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

Anders Rehfeld[1,2]*, A. M. Andersson[1,2] and N. E. Skakkebæk[1,2]

[1] Department of Growth and Reproduction, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark,
[2] International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

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$_2$PO$_4$, 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$_2$PO$_4$, 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 ($10 \mu M$) for 45 min at $37°C$. Excess dye was removed by centrifugation ($700 \times$ g, 10 min, RT) and the sperm pellet was resuspended in HTF^+ to 5×10^6 sperm cells/mL. Aliquots of $50 \mu L$ 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 $25 \mu L$ bisphenol solutions, negative control (buffer with vehicle), positive control (progesterone, $5 \mu M$ 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 ($10 \mu M$) for 15 min at $37°C$. Excess dye was removed by centrifugation ($700 \times$ g, 10 min, RT) and the sperm pellet was resuspended in HTF^+ to 5×10^6 sperm/ml. Aliquots of $50 \mu L$ 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 $25 \mu L$ 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 $\times$ 2.0 mm $\times$ 10 cm (VitroCom, USA)] by capillary forces, by placing the glass tubes vertically in a 1.5 mL microfuge tube with $750 \mu L$ methylcellulose (1% w/v) for 15 min. 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 $200 \mu L$ non-capacitated sperm sample (10×10^6/ml in HTF^+). Just prior to the insertion of the glass tubes, either bisphenols ($10 \mu M$), $5 \mu M$ progesterone (positive control), $5 \mu M$ 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 $\times 200$ (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 $5 \mu g/mL$ FITC-PSA, $0.5 \mu g/mL$ PI, and $10 \mu g/mL$ Hoechst in HTF^+. Bisphenols ($10 \mu M$) were added to the aliquots of stained capacitated sperm cells together with the positive controls, ionomycin ($10 \mu M$), and progesterone ($10 \mu M$). 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 $50 \mu L$ sample was drawn and added to a $100 \mu L$ 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 $\geq 100\%$ 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
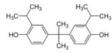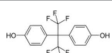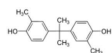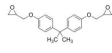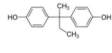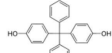
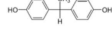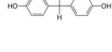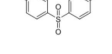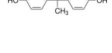comparison type I error inflation by Dunnett's method. Statistical analyses were performed using GraphPad Prism 8.3.1 (GraphPad Software Inc., USA).

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 $10\,\mu M$, along a positive control (progesterone, $5\,\mu M$), 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 $10\,\mu M$, i.e., the peak Ca^{2+} signal induced by the bisphenol at $10\,\mu M$ divided by the peak Ca^{2+} signal induced by progesterone at $5\,\mu M$ in the same experiment.

Rank		Name	CAS number	Abbreviation	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) $\pm 3 \times SD$ ($0.0 \pm 3 \times 2.3 = 6.9\%$) 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) $\pm 3 \times$ SD (0.0 $\pm$ 3 $\times$ 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

