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Letter to the Editor

In vitro Exposure to CPF Affects Bovine Sperm Epigenetic Gene Methylation Pattern and the Ability of Sperm to Support Fertilization and Embryo Development

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Several studies have demonstrated that overexposure to pesticides can reduce mammalian sperm quality, impairing male fertility. Chlorpyrifos (CPF), a widely used organophosphate pesticide, was shown to impair spermatogenesis by inducing the formation of highly reactive toxic intermediates. To gain further insight into the mechanisms underlying the cytotoxicity and genotoxicity of CPF, bovine spermatozoa were exposed *in vitro* to environmental CPF concentrations and the motility, *in vitro* fertilization rates, DNA fragmentation, chromatin alterations, and methylation patterns were assessed. Motility and *in vitro* fertilization rates were significantly reduced in spermatozoa exposed to CPF, while DNA fragmentation and putative chromatin deconstruction appeared to increase at higher pesticide concentrations. *In situ* hybridization was carried out with X and Y probes on sperm samples exposed to different CPF concentrations, and subsequent analysis highlighted a significant percentage of spermatozoa with a peculiar morphological malformation, in which a narrowing occurred at the level of the hybridization. Analysis of potential abnormalities in the methylation pattern of *NESP55-GNAS* and *XIST* promoters displayed no differentially methylated regions in GNAS promoter relative to the control, whereas spermatozoa exposed to 10 μg/mL CPF had increased methylation variance in one region of imprinted *XIST* promoter. Our results provide support that CPF can induce a genotoxic effect on spermatozoa, impairig their ability to fertilize and support preimplantation embryo development *in vitro*. These observations are worrying since altered levels of sporadic methylation in genes of male gametes may affect the success of reproduction and contribute to infertility. Environ. Mol. Mutagen. 2018. © 2018 Wiley Periodicals, Inc.

Key words: chlorpyrifos; organophosphate pesticides; *Bos taurus*; DNA methylation; Xist gene; infertility

INTRODUCTION

In recent years, studies have repeatedly reported that environmental pollutants interfere at different levels with the physiology of organisms, inducing alterations in development and reproduction (Salazar-Aredondo et al., 2008; Capriglione et al., 2011; Perry et al., 2011; ACOG, 2013; Tussellino et al., 2016; Pallotta et al., 2017). Overexposure to chemical agents, heavy metals and pesticides, present in the environment or introduced into the bodies by feeding, has been demonstrated to reduce sperm quality, acting on spermatozoa counts and lowering percentages of normal mammalian sperm (Selvaraju et al., 2011; Ebadi Manas, 2013; Chiu et al., 2014).

In the last decade, occurrences of infertility have significantly risen, and male factors have accounted for roughly 50% of these cases (Agarwall et al., 2014). Pollutants have been shown to contribute to infertility by causing defects during spermatogenesis and impairing reproductive capacities in exposed populations (Carré et al., 2017).

High levels of pesticides have been found both in human and farm animal's sperm (Dallinga et al., 2002; Kamarianos et al., 2003) and a higher concentration of chlorinated pesticides was found in the semen of infertile men (Abell et al., 2000; Pant et al., 2004; Singh et al., 2008).

The high surface area to volume ratio (approximately 50:1) render sperm highly susceptible to the negative

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effects of pollutants that can impair sperm count, motility, morphology, and fertilization rates (Salazar-Arredondo et al., 2008; Selvaraju et al., 2011; Alaa-Eldin et al., 2017).

Organophosphorus pesticides (OPs) are among the more commonly found pollutants in human body fluids. Although, OPs are known for their neurotoxic effects through inhibition of acetylcholinesterase (AChE) (Del Pino et al., 2015; Pallotta et al., 2017), recent evidence indicate that they also exert adverse effects upon the mammalian male reproductive system (Selvaraju et al., 2011; Dhanuskae and Peiris, 2017).

Chlorpyrifos (CPF) is one of the most used and toxic OPs and its oxidative desulfurization has been reported in various tissues, including testes. The presence of Cytochrome p 450 (CYP) isoforms in the testes and epididymis of rats suggests that the male reproductive tract has the ability to bioactivate CPF *in situ* (Dalvi et al., 2004).

Furthermore, CPF can cause serious defects in spermatogenesis, reducing semen quality in a murine animal model *in vivo* (Sai et al., 2014; Alaa-Eldin et al., 2017; Peiris and Dhanushka, 2017), and interfering as an endocrine disruptor (ED) (Ventura et al., 2016) with the hypothalamic–pituitary endocrine function, affecting spermatogenesis in exposed workers (Recio et al., 2005; Mandal et al., 2012).

Although male infertility has recently been shown to correlate with epigenetic modifications that occur during mammalian spermatogenesis (Congras et al., 2014; Vecoli et al., 2016; Jenkins et al., 2017), the mechanisms involved in determining these epigenetic changes are still unknown and require further in-depth investigation. It is worth noting that CPF has several genotoxic effects, including interference with methylation levels of the sperm DNA (Shin et al., 2015).

The main aim of our work was to study the mechanisms underlying the cytotoxicity and genotoxicity of CPF, by exposing bovine spermatozoa to environmental CPF concentrations *in vitro* (Brancato et al., 2017). The following parameters were evaluated: motility, DNA fragmentation, *in vitro* fertilization rates, X and Y chromosome *in situ* hybridization, and global and sequence-specific DNA methylation patterns.

MATERIALS AND METHODS

The study was conducted in conformity with the animal study guidelines of the Department of Biology, University of Naples Federico II.

Chemicals

CPF (C9H11Cl3NO3PS) with >99.8% purity, purchased from Sigma Aldrich (St. Louis, MO), was dissolved in acetone at a final concentration of 50 μg/ml. Exposure dosages are similar to exposure levels experienced in environmental conditions (Brancato et al., 2017).

Paraformaldehyde, Triton X-100, sodium citrate, Hoechst 33342, polyvinyl alcohol (PVA), M 199 (cod. 4530), gentamycin, amphotericine B, fetal calf serum (FCS), epidermal growth factor, HEPES sodium salt, heparin sodium salt (H3393), and reagents and water (cell culture tested) for preparation of salines and culture media were purchased from Sigma Chemical Company (Milan, Italy). *In situ* cell death detection kit, fluorescein, and DNase I were purchased from Roche Diagnostics (Milan, Italy).

