REPRODUCTION

RESEARCH

Acute effects on human sperm exposed *in vitro* to cadmium chloride and diisobutyl phthalate

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Abstract

Epidemiological studies reported a negative relationship between concentrations of heavy metals and phthalates in seminal fluid and semen quality, likely compromising male fertility potential. The aim of this study was to investigate the *in vitro* effects of cadmium chloride ($CdCl_2$), a common heavy metal, and diisobutyl phthalate (DIBP), a common phthalate ester, on human sperm functions necessary for fertilization. After *in vitro* incubation of spermatozoa with 10 µM $CdCl_2$ or 100 and 200 µM DIBP for 24 h, a significant decrease of sperm progressive and hyperactivated motility was observed. The exposure to each of the two toxic agents also induced spontaneous sperm acrosome reaction and blunted the physiological response to progesterone. Both agents induced an increase of caspase activity suggesting triggering of an apoptotic pathway. Our results suggest that acute exposure of spermatozoa to these pollutants may impair sperm ability to reach and fertilize the oocyte.

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Introduction

Biomonitoring studies indicate that the levels of environmental contaminants rose substantially in last 3–4 decades exposing the population to toxicity of such compounds, which tend to accumulate in the organism and to induce multiple organ alterations. These exogenous agents are represented by a heterogeneous group of substances, normally present in the environment (air, water, soil etc.) but also in mass-consumer products and food, which can behave as endocrine disruptors interfering with normal hormonal activity. Among the endocrine disruptors, heavy metals, bisphenol A, phthalates, parabens and pesticides can be found in biological fluids at various concentrations. Epidemiological studies demonstrated that such chemicals can impact on human health disrupting the normal function of the endocrine system, and subsequently, produce disorders at developmental, neurological, cardiovascular, metabolic, immune and also reproductive level (Wang *et al.* 2016*a*, 2019). Indeed, environmental and occupational exposure of the population could be one of the risk factors of the increased incidence of male infertility and the decline of human semen quality seen in the last decades (Levine *et al.* 2017, Mínguez-Alarcón *et al.* 2018). In general, infertility affects 15% of the couples and the male factor contributes for about 50% of them. Besides couple infertility, a decline of male reproductive health could have wider public health implications, as the deterioration of the semen quality is associated to increased morbidity and mortality (Jensen *et al.* 2009, Eisenberg *et al.* 2014, 2016, Latif *et al.* 2017).

Recent epidemiological evidence reported increased risk of reproductive disorders following prenatal and postnatal persistent exposure to environmental chemicals (Bonde *et al.* 2016, Wang *et al.* 2016*a*, Sifakis *et al.* 2017). For example, alterations of semen parameters have been reported following exposure to phthalates, commonly used as plasticizers (Radke *et al.* 2018) and in subjects with high semen concentrations of heavy metals, widely used in industry (Sun *et al.* 2017). In addition, high levels of heavy metals in follicular fluid and high concentrations of phthalate metabolites in female urine decrease assisted reproduction outcomes (Wdowiak *et al.* 2017, Al-Saleh *et al.* 2019).

Data from animal and epidemiological studies on the effects of toxicants on male reproductive health are still insufficient and conflicting and the few experimental studies on human beings do not allow establishing definite evidence (Tavares *et al.* 2016, de Angelis *et al.* 2017). Pant *et al.* (2014) found that seminal levels of cadmium and phthalate esters are negatively associated with sperm motility and concentration and positively

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related with DNA damage in non-occupationally exposed subjects. Also, in animal models, reduced plasma and testis testosterone levels, steroidogenic enzyme expression and semen quality, have been shown after exposure of adult male mice to diisobutyl phthalate (Pan *et al.* 2017) as well as testis toxicity and morphological and functional alterations of spermatozoa following cadmium administration in rats (Medina *et al.* 2017). However, considering that spermatozoa must 'travel' in the female genital tract where they undergo selection as well as profound modifications of motility (development of hyperactivated motility necessary to penetrate oocyte vestments) and acrosome reaction in response to physiological stimuli in order to fertilize the oocyte (Stival *et al.* 2016), it is crucial also to understand the possible effect of the environmental contaminants on these functions.

The present study was designed to investigate the acute effect of compounds representative of two categories of environmental pollutants on sperm quality and function. In particular, we analyzed the effects of *in vitro* exposure of human spermatozoa to cadmium chloride ($CdCl_2$), one of the most common heavy metal contaminants, present at high levels in batteries, computer components and tobacco smoke, and diisobutyl phthalate (DIBP), a common environmental phthalate ester, contained in plastic goods for food preservation, inks, paints and personal care products.

The effect of the two contaminants on sperm progressive and hyperactivated motility, sperm acrosome reaction and sperm intracellular calcium levels, parameters essential for a successful fertilization, were evaluated in swim-up selected spermatozoa in order to mimic the natural selection occurring in the female genital tract.

