Human Reproduction, Vol.32, No.2 pp. 290–298, 2017
Advanced Access publication on December 28, 2016 doi:10.1093/humrep/dew332

human reproduction

ORIGINAL ARTICLE *Andrology*

Diethylstilbestrol activates CatSper and disturbs progesterone actions in human spermatozoa

Qian-xing Zou[1,†], Zhen Peng[1,†], Qing Zhao[1], Hou-yang Chen[2], Yi-min Cheng[1], Qing Liu[1], Yuan-qiao He[1], Shi-qi Weng[1], Hua-feng Wang[1], Tao Wang[1], Li-ping Zheng[1], and Tao Luo[1,*]

[1]Institute of Life Science and School of Life Science, Nanchang University, Nanchang, Jiangxi 330031, PR China [2]Reproductive Medical Center, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi 330006, PR China

*Correspondence address. Institute of Life Science and School of Life Science, Nanchang University, Nanchang, Jiangxi 330031, PR China. E-mail: luotao@ncu.edu.cn

Submitted on June 24, 2016; resubmitted on November 24, 2016; accepted on December 16, 2016

STUDY QUESTION: Is diethylstilbestrol (DES), a prototypical endocrine-disrupting chemical (EDC), able to induce physiological changes in human spermatozoa and affect progesterone actions?

SUMMARY ANSWER: DES promoted Ca^{2+} flux into human spermatozoa by activating the cation channel of sperm (CatSper) and suppressed progesterone-induced Ca^{2+} signaling, tyrosine phosphorylation and sperm functions.

WHAT IS KNOWN ALREADY: DES significantly impairs the male reproductive system both in fetal and postnatal exposure. Although various EDCs affect human spermatozoa in a non-genomic manner, the effect of DES on human spermatozoa remains unknown.

STUDY DESIGN, SIZE, DURATION: Sperm samples from normozoospermic donors were exposed *in vitro* to a range of DES concentrations with or without progesterone at 37°C in a 5% CO_2 incubator to mimic the putative exposure to this toxicant in seminal plasma and the female reproductive tract fluids. The incubation time varied according to the experimental protocols. All experiments were repeated at least five times using different individual sperm samples.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human sperm intracellular calcium concentrations ($[Ca^{2+}]_i$) were monitored with a multimode plate reader following sperm loading with Ca^{2+} indicator Fluo-4 AM, and the whole-cell patch-clamp technique was performed to record CatSper and alkalinization-activated sperm K^+ channel (KSper) currents. Sperm viability and motility parameters were assessed by an eosin–nigrosin staining kit and a computer-assisted semen analysis system, respectively. The ability of sperm to penetrate into viscous media was examined by penetration into 1% methylcellulose. The sperm acrosome reaction was measured using chlortetracycline staining. The level of tyrosine phosphorylation was determined by western blot assay.

MAIN RESULTS AND THE ROLE OF CHANCE: DES exposure rapidly increased human sperm $[Ca^{2+}]_i$ dose dependently and even at an environmentally relevant concentration (100 pM). The elevation of $[Ca^{2+}]_i$ was derived from extracellular Ca^{2+} influx and mainly mediated by CatSper. Although DES did not affect sperm viability, motility, penetration into viscous media, tyrosine phosphorylation or the acrosome reaction, it suppressed progesterone-stimulated Ca^{2+} signaling and tyrosine phosphorylation. Consequently, DES (1–100 μM) significantly inhibited progesterone-induced human sperm penetration into viscous media and acrosome reaction.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Although DES has been shown to disturb progesterone actions on human spermatozoa, this study was performed *in vitro*, and caution must be taken when extrapolating the results in practical applications.

WIDER IMPLICATIONS OF THE FINDINGS: The present study revealed that DES interfered with progesterone-stimulated Ca^{2+} signaling and tyrosine phosphorylation, ultimately inhibited progesterone-induced human sperm functions and, thereby, might impair sperm

[†]Joint first authors.

fertility. The non-genomic manner in which DES disturbs progesterone actions may be a potential mechanism for some estrogenic endocrine disruptors to affect human sperm function.

STUDY FUNDING/COMPETING INTEREST(S): National Natural Science Foundation of China (No. 31400996); Natural Science Foundation of Jiangxi, China (No. 20161BAB204167 and No. 20142BAB215050); open project of National Population and Family Planning Key Laboratory of Contraceptives and Devices Research (No. 2016KF07) to T. Luo; National Natural Science Foundation of China (No. 81300539) to L.P. Zheng. The authors have no conflicts of interest to declare.

Key words: acrosome reaction / intracellular calcium concentration / CatSper / diethylstilbestrol / endocrine-disrupting chemical / human sperm / hyperactivation / progesterone / tyrosine phosphorylation

Introduction

The increase in rates of male infertility worldwide is thought to be closely related to environmental pollution (Auger *et al.*, 2001). Endocrine-disrupting chemicals (EDCs) are the major pollutants influencing male reproductive health by interfering with normal endocrine functions (Daston *et al.*, 1997). Diethylstilbestrol (DES), a prototypical EDC and environmental estrogen, is a synthetic non-steroidal estrogen that was originally used to prevent miscarriage and premature deliveries in pregnant women (Newbold, 2008). Because it has toxic effects on the reproductive systems of both the mother and her offspring, the use of DES for pregnancy support was banned (Hoover *et al.*, 2011). Nevertheless, DES continues to be used to treat prostate and breast cancers (Bosset *et al.*, 2012). It was also frequently used in non-medical applications such as cosmetics, lotions and shampoo and as a growth promoter in livestock feed that was present as a contaminant in food sources (Patisaul and Adewale, 2009). Therefore, a significant source of DES exposure to humans may be present in daily life, and DES may accumulate in the body and affect human health.

Early epidemiological investigations and laboratory research have demonstrated the long-term male reproductive toxicity of DES. *In utero* exposure to DES decreases fertility by causing multiple reproductive tract abnormalities, including testicular hypoplasia, cryptorchidism, hypospadias, epididymal cysts, inhibition of gubernaculum development, tumors of the rete testis and interstitial cells and impaired spermatogenesis (Whitehead and Leiter, 1981; Fielden *et al.*, 2002; Klip *et al.*, 2002). In addition to the toxicity of *in utero* exposure, postnatal exposure to DES also causes male reproductive defects (Wilcox *et al.*, 1995; Visser *et al.*, 1998). In male rats that had been administered a DES (8–1000 μg per day) injection for 12 days, the relative weight of the epididymis, sperm numbers and sperm motility parameters were significantly reduced (Goyal *et al.*, 2001). In hamsters, the testicular weight and seminiferous tubular area were decreased after 1 mg/kg DES injections for 1 week, and increased oxidative stress resulted in spermatogenic cell apoptosis (Ma *et al.*, 2008). The long-term effect of DES on the male reproductive system has been elucidated both in fetal and postnatal exposure. However, the effect of DES on human spermatozoa remains unclear.