Sperm Preparation

Frozen bovine semen from ejaculates of eight bulls (0.5 mL straws; 40 x10 (Blottner et al., 1990) spermatozoa per straw; motility after thawing 70%), obtained from Inseme (San Giuliano Saliceta, Modena, Italy), was used in all experiments. Straws were thawed in a water bath at 38°C for 30 sec and washed in 10 mL sperm Tyrode's albumin lactate pyruvate (TALP) medium by centrifugation at 170 g for 10 min. After resuspension in a fresh medium, the recovered spermatozoa were assessed for concentration and percent motility using a Makler chamber placed on a microscope stage heated to 39°C (Gualtieri et al., 2014).

Effects of CPF on Sperm Motility and Kinetics

Sperm suspensions in sperm TALP were treated with different concentrations of CPF (1, 5, 10, 25, and 50 μg/mL). In all experiments, controls (CTRLs) were added with identical concentrations of vehicle (acetone) present in CPF treated samples. Samples were loaded on to a Makler chamber and analyzed on a heated stage at 39°C every hour until 2 hr using a Nikon TE 2000 inverted microscope connected to a Basler Vision Technology A312 FC camera with a positive phase contrast 10x objective lens. For each time point, at least 400 cells and four fields were acquired and analyzed. Progressive motility and kinetic parameters were evaluated: curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). These parameters were evaluated by a Sperm Class analyzer (SCA Microptic S.L. Barcelona, Spain) with the following software setting: frame rates: 25 frames/s, number of frames: 10 frames/object, velocity limit for slow spermatozoa: 10 mm/s, velocity limit for medium spermatozoa, 25 mm/s, velocity limit for rapid spermatozoa 50 mm/s, minimal linearity 50%, and straightness for progressive fast spermatozoa 70%.

TUNEL Assay

DNA fragmentation in spermatozoa was measured by the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (*in situ* cell death detection kit, fluorescein; Roche Diagnostics, Milan, Italy) as described in Gualtieri et al., 2014. The percentages of TUNEL-positive spermatozoa were determined on at least 200 cells for each sample.

Oocyte Collection, In Vitro Fertilization (*IVF*), *and Embryo Culture*

Bovine ovaries were collected at the Slaughterhouse Straccione (San Marcellino, Caserta, Italy; CEE accreditation number 1403/M) and transported to the laboratory in Leibovitz's L-15, 1% penicillin-streptomycin (Pen-Strep), 1 μg/mL Amphotericin-B, at 30°C within 2–3 hr. Cumulus–oocyte complexes (COCs) were collected by aspiration of individual follicles with a 19-gauge needle. COCs were matured for 22–24 hr in M199 supplemented with 50 mg/mL gentamycin, 1 mg/mL amphotericine B, 10% FCS, and 10 ng/mL epidermal growth factor at 39°C, 5% CO_2 in air, and 95% humidity. Sperm suspensions, 1 mL at 2×10^6/mL (Blottner et al., 1990), were treated for 2 hr with 1, 5, 10, 25, and 50 μg/mL CPF or with vehicle (CTRL) in sperm TALP. At the end of treatment, sperm suspensions were diluted to 15 mL with sperm TALP and centrifuged at 170 g for 10 min, and the pellets (50 mL) were resuspended in 1 mL of IVF-TALP. For fertilization, 50 in vitro-matured COCs in 250 mL IVF-TALP were inseminated with 250 mL of each sperm suspension (sperm final concentration, 1×10^6/mL) and added with heparin at a final concentration of 10 mg/mL. To understand whether the residual concentration of CPF passively transported in the fertilization wells at insemination had an effect on embryo development, each experiment included three experimental COC groups: CTRL, treated, and residue. In particular, one COC group

was inseminated with treated and two groups with CTRL spermatozoa. One served as vehicle CTRL and the other, termed "residue," was supplemented with identical residual concentrations of the CPF present in the treated COCs group. After 18–20 hr of coincubation at 39°C and 5% CO_2, the COCs were transferred in Hepes-TALP and cumulus cells were removed by vortexing. Presumptive zygotes were collected, washed in synthetic oviduct fluid (SOF) supplemented with 5% FCS, and incubated in 700 mL of fresh SOF for 7 d at 39°C, 5% CO_2, 5% O_2, and 90% N_2. The cleavage rates and percentages of eight cell embryos were determined at Day 3 post-insemination (pi).

Fluorescence In Situ Hybridization (FISH)

The spermatozoa were decondensed according to Blottner et al. (1990) with some modifications. After thawing, the spermatozoa were washed twice in 0.9% NaCl, spun down, and diluted in a small volume of 0.9% NaCl. After soaking overnight in absolute ethanol, the preparations were air-dried vertically. Decondensation was achieved by treatment for 1–4 min in papain-DTT (dithiothreitol) solution (1.250 g papain, Merck 1.07144, plus 0.155 g DTT, Sigma D-9779), dissolved in 100 mL 0.2 M Tris-buffer, pH 8.6). After decondensation, the slides were dipped twice in a 0.2 M Tris-buffer, air-dried vertically, and washed in three changes of absolute ethanol. After drying the slides vertically, the result of the decondensation treatment was checked with a phase contrast microscope. The most appropriate part of the preparation was chosen for the in situ hybridization. The preparations were fixed in methanol-glacial acetic acid (3:1), kept in the freezer (−22°C) overnight and then dried. If decondensation was insufficient, the decondensation process was repeated before fixation in methanol-acetic acid. The Probes used were prepared as described in Pinton et al. (2003). In situ hybridization followed the protocol by Pinkel et al. (1986) with some modifications. Prior to hybridization, sperm slides were treated with RNAse (1 mg/mL) at 37°C for 60 min, sequentially washed in 3 X SSC and then dehydrated in 70, 80, 90, and absolute ethanol for 2 min each. Sperm slides were denaturated by immersion in 70% formamide/2 X SCC at 70°C for 2 min, and then dehydrated by passing through a cold ethanol series. Just before hybridization, the Y-probe (60 ng), the X-probe (100 ng), sonicated bovine DNA (100 ng), and sonicated salmon sperm DNA (100 ng) were pooled together, dried down to 4.5 μL and mixed with 10.5 μL preincubated MMI (50% formamide, 10% dextran sulfate, and 3XSSC, pH 7.0). The hybridization mixture was denatured at 75°C for 10 min in a dry block and immediately chilled on ice for 5 min. Four microliters were applied on each slide under a coverslip, mounted with rubber cement, and kept overnight in a moist, dark chamber at 37°C. Post hybridization washing was carried out at 43–45°C and the slides were washed three times for 8 min each in 50% formamide/2 X SCC, pH 7.0. Biotinylated and digoxigenin-labeled probes were simultaneously detected as described earlier (Scherthan et al., 1994; Frönicke et al., 1996). Sperm nuclei slides were counterstained using 10 μL of antifade solution (Vector Lab Inc., Burlingame, CA) containing 20 ng/mL DAPI (4,6,-diamidino-2-phenylindole).