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.
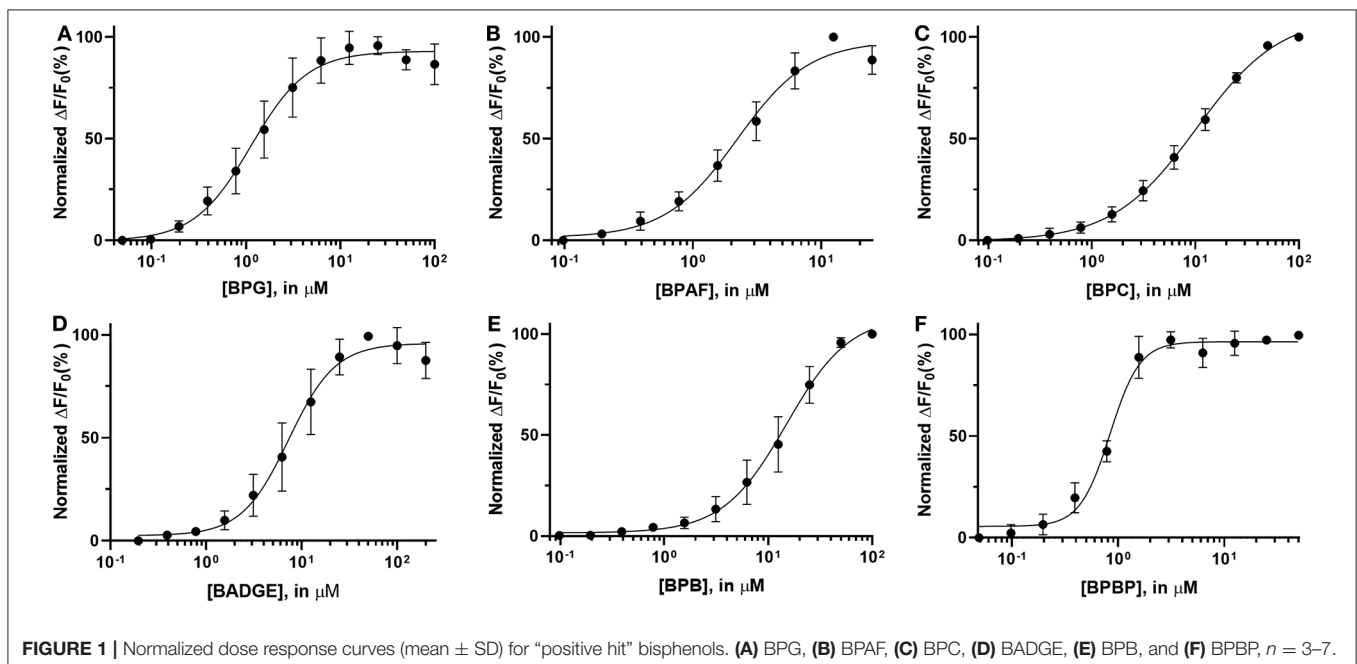
0.79–15.87 μM and mean EC_{05}-values within the concentration range 0.18–2.37 μM (**Table 2**, **Figure 1**).

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 $\pm$ 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