Recently, the non-genomic actions of EDCs on mammalian spermatozoa have been studied by many researchers (Tavares *et al.*, 2016). Various EDCs increase human spermatozoa intracellular calcium concentrations ($[Ca^{2+}]_i$) by activating the cation channel of sperm (CatSper) and affecting Ca^{2+}-related sperm functions (Schiffer *et al.*, 2014). Interestingly, the effects of EDCs on human spermatozoa are quite similar to progesterone, an important product of the oviduct secreted from cumulus cells. In humans, progesterone stimulates Ca^{2+} influx by activating CatSper and consequently induces Ca^{2+}-related sperm functions including capacitation, chemotaxis, hyperactivation and the acrosome reaction (Lishko *et al.*, 2011; Strünker *et al.*, 2011). Although various EDCs can compete with progesterone for CatSper activation (Schiffer *et al.*, 2014), the effect of EDCs on progesterone-induced human sperm functions remains unclear.

Therefore, the present study aimed to examine the *in vitro* effect of DES on human spermatozoa and elucidate whether DES disturbs progesterone actions, thus potentially affecting human male fertility. The results may provide new insight into a non-genomic mechanism by which estrogenic endocrine disruptors impair male fertility.

Materials and Methods

Chemicals

Human tubal fluid (HTF) medium, human serum albumin (HSA) and anti-phosphotyrosine monoclonal antibody 4G10 were purchased from Merck Millipore Corporation (Billerica, MA, USA). The mouse anti-GAPDH monoclonal antibody was obtained from Proteintech Group, Inc. (Wuhan, China). The horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody was from Thermo Fisher Scientific (Waltham, MA, USA). Fluo-4 AM and Pluronic F-127 were obtained from Molecular Probes (Eugen, OR, USA). DES, mibefradil, progesterone (P4), methylcellulose, chlortetracycline (CTC) hydrochloride, phosphatase inhibitor cocktail, protease inhibitor cocktail and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Ethical approval

The collection of semen samples and experiments in this study were approved by the Institutional Ethics Committee on human subjects of Jiangxi Maternal and Child Health Hospital.

Sperm sample collection and treatments

Semen samples were obtained by masturbation after 3–5 days of sexual abstinence from healthy donors who had reproductive history during the preceding 2 years and normal sperm quality according to the World Health Organization laboratory manual for the examination and processing of human semen (http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/). After liquefaction, the spermatozoa were purified by direct swim-up in high-saline (HS) solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid and 1 mM Na-pyruvate at pH 7.4 with NaOH) as

the non-capacitating medium or HTF/HSA medium (a capacitating medium containing 25 mM $NaHCO_3$ and 3% HSA) as described previously (Luo *et al.*, 2015). These mediums can also mimic the ionic environments in the male or female reproductive tract. In this study, we set a range of concentrations (10 pM–100 μM) to examine the effect of DES on ejaculated human spermatozoa based on the concentrations of DES detected in surface water from various locations (up to 100 pM) and in the plasma (25 nM) of patients who received three times daily treatments of 1 mg DES (Kemp *et al.*, 1981; Lei *et al.*, 2016). Thus, the spermatozoa were resuspended in HS or HTF/HSA medium containing different concentrations of DES and incubated at 37°C in a 5% CO_2 incubator for different times according to the experimental protocols.

Assessment of sperm viability and motility

Sperm suspensions were treated with different concentrations of DES for 1 h in HS medium (a non-capacitating medium) and for 4 h in HTF/HSA medium (capacitating medium), which represent non-capacitating and capacitating stages, respectively. Sperm viability was determined by eosin–nigrosin staining kits (Nanchang Royo Biotech Co. Ltd., Nanchang, PR China) according to the user manual. The heads of dead spermatozoa were stained red, while viable spermatozoa were not stained. Sperm motility was measured by a computer-assisted sperm analysis (CASA) system (WLJY-9000, WeiLi. Co., Ltd., Beijing, China). After incubation, an aliquot of 10 μl of each sperm sample was transferred to a sperm-counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) and sperm motility parameters including total motility, progressive motility, curvilinear velocity (VCL) and straight-line velocity (VSL) were recorded. At least, 200 spermatozoa were counted for each assay.

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

The change of human sperm $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fluo-4 AM with the EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) as previously described (Strünker *et al.*, 2011). To evaluate the effect of DES on $[Ca^{2+}]_i$, human spermatozoa were exposed to different DES concentrations diluted in HS. To further examine whether CatSper was involved in the DES effect on human sperm $[Ca^{2+}]_i$, 6 mM 1,2-bis (o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-buffered HS medium or 30 μM CatSper blocker mibefradil were added before DES treatment. Moreover, to determine the effect of DES on progesterone-stimulated $[Ca^{2+}]_i$ influx, two methods were used: first, human spermatozoa were pretreated with DES for 30 min, then 1 μM progesterone was added; second, DES and progesterone were added together to test the change in sperm $[Ca^{2+}]_i$. The change of sperm $[Ca^{2+}]_i$ was calculated by $\Delta F/F_0$ (%) indicating the percent (%) of fluorescent changes (ΔF) normalized to the mean basal fluorescence before the application of any chemicals (F_0). $\Delta F/F_0$ (%) = $(F - F_0)/F_0 \times 100\%$, F indicates the fluorescent intensity at each recorded time point.

Sperm patch-clamp recordings

The whole-cell patch-clamp technique was applied to record human sperm CatSper and the alkalinization-activated sperm K^+ channel (KSper) currents as previously described (Lishko *et al.*, 2011; Zeng *et al.*, 2011). Seals were formed at the sperm cytoplasmic droplet or the neck region by a 15–30 MΩ pipette. Then, the transition into whole-cell mode was made by the application of short (1 ms) voltage pulses (400–650 mV) combined with light suction. The currents were stimulated by 1 s voltage ramps from −100 to +100 mV from a holding potential of 0 mV. For recording the monovalent current of CatSper, divalent-free (DVF) solution (150 mM NaCl, 20 mM HEPES, and 5 mM EDTA, pH 7.4) was used to record basal CatSper monovalent currents. Then, 10 μM DES, 1 μM progesterone and 10 μM DES together with 1 μM progesterone in DVF were perfused to record CatSper currents. For KSper recording, cells were perfused with the high-K^+ HS (160 K^+) solution (160 mM KOH, 10 mM Hepes, 150 mM methanesulfonic acid (Mes), and 2 mM $Ca(Mes)_2$, adjusted to pH 7.4 with Mes) and 10 μM DES in 160 K^+ solution to record the basal and DES-induced KSper currents. Data were analyzed with Clampfit version 10.4 software (Axon, Gilze, the Netherlands).