Materials and methods

Chemicals

Human tubal fluid (HTF) medium and human serum albumin (HSA) were purchased from Biocare Europe (Rome, Italy). Cadmium chloride hydrate ($CdCl_2$), diisobutyl phthalate (DIBP), FITC-labeled *Arachis hypogea* (peanut) lectin and progesterone were obtained from Sigma Aldrich. $CdCl_2$ was dissolved in water to a stock concentration of 100 mM. DIBP original stock solution (3.6 M) was diluted with ethanol to obtain 100 mM working solution. The lectin was diluted 1 mg/mL in phosphate-buffered saline, pH 7.2. Progesterone was dissolved in DMSO to obtain 0.1 M working solution.

Fura 2/AM Cell Permeant and Vybrant FAM Caspase 3 and 7 Assay Kit were purchased from Life Technologies Italia.

Human semen samples

Semen samples were obtained by masturbation from normozoospermic patients undergoing routine semen analysis for couple infertility, in the Andrology Laboratory of the University of Florence, Italy. The study was approved by the local ethical committee (protocol n. 8607/2018, cod. 12186/OSS). Patients were informed about the purpose of the study and a written informed consent to use the semen remaining after routine analysis was obtained. Semen analysis was carried out according to guidelines of the World Health Organization (World Health Organization (WHO) 2010). Semen samples with leukocytes were excluded from the study, after assessment of their presence according to WHO (2010). The Laboratory of Andrology of the Azienda Ospedaliera-Universitaria Careggi of Florence has been participating in the UK-NEQAS (United Kingdom National External Quality Assessment Service) external quality control program for semen analysis since 2005. The mean (±S.D.) percent biases of the laboratory for the years 2018 were 8.9 (±6.6) and 15.9 (±12.8), respectively, for total and progressive motility and 9.1 (±5.3) for sperm concentration ($n = 20$, data from UK-NEQAS). For the study purpose, sperm were selected by direct swim-up procedure (WHO 2010) from 46 subjects (sperm concentration median value: 114.0×10^6/mL (IQR: 68.8–165.5)); progressive motility median value: 58.0% (IQR: 48.8–65.0); total motility median value: 65.0% (IQR: 58.0–71.3)) in order to obtain a sufficient number of selected spermatozoa to perform the experiments. Briefly, 1 mL of HTF −10% HSA was gently layered on an equal volume of semen sample and incubated at 37°C. After 50 min, 800 µL of the upper medium phase, containing the motile fraction of spermatozoa, was collected. In all samples, motility was evaluated and the obtained sperm number was counted according to WHO (2010). Only those samples with a progressive motility >90% were used for further experiments.

Dose and time response experiments

In order to choose the concentrations of $CdCl_2$ and DIBP as well as the time of exposure to use in the following experiments, we tested the effect of increasing concentrations and incubation times of the two toxicants on sperm motility. Swim-up selected spermatozoa (10 millions) were incubated in 1 mL of HTF −10% HSA with $CdCl_2$ at the doses of 2, 5 and 10 µM or with DIBP at the doses of 25, 50, 100 and 200 µM for 1, 3 and 24 h at 37°C, 5% CO_2. $CdCl_2$ concentrations were chosen on the basis of previous studies evaluating cadmium levels in seminal fluid (Pant *et al.* 2013) and its *in vitro* effect on human spermatozoa (Pant *et al.* 2013). DIBP concentrations were chosen on the basis of the levels of phthalates detected in semen (Pant *et al.* 2011) and up to the high doses within the range of phthalates concentrations tested in prior *in vitro* studies (Pant *et al.* 2011, Adir *et al.* 2017*a*,*b*).

For each sample a control aliquot containing HTF −10% HSA and the solvent (water for $CdCl_2$ and ethanol for DIBP) was carried out. After incubation, the percentage of progressive and total sperm motility were checked by phase-contrast microscope (Leica DMLS; Leica, Wetzlar, Germany). As shown in the Supplementary Fig. 1 (see section on supplementary data given at the end of this article), $CdCl_2$ at the concentrations of 2 and 5 µM had no statistically significant effects on sperm motility, even after a long incubation (24 h), whereas 10 µM $CdCl_2$ significantly decreased both progressive and total motility, starting from the first hour of incubation

Table 1 Number of analyzed samples for each sperm parameter.

Number of samples	Sperm parameter
46	Progressive and total motility
33	Computer-assisted sperm analysis
23	Viability
7	Caspase activity
10	Spontaneous and progesterone-induced acrosome reaction
9	Spontaneous and progesterone-induced intracellular calcium levels

(Supplementary Fig. 1A and B). Regarding incubation with DIBP, no effects were observed after both 1 and 3 h of treatment, even at the maximum dose (200 µM). After 24 h, the concentration of 100 µM DIBP induced a notable reduction of progressive motility and a slight decrease of total motility (Supplementary Fig. 1C and D). At the concentration of 200 µM the effect of DIBP on both total and progressive motility was considerably more pronounced (Supplementary Fig. 1C and D).

In light of these results, we decided to perform the subsequent experiments using the doses of 10 µM for $CdCl_2$ and 100 µM and 200 µM for the DIBP and the incubation time of 24 h (the number of analyzed samples for each sperm parameter is reported in Table 1).