FIGURE 3 | Changes (mean ± SD) in pH$_{(i)}$ induced by the bisphenols (5–50 μM), HTF$^+$ buffer and positive control NH$_4$Cl at 30 mM ($n = 3$).
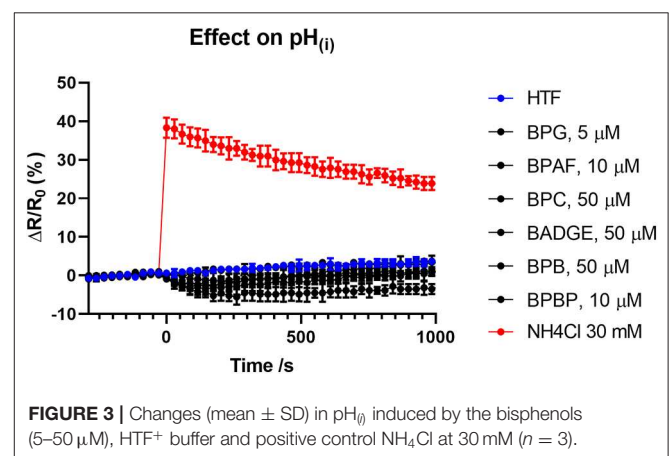
sperm cells (**Figure 6**), similar to the effect of the endogenous CatSper ligand progesterone at 10 μM, in capacitated human sperm cells.
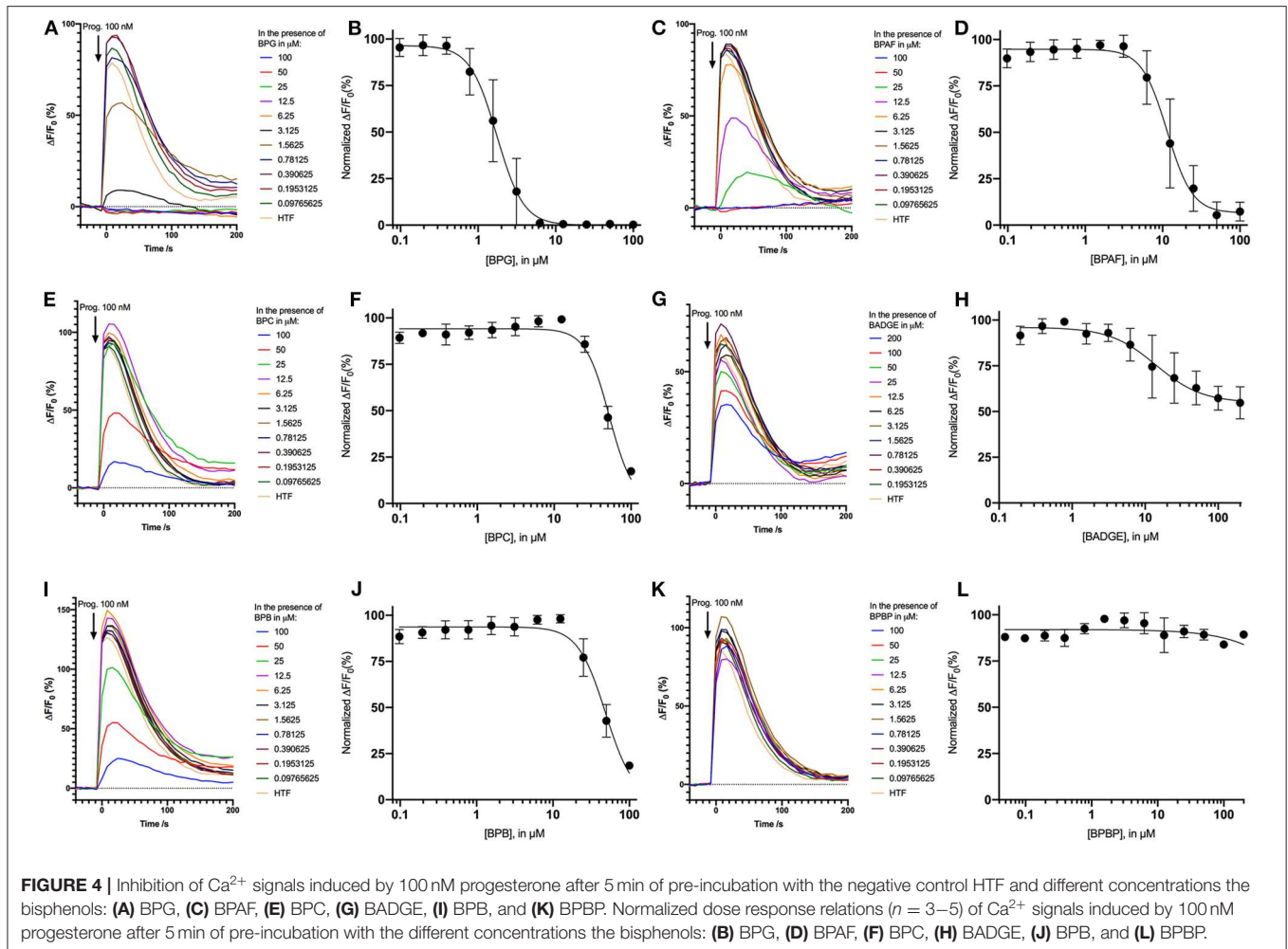
FIGURE 4 | Inhibition of Ca^{2+} signals induced by 100 nM progesterone after 5 min of pre-incubation with the negative control HTF and different concentrations the bisphenols: (A) BPG, (C) BPAF, (E) BPC, (G) BADGE, (I) BPB, and (K) BPBP. Normalized dose response relations ($n = 3–5$) of Ca^{2+} signals 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 ($\geq$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.
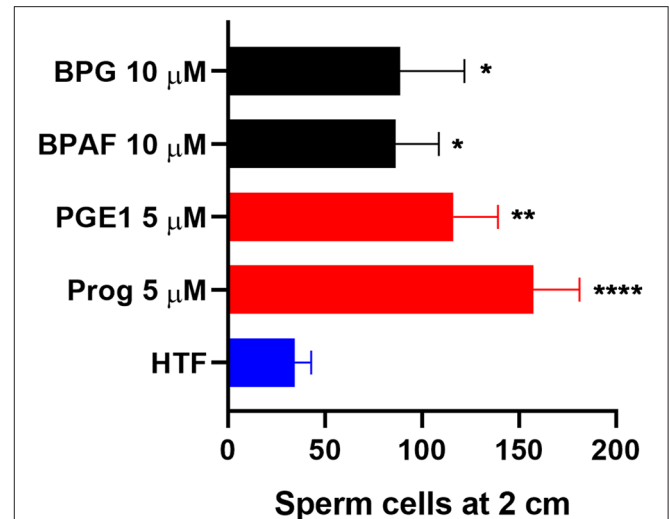