Penetration of artificial viscous media

The examination of human sperm penetration into viscous media (1% methylcellulose) was performed as previously described (Alasmari *et al.*, 2013). The methylcellulose was prepared using HTF/HSA medium and introduced into 7.5-cm flattened capillary tubes with 1.0-mm inner depth (Elite Medical Co., Ltd., Nanjing, China). Before penetration, human spermatozoa were capacitated in HTF/HSA medium containing different concentrations of DES for 4 h. To determine the effect of DES on progesterone-induced sperm penetration ability, the spermatozoa were capacitated in HTF/HSA medium for 4 h and capacitated spermatozoa were pretreated with DES for 30 min before 10 μM progesterone was added. Then, the open ends of the capillary tubes were inserted. Following 1 h of incubation, the tubes were removed, wiped and observed using a CASA system. Three fields at 1 and 2 cm from the base of the tube were counted, and the average number of cells per field was calculated. The cell numbers were normalized to values from parallel, untreated controls.

Evaluation of the acrosome reaction

The acrosome reaction of human spermatozoa was detected by CTC staining as previously described (DasGupta *et al.*, 1993). To determine the effect of DES on the spontaneous acrosome reaction, human spermatozoa were treated with different concentrations of DES in HTF/HSA medium for 4 h. To evaluate the effect of DES on progesterone-induced acrosome reaction, human spermatozoa were first capacitated in HTF/HSA medium for 4 h, then pretreated with DES for 30 min before 20 μM progesterone was added, and incubated an additional 1 h. After CTC staining, human spermatozoa were imaged with a Leica DM2500 Upright Fluorescence Microscope (Leica Microsystems, Wetzlar, Germany). The absence of fluorescence from the head is characteristic of acrosome-reacted sperm. A total of 200 spermatozoa were counted to evaluate sperm acrosome reaction.

Tyrosine phosphorylation analysis

To examine DES effects on the level of tyrosine phosphorylation, human spermatozoa were treated with different concentrations of DES in HTF/HSA medium for 4 h. To examine DES effects on progesterone-induced tyrosine phosphorylation, human spermatozoa were first capacitated in HTF/HSA medium for 4 h, then pretreated with DES for 30 min before 20 μM progesterone was added, and incubated an additional 1 h. Tyrosine phosphorylation was examined by western blotting using anti-phosphotyrosine antibody according to Luo *et al.* (2015).

Statistical analysis

Data are expressed as the mean ± SEM. Differences between the controls and the treated samples were assessed using one-way ANOVA. Statistically significant differences were determined at $P < 0.05$ using the statistical software GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA, USA).

Results

DES increases human sperm $[Ca^{2+}]_i$ via extracellular Ca^{2+} influx

DES had no effect on viability and motility parameters including total motility, progressive motility, VCL and VSL in both the non-capacitating stage (in HS for 1 h) and capacitating stage (in HTF/HSA for 4 h, Supplementary Table SI). However, it caused a transient increase in sperm $[Ca^{2+}]_i$ in a dose-dependent manner, followed by a gradual reduction in $[Ca^{2+}]_i$ to near resting levels (Fig. 1A). According to the statistical analysis, DES significantly increased sperm $[Ca^{2+}]_i$ even at environmentally relevant concentrations (100 pM) (Fig. 1B). The elevated $[Ca^{2+}]_i$ was intensified with increased DES concentrations up to 100 μM, at ~80% of control values (Fig. 1B).

To determine whether the DES-induced increase in $[Ca^{2+}]_i$ was due to Ca^{2+} influx or the mobilization of Ca^{2+} stores, we measured $[Ca^{2+}]_i$ of spermatozoa exposed to a Ca^{2+}-free medium containing DES and the Ca^{2+} chelator BAPTA as previously described (Strünker *et al.*, 2011). In this condition, a well-defined extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is rapidly adjusted, which prevents depletion of intracellular Ca^{2+} stores before DES stimulation. Sperm $[Ca^{2+}]_i$ was decreased upon $[Ca^{2+}]_o$ rapidly adjusted to <50 nM (BAPTA, Fig. 1C and D) and 100 μM DES did not cause an increase of sperm $[Ca^{2+}]_i$ at this $[Ca^{2+}]_o$ (DES + BAPTA, Fig. 1C and D). These results indicate that DES-induced human sperm $[Ca^{2+}]_i$ is due to extracellular Ca^{2+} influx.

DES enhances human sperm CatSper currents

In this study, we found that DES increases human sperm $[Ca^{2+}]_i$ via extracellular Ca^{2+} influx (Fig. 1). In human spermatozoa, CatSper is the predominant Ca^{2+} channel responsible for Ca^{2+} influx (Hildebrand *et al.*, 2010; Williams *et al.*, 2015). Therefore, it is reasonable to determine whether CatSper is involved in the DES-induced intracellular Ca^{2+} response. Thirty μM mibefradil, a CatSper blocker, significantly impaired the DES-induced Ca^{2+} response ($P < 0.001$, Supplementary Fig. S1A and B) although mibefradil itself could cause a slight increase in $[Ca^{2+}]_i$ (Supplementary Fig. S1A and B) as described by Strünker *et al.* (2011). These results imply that CatSper may be involved in DES-induced increase in sperm $[Ca^{2+}]_i$. To confirm this, the whole-cell patch-clamp technique was applied to examine the effect of DES on CatSper currents. The results showed that 10 μM DES increased both outward and inward monovalent CatSper currents (Fig. 2A). Statistical analysis of mean currents at +100 and −100 mV showed that DES increased absolute value of currents from 95.5 to 143.1 pA ($P < 0.05$) and 44.5 to 70.5 pA ($P < 0.05$), respectively (Fig. 2B). Additionally, it was reported that the driving force for Ca^{2+} entry via CatSper can be increased by