Assessment of sperm motility

Sperm motility was measured by evaluating 200 spermatozoa under a phase-contrast microscope (Leica DM LS; Leica) and graded according to WHO (2010) criteria. Sperm kinematic motility parameters were determined on motile spermatozoa by using the CASA system (Computer-Assisted Sperm Analysis, Hamilton Thorn Research, Beverly, MA, USA). The settings used during CASA procedures were: analysis duration of 1 s (30 frames); minimum contrast, 80; minimum size, 3; low size and high size gates, 0.7 and 2.6; low-intensity and high-intensity gates, 0.34 and 1.40 (Luconi *et al.* 2004). Average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity of progression (LIN, %) were recorded. A sort fraction representing the percentage of hyperactivated spermatozoa (HA, %) was identified setting manually the following threshold values: VCL ≥150 µm/s, ALH ≥7 µm and LIN ≤50% (Mortimer *et al.* 1998). A minimum of 200 motile cells and 5 fields were analyzed for each aliquot. All analyses were performed at 37°C.

Assessment of sperm viability

Sperm viability was evaluated by using eosin-nigrosin staining (WHO 2010). Sperm suspension and eosin-nigrosin stain (1:1) were mixed, incubated 30 s at room temperature, and then smeared on the slide. Spermatozoa were evaluated by optical microscopy using 100× oil immersion objective. A total of 200 spermatozoa for each aliquot were evaluated distinguishing between viable (unstained) or dead (stained with eosin) spermatozoa.

Assessment of 3, 7 caspases activity

Caspases activity was evaluated using Vybrant FAM Caspase-3 and -7 Assay Kit based on a fluorescent inhibitor of caspases (FLICA™) (Marchiani *et al.* 2014).

After incubation with the two toxicants (see above), each sample (4×10^6 spermatozoa) was split into two aliquots, one was resuspended in 300 µL of HTF medium and 10 µL of 30× FLICA working solution was added (test sample) and the other was incubated only with HTF medium (negative control). After 1 h of incubation at 37°C, samples were washed with Wash Buffer 1× and fixed with 40 µL of 10% formaldehyde for 10 min at room temperature. Wash and fixative solutions were supplied by the kit. Sperm samples were washed again twice and resuspended in 400 µL of Wash Buffer 1× containing 6 µL of propidium iodide solution (PI, 50 µg/mL in PBS).

Samples were acquired by a FACScan flow cytometer equipped with a 15-mW argon-ion laser for excitation. Green fluorescence of caspases and red fluorescence of PI were revealed by the FL-1 (515–555-nm wavelength band) and FL-2 (563–607-nm wavelength band) detector respectively. Nucleated events in the gate (i.e. the events labeled with PI, $n = 8000$) of the characteristic forward scatter/side scatter region of sperm cells were acquired (Muratori *et al.* 2008). A marker was established in the dot plot of fluorescence distribution of the negative sample, including 99% of total events. This marker was translated in the corresponding test sample and all the events beyond the marker were considered positive for caspase activity. CellQuest-Pro software program (Becton–Dickinson) was used for acquisition and analysis.

Assessment of acrosome reaction

Acrosome reaction was evaluated by fluorescent microscopy as previously reported (Krausz *et al.* 1995, 1996). Briefly, both control and treated samples were divided into two aliquots. An aliquot was incubated with progesterone (10 µM) and the other with 0.1% DMSO (vehicle control), for 1 h at 37°C. Afterward, spermatozoa were washed by centrifugation and resuspended in 500 µL of hypo-osmotic swelling medium in order to evaluate acrosome reaction only in live spermatozoa. After 1 h at 37°C, spermatozoa were washed again and fixed in 50 µL ice-cold methanol. The sperm were layered on a slide, air-dried and stored at −20°C. For acrosome staining, sperm were incubated for 20 min, in the dark, with FITC-labeled *Arachis hypogaea* (peanut) lectin and green fluorescence was observed under an Axiolab A1 FL fluorescence microscope (Carl Zeiss, Jena, Germany), equipped with filter set 49 and an oil immersion 100× magnification objective. For each condition, 200 curled-tail (viable) cells were analyzed for their acrosomal status. For each different experimental conditions, the percentage of acrosome-reacted spermatozoa was reported.

Evaluation of intracellular calcium levels

Intracellular calcium levels were evaluated by a spectrofluorimetric method as previously reported (Luconi *et al.* 1999). Briefly, 10×10^6 spermatozoa were incubated with the Fura2/AM probe for 45 min at 37°C. Then, samples were

washed by centrifugation and incubated in 1 mL FM buffer (125 mM NaCl, 10 mM KCl, 2.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 19 mM sodium lactate, 2.5 mM sodium pyruvate, 20 mM HEPES/NaOH) for 30 min at 37°C. Samples were washed and resuspended again in 1 mL FM buffer. Spermatozoa were stimulated with progesterone (10 µM) which was added directly in the cuvette. $[Ca^{2+}]_i$ was measured by using a spectrofluorimetric method with a Perkin–Elmer LS50B instrument equipped with a fast rotary filter shuttle for alternate 340 and 380 nm excitation. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence with digitonin (0.01% (w/v) final concentration) followed by minimal fluorescence with 10 mM EGTA, pH 10. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* (1985) assuming a dissociation constant of Fura 2 for calcium of 224 nM. Three determinations were made for each sample and the mean value was reported.