The slides were observed at 1003 magnification with an Olympus BX 60 fluorescence microscope equipped with a FITC (avidin-fluorescein isothiocyanate)/rhodamin double band pass filter, and a DAPI single band pass filter. Representative sperm were captured using ISIS software (MetaSystems Hard&Software, Altlussheim, Germany). Ten thousand cells were counted for each experimental group.

Statistical Analysis

FISH, TUNEL positivity in spermatozoa, rates of cleavage and eight cell embryos percentages were analyzed by a Chi-square test. Sperm motility and kinetics were analyzed by the estimate model of ANOVA followed by the Tukey's honestly significant difference test for pairwise comparisons when the overall significance was detected. Percent data were transformed into arcsine before statistical analysis.

DNA Extraction

DNA extraction for each individual sample was performed according to the protocol described by Marques et al. (2017). Briefly, the sperm pellets were overlaid with 100 μL of lysis solution (10 mM Tris–HCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 75 mM NaCl) in the presence of 0.2 mg/mL proteinase K, 50 mM DTT and 0.5 μg glycogen (Invitrogen, Cergy-Pontoise, France) and incubated overnight at 50°C. DNA was isolated by standard phenol chloroform extraction.

Global Methylation Level Luminometric Methylation Assay

The global methylation level of each DNA sample was measured using Luminometric Methylation Assay (LUMA), a pyrosequencing-based method (Johansson et al., 2006), in two independent experiments. First, 500 ng of genomic DNA was digested by EcoRI and by either HpaII or MspI. Enzymatic digestion of DNA was performed using an excess of restriction enzymes and 4 h of incubation to guarantee the efficiency of the reaction. Digestion efficiencies were checked on an agarose gel. Each digested DNA sample was then diluted in Pyromark Annealing Buffer (Qiagen, Hilden, Germany) and then pyrosequenced on a PyroMark Q24 sequencer (Qiagen; product no. 9001514) using PyroMark Gold Q24 Reagents (Qiagen; product no. 970802). The isoschizomers HpaII and MspI target the same DNA CCGG sequence. HpaII is methylation sensitive and does not cleave methylated sites, while MspI is methylation insensitive. Pyrosequencing is used to sequence the overhangs left by both enzymes. During pyrosequencing, the proportion of incorporated C and G nucleotides, at 5'-CG overhangs, is directly related to the number of digested sites in the sample. The nucleotide dispensation order is defined as A;C + G;T;C + G;G;A, where the adenosine and thymine incorporation reflect the EcoRI digestion efficiency and the (C + G) simultaneous incorporation reflects both HpaII and MspI digestions. The peak height of C + G incorporation was normalized by the peak height of A + T incorporation to normalize for digestion efficiencies. The calculated ratio between peak height of simultaneous C + G incorporations in HpaII and MspI digests is, therefore, representative of the DNA methylation level of the sample. The ratio is close to 1 when the sample is fully unmethylated. Differences in global methylation level between the three treated groups of sperm (control, 10 μg/mL, 25 μg/mL) were assessed by a t-test, with a P value <0.05 threshold considered as significant.

Analysis of XIST (*X-inactive Specific Transcript*) and GNAS (*Guanine Nucleotide Binding Protein, Alpha Stimulating*) Promoter Region Methylation by Bisulfite Conversion and Pyrosequencing

Bisulfite conversion using 1 μg of sperm DNA was performed as previously described (Dupont et al., 2004). After ethanol precipitation, the DNA pellet was resuspended in 20 μL H_2O. The targeted regions were amplified by PCR with PyroMark PCR kit (Qiagen; product no. 978703) from 1 μl treated DNA (50 ng) in 25 μl reaction volume, according to the manufacturer's instructions with variable $MgCl_2$ concentrations. The PCR program was: 3 min at 94°C followed by 45 cycles of 30 sec at 94°C, 1 min at 60°C for hydridization and 1 min at 72°C for elongation, with a final extension of 10 min at 72°C. In order to analyze the methylation status of *XIST* and *GNAS* promoter region, primer pairs for PCR amplification and pyrosequencing primers were designed using the PyroMark Assay Design software version 2.0 (Qiagen, Hilden, Germany; product no. 9019062) and are listed in Table I.