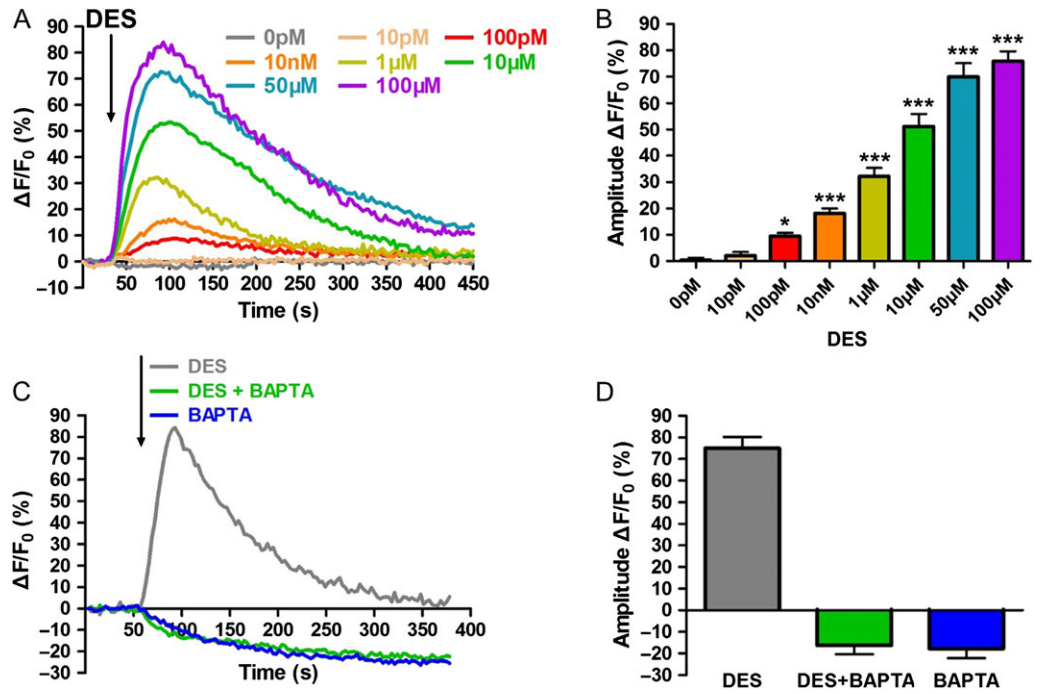

Figure 1 Human sperm intracellular Ca^{2+} levels during DES exposure. (**A**) The fluorescence time-course traces representing intracellular Ca^{2+} changes after exposure to different concentrations of DES. Arrows indicate the exact time points of DES (0–100 μM) addition into sperm samples. (**B**) Magnitudes of Ca^{2+} response caused by DES were calculated as the amplitude $\Delta F/F_0$ from time-course traces by statistical analysis. (**C**) Effect of 100 μM DES on $[Ca^{2+}]_i$ in Ca^{2+}-free medium. Sperm $[Ca^{2+}]_i$ was rapidly examined by mixing of sperm suspension (in HS solution containing 2 mM $[Ca^{2+}]_o$) 1:1 (v/v) with a Ca^{2+}-free HS solution containing 12 mM BAPTA (BAPTA) or 12 mM BAPTA and 200 μM DES (DES + BAPTA) or 200 μM DES (DES). (**D**) Statistical analysis of the amplitude of the Ca^{2+} changes from (C) time-course traces. Bar: mean ± SEM. *$P < 0.05$ and ***$P < 0.001$, $n = 5$. DES, diethylstilbestrol; HS, high saline; BAPTA, 1,2-bis (o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

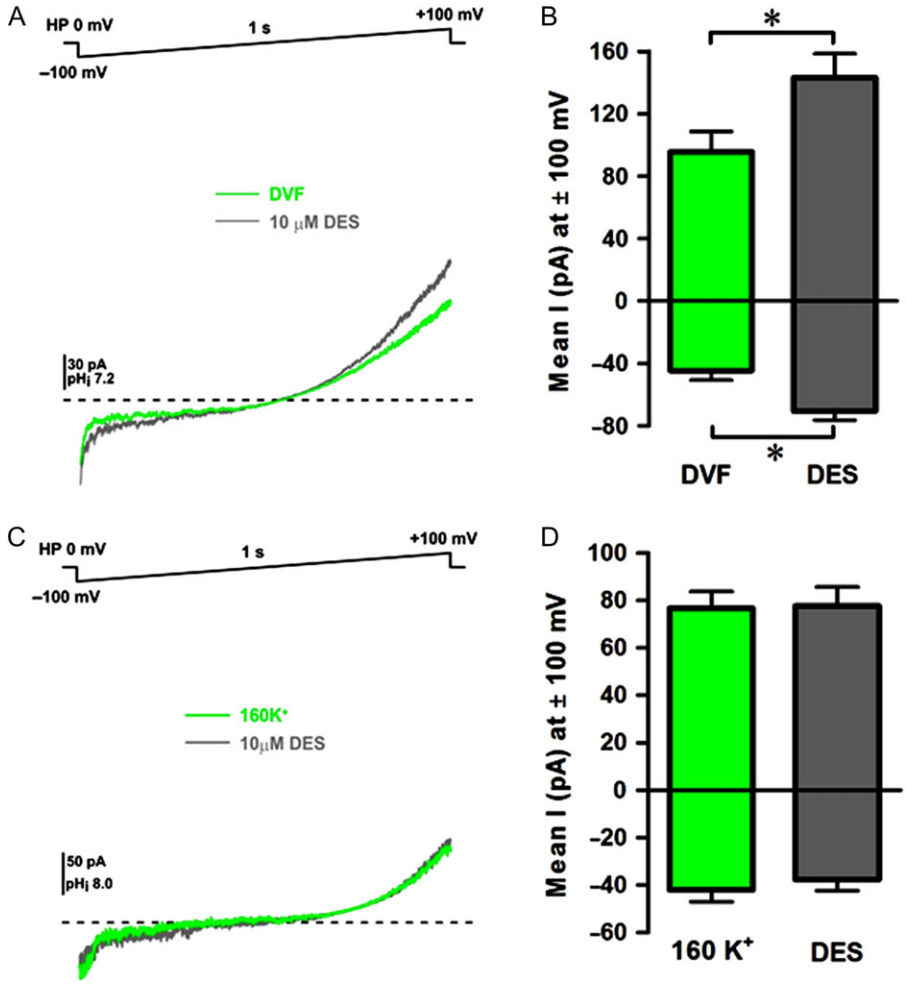

Figure 2 DES increases human sperm CatSper current but not KSper current. The effects of DES on human sperm CatSper (**A**) and KSper (**C**) currents were examined by voltage-clamp ramp protocol from −100 to +100 mV as described in Materials and Methods. DVF and high-K^+ HS (160 K^+) solutions were used to record baseline monovalent CatSper and KSper currents. Then, 10 μM DES diluted in DVF and 160 K^+ was perfused to record DES-induced CatSper and KSper currents. Statistical analysis of the mean CatSper (**B**) and KSper (**D**) currents at +100 mV (positive) and −100 mV (negative). Bar: mean ± SEM. *$P < 0.05$, $n = 5$. DVF, divalent free.

hyperpolarization when activation of KSper, the predominant K^+ channel in mammalian spermatozoa (Navarro *et al.*, 2007). Meanwhile, the mibefradil, which we used to inhibit CatSper, also blocks KSper current (Mansell *et al.*, 2014). Therefore, we further examined the effect of DES on KSper and found that 10 μM DES did not change KSper current (Fig. 2C and D) although it significantly increase CatSper current ($P < 0.05$, Fig. 2B). These results indicate that DES increases human sperm $[Ca^{2+}]_i$ mainly by activating the CatSper channel.