Statistical analysis

Statistical analysis was performed using the Statistical package for the Social Sciences version 25.0 (SPSS) for Windows. The Kolmogorov–Smirnov test was used to test the data distribution. Data are expressed as mean (±S.D.) when normally distributed and as median (interquartiles, IQR) when non-normally distributed. Since the two solvents showed similar values for each of the measured parameters, they were averaged in the figures. For normally distributed parameters, differences between groups were evaluated by paired two-sided Student's *t*-test, whereas, for non-normally distributed parameters, the Wilcoxon signed-rank test was performed. A *P* value of 0.05 was considered significant.

Results

$CdCl_2$ and DIBP effects on sperm motility and viability

On the basis of preliminary results (Supplementary Fig. 1), the doses of 10 µM for $CdCl_2$ and of 100 and 200 µM for DIBP, and the incubation time of 24 h, were chosen to perform the subsequent experiments. Progressive (Fig. 1A) and total (Fig. 1B) sperm motility, evaluated on 38 samples, decreased significantly after exposure to $CdCl_2$ and both DIBP concentrations. The percentage decrease of progressive motility was 30.0 (IQR: 17.0–42.4), 12.7 (IQR: 3.6–18.1] and 27.1 (IQR: 14.8–41.8) after incubation with 10 µM $CdCl_2$, 100 and 200 µM DIBP respectively, whereas the percentage decrease of total motility was 14.3 (IQR: 6.5–31.3) in 10 µM $CdCl_2$-treated samples and 7.3 (IQR: 0.9–12.3) and 20.3 (IQR: 12.7–36.6) in those treated with 100 µM and 200 µM DIBP. Although not statistically significant, an increase of the percentage of non-progressive motile spermatozoa after exposure to both pollutants was also observed (median value of non-progressive motility: 5.0% (IQR: 2.0–7.3) in CTRL samples, 8.0% (IQR: 5.0–10.0) in 10 µM $CdCl_2$-treated samples, 6.5% (IQR: 3.0–10.0) in 100 µM DIBP-treated samples and 6.0% (IQR: 3.8-12.5) in 200 µM DIBP-treated samples).

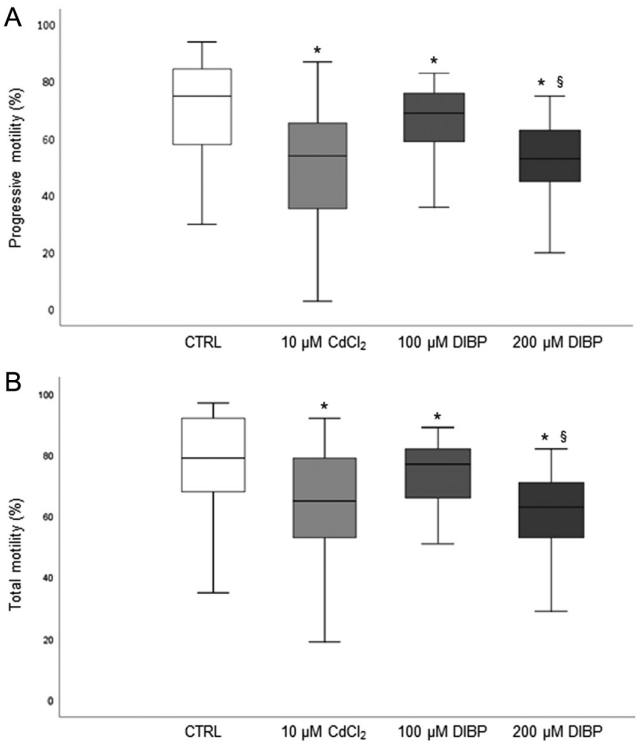

Figure 1 Box plots represent percentage of sperm progressive (A) or total (B) motility in control samples (CTRL) and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or 200 µM DIBP for 24 h ($n = 38$). Wilcoxon test: $^*P < 0.05$ vs CTRL; $^§P < 0.05$ vs 100 µM DIBP.

Most of the kinematic parameters measured by CASA system on 25 samples, underwent a significant decrease after incubation with $CdCl_2$ and DIBP (Table 2). In particular, the percentage of hyperactivated spermatozoa was reduced by 55.0 (IQR: 22.9–71.9), 16.7 (IQR: 4.3–45.8) and 51.8 (IQR: 1.9–79.3) after incubation with 10 µM $CdCl_2$, 100 and 200 µM DIBP, respectively.

The percentage of viable spermatozoa was reduced by 6.3 (IQR, 0.0–13.2) in samples treated with 10 µM $CdCl_2$, and of 11.3 (IQR: 5.4–18.9) in samples incubated with 200 µM DIBP (Fig. 2, $n = 15$). 100 µM DIBP did not affect sperm viability (Fig. 2, $n = 15$).

