sperm density and survival rate	
BPA	5.0 mg/kgbw	Holtzman rat (8 weeks)	Increased sperm DNA damage	(123)
			Decreased motility	
			Decreased sperm count	
BPA	2.4 μg/pup	Holtzman rat	Induction of hypomethylation	(124)
BPA	30 mg/kg/day	Kunming mice (8 weeks)	Induction of apoptosis in germ cells	(125)
BPA, BPE, and BPS	0.5, 20, or 50 μg/kg/day	CD-1 mice (Post-natal day 12 and 16)	Disrupted progression of germ cell development	(126)
			Decreased sperm motility	
			Induction of oxidative stress and apoptosis of germ cells	
			Spermatogenic defect	
BPA (10 mg/kgbw)	50 or 10 mg/kgbw	CD-1 mice (5–6 weeks)	Meiotic errors during spermatogenesis	(127)
			Reduced sperm production and quality	
			Disrupted male germ cell development	
BPE (50 μg/kgbw)			Induction of germ cell apoptosis and DNA breaks in pachytene spermatocytes	
BPS (10 mg/kgbw			Delayed cycle in germ cell development	
BPA, BPB, BPF, and BPS	50 μg/L	Rat (22 day old)	Reduced sperm motility	(128)
			Reduced daily sperm production	
			Reduced number of epididymal sperm	
BPA	2 and 20 mg/kgbw	Wistar rat	Abnormalities in sperm morphology	(129)
			Decreased epididymal sperm counts and motility	
			Induction of oxidative stress in epididymal sperm	
BPA	50 mg/kg/day)	FXRα^{-1-} mice	Reduced number of germ cells	(130)
BPS	50 μg/L	Sprague Dawley rats (70–80 days)	Generation of reactive oxygen species (ROS)	(131)
			Induction of apoptosis	
			Reduced number of germ cells	
BPA	1, 5, and 100 mg/kgbw	Sprague Dawley rat (Postnatal day 21)	Undifferentiated germ cells	(132)
			Empty epididymal tubules	
			Sloughing of germ cells	
			Altered germ cell maturity	
BPA	2 mg/kgbw	Chicken (white leghorn)	Constrained spermatogenesis	(133)
BPA	5 mg/kg/day	ICR mice (4 weeks)	Lower seminiferous tubule and mature spermatids	(134)
			Disruption of spermatogenesis	
BPA	1.2 and 2.4 μg/kg/day	Holtzman mice (Postnatal day 75)	Increased time taken for copulation	(135)
			Degeneration of the germ cell	
			Sertoli cell only syndrome	
			Sloughing of germ cells	
BPA	100 μM	ICR mice	Decreased number of motile sperm	(136)
			Altered spermatozoa mitochondria activities	
BPA	50 mg/kg bw/day)	ICR mice (8 weeks old)	Alteration of capacitated spermatozoa function and the proteomic profile	(137)
			Compromised fertilization capabilities of Spermatozoa	

(Continued)

oxidative stress (72, 136, 141–148). A description of the effect of bisphenols on germ cells is shown in **Figure 4**.

Bisphenols Are Related to Transgenerational Male Reproductive Health Disorders

Bisphenols are both genotoxic and epigenotoxic (149). The transgenerational inheritance of the effects of endocrine disruptors has been demonstrated in a series of studies (135, 137, 147, 149, 150). A recently published study demonstrated that paternal exposure to BPA during spermatogenesis markedly modified sperm genetic materials and F1 embryo (149). Our previous study indicated that effects of BPA were vertically transferred to sperm of F1 mice after gestational exposure (143). In another study (137), gestational exposure to BPA caused functional and proteomic modifications in F1 capacitated spermatozoa of adult mice. Epigenetic mechanisms are believed to be responsible for these transgenerational effects (151). Epigenetic modification in spermatozoa and other testicular cells could occur via histone modifications, DNA methylation, and noncoding RNAs (151, 152). Recently, it was discovered that the impacts of BPA could be transgenerational and multigenerational via DNA methylation (141). During DNA methylation, a methyl group is added to the cytosine base within a CpG dinucleotide, and methylation is always connected to transcription subjugation (153). Histone modification involves changing the chromatin organization by substituting DNA interaction with other histones, thereby accelerating or reducing transcription chances (154). Noncoding RNAs, including small and long, are involved in chromatin function and the modulation of gene expression through gene silencing or activation (155, 156). Global hypomethylation in human spermatozoa (157) and zebrafish testes have been reported to be caused by BPA (157, 158). In the same manner, BPA has been implicated in hypermethylation of mouse spermatocytes (159). A decrease in histone acetylation in rat testes was observed following long-term exposure to a low concentration of BPA (160), while increase in histone acetylation in zebrafish testes accompanied exposure to high dose of BPA (161). These reports indicate that bisphenols, especially BPA, threaten the reproductive health of direct contact males and successive generations.