DES affects progesterone-stimulated Ca^{2+} influx

It is well known that progesterone increases human sperm $[Ca^{2+}]_i$ through activating CatSper (Lishko *et al.*, 2011; Strünker *et al.*, 2011; Miller *et al.*, 2016). In this study, we found that DES increased human sperm $[Ca^{2+}]_i$ in a similar manner to progesterone (Figs 1 and 2). Thus, we further examined whether DES affects progesterone-induced Ca^{2+} signaling. Human spermatozoa were pretreated with different concentrations of DES for 30 min, which caused a transient increase in $[Ca^{2+}]_i$ in a dose-dependent manner, followed by a gradual reduction in $[Ca^{2+}]_i$ to near resting levels (Fig. 3A). Subsequently, 1 μM progesterone was added and the results showed that progesterone-induced $[Ca^{2+}]_i$ rise was inhibited by DES in a dose-dependent manner (Fig. 3A and B). Furthermore, we compared the amplitude of $[Ca^{2+}]_i$ induced by DES and progesterone alone or combined. The results showed that the 100 μM DES- and 1 μM progesterone-induced Ca^{2+} responses were 74.2% and 94.2%, respectively (Fig. 3C and D). The Ca^{2+} response induced by the combination of DES and progesterone was 99.0% (Fig. 3C and D), obviously lower than the sum of the separate inductions of the two chemicals, suggesting that DES and progesterone may utilize identical pathways to increase human sperm $[Ca^{2+}]_i$. Whole-cell patch-clamp recordings also support this, as both outward and inward monovalent CatSper currents stimulated by DES together with progesterone

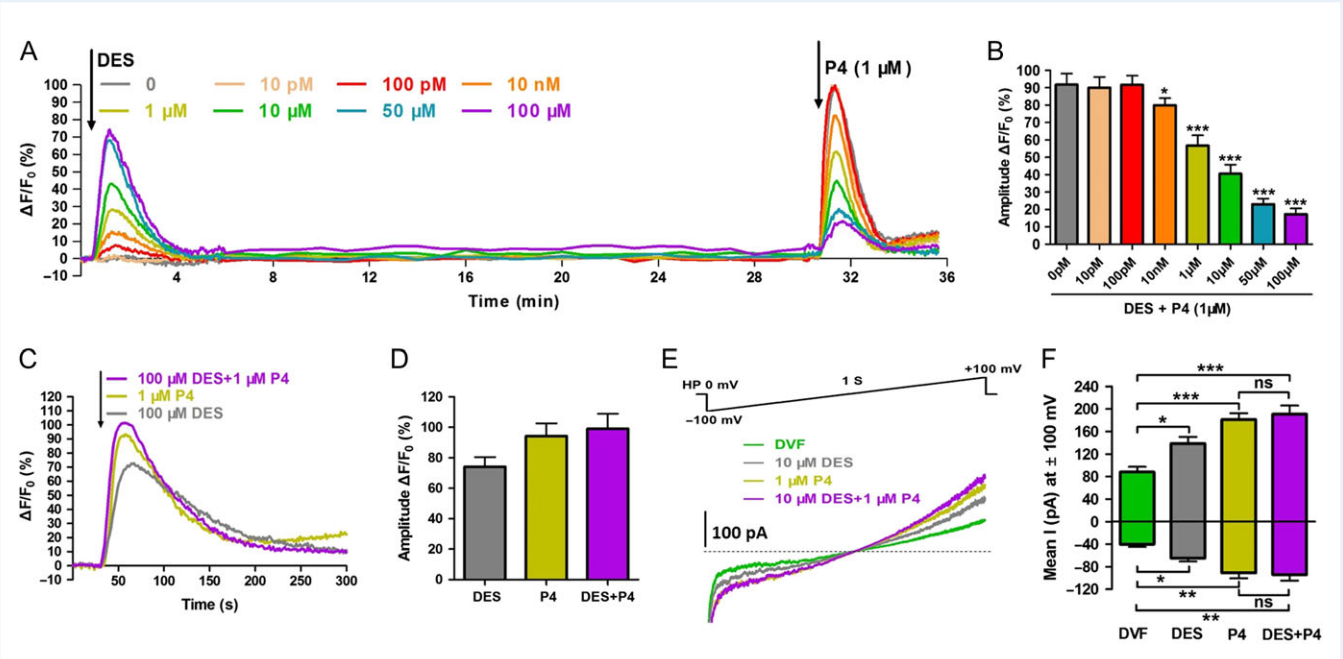

Figure 3 The effect of DES on progesterone-stimulated Ca^{2+} influx. (**A**) The time-course traces representing intracellular Ca^{2+} changes stimulated by 1 μM P4 (progesterone) after human sperm were pretreated for 30 min with different concentrations of DES. (**B**) Statistical analysis of the amplitude of the Ca^{2+} changes stimulated by 1 μM P4 from A. The amplitude $\Delta F/F_0$ (%) = maximum $\Delta F/F_0$ (%) stimulated by 1 μM P4–$\Delta F/F_0$ (%) at the point of adding P4. (**C**) The time-course traces representing intracellular Ca^{2+} changes stimulated by 100 μM DES, 1 μM P4 and 100 μM DES together with 1 μM P4. (**D**) Statistical analysis of the amplitude of the Ca^{2+} changes from (C). (**E**) CatSper currents activated by 100 μM DES, 1 μM P4 and 100 μM DES together with 1 μM P4. (**F**) Statistical analysis of the mean CatSper currents at +100 mV (positive) and −100 mV (negative). Bar: mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$, ns (not significant), $n = 5$.

were obviously lower than the sum of the separate activations caused by the two chemicals (Fig. 3E and F).

DES inhibits progesterone-induced human sperm functions

Progesterone activation of CatSper to increase $[Ca^{2+}]_i$ has been proposed to control hyperactivation and the acrosome reaction. Thus, we next examined whether DES affects progesterone-induced human sperm functions. The ability of human spermatozoa to penetrate into artificial viscous media, which mimics the viscous environment in the female reproductive tract, is a comprehensive evaluation of sperm motility, especially hyperactivation (Alasmari *et al.*, 2013). We found that 10 μM progesterone increased the mean cell numbers both at 1 and 2 cm from the base of the tube compared to the controls (Fig. 4A and B). This action can be significantly inhibited by 1–100 μM DES (Fig. 4A and B). Moreover, DES (1–100 μM) can also inhibit 20 μM progesterone-induced acrosome reaction (Fig. 4D). In contrast, DES itself had no effect on sperm penetration through viscous media and the spontaneous acrosome reaction (Fig. 4A–C).

DES lowers progesterone-induced tyrosine phosphorylation in human spermatozoa

While DES increases $[Ca^{2+}]_i$ through the activation of CatSper (Figs 1 and 2), unlike progesterone it did not affect human sperm functions including motility, penetration ability and the spontaneous acrosome reaction (Fig. 4A–C). We hypothesized that DES and progesterone may affect human sperm functions through different intracellular signaling pathways, although they may utilize similar pathways to increase human sperm $[Ca^{2+}]_i$. In mammalian spermatozoa, intracellular tyrosine phosphorylation is a universal modification pattern and serves as an important regulator of sperm function (Urner and Sakkas, 2003; Naz and Rajesh, 2004). Previous studies have reported that progesterone can increase the level of tyrosine phosphorylation in human spermatozoa (Sagare-Patil *et al.*, 2012). Therefore, we tested the effect of DES on human sperm intracellular protein tyrosine phosphorylation. We found that DES did not affect the level of tyrosine phosphorylation (Fig. 5A and B). However, 1–100 μM DES dose dependently inhibited progesterone-induced tyrosine phosphorylation (Fig. 5C and D).