FIGURE 5 | Human sperm cells at 2 cm into a viscous medium (mean ± SEM) after treatment with negative control (HTF$^+$ with 0.1% DMSO "HTF"), positive controls (5 μM progesterone "Prog" and prostaglandin E1 "PGE1"), 10 μM BPG, and 10 μM BPAF ($n \geq 5$). Statistics from multiple comparison between negative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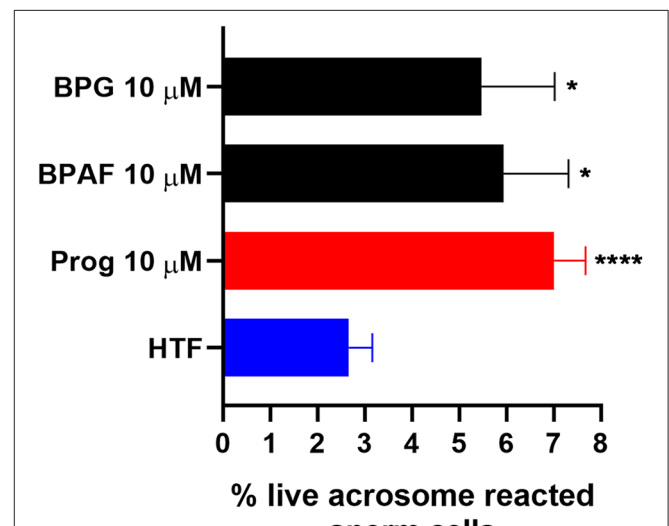

FIGURE 6 | Percentage live acrosome reacted sperm cells (mean ± SEM) after 30 min treatment of capacitated human sperm cells with negative control (HTF$^+$ with 0.2% DMSO "HTF"), positive control (10 μM progesterone "Prog"), 10 μM BPG, and 10 μM BPAF ($n \geq 8$). Statistics from multiple comparison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

ACKNOWLEDGMENTS

REFERENCES

1. Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJR, Schoenfelder G. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environ Health Perspect.* (2010) 118:1055–70. doi: 10.1289/ehp.0901716

2. Chamorro-García R, Kirchner S, Li X, Janesick A, Casey SC, Chow C, et al. Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stromal stem cells through a peroxisome proliferator-activated receptor gamma-independent mechanism. *Environ Health Perspect.* (2012) 120:984–9. doi: 10.1289/ehp.1205063

3. Frederiksen H, Nielsen O, Koch HM, Skakkebaek NE, Juul A, Jørgensen N, et al. Changes in urinary excretion of phthalates, phthalate substitutes, bisphenols and other polychlorinated and phenolic substances in young Danish men; 2009-2017. *Int J Hyg Environ Health.* (2020) 223:93–105. doi: 10.1016/j.ijheh.2019.10.002