$CdCl_2$ and DIBP induce caspases activation

In order to understand whether the decrease of sperm viability and motility observed after incubation with the two environmental pollutants was due to induction of an apoptotic pathway, we evaluated the percentage of spermatozoa expressing activated caspases 3 and 7, considered the main effectors of apoptosis (Grunewald *et al.* 2009). We found that exposure to 10 µM $CdCl_2$ or 200 µM DIBP for 24 h resulted in a significant increase of caspases activity (Fig. 3, $n = 7$).

Table 2 Median values (IQR) of kinematic sperm parameters, evaluated by CASA system, are reported in control samples and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or 200 µM DIBP for 24 h ($n = 25$).

CASA parameter	CTRL	10 µM $CdCl_2$	100 µM DIBP	200 µM DIBP
VAP (µm/s)	69.2 (63.7–74.4)	64.6* (60.3–71.6)	65.0§ (60.2–71.9)	57.2^° (54.5–65.1)
VSL (µm/s)	56.4 (51.5–62.7)	57.5 (50.8–59.5)	53.7§ (49.2–55.5)	47.8^° (44.4–52.5)
VCL (µm/s)	118.3 (104.5–129.3)	111.4* (99.5–117.7)	108.8§ (100.1–136.5)	102.1^ (91.8–123.1)
ALH (µm)	5.4 (4.7–5.8)	5.1 (4.4–5.7)	5.0 (4.6–6.0)	5.2 (4.3–6.0)
BCF (Hz)	20.1 (18.4–22.7)	21.7 (16.9–24.0)	18.9 (17.6–21.2)	18.2^° (17.0–18.9)
STR (%)	84.0 (80.9–86.0)	86.5* (82.8–89.0)	81.0 (77.3–85.8)	82.0^ (79.3–85.0)
LIN (%)	50.0 (47.0–53.1)	52.5 (49.3–57.3)	48.0 (43.5–53.0)	47.8^ (45.3–53.1)
HA (%)	15.0 (5.0–24.0)	5.0* (0.0–11.3)	11.5§ (4.0–27.5)	6.0^° (2.6–21.0)

Wilcoxon test: *$P < 0.05$ 10 µM $CdCl_2$ vs CTRL; §$P < 0.05$ 100 µM DIBP vs CTRL; ^$P < 0.05$ 200 µM DIBP vs CTRL; °$P < 0.05$ 200 µM DIBP vs 100 µM DIBP.
ALH, amplitude of lateral head displacement; BCF, beat cross frequency; HA, hyperactivated motility; LIN, linearity of progression; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

$CdCl_2$ and DIBP effects on sperm acrosome reaction

Next, we evaluated the effect of the two contaminants on the ability of sperm to undergo acrosome reaction in basal conditions and in response to the steroid progesterone, considered a physiological inducer of the process (Baldi *et al.* 2009). As can be observed in Fig. 4, spontaneous (basal) acrosome reaction in viable spermatozoa was significantly increased after exposure to 10 µM $CdCl_2$ and 200 µM DIBP, $n = 10$. As expected, in untreated samples, the percentage of acrosome-reacted spermatozoa increased after stimulation with progesterone (Fig. 4). Interestingly, in $CdCl_2$- and DIBP-treated samples, incubation with the steroid hormone did not result in an increase of the percentage of acrosome-reacted spermatozoa compared to respective basal condition (Fig. 4). These data suggest that exposure to toxic agents may induce an early acrosome reaction of human spermatozoa blunting the physiological response to progesterone.

$CdCl_2$ and DIBP effects on sperm intracellular calcium levels

Because the entry of calcium is involved in triggering acrosome reaction, we evaluated the intracellular levels ($[Ca^{2+}]_i$) of such ion after incubation with the two pollutants. We found that 24 h incubation with $CdCl_2$ resulted in an increase in basal $[Ca^{2+}]_i$ levels (Fig. 5A and B, $n = 9$). Conversely, incubation with DIBP did not affect basal $[Ca^{2+}]_i$ levels (Fig. 5B). After stimulation with progesterone, the expected increase of $[Ca^{2+}]_i$ levels was observed both in untreated and treated samples.

Combined effect of $CdCl_2$ and DIBP on sperm motility

In order to evaluate if a synergistic effect between the two toxicants may occur, spermatozoa were incubated with the mixture of both compounds for 24 h ($n = 8$). On the basis of results on sperm motility and viability (Figs 1, 2 and Supplementary Fig. 1), we chose the doses of 5 and 10 µM for $CdCl_2$ and 100 µM for DIBP. As shown in Fig. 6, a significant decrease of progressive motility was observed for 10 µM $CdCl_2$ + 100 µM DIBP (MIX 1) compared both to control and to single treatments, whereas no significant effect was observed for 5 µM $CdCl_2$ + 100 µM DIBP (MIX 2, not shown). MIX 1 also resulted in a significant reduction of sperm viability both compared to control and to 10 µM $CdCl_2$ (median values: control, 87.0% (IQR: 78.5–90.0); 10 µM $CdCl_2$,

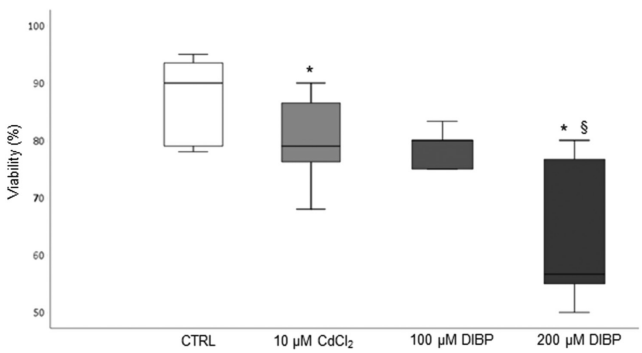

Figure 2 Box plots represent percentage of sperm viability in control samples (CTRL) and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or 200 µM DIBP for 24 h ($n = 15$). Wilcoxon test: *$P < 0.05$ vs CTRL; §$P < 0.05$ vs 100 µM DIBP.