Discussion

Although the long-term reproductive toxicities of DES are notorious, the *in vitro* effect of DES on mammalian spermatozoa remains unknown. In this study, we found that DES significantly disturbed progesterone-induced Ca^{2+} signaling, tyrosine phosphorylation, ability to penetrate into viscous media and the acrosome reaction in human spermatozoa. These results suggest that DES may affect male reproduction once it accumulates in the environment of spermatozoa, such as the seminal plasma and female reproductive tract fluids. Our

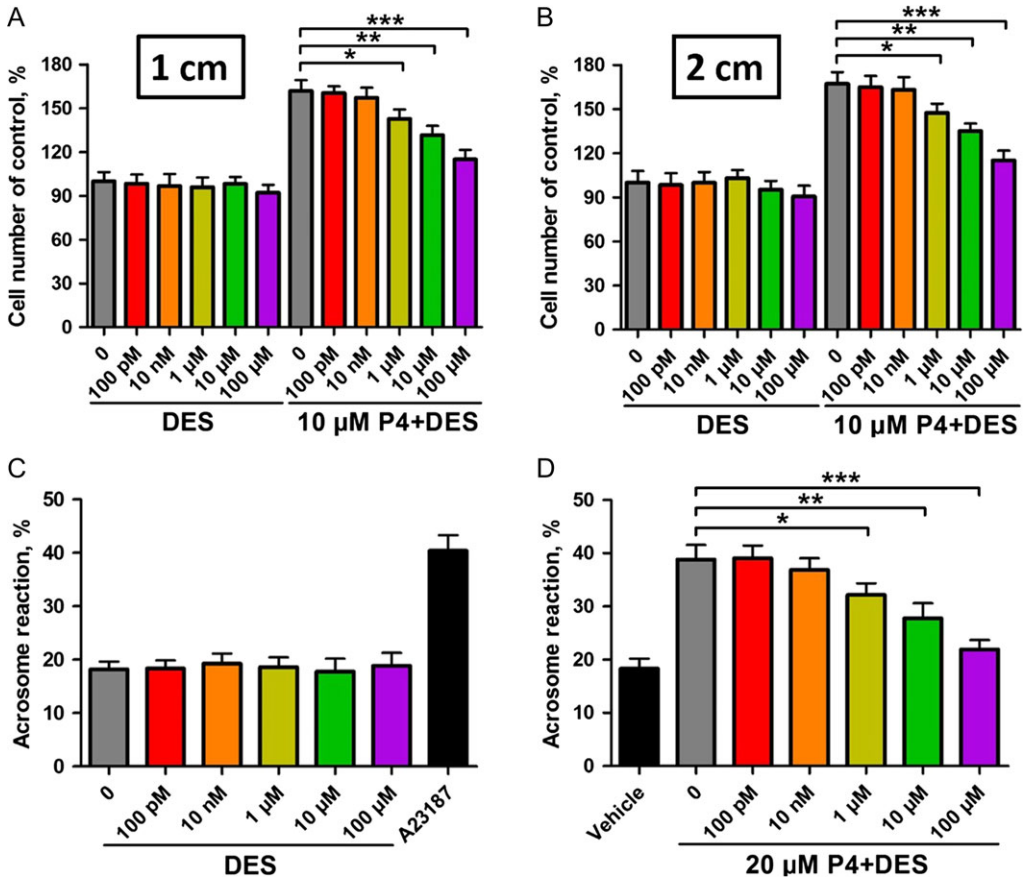

Figure 4 DES inhibited progesterone-induced sperm penetration into viscous media and acrosome reaction. (**A**) and (**B**) The effect of DES on human sperm penetration into viscous media and 10 μM progesterone-induced penetration ability. Sperm cell numbers at 1 and 2 cm from the base of the tube were calculated and normalized to values of untreated controls. (**C**) The effect of DES on the spontaneous acrosome reaction. Human sperm were treated with different concentrations of DES in HTF/HSA medium for 4 h; 10 μM A23187 (a calcium ionophore) was used as a positive control. (**D**) The effect of DES on progesterone-induced acrosome reaction. Human sperm were first capacitated in HTF/HSA for 4 h, then treated with different concentrations of DES for 30 min and 20 μM progesterone was added and incubated an additional 1 h. The vehicle (0.1% dimethylsulfoxide) acted as a negative control. Bar: mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, $n = 8$. HTF, human tubal fluid; HSA, human serum albumin.

findings demonstrate how DES may increase the risk of infertility in those who have had prolonged exposure to DES-polluted areas or accept DES therapies in the clinic.

The inhibitory effects of DES on progesterone-induced human sperm functions were similar to 17β-estradiol which was reported to suppress progesterone-induced sperm hyperactivation and acrosome reaction (Baldi *et al.*, 2000; Fujinoki, 2010). These phenomena indicate that DES may play an estrogen-like role in inhibition of progesterone actions. Herein, the present study further explored the mechanism underlying the inhibitory effect of DES. The results demonstrated that DES might suppress progesterone-induced human sperm functions by disturbance of progesterone-stimulated Ca^{2+} signaling and tyrosine phosphorylation. Therefore, once DES accumulates in the environment of spermatozoa, such as the seminal plasma and female reproductive tract fluids, it can contact human spermatozoa prior to progesterone and occupy the pathway(s) by which progesterone increases human sperm $[Ca^{2+}]_i$, preventing the progesterone-induced increase in $[Ca^{2+}]_i$. Additionally, our data showed that DES did not stimulate tyrosine phosphorylation in human spermatozoa. Thus, when DES contacts human spermatozoa prior to progesterone, progesterone-induced tyrosine phosphorylation is suppressed. Hence, DES may inhibit progesterone actions in physiological conditions in these ways and thereby compromise human sperm fertility.