Bisulfite converted DNA (50 ng) were used to produce PCR products using the PyroMark PCR kit (Qiagen, Hilden, Germany; product no. 978703). Amplicons were then pyrosequenced on a PyroMark Q24 pyrosequencer (Qiagen; product no. 9001514) with the appropriate buffers and solutions (Qiagen; product nos. 979009, 979006, 979007, 979008,

TABLE I. Primers Used on Sequences of Interest to Amplify Specifically Converted and for Sequencing

Gene name	Oligo sequence
XIST1 FOR	GGGTGTTTTTGTTTTAGTGTGTAG
XIST1 REV	ACAAAATCAACCATATTATCCCTACAA
XIST2 FOR	TTTTTGATAGGTTTTGTTGTAGGGATAAT
XIST2 REV	TAACATTACCAATCCCTCCTTTCACT
GNAS1 FOR	AGAGAAATTAGTTTTAAATTAGTTGGTTAT
GNAS1 REV	AAGTATTTGGAGTTTGGAGTTTAGGATT
GNAS2 FOR	TGGAGTTTGGAGTTTAGGATT
GNAS2 REV	AATTATTTGTGTTTGGAGGGTAAG
GNAS3 FOR	TTTGTGTTTGGAGGGTAAGTAGAG
GNAS3 REV	TGGATATATTTTGGTTGGAGAG
XIST1 seq	GGTAGAAATATTTTGTTATATAG
XIST2 seq	GATATTGTGGTAGGGGTG
GNAS1 seq	GGAAAGTAGGGT
GNAS2 seq	GGGGTTAGAGAT
GNAS3 seq	TAGTTGGTTTGATGG

and 970802). The reverse primers were biotinylated for all amplified regions in order to allow the PCR product purification by binding on Streptavidine Sepharose beads (GE Healthcare 17–5113-01). Twenty microliters of the PCR reaction were used as a template for pyrosequencing with 0.3 μM pyrosequencing primer, using the Pyromark Q24 device and Pyromark Gold Q96 reagents (Qiagen, Hilden, Germany). Each analyzed region was assayed in duplicate and the methylation value for each CpG was also obtained in duplicate and inconsistent duplicates (more than 5% difference) were repeated (Kiefer et al., 2016). The methylation percentage per CpG was then obtained by calculating the mean of all replicates that passed the control quality of the Pyromark Q24 software. The average methylation percentage of all CpGs analyzed in a given region was computed for each individual. Groups were then compared using a permutation test for k independent samples (Monte-Carlo sampling of 100,000 permutations, coin R package) followed by pairwise comparisons (1000 permutations; Benjamini–Hochberg correction).

RESULTS

Effects of CPF on Sperm Motility and Kinetics

The analysis of total and progressive motility, carried out using the computerized SCA system, showed a significant reduction in both motility and kinetics in control samples incubated for 1 and 2 hr at 37°C. Sample 0 (total motility: CTRL time 0 = 78.9% ±6.6%, CTRL 1H = 65.4% ±9.8%, CTRL 2H = 59.2 ±9.9%, 0 vs. 1H and 0 vs. 2H, $P < 0.01$, Progressive Motility: CTRL Time 0 = 74.2 ±7.7%, CTRL 1H = 61.9 ±10.5%, CTRL 2H = 55.8 ±10.3%, 0 vs. 1H $P < 0.05$ and 0 vs. 2H $P < 0.01$). The decrease in motility (starting from an hour of incubation) observed in sperm control is attributable to the typical behavior of bovine sperms undergoing freezing/thawing.

After 1 hr, only the sample treated with the highest pesticide concentration displayed a highly significant decrease of total and progressive motility, whereas, after 2 hr, a relevant decrease was found in sperm samples treated with 10, 25, and 50 μg/mL of CPF (total motility: CTRL 1H = 65.4% ±9.8% vs. CPF 50 μg/mL 1H = 41.3% ±16.7% $P < 0.01$; CTRL 2H = 59.2% ±9.9% CPF 10 μg/mL 2H = 40.3% ±11.3%, CPF 25 μg/mL 2H = 38.6% ±11%, CPF 50 μg/mL 2H = 26% ±13.6%, CTRL 2H vs. CPF 10, 25, and 50 μg/mL $P < 0.01$, progressive motility: CTRL 1H = 61.9 ±10.5% vs. CPF 50 μg/mL 1H = 35.3 ±18.2% $P < 0.01$, CTRL 2H = 55.8 ±10.3%, CPF 10 μg/mL 2H = 36.8% ±12.8%, CPF 25 μg/mL 2H = 34.3% ±11.3%, CPF 50 μg/mL 2H = 19, 5% ±14%, CTRL 2H vs. CPF 10, 25, and 50 μg/mL $P < 0.01$) (Fig. 1, Table II).

Kinetic parameter analysis showed again a significant increase in VSL and VAP for control sample incubated for 1 hr at 37°C relative to the control at 0 hr (VSL: CTRL time 0 = 80.6 ±12, CTRL 1H = 100, 5 ±15.5, CTRL 2H = 99.7 ±14; 0 vs. 1H and 0 vs. 2H, $P < 0.01$; VAP: CTRL time 0 = 90.5 ±18, CTRL 1H = 106.6 ±15, CTRL 2H = 103.1 ±15.3, 0 vs. 1H and 0 vs. 2H, $P < 0.05$) (Table III).

Among the samples exposed to CPF, a highly significant decrease of all three kinetic parameters relative to the corresponding control was observed only after treatment with the highest pesticide concentration (50 μg/mL). The decrease is more evident in the samples incubated for 2 hr (VSL: CTRL 1H = 100.5 ±15.5, 50 μg/mL 1H = 65.7 ±18.8, CTRL 2H = 99.7 ±14, 50 μg/mL 2H = 58.7 ±22.6, CTRL 1H vs. 50 μg/mL 1H, $P < 0.01$, CTRL 2H vs. 50 μg/mL 2H, $P < 0.01$, VAP: CTRL

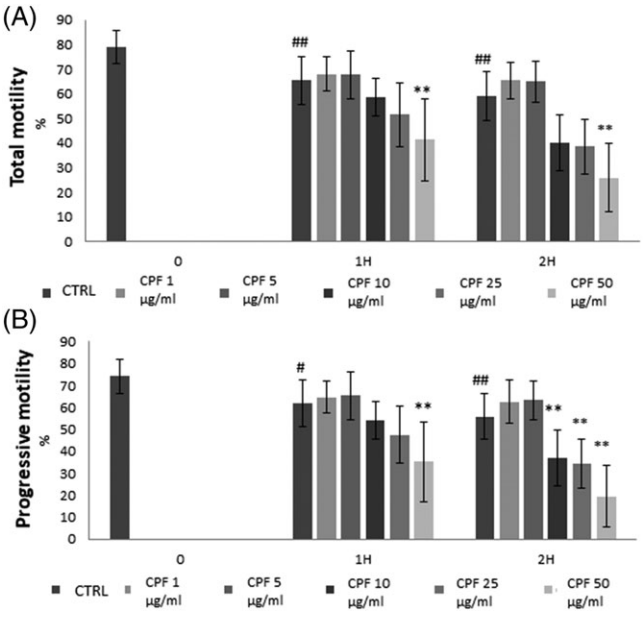

Fig. 1. Effects of CPF on total (A) and progressive motility (B) of sperm. #,##Significant and highly significant differences relative to control at 0 hr ($P < 0.05$; $P < 0.01$); **Highly significant compared to the corresponding control (1 hr and 2 hr) ($P < 0.01$).