4. Sartain CV, Hunt PA. An old culprit but a new story: bisphenol A and "NextGen" bisphenols. *Fertil Steril.* (2016) 106:820–6. doi: 10.1016/j.fertnstert.2016.07.1114

5. Siracusa JS, Yin L, Measel E, Liang S, Yu X. Effects of bisphenol A and its analogs on reproductive health: a mini review. *Reprod Toxicol.* (2018) 79:96–123. doi: 10.1016/j.reprotox.2018.06.005

6. Matuszczak E, Komarowska MD, Debek W, Hermanowicz A. The impact of bisphenol A on fertility, reproductive system, and development: a review of the literature. *Int J Endocrinol.* (2019) 2019:4068717. doi: 10.1155/2019/4068717

7. Cariati F, D'Uonno N, Borrillo F, Iervolino S, Galdiero G, Tomaiuolo R. "Bisphenol a: an emerging threat to male fertility." *Reprod Biol Endocrinol.* (2019) 17:6. doi: 10.1186/s12958-018-0447-6

8. Desdoits-Lethimonier C, Lesné L, Gaudriault P, Zalko D, Antignac JP, Deceuninck Y, et al. Parallel assessment of the effects of bisphenol A and several of its analogs on the adult human testis. *Hum Reprod.* (2017) 32:1465–73. doi: 10.1093/humrep/dex093

9. Publicover S, Harper CV, Barratt C. [Ca^{2+}]i signalling in sperm–making the most of what you've got. *Nat Cell Biol.* (2007) 9:235–42. doi: 10.1038/ncb0307-235

10. Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, et al. The CatSper channel mediates progesterone-induced Ca^{2+} influx in human sperm. *Nature.* (2011) 471:382–6. doi: 10.1038/nature09769

11. Lishko PV, Kirichok Y, Ren D, Navarro B, Chung J-J, Clapham DE. The control of male fertility by spermatozoan ion channels. *Annu Rev Physiol.* (2012) 74:453–75. doi: 10.1146/annurev-physiol-020911-153258

12. Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature.* (2011) 471:387–91. doi: 10.1038/nature09767

13. Rennhack A, Schiffer C, Brenker C, Fridman D, Nitao ET, Cheng Y-M, et al. A novel cross-species inhibitor to study the function of CatSper Ca^{2+} channels in sperm. *Br J Pharmacol.* (2018) 175:3144–61. doi: 10.1111/bph.14355

14. Krausz C, Bonaccorsi L, Luconi M, Fuzzi B, Criscuoli L, Pellegrini S, et al. Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with *in-vitro* fertilization. *Hum Reprod.* (1995) 10:120–4. doi: 10.1093/humrep/10.1.120

15. Oehninger S, Blackmore P, Morshedi M, Sueldo C, Acosta AA, Alexander NJ. Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. *Fertil Steril.* (1994) 61:349–54. doi: 10.1016/S0015-0282(16)56530-3

16. Shimizu Y, Nord EP, Bronson RA. Progesterone-evoked increases in sperm [Ca^{2+}]i correlate with the egg penetrating ability of sperm from fertile but not infertile men. *Fertil Steril.* (1993) 60:526–32. doi: 10.1016/S0015-0282(16)56172-X

17. Falsetti C, Baldi E, Krausz C, Casano R, Failli P, Forti G. Decreased responsiveness to progesterone of spermatozoa in oligozoospermic patients. *J Androl.* (1993) 14:17–22.

18. Williams HL, Mansell S, Alasmari W, Brown SG, Wilson SM, Sutton KA, et al. Specific loss of CatSper function is sufficient to compromise

fertilizing capacity of human spermatozoa. *Hum Reprod.* (2015) 30:2737–46. doi: 10.1093/humrep/dev243

19. Forti G, Baldi E, Krausz C, Luconi M, Bonaccorsi L, Maggi M, et al. Effects of progesterone on human spermatozoa: clinical implications. *Ann Endocrinol (Paris).* (1999) 60:107–10.