Action Mechanisms of Bisphenols on Germ Cells

BPA and its derivatives exert their effects on different cells and tissues via varying mechanisms and signaling pathways. The effect of exposure to BPA is instant and prompt although observed consequence on gene expression could be delayed by action of the nuclear hormone receptor (36, 162). The mechanism involved in the action of bisphenols on varying cell types is becoming clearer based on recent research findings. The quality and features of receptors implicated in the onset of effect signaling sometimes differ from one cell to the other but are usually associated with those of nuclear hormone receptor-like protein (36). It is generally known that bisphenols possess estrogenic and antiandrogens properties competent of interfering with hypothalamic-pituitary-gonadal axis, especially BPA (11, 32, 52, 163). The structural characteristics of BPA aid its capability to bind with both estrogen receptor types (ERα and ERβ) (17, 164, 165). Germ cells of all developmental stages of rodents and man are known to express ERα and ERβ. As a selective ER modulator, BPA can behave as estrogen agonist or antagonist depending on cell type (166). The activities of bisphenols via estrogen receptors is supported by several studies (36, 167, 168). Through binding

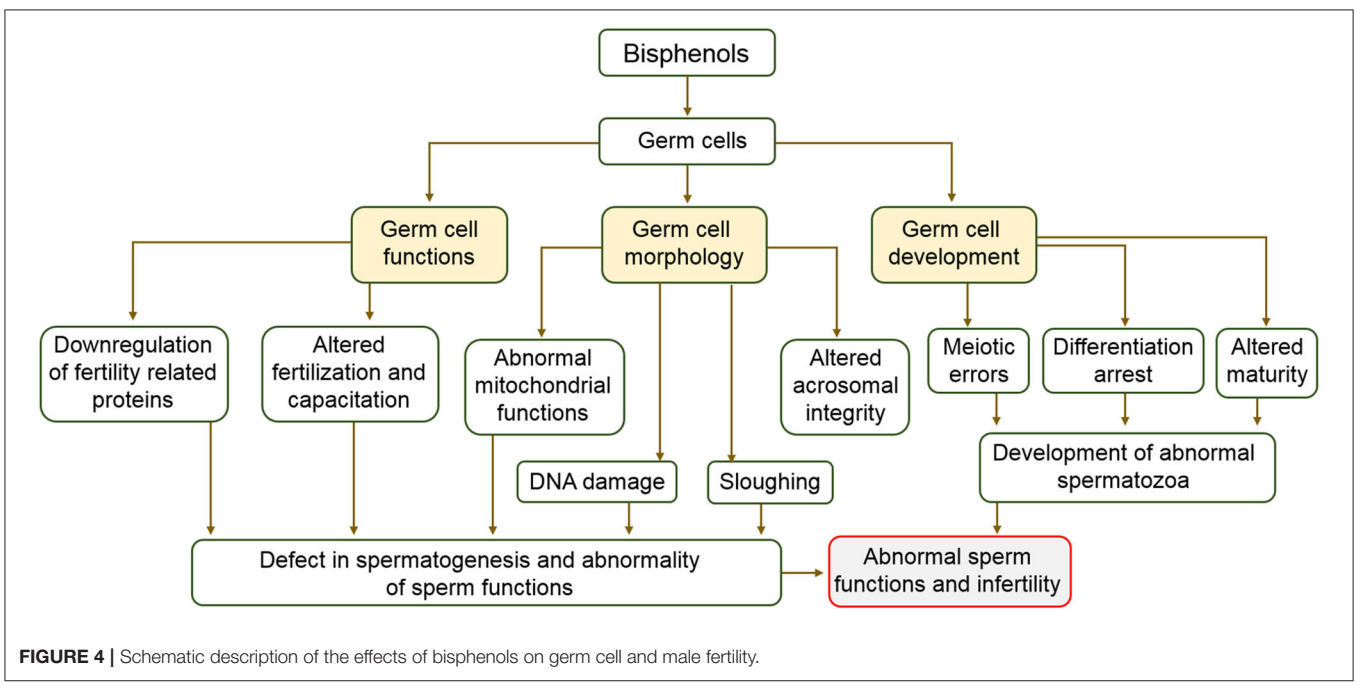

FIGURE 4 | Schematic description of the effects of bisphenols on germ cell and male fertility.