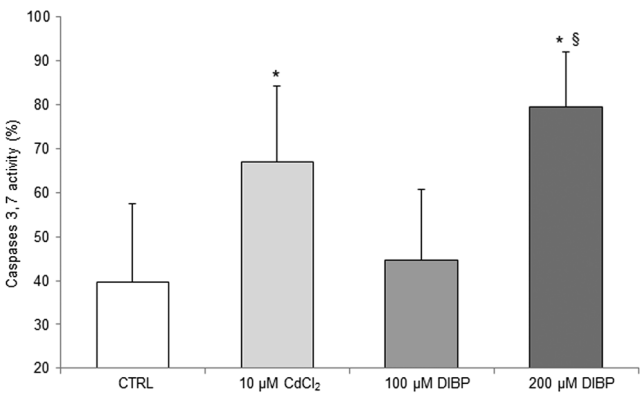

Figure 3 Histograms represent percentage of sperm caspases 3 and 7 activity in control samples (CTRL) and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or 200 µM DIBP for 24 h ($n = 7$). Paired *t*-test: *$P < 0.05$ vs CTRL; §$P < 0.05$ vs 100 µM DIBP.

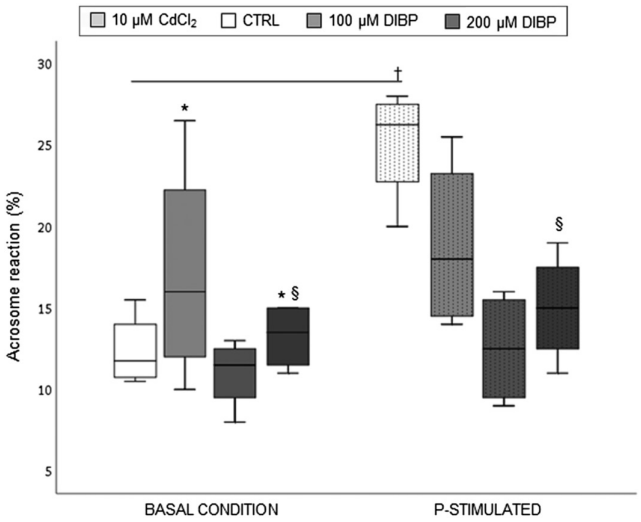

Figure 4 Box plots represent percentage of acrosome-reacted spermatozoa in basal condition (left) and after stimulation with progesterone (P, right) in control samples (CTRL) and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or 200 µM DIBP for 24 h ($n=10$). Wilcoxon test: $^{\dagger}P<0.05$, P-stimulated vs basal condition in CTRL samples; $^{*}P<0.05$ vs CTRL; $^{\S}P<0.05$ vs 100 µM DIBP.

69.5% (IQR: 63.5–87.5); MIX 1, 57.5% (IQR: 52.3–64.8); $P<0.05$). Finally, MIX 1 determined a further decrease of HA of 20.0% (IQR: 18.2–45.5) compared to 10 µM $CdCl_2$ ($P=$ns) and of 50.0% (IQR: 6.3–71.0) compared to 100 µM DIBP ($P<0.05$).

Discussion

The general population is daily exposed to several environmental contaminants by inhalation, ingestion and through the skin. The long-term exposure to environmental chemicals can lead to their accumulation in the organism, damaging organs and tissues. Reproductive systems appear to be particularly sensitive to the endocrine-disrupting action of these agents (Sifakis *et al.* 2017), which may provoke alterations of gonadal function and of other structures of the reproductive tracts as well as impact on gamete functions. In the present study, we demonstrate the occurrence of detrimental effects on human sperm functions necessary for fertilization after acute *in vitro* exposure to two toxic agents ($CdCl_2$ and DIBP) representative of different categories of environmental pollutants, heavy metals and phthalates. Both compounds significantly reduced the percentage of sperm progressive and hyperactivated motility and alter the ability of spermatozoa to undergo acrosome reaction in response to the physiological stimulus progesterone (Baldi *et al.* 2009). Overall, our results indicate that environmental toxic agents such as $CdCl_2$ and DIBP could damage the sperm fertilization potential. Indeed, progressive and hyperactivated motility are necessary, respectively, to