20. Krausz C, Bonaccorsi L, Maggio P, Luconi M, Criscuoli L, Fuzzi B, et al. Two functional assays of sperm responsiveness to progesterone and their predictive values in *in-vitro* fertilization. *Hum Reprod.* (1996) 11:1661–7. doi: 10.1093/oxfordjournals.humrep.a019466

21. Avenarius MR, Hildebrand MS, Zhang Y, Meyer NC, Smith LLH, Kahrizi K, et al. Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet.* (2009) 84:505–10. doi: 10.1016/j.ajhg.2009.03.004

22. Smith JF, Syritsyna O, Fellous M, Serres C, Mannowetz N, Kirichok Y, et al. Disruption of the principal, progesterone-activated sperm Ca^{2+} channel in a CatSper2-deficient infertile patient. *Proc Natl Acad Sci U S A.* (2013) 110:6823–8. doi: 10.1073/pnas.1216588110

23. Hildebrand MS, Avenarius MR, Fellous M, Zhang Y, Meyer NC, Auer J, et al. Genetic male infertility and mutation of CATSPER ion channels. *Eur J Hum Genet.* (2010) 18:1178–84. doi: 10.1038/ejhg.2010.108

24. Avidan N, Tamary H, Dgany O, Cattan D, Pariente A, Thulliez M, et al. CATSPER2, a human autosomal nonsyndromic male infertility gene. *Eur J Hum Genet.* (2003) 11:497–502. doi: 10.1038/sj.ejhg.5200991

25. Zhang Y, Malekpour M, Al-Madani N, Kahrizi K, Zanganeh M, Mohseni M, et al. Sensorineural deafness and male infertility: a contiguous gene deletion syndrome. *BMJ Case Rep.* (2009) 2009:bcr08.2008.0645. doi: 10.1136/bcr.08.2008.0645

26. Jaiswal D, Singh V, Dwivedi US, Trivedi S, Singh K. Chromosome microarray analysis: a case report of infertile brothers with CATSPER gene deletion. *Gene.* (2014) 542:263–5. doi: 10.1016/j.gene.2014.03.055

27. Brown SG, Miller MR, Lishko PV, Lester DH, Publicover SJ, Barratt CLR, et al. Homozygous in-frame deletion in CATSPERE in a man producing spermatozoa with loss of CatSper function and compromised fertilizing capacity. *Hum Reprod.* (2018) 33:1812–816. doi: 10.1093/humrep/dey278

28. Luo T, Chen H-Y, Zou Q-X, Wang T, Cheng Y-M, Wang H-F, et al. A novel copy number variation in CATSPER2 causes idiopathic male infertility with normal semen parameters. *Hum Reprod.* (2019) 34:414–23. doi: 10.1093/humrep/dey377

29. Schiffer C, Rieger S, Brenker C, Young S, Hamzeh H, Wachten D, et al. Rotational motion and rheotaxis of human sperm do not require functional CatSper channels and transmembrane Ca^{2+} signaling. *EMBO J.* (2020) 39:e102363. doi: 10.15252/embj.2019102363

30. Brenker C, Goodwin N, Weyand I, Kashikar ND, Naruse M, Krähling M, et al. The CatSper channel: a polymodal chemosensor in human sperm. *EMBO J.* (2012) 31:1654–65. doi: 10.1038/emboj.2012.30

31. Brenker C, Schiffer C, Wagner IV, Tüttelmann F, Röpke A, Rennhack A, et al. Action of steroids and plant triterpenoids on CatSper Ca^{2+} channels in human sperm. *Proc Natl Acad Sci U S A.* (2018) 115:E344–E6. doi: 10.1073/pnas.1717929115

32. Mannowetz N, Miller MR, Lishko PV. Regulation of the sperm calcium channel CatSper by endogenous steroids and plant triterpenoids. *Proc Natl Acad Sci U S A.* (2017) 114:5743–8. doi: 10.1073/pnas.1700367114