In this study, we found that DES induced a transient $[Ca^{2+}]_i$ rise in human spermatozoa by activating the CatSper channel. This rapid non-genomic action of EDCs exposure is regarded to be the underlying mechanism to influence human sperm functions (Tavares *et al.*, 2013; Schiffer *et al.*, 2014). However, our results showed that DES itself did not affect sperm motility, penetration into viscous media and the spontaneous acrosome reaction. These results were apparently not consistent with the previous report that activation of CatSper by several EDCs and progesterone promotes human sperm hyperactivation and acrosomal exocytosis (Lishko *et al.*, 2011; Strünker *et al.*, 2011; Schiffer *et al.*, 2014). Interestingly, other cases like DES effects have been also reported previously. For example, bourgeonal, an odorant, did not affect spontaneous acrosome reaction although it activated CatSper (Spehr *et al.*,

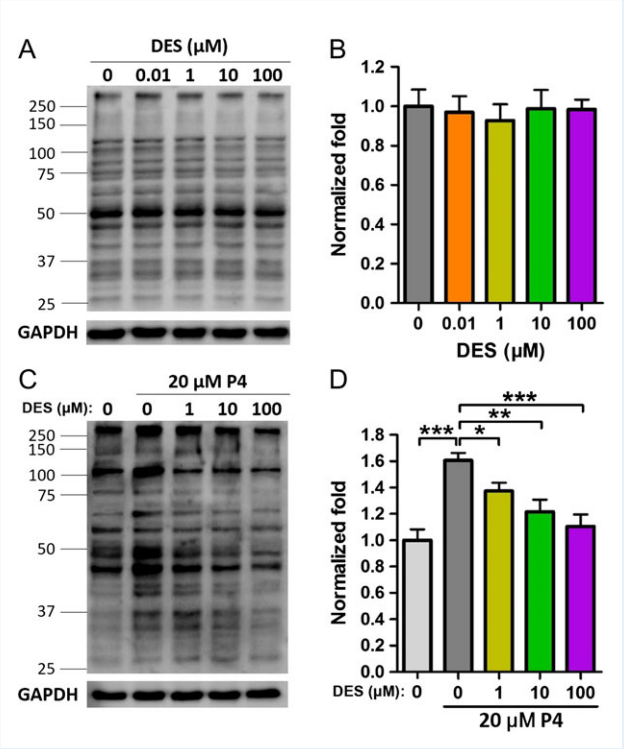

Figure 5 The effect of DES on human sperm intracellular protein phosphorylation. (**A**) DES effects on intracellular protein tyrosine phosphorylation (P-Tyr). Human sperm were treated with 10 nM–100 μM DES for 4 h in HTF/HSA medium, and then sperm proteins were isolated. (**C**) DES effects on progesterone-induced tyrosine phosphorylation. Human sperm were first capacitated in HTF/HSA medium for 4 h, then the capacitated sperm were pretreated with 1–100 μM DES for 30 min and 20 μM progesterone was added for an additional 1 h incubation. The tyrosine phosphorylation was measured by western blot using anti-phosphotyrosine 4G10 (1:1000 dilution) and anti-GAPDH (1:20 000 dilution, loading control) primary antibodies. (**B**) and (**D**) Statistical analysis of the level of tyrosine phosphorylation by quantifying with the total intensities of the bands normalized to the loading control from (A) and (C) using Image J software (version 1.44, National Institutes of Health, download page: https://imagej.nih.gov/ij/download.html), respectively. Bar: mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, $n = 5$.

2004). The *p*, *p*′-dichlorodiphenyldichloroethylene, an environmental endocrine disruptor, had no effect on spontaneous acrosome reaction within 1 day incubation but it activates CatSper in a few minutes (Tavares *et al.*, 2013). These results implicate that activation of CatSper by some chemicals does not influence human sperm function. Although Ca^{2+} signaling mediated by CatSper plays a vital role in regulation of human sperm functions, the downstream intracellular signaling pathways dependent/independent Ca^{2+} signaling mediated by CatSper are also involved (Torres-Flores *et al.*, 2008; Fujinoki, 2013; Sagare-Patil *et al.*, 2013). Previous studies showed that tyrosine phosphorylation is an important event in modulating human sperm functions (Urner and Sakkas, 2003; Naz and Rajesh, 2004). Interestingly, we found that progesterone can enhance tyrosine phosphorylation in human spermatozoa but DES cannot activate this process. These results imply that progesterone regulates sperm functions by tyrosine phosphorylation of related proteins in addition to activating CatSper. However, DES cannot affect sperm functions when only activating CatSper. Although both DES and progesterone increase $[Ca^{2+}]_i$ by activating CatSper, only progesterone can stimulate the downstream signaling pathways to modulate tyrosine phosphorylation of sperm function-related proteins. These theories may be the possible explanations for the different effects between DES and progesterone on human sperm function.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We thank Prof. Xu-hui Zeng (Institute of Life Science and School of Life Science, Nanchang University) for sperm patch-clamp recordings. We thank Na Li and Xiang Kang (Institute of Life Science and School of Life Science, Nanchang University) for help with sperm functional analysis.

Authors' roles

Q.Z., Z.P. and Q.Z. performed sperm $[Ca^{2+}]_i$ measurement and western blot assay. H.C. collected the semen samples and performed the semen analysis. Y.C., H.W. and T.W. performed sperm patch-clamp recordings and analyzed the detailed data. Z.P., H.Q., Q.L. and S.W. were involved in penetration of artificial viscous media assay and evaluation of the acrosome reaction. Q.Z., L.Z. and T.L. collected the data and performed the statistical analysis and were responsible for manuscript preparation. T.L. designed and coordinated the study and wrote the manuscript. All the authors made substantial contributions in critically revising the manuscript. All the authors approved the final manuscript for submission.

Funding

National Natural Science Foundation of China (No. 31400996) to T. Luo; Natural Science Foundation of Jiangxi, China (No. 20161BAB204167 and No. 20142BAB215050) to T. Luo; the open project of National Population and Family Planning Key Laboratory of Contraceptives and Devices Research (No. 2016KF07) to T. Luo; National Natural Science Foundation of China (No. 81300539) to L.P. Zheng.

Conflict of interest

None declared.

References

Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, Ramalho-Santos J, Kirkman-Brown J, Michelangeli F, Publicover S *et al.* Ca^{2+} signals generated by CatSper and Ca^{2+} stores regulate different behaviors in human sperm. *J Biol Chem* 2013;**288**:6248–6258.

Auger J, Eustache F, Andersen AG, Irvine DS, Jorgensen N, Skakkebaek NE, Suominen J, Toppari J, Vierula M, Jouannet P. Sperm morphological defects related to environment, lifestyle and medical history of 1001

male partners of pregnant women from four European cities. *Hum Reprod* 2001;**16**:2710–2717.

Baldi E, Luconi M, Muratori M, Forti G. A novel functional estrogen receptor on human sperm membrane interferes with progesterone effects. *Mol Cell Endocrinol* 2000;**161**:31–35.

Bosset PO, Albiges L, Seisen T, de la Motte Rouge T, Phe V, Bitker MO, Roupret M. Current role of diethylstilbestrol in the management of advanced prostate cancer. *BJU Int* 2012;**110**:E826–829.

DasGupta S, Mills CL, Fraser LR. Ca^{2+}-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. *J Reprod Fertil* 1993;**99**:135–143.

Daston GP, Gooch JW, Breslin WJ, Shuey DL, Nikiforov AI, Fico TA, Gorsuch JW. Environmental estrogens and reproductive health: a discussion of the human and environmental data. *Reprod Toxicol* 1997;**11**:465–481.