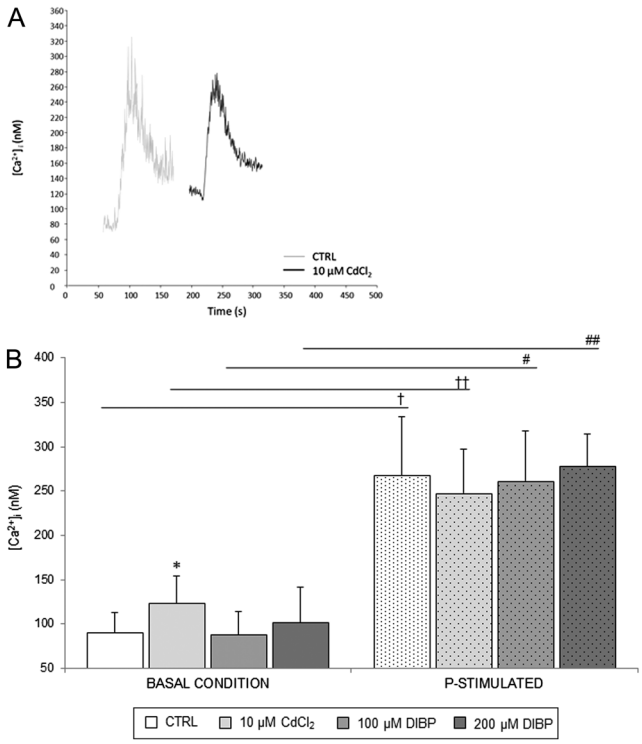

Figure 5 (A) Typical $[Ca^{2+}]_i$ changes in response to progesterone in control samples and in samples exposed to 10 µM $CdCl_2$. (B) Histograms represent $[Ca^{2+}]_i$ in basal condition (left) and after stimulation with progesterone (P, right) in control samples and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or 200 µM DIBP for 24 h ($n=9$). Paired *t*-test: $^{\dagger}P<0.05$, P-stimulated vs basal condition in CTRL samples; $^{\dagger\dagger}P<0.05$, P-stimulated vs basal condition in 10 µM $CdCl_2$-treated samples; $^{\#}P<0.05$, P-stimulated vs basal condition in 100 µM DIBP samples; $^{\#\#}P<0.05$, P-stimulated vs basal condition in 200 µM DIBP samples; $^{*}P<0.05$ vs CTRL.

reach the oocyte and to penetrate its vestments (Freitas *et al.* 2017), whereas acrosome reaction is needed to facilitate the fertilization process (Jin *et al.* 2011).

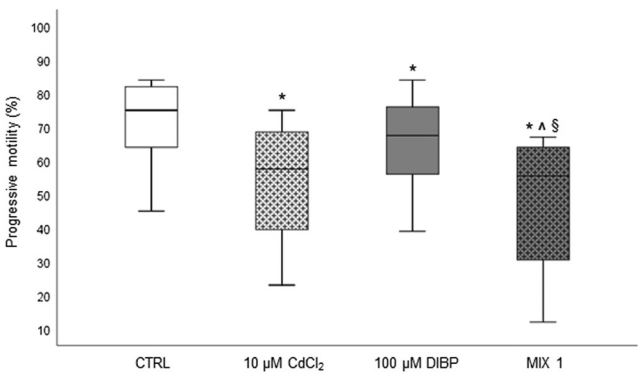

Figure 6 Box plots represent percentage of sperm progressive motility in control samples (CTRL) and in samples exposed to 10 µM $CdCl_2$ or 100 µM DIBP or MIX 1 (10 µM $CdCl_2$ + 100 µM DIBP) for 24 h ($n=8$). Wilcoxon test: $^{*}P<0.05$ vs CTRL; $^{\wedge}P<0.05$ vs 10 µM $CdCl_2$; $^{\S}P<0.05$ vs 100 µM DIBP.

In particular, progesterone-induced acrosome reaction is highly related to fertilization ability of spermatozoa as demonstrated by numerous studies (Xu *et al.* 2018*a*). Although evaluating the effect of these compounds on the ability to fertilize of human spermatozoa is not possible for ethical reasons, it is worth mentioning that a previous study demonstrated that mouse spermatozoa exposed *in vitro* to cadmium produced a lower number of pronuclei with respect to controls during *in vitro* fertilization (Zhao *et al.* 2017).

A detrimental effect of cadmium on sperm motility was observed previously in other *in vitro* studies, where human and mouse spermatozoa were incubated with the metal at a concentration similar or higher to those used in the present study (Wang *et al.* 2016*b*, Zhao *et al.* 2017, Hardneck *et al.* 2018). A severe reduction of sperm motility and kinematic parameters were also shown in a rat model exposed to cadmium *in vivo* (Adamkovicova *et al.* 2016). Our study extends the toxic effects of the heavy metal to hyperactivated motility, which develops in the female genital tract or following *in vitro* incubation in capacitating media (Freitas *et al.* 2017). Interestingly, such effects were observed at a concentration of cadmium similar or slightly higher than those found in seminal plasma of non-professionally exposed subjects (Pant *et al.* 2013).