TABLE II. Motility Values

	CTRL time 0	CTRL 1H	CPF 10μG/mL 1H	CPF 25μG/mL 1H	CPF 50μG/mL 1H	CTRL 2H	CPF 10μG/mL 2H	CPF 25μG/mL 2H	CPF 50μG/mL 2H
Total motility (%) mean ± SD	78.9 ± 6.6	65.4 ± 9.8[b]	58.7 ± 7.8	51.5 ± 12.9	41.3 ± 16.7[c]	59.2 ± 9.9[b]	40.3 ± 11.3[c]	38.6 ± 11[c]	26 ± 13.6[c]
Progressive motility (%) mean ± SD	74.2 ± 7.7	61.9 ± 10.5[a]	54 ± 8.6	47.4 ± 12.9	35.3 ± 18.2[c]	55.8 ± 10.3[b]	36.8 ± 12.8[c]	34.3 ± 11.3[c]	19.5 ± 14[c]

[a,b]Significant and highly significant compared to the control 0 h ($P < 0.05$; $P < 0.01$); [c]Highly significant relative to the corresponding control (1 hr; 2 hr) ($P < 0.01$).

1H = 106.6 ±15, 1, 50 μg/mL 1H = 69.9 ±20.1, CTRL 2H = 103.1 ±15.3, 50 μg/mL 2H = 62.5 ±23.4, CTRL 1H vs. 50 μg/mL 1H, $P < 0.01$, CTRL 2H vs. 50 μg/mL 2H, $P < 0.01$, VCL: CTRL 2H = 131.5 ±12.7 vs. 50 μg/ mL 2H = 80 ±29.4, $P < 0.01$) (Fig. 2).

DNA Fragmentation

The effects of CPF were evaluated not only on sperm motility and kinetics, but also on DNA fragmentation. For this purpose, two control suspensions (0 and 2 hr), together with the samples incubated for 2 hr with 10 μg/mL, 25 μg/mL, and 50 μg/mL of the pesticide, were subjected to Tunel Assay. Data showed that the percentage of spermatozoa with fragmented DNA in the control suspensions increased from 10.1% at 0 hr to 16.4% after 2 hr of incubation ($P < 0.01$). Among samples exposed to the pesticide for 2 hr, only the highest concentrations of CPF, 25 and 50 μg/mL, displayed a significant increase in the percentage of fragmented nuclear DNA relative to the control ($P < 0.01$; $P < 0.05$) (Fig. 3).

Percentages of 8 Cell Embryos at Day 3

After 3 days, *in vitro* fertilization experiments carried out on COCs using spermatozoa samples treated for 2 hr with 1 μg/mL, 10 μg/mL, 25 μg/mL, and 50 μg/ml of CPF showed that the sperm suspensions exposed to pesticide produced a lower percentage of fertilized eggs, as well as a reduced number of 8-cell embryos. In particular, 50 μg/mL CPF treatment showed a fertilization rate significantly lower (28.2%) ($P < 0.01$) than the corresponding control (69.21%) (Fig. 4). Furthermore, the number of 8-cell stage embryos, developed from oocytes *in vitro* fertilized with treated spermatozoa, was also significantly reduced using a similar concentration (50 μg/mL: 25.76%, CTRL: 54.63%) (Fig. 4).

Effects of Sperm CPF Exposure on Chromosome Structural Integrity

To evaluate the structural integrity of the chromosomes after CPF exposure, fluorescence *in situ* hybridization (FISH) was performed using chromosome painting probes specific for X and Y sex chromosomes. This type of probes recognizes specific sequences for each individual chromosome located along its entire axis. Analysis of spermatozoa hybridized with X or Y probes showed no loss of X and Y chromosome hybridization signals in all the slides analyzed (10,000 cells for each sample). Interestingly, results revealed a significant percentage of sperm possessing a peculiar morphological malformation, in which a narrowing at the level of the hybridization signal was apparent (Fig. 5). In fluorescence, the morphology of altered heads appeared as a figure of eight shape. Among spermatozoa treated with CPF at concentrations of 10, 25 and 50 μg/mL, the percentage of

TABLE III. Kinetic Parameter Values

	CTRL time 0	CTRL 1H	CPF 10μG/mL 1H	CPF 25μG/mL 1H	CPF 50μG/mL 1H	CTRL 2H	CPF 10μG/mL 2H	CPF 25μG/mL 2H	CPF 50μG/mL 2H
VCL	132.4 ± 13.2	138.7 ± 12.6	122.4 ± 19.5	122.2 ± 19.6	91.9 ± 28.3[c]	131.5 ± 12.6	119.5 ± 19.9	114.7 ± 18	80 ± 29.9[c]
VSL	80.6 ± 12	100.5 ± 15.2[b]	88.8 ± 14.8	89.9 ± 17.4	65.7 ± 18.8[c]	99.7 ± 14[b]	91.3 ± 19.8	88.1 ± 16.6	58.7 ± 22.6[c]
VAP	90.5 ± 18	106.6 ± 15.1[a]	95.1 ± 16	95.2 ± 17.7	69.9 ± 20.1[c]	103.1 ± 15.3[a]	96.4 ± 19.8	92.9 ± 17.1	68.5 ± 23.4[c]

[a,b]Significant and highly significant compared to the control 0 h ($P < 0.05$; $P < 0.01$); [c]Highly significant relative to the corresponding control (1 hr; 2 hr) ($P < 0.01$).

sperm presenting this type of malformation was significantly higher than the CTRL.