33. Martins da Silva SJ, Brown SG, Sutton K, King LV, Ruso H, Gray DW, et al. Drug discovery for male subfertility using high-throughput screening: a new approach to an unsolved problem. *Hum Reprod.* (2017) 32:974–84. doi: 10.1093/humrep/dex055

34. Schiffer C, Müller A, Egeberg DL, Alvarez L, Brenker C, Rehfeld A, et al. Direct action of endocrine disrupting chemicals on human sperm. *EMBO Rep.* (2014) 15:758–65. doi: 10.15252/embr.201438869

35. Rehfeld A, Dissing S, Skakkebæk NE. Chemical UV filters mimic the effect of progesterone on Ca^{2+} signaling in human sperm cells. *Endocrinology.* (2016) 157:4297–308. doi: 10.1210/en.2016-1473

36. Zou Q-X, Peng Z, Zhao Q, Chen H-Y, Cheng Y-M, Liu Q, et al. Diethylstilbestrol activates CatSper and disturbs progesterone actions in human spermatozoa. *Hum Reprod.* (2017) 32:290–8. doi: 10.1093/humrep/dew332

37. Tavares RS, Mansell S, Barratt CLR, Wilson SM, Publicover SJ, Ramalho-Santos J. p,p′-DDE activates CatSper and compromises human sperm

function at environmentally relevant concentrations. *Hum Reprod.* (2013) 28:3167–77. doi: 10.1093/humrep/det372

38. Yuan Y, Ding X, Cheng Y, Kang H, Luo T, Zhang X, et al. PFOA evokes extracellular Ca^{2+} influx and compromises progesterone-induced response in human sperm. *Chemosphere.* (2020) 241:125074. doi: 10.1016/j.chemosphere.2019.125074

39. Shannon M, Rehfeld A, Frizzell C, Livingstone C, McGonagle C, Skakkebaek NE, et al. *In vitro* bioassay investigations of the endocrine disrupting potential of steviol glycosides and their metabolite steviol, components of the natural sweetener Stevia. *Mol Cell Endocrinol.* (2016) 427:65–72. doi: 10.1016/j.mce.2016.03.005

40. Kotwicka M, Skibinska I, Piworun N, Jendraszak M, Chmielewska M, Jedrzejczak P. Bisphenol A modifies human spermatozoa motility *in vitro*. *J Med Sci.* (2016) 85:39–45. doi: 10.20883/jms.2016.5

41. Rehfeld A, Egeberg Palme DL, Almstrup K, Juul A, Skakkebaek NE. Medium-throughput screening assays for assessment of effects on Ca^{2+}-Signaling and acrosome reaction in human sperm. *J Vis Exp.* (2019). doi: 10.3791/59212

42. Egeberg DL, Kjaerulff S, Hansen C, Petersen JH, Glensbjerg M, Skakkebaek NE, et al. Image cytometer method for automated assessment of human spermatozoa concentration. *Andrology.* (2013) 1:615–23. doi: 10.1111/j.2047-2927.2013.00082.x

43. Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, et al. Ca^{2+} signals generated by CatSper and Ca^{2+} stores regulate different behaviors in human sperm. *J Biol Chem.* (2013) 288:6248–58. doi: 10.1074/jbc.M112.439356

44. König K, Tadir Y, Patrizio P, Berns MW, Tromberg BJ. Effects of ultraviolet exposure and near infrared laser tweezers on human spermatozoa. *Hum Reprod.* (1996) 11:2162–4. doi: 10.1093/oxfordjournals.humrep.a019069

45. Padberg F, Tarnow P, Luch A, Zellmer S. Minor structural modifications of bisphenol A strongly affect physiological responses of HepG2 cells. *Arch Toxicol.* (2019) 93:1529–541. doi: 10.1007/s00204-019-02457-y