To our knowledge, no previous studies explored the *in vitro* effect of DIBP on spermatozoa probably because the use of this compound as plasticizer has been introduced only recently. In particular, DIBP is now used as a substitute of other phthalates, such as its isomer dibutylphthalate (DBP), because it is considered less harmful for human health (Zota *et al.* 2014). Our results indicate that, at high concentrations, DIBP produces a decline in the percentage of motile cells, as reported in previous studies using DBP *in vitro* (Pant *et al.* 2011, Adir *et al.* 2017*a*,*b*). An *in vivo* study on adult mice exposed to 450 mg/kg/day DIBP (Pan *et al.* 2017) also demonstrated a decrease of sperm motility.

The decrease of sperm viability observed in samples treated with 10 µM $CdCl_2$ or 200 (but not 100) µM DIBP, could explain, together with the increase of the percentage of non-progressive motile spermatozoa, the observed reduction of progressive motility. Of note, the effect of the two toxicants on sperm viability does not affect the measure of hyperactivated motility and all the kinematic parameters evaluated by CASA system, because they are determined on motile spermatozoa, nor influences results of acrosome reaction, evaluated in viable spermatozoa. The decrease in sperm viability appears to be partly due to the triggering of an apoptotic cascade (Muratori *et al.* 2004, Said *et al.* 2004), as we found a significant increase of caspases 3 and 7 activity, two effectors of the apoptotic pathway, in exposed spermatozoa. Programmed cell death induction, following *in vitro* incubation with phthalates, has been demonstrated also in a mouse spermatocyte-derived cell line (Fu *et al.* 2017). We cannot exclude that the two toxicants induce an imbalance of sperm oxidative status leading to apoptosis (Bu *et al.* 2011, Zhou *et al.* 2011). Whether spermatozoa can undergo apoptosis after ejaculation is still discussed. However, recently, it has been reported that the *in vitro* treatment of human spermatozoa with apoptotic stimuli provokes an increase of caspase activity and typical morphological changes of apoptosis (Engel *et al.* 2018).

Another effect observed in spermatozoa treated with $CdCl_2$ or 200 µM DIBP is an increase of the percentage of spontaneous acrosome reaction. Since spontaneous acrosome reaction is considered a marker of acrosome stability (Xu *et al.* 2018*b*), its increase following *in vitro* exposure to both toxicants, could suggest an alteration of the acrosomal structure due to the two agents. It has been demonstrated, in rabbit spermatozoa, that *in vitro* exposure to heavy metals, including cadmium, can produce the formation of macrovesicles or large holes in the acrosome of sperm membrane (Castellini *et al.* 2009) and *in vivo* studies in mouse reported a premature sperm acrosome reaction following exposure to cadmium (Oliveira *et al.* 2009, Wang *et al.* 2017). Alterations in the acrosomal membrane could be involved also in modifying the responsiveness to progesterone. Indeed, after treatment with $CdCl_2$ and DIBP, sperm responsiveness to progesterone was totally blunted. Although no previous studies investigated the effects of phthalates on sperm acrosome reaction, endocrine disruptors have been reported to affect such sperm function (Tavares *et al.* 2013, Wang *et al.* 2016*a*). At least for $CdCl_2$, the increase of spontaneous acrosome reaction could be related also to the observed increase of basal intracellular calcium levels produced by the metal. However, since DIBP does not provoke calcium changes, such hypothesis is less plausible. Similarly, the lack of any effect of the two toxicants on $[Ca^{2+}]_i$ levels induced by progesterone suggests that other mechanisms are involved in blunting acrosome reaction in response to the steroid. It should be noted that a study evaluating concomitantly acrosome reaction and intracellular calcium increase in response to progesterone in human spermatozoa (Sánchez-Cárdenas *et al.* 2014) demonstrated that the initial calcium peak in response to the steroid is not sufficient to trigger acrosomal exocytosis. Moreover, other signaling pathways are involved in acrosome reaction in response to progesterone (Baldi *et al.* 2009).

This study presents typical limitation of an *in vitro* study that cannot exactly reproduce the *in vivo* situation. Indeed, it is difficult to completely reflect the exposure to environmental contaminants to which the human body is subjected. However, short-term exposure (in our case 24 h of incubation) could represent the condition to which the spermatozoa are subjected once ejaculated and entered in the female genital tract, where they can survive for few days. It is here that, physiologically, the

main sperm functions required for oocyte fertilization, take place. Moreover, it is unlikely that sperm cells are exposed to a single contaminant as it is more conceivable a simultaneous exposure to many toxic substances (Smarr *et al*. 2018). In addition, it should be considered that spermatozoa are virtually exposed to the toxicants present both in the testicular environment, seminal plasma and in the female reproductive tract, and it cannot be excluded from an additive effect. As observed in our study, incubation of spermatozoa with a mixture of $CdCl_2$ and DIBP determined a further decrease of progressive and hyperactivated motility compared to the single exposures, suggesting that a significant interaction and a synergistic effect between the two agents may occur.

In summary, our results showed that acute *in vitro* exposure to $CdCl_2$ or DIBP can compromise sperm quality and functions necessary for successful oocyte fertilization. Such findings suggest that heavy metals and phthalates might be contributing factors, among environmental pollutants, associated with progressive deterioration of male fertility potential.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0207.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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