The percentage of sperm with malformations ranges from 1.4% (CTRL) to 22% (CPF 50 μg/mL) for chromosome X and 1.5% (CTRL) to 16% (CPF 50 μg/mL) for chromosome Y, with a dose-dependent pattern (Fig. 5). All the values obtained at the three different concentrations were extremely significant relative to the control ($P < 0.01$).

CPF Effects on Methylation Status of Promoter Region of XIST and GNAS Genes

Potential abnormalities in the methylation pattern of *XIST* and *NESP55-GNAS* promoter regions were analyzed by bisulfite pyrosequencing on sperm samples exposed for 2 hr to 10 μg/mL and 25 μg/mL of CPF, as well as the relative CTRL. Our analysis covered 22 CpGs for promoter region of *GNAS* and 10 CpGs for promoter region of *XIST* gene.

NESP55-GNAS locus, which was found to be imprinted in cattle (Khatib et al., 2004), mice (Fröhlich et al., 2010), humans (Grybek et al., 2014), and pigs (Oczkowicz et al., 2012), covers more than 11 Kb on bovine chromosome 13. Congras et al. (2014) have previously described an altered methylation at this locus in infertile boars. A high sequence homology was found between pig and bovine sequences, so we opted to analyze the same region. No differentially methylated regions were identified in GNAS promoter (Fig. 6) relative to the CTRL, whereas spermatozoa from eight samples exposed to 10 μg/mL CPF had increased methylation variance in region five of imprinted gene XIST ($P < 0.05$) (Fig. 7).

Global Methylation Level Evaluated by LUminometric Methylation Assay (LUMA)

LUMA was used to detect variations in the global DNA methylation pattern of the 10 and 25 μg/mL CPF exposed spermatozoa samples used for the analysis reported above. However, the observed epigenetic profiles did not show significant levels of differentiation between samples exposed to pesticide and the relative control (Fig. 8).

DISCUSSION

In this study, we have investigated the effects of *in vitro* exposure to CPF on various functional parameters of bovine spermatozoa (Moce and Graham, 2008) and examined the possible causes of the alterations observed. Results revealed a significant correlation between *in vitro* exposure of spermatozoa to CPF and adverse effects on total and progressive motility which are in agreement with sperm reported in humans, bovines and rats (Salazar-Arredondo et al., 2008; Selvaraju et al., 2011; Alaa-Eldin et al., 2017).

In this study, *in vitro* exposure of bovine sperm to pesticide concentrations, similar to those residually found in

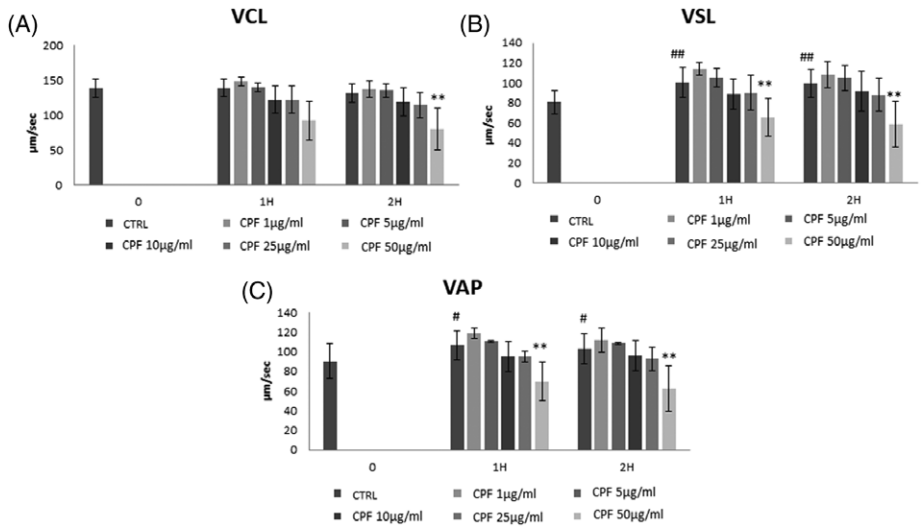

Fig. 2. Chlorpyrifos effects on sperm kinetics. (A) Curvilinear velocity (VCL), (B) Straight line velocity (VSL), (C) Path velocity (VAP). $^{\#,\#\#}$Significant and highly significant differences relative to control 0 hr ($P < 0.05$; $P < 0.01$); **Highly significant compared to the corresponding control (1 hr and 2 hr) ($P < 0.01$).

nature (Brancato et al., 2017), caused reduced fertilization and a reduced number of 8-cell embryo development, relative to control. Selvaraju et al. (2011) showed that frozen/thawed buffalo sperm incubated with various concentrations of pesticides, including CPF, displayed a lowering of various kinetic parameters, a reduction of plasma membrane integrity, decreased mitochondrial membrane potential and reduced fertility. Lower motility rates could be due to decreased mitochondrial activity and ATP content, altered fructose synthesis and impairment of microtubular sperm structures, all of which are fundamental to sperm physiology (Uzunhisarcikli et al., 2007; Heikal et al., 2014). Namely, the ATP assay was shown to be potentially useful for studying the mechanism of toxicity, because ATP generation is geared to sperm motility (Kemp et al., 1990).

Salazar-Arredondo et al. (2008) suggested that acute exposure of agricultural workers to CPF during the "spraying season" could affect fertility and/or the development of conceived embryos. This observation is in agreement with our data that showed a reduced percentage of embryos reaching the 8-cell stage. The higher proportion of sperm DNA fragmentation, observed after *in vitro* exposure to CPF, could cause the reduction of fertility and embryo development that we have observed. Interestingly, sperm DNA fragmentation correlates with the negative outcomes

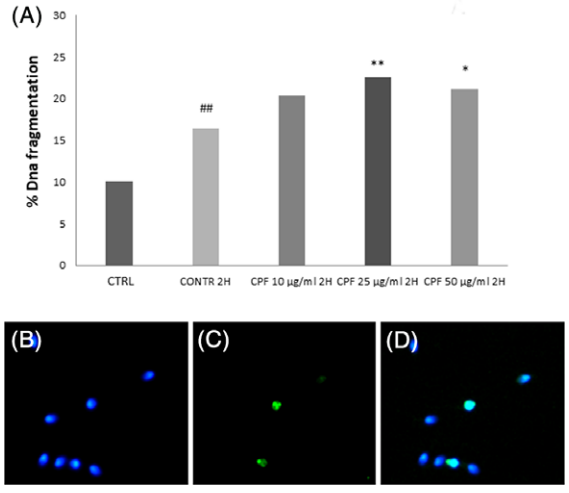

Fig. 3. Effects of different chlorpyrifos concentrations (10 μg/mL, 25 μg/mL, and 50 μg/mL) on sperm DNA fragmentation (A). $^{\#\#}$Highly significant differences relative to the control 0 hr ($P < 0.01$); *,**Significant and highly significant compared to the corresponding control (1 hr and 2 hr) ($P < 0.05$; $P < 0.01$). (B) Hoechst stained spermatozoa; (C) Tunnel positive spermatozoa; (D) Merge.