Fielden MR, Halgren RG, Fong CJ, Staub C, Johnson L, Chou K, Zacharewski TR. Gestational and lactational exposure of male mice to diethylstilbestrol causes long-term effects on the testis, sperm fertilizing ability in vitro, and testicular gene expression. *Endocrinology* 2002;**143**:3044–3059.

Fujinoki M. Suppression of progesterone-enhanced hyperactivation in hamster spermatozoa by estrogen. *Reproduction* 2010;**140**:453–464.

Fujinoki M. Progesterone-enhanced sperm hyperactivation through IP3-PKC and PKA signals. *Reprod Med Biol* 2013;**12**:27–33.

Goyal HO, Braden TD, Mansour M, Williams CS, Kamaleldin A, Srivastava KK. Diethylstilbestrol-treated adult rats with altered epididymal sperm numbers and sperm motility parameters, but without alterations in sperm production and sperm morphology. *Biol Reprod* 2001;**64**:927–934.

Hildebrand MS, Avenarius MR, Fellous M, Zhang Y, Meyer NC, Auer J, Serres C, Kahrizi K, Najmabadi H, Beckmann JS *et al*. Genetic male infertility and mutation of CATSPER ion channels. *Eur J Hum Genet* 2010;**18**:1178–1184.

Hoover RN, Hyer M, Pfeiffer RM, Adam E, Bond B, Cheville AL, Colton T, Hartge P, Hatch EE, Herbst AL *et al*. Adverse health outcomes in women exposed in utero to diethylstilbestrol. *N Engl J Med* 2011;**365**:1304–1314.

Kemp HA, Read GF, Riad-Fahmy D, Pike AW, Gaskell SJ, Queen K, Harper ME, Griffiths K. Measurement of diethylstilbestrol in plasma from patients with cancer of the prostate. *Cancer Res* 1981;**41**:4693–4697.

Klip H, Verloop J, van Gool JD, Koster ME, Burger CW, van Leeuwen FE, Group OP. Hypospadias in sons of women exposed to diethylstilbestrol in utero: a cohort study. *Lancet* 2002;**359**:1102–1107.

Lei B, Peng W, Li W, Yu Y, Xu J, Wang Y. Diethylstilbestrol at environmental levels affects the development of early life stage and target gene expression in Japanese Medaka (*Oryzias latipes*). *Ecotoxicology* 2016;**25**:563–573.

Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature* 2011;**471**:387–391.

Luo T, Li N, He YQ, Weng SQ, Wang T, Zou QX, Zeng XH. Emodin inhibits human sperm functions by reducing sperm $[Ca^{2+}]_i$ and tyrosine phosphorylation. *Reprod Toxicol* 2015;**51**:14–21.

Ma A, Yang X, Wang Z, Shi D, Chen Y. Adult exposure to diethylstilbestrol induces spermatogenic cell apoptosis in vivo through increased oxidative stress in male hamster. *Reprod Toxicol* 2008;**25**:367–373.

Mansell SA, Publicover SJ, Barratt CL, Wilson SM. Patch clamp studies of human sperm under physiological ionic conditions reveal three functionally and pharmacologically distinct cation channels. *Mol Hum Reprod* 2014;**20**:392–408.

Miller MR, Mannowetz N, Iavarone AT, Safavi R, Gracheva EO, Smith JF, Hill RZ, Bautista DM, Kirichok Y, Lishko PV. Unconventional endocannabinoid signaling governs sperm activation via the sex hormone progesterone. *Science* 2016;**352**:555–559.

Navarro B, Kirichok Y, Clapham DE. KSper, a pH-sensitive K+ current that controls sperm membrane potential. *Proc Natl Acad Sci U S A* 2007;**104**:7688–7692.

Naz RK, Rajesh PB. Role of tyrosine phosphorylation in sperm capacitation/ acrosome reaction. *Reprod Biol Endocr* 2004;**2**:75.

Newbold RR. Prenatal exposure to diethylstilbestrol (DES). *Fertil Steril* 2008;**89**:e55–56.

Patisaul HB, Adewale HB. Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior. *Front Behav Neurosci* 2009;**3**:10.

Sagare-Patil V, Galvankar M, Satiya M, Bhandari B, Gupta SK, Modi D. Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa. *Int J Androl* 2012;**35**:633–644.

Sagare-Patil V, Vernekar M, Galvankar M, Modi D. Progesterone utilizes the PI3K-AKT pathway in human spermatozoa to regulate motility and hyperactivation but not acrosome reaction. *Mol Cell Endocrinol* 2013;**374**:82–91.

Schiffer C, Muller A, Egeberg DL, Alvarez L, Brenker C, Rehfeld A, Frederiksen H, Waschle B, Kaupp UB, Balbach M *et al*. Direct action of endocrine disrupting chemicals on human sperm. *EMBO Rep* 2014;**15**:758–765.

Spehr M, Schwane K, Riffell JA, Barbour J, Zimmer RK, Neuhaus EM, Hatt H. Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. *J Biol Chem* 2004;**279**:40194–40203.

Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced Ca^{2+} influx in human sperm. *Nature* 2011;**471**:382–386.

Tavares RS, Escada-Rebelo S, Correia M, Mota PC, Ramalho-Santos J. The non-genomic effects of endocrine-disrupting chemicals on mammalian sperm. *Reproduction* 2016;**151**:1–13.

Tavares RS, Mansell S, Barratt CL, 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**:3167–3177.

Torres-Flores V, Hernandez-Rueda YL, Neri-Vidaurri PC, Jimenez-Trejo F, Calderon-Salinas V, Molina-Guarneros JA, Gonzalez-Martinez MT. Activation of protein kinase A stimulates the progesterone-induced calcium influx in human sperm exposed to the phosphodiesterase inhibitor papaverine. *J Androl* 2008;**29**:549–557.

Urner F, Sakkas D. Protein phosphorylation in mammalian spermatozoa. *Reproduction* 2003;**125**:17–26.

Visser JA, McLuskey A, Verhoef-Post M, Kramer P, Grootegoed JA, Themmen AP. Effect of prenatal exposure to diethylstilbestrol on Mullerian duct development in fetal male mice. *Endocrinology* 1998;**139**:4244–4251.

Whitehead ED, Leiter E. Genital abnormalities and abnormal semen analyses in male patients exposed to diethylstilbestrol in utero. *J Urol* 1981;**125**:47–50.

Wilcox AJ, Baird DD, Weinberg CR, Hornsby PP, Herbst AL. Fertility in men exposed prenatally to diethylstilbestrol. *N Engl J Med* 1995;**332**:1411–1416.

Williams HL, Mansell S, Alasmari W, Brown SG, Wilson SM, Sutton KA, Miller MR, Lishko PV, Barratt CL, Publicover SJ *et al*. Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. *Hum Reprod* 2015;**30**:2737–2746.

Zeng XH, Yang C, Kim ST, Lingle CJ, Xia XM. Deletion of the Slo3 gene abolishes alkalization-activated K+ current in mouse spermatozoa. *Proc Natl Acad Sci U S A* 2011;**108**:5879–5884.