46. Cao L-Y, Ren X-M, Li C-H, Zhang J, Qin W-P, Yang Y, et al. Bisphenol AF and bisphenol B exert higher estrogenic effects than bisphenol A via G protein-coupled estrogen receptor pathway. *Environ Sci Technol.* (2017) 51:11423–30. doi: 10.1021/acs.est.7b03336

47. Asano S, Tune JD, Dick GM. Bisphenol A activates Maxi-K ($K_{Ca}1.1$) channels in coronary smooth muscle. *Br J Pharmacol.* (2010) 160:160–70. doi: 10.1111/j.1476-5381.2010.00687.x

48. Deutschmann A, Hans M, Meyer R, Häberlein H, Swandulla D. Bisphenol A inhibits voltage-activated Ca^{2+} channels *in vitro*: mechanisms and structural requirements. *Mol Pharmacol.* (2013) 83:501–11. doi: 10.1124/mol.112.081372

49. Zhang B, He Y, Zhu H, Huang X, Bai X, Kannan K, et al. Concentrations of bisphenol A and its alternatives in paired maternal-fetal urine, serum and amniotic fluid from an e-waste dismantling area in China. *Environ Int.* (2020) 136:105407. doi: 10.1016/j.envint.2019.105407

50. Russo G, Barbato F, Mita DG, Grumetto L. Simultaneous determination of fifteen multiclass organic pollutants in human saliva and serum by liquid chromatography-tandem ultraviolet/fluorescence detection: a validated method. *Biomed Chromatogr.* (2019) 33:e4427. doi: 10.1002/bmc.4427

51. Song S, Duan Y, Zhang T, Zhang B, Zhao Z, Bai X, et al. Serum concentrations of bisphenol A and its alternatives in elderly population living around e-waste recycling facilities in China: associations with fasting blood glucose. *Ecotoxicol Environ Saf.* (2019) 169:822–8. doi: 10.1016/j.ecoenv.2018.11.101

52. Li A, Zhuang T, Shi W, Liang Y, Liao C, Song M, et al. Serum concentration of bisphenol analogues in pregnant women in China. *Sci Total Environ.* (2020) 707:136100. doi: 10.1016/j.scitotenv.2019.136100

53. Wang L, Xue J, Kannan K. Widespread occurrence and accumulation of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives in human blood and adipose fat. *Environ Sci Technol.* (2015) 49:3150–7. doi: 10.1021/acs.est.5b00096

54. Miller MR, Mannowetz N, Iavarone AT, Safavi R, Gracheva EO, Smith JF, et al. Unconventional endocannabinoid signaling governs sperm

activation via the sex hormone progesterone. *Science.* (2016) 352:555–9. doi: 10.1126/science.aad6887

55. Brenker C, Rehfeld A, Schiffer C, Kierzek M, Kaupp UB, Skakkebæk NE, et al. Synergistic activation of CatSper Ca^{2+} channels in human sperm by oviductal ligands and endocrine disrupting chemicals. *Hum Reprod.* (2018) 33:1915–23. doi: 10.1093/humrep/dey275

56. Rehfeld A, Egeberg D, Almstrup K, Holm Petersen J, Dissing S, Skakkebæk NE. Chemical UV filters can affect human sperm function in a progesterone-like manner. *Endocr Connect.* (2017) 7:16–25.". doi: 10.1530/EC-17-0156

57. Barbonetti A, Castellini C, Di Giammarco N, Santilli G, Francavilla S, Francavilla F. *In vitro* exposure of human spermatozoa to bisphenol A induces pro-oxidative/apoptotic mitochondrial dysfunction. *Reprod Toxicol.* (2016) 66:61–7. doi: 10.1016/j.reprotox.2016.09.014

58. Di Renzo GC, Conry JA, Blake J, DeFrancesco MS, DeNicola N, Martin JN, et al. International federation of gynecology and obstetrics opinion on reproductive health impacts of exposure to toxic environmental chemicals. *Int J Gynaecol Obstet.* (2015) 131:219–25. doi: 10.1016/j.ijgo.2015.09.002