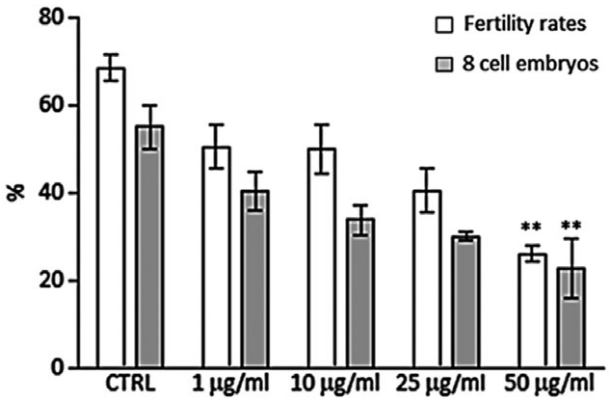

Fig. 4. Effects of CPF (1 μg/mL, 10 μg/mL, 25 μg/mL, and 50 μg/mL) exposure on fertility rates and on eight cell embryos at Day 3. **Highly significant compared to the corresponding control (2 hr) ($P < 0.01$).

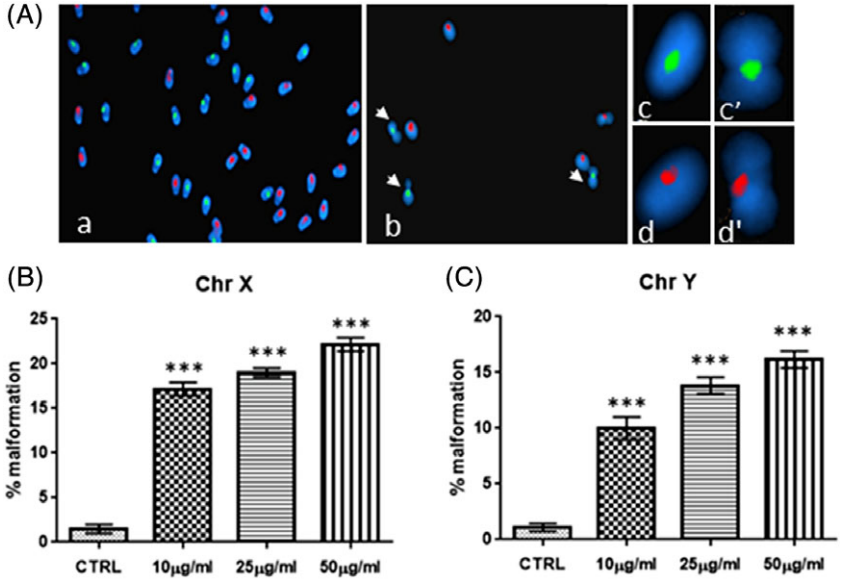

Fig. 5. (A) Fluorescence *in situ* hybridization (FISH) with Y-(green) and X-(red) chromosome specific probes on CTRL (a) and CPF treated spermatozoa (b). Magnifications of normal and altered sperm nuclei hybridized with X (d,d') and Y (c,c') probes. Percentages of sperm cells with malformations after CPF exposure, hybridized with: (B) X chromosome and (C) Y chromosome. White arrows indicate altered sperm nuclei.

of natural pregnancy and *in vitro* fertilization, as well as with the increased risk of premature spontaneous abortion (Evenson et al., 2006).

Several studies, both on exposed laboratory animals and *in vitro*, have highlighted that induction of oxidative stress, the production of reactive oxygen species (ROS) and the subsequent DNA damage can be among the potential genotoxicity mechanisms of organophosphates (Bian et al., 2004; Salazar-Arredondo et al., 2008). Furthermore, Piña-Guzmán et al. (2005) suggested that organophosphates could be considered powerful phosphorylating agents, potentially genotoxic to mouse sperm, because they lead to alteration of the chromatin structure by acting on DNA and protamines, making the former more susceptible to denaturation. In this framework, the results of FISH on sex chromosomes are intriguing because we found a positive correlation between CPF concentration and the proportion of spermatozoa with modified "figure of 8 shaped" nuclei after *in situ* hybridization. This modification was particularly evident in sperm nuclei hybridized with chromosome X, which

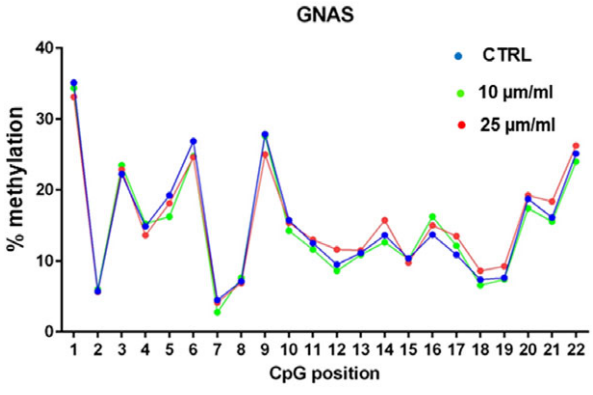

Fig. 6. Comparison of percentages of methylation in the bovine GNAS complex locus of spermatozoa *in vitro* exposed to CPF (10, 25 µg/mL) relative to the control.