to GPER/GPR30, BPA can produce rapid and impactful effects in germ cells (90, 169, 170). Moreover, it was recently reported that IFNβ-XAF1-XIAP pathways mediated BPA induced male germ cell apoptosis in adult mice (125). The multiple pathways involved in the effect of bisphenols, especially BPA, on male germ cells may be why it affects both neonatal and adult rat germ cells, while significant effects of BPA were mainly observed in the Sertoli cells of neonatal rats in most studies.

BISPHENOLS EFFECT ON PERITUBULAR MYOID CELLS

Peritubular myoid cells are smooth muscle cells that surround the seminiferous tubule in the testis. They are contractile cells which propel spermatozoa to the caput portion of the epididymis (171, 172). Earlier finding also indicated that testosterone-regulated glial cell line-derived neurotrophic factor (GDNF) expression by peritubular myoid cells contributes to the maintenance of spermatogonial stem cell (172). They secrete components of the basement membrane such as fibronectin, collagens, proteoglycans, and entactin (173). In addition, communication between the peritubular myoid and the Sertoli cells is required for the formation of basal lamina during postnatal development (174).

Meanwhile, it has been reported that gestational exposure of female ICR mice to 100 nmol/l per day from gestational day 0 to the end of lactation resulted in apoptosis and mitochondrial vacuolation of peritubular myoid cells of the male pups (175). In another study, an ultrastructural analysis of the testes of adult monkeys (marmosets) exposed to 12.5 and 25 μg/kgbw/day showed the presence of vacuoles in mitochondria of peritubular myoid cells. These reports suggest BPA could interfere with the sperm transport in exposed males. While the effect of bisphenols on Leydig, Sertoli, germ, and peritubular myoid cells are becoming better understood, the impacts on other testicular cell types such as nerve, blood, and lymphatic endothelial cells have not been studied. Therefore, investigation into the effects of bisphenols on these cells is needed, thus providing a roadmap for future studies.

FUTURE PERSPECTIVES

For decades, the impact of low doses of bisphenols on male reproduction has been disputed. While some studies have reported that the administration of bisphenols at low doses does not impact vital alteration in reproductive qualities, other revealed varying degree of harm they unleash on male fertility. In this review, we have shown that bisphenols represent threats to male reproductive health through their actions on Leydig, Sertoli, and germ cells. However, bisphenols are crucial chemicals in household chemical products and plastics industry and are still being used in the production of consumer products around the world due to difficulties in developing economical and safe alternatives (176). It is worthy of note that some of the evidence reported in animal studies may not have same effects on humans for reasons such as stage of development at exposure, cocktail effect, and difference in metabolic process. Therefore, future research should focus on the clarification of the extrapolation of human risk assessment data from rodents due to varying responses to bisphenols among different strains. Secondly, testes are complex organs housing distinct cells that respond to bisphenols differently via varying mechanisms, therefore, holistic studies that simultaneously evaluate the response of Leydig, Sertoli, and germ cells to the same dosages of bisphenols and mechanisms involved are greatly needed. In addition, other testicular cells such as peritubular cells, macrophages, other immune cells, and vasculature should be included among priorities in the study of effect of bisphenols on testicular cells. Fortunately, some approaches have been suggested toward the suppression of BPA toxicities. The pharmacological inhibition of ERKI/2 could be considered a target for mitigating the effects of bisphenols in testicular cells. In addition, producers in real situation do not strictly adhere to a prescribed quantity or dosage by regulatory bodies because they may not provide the desired quality. While the scientific consensus indicates that at a cellular level, BPA, and its analogs alter testicular cell development and functions at low, environmentally relevant doses, future studies should investigate mitigation to protect human health and the environment.

AUTHOR CONTRIBUTIONS

EA and MR wrote the manuscript. M-GP conceived the innovations in the manuscript and edited it. All authors thoroughly revised the manuscript and approved its submission.

FUNDING

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (Project No. NRF2018R1A6A1A03025159). EA was supported by the Korea Research Fellowship Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (Grant No. 2019H1D3A1A01071117).

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