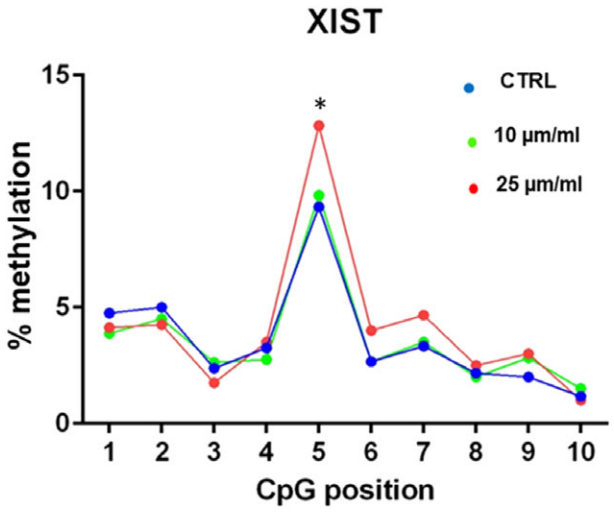

Fig. 7. Methylation pattern in the bovine XIST locus of spermatozoa *in vitro* exposed to CPF (10, 25 µg/mL). $^{*}P < 0.05$.

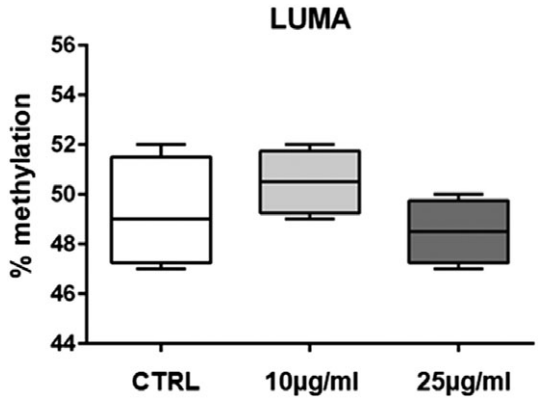

Fig. 8. Global methylation level of bovine sperm DNA treated with 10 and 25 µg/mL of CPF relative to the control.

seemed to be more sensitive to alterations induced by the pesticide. It has been shown, in both diploid and haploid cells, that chromosomes occupy defined, non-random, positions inside the nucleus and, along with interchromatin compartments, the nuclear matrix and nucleoplasm create the intranuclear architecture territories. In human sperm cells, changes in the intranuclear topology of sperm chromosomes 15, 18 X, and Y were correlated with aneuploidy and an increase of infertility (Olszewska et al., 2008). The alteration observed may have been influenced by the hybridization procedure which was performed on a sperm chromatin structure already compromised by exposure to the pesticide. Furthermore, we hypothesize that the larger chromosome X size, and its likely position in the sperm nucleus, would make it more accessible than chromosome Y to pesticide action (Cremer et al., 2010). Finally, it is possible that DNA methylation changes, as that observed for one CpG of XIST gene may also have a role in the abnormal morphology reported.

These last data are compelling because the structural and functional integrity of sperm DNA is a crucial factor during reproduction, hence chromatin alterations or damage to germinal DNA can result in male fertility problems (Evenson et al., 2002). In this context, the variation in the methylation levels of the XIST gene promoter is an interesting result because this gene is a major effector of the X inactivation process. So far, there is no evidence in the literature of changes in the percentage of methylation of the XIST gene following exposure to organophosphate pesticides, although epigenetic modifications were described for other genes after *in vitro* treatment with OPs. Zhang et al. (2012) reported methylation changes *in vitro* of the promoter regions of 712 human genes, studied after exposure to three organophosphate pesticides. Furthermore, an agricultural health investigation examining male pesticide applicators showed that even at low concentrations CPF has the potential to affect mitochondrial DNA in humans. Of particular interest was the report of hypermethylation of the promoter region of GSTp1, a gene consistently associated with prostate cancer, which occurs abnormally frequently in these farmer populations (Rusiecki et al., 2017).

We hypothesize that the partial hypermethylation of the XIST promoter, which we have displayed, may correlate either with a decrease of the gene transcripts (Chureau et al., 2010) or with a silencing of the XIST gene. These modifications may affect paternal X chromosome inactivation, altering genic dosage during embryo development. Dosage compensation failure has an impact as early as the blastocyst stage (Borensztein et al., 2017) and paternal inheritance of an Xist deletion (XpΔXist) is embryonic lethal to female embryos, due to iXCI abolishment (Federici et al., 2016).

Interestingly, some of the alterations induced by the pesticide on sperm samples are active even at low CPF concentrations. This could be justified given that CPF can also act as an ED. These agents do not follow the classical dose/response effect but can induce deleterious damages even at low doses (Kavlock et al., 1996).

Our data is of particular interest because, besides demonstrating the potential toxic effect of CPF, it also highlights the possible risk of generating (transgenerational) heritable DNA damage, when the pesticide is in the seminal fluid. Our hypothesis builds on recent epigenetic studies which have demonstrated a correlation between fathers with faulty spermatozoa methylation (Kobayashi et al., 2009) and methylation defects in their children conceived by *in vitro* fertilization techniques.

However, in contrast, genome-wide DNA methylation profiles of spermatozoa DNA, as well as methylation analysis on the GNAS promoter region of sperm treated with the pesticide, did not show evident alterations at the concentrations used for the experimental design. Nonetheless, our interest for GNAS was still justified by evidence reported in previous studies; higher methylation levels of GNAS promoter were found in sterile boars compared to fertile boars (Congras et al., 2014).

CONCLUSIONS

Our results have shown that CPF can induce even *in vitro* a negative effect on spermatozoa, supporting the data already available in the literature for organophosphates.

These data, although valid and consistent, need to be further investigated and analyzed, as the mechanisms underlying the alterations that CPF causes require a more complete understanding. In particular, it is important to have a better understanding of the mechanism that could induce altered methylation pattern because it could affect success of reproduction.

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AUTHOR CONTRIBUTIONS

All the authors designed the study and interpreted all results. Drs. Capriglione and Pallotta wrote the manuscript with intellectual input of Drs. Acloque, Gualtieri, Jammes, Pinton, and Talevi.

Drs. Barbato and Pallotta prepared draft figures and tables. All authors approved the final manuscript.

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