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Bulletin of Environmental Contamination and Toxicology

ISSN 0007-4861
Volume 91
Number 2

Bull Environ Contam Toxicol (2013)
91:197-201
DOI 10.1007/s00128-013-1032-5

Springer

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Received: 20 December 2012 / Accepted: 1 June 2013 / Published online: 11 June 2013

Abstract In vitro effects of lead (Pb) on ram (*Ovis aries*) spermatozoa were studied to establish a threshold level that affects sperm function. Spermatozoa were incubated between 15 and 180 min with Pb concentrations ranging from 0 to 5,000 ng/mL. Sperm motility, acrosome integrity, membrane functionality and sperm viability were all negatively affected by Pb and incubation time. Acrosome integrity was linearly affected by Pb levels at an incubation time of 30 min, and 50 ng/mL was the lowest Pb level producing such effect. These experimental conditions can be appropriate for in vitro studies of the mechanisms of action of Pb on spermatozoa.

Keywords Lead · Spermatozoa · Semen quality · Acrosome integrity

Several studies have suggested that the semen quality in humans is globally declining due to different environmental factors such as lifestyle, nutrition or exposure to chemical substances (Skakkebaek et al. 2006; Deonandan and Jaleal 2012). The potential deleterious role of toxic compounds in this progressive alteration of men reproduction function has been supported by experimental studies with animals and similar evidences observed in wildlife (Auger 1997). Particularly, the increase of heavy metal emissions in developed countries and the occupational exposure to lead (Pb) have been associated with adverse effects on male reproductive function. Pant et al. (2003) showed a significant negative association between Pb concentrations in semen and sperm quality in humans, having found 60 ng/mL of Pb in seminal plasma of fertile men and 125 ng/mL in infertile oligospermic, asthenospermic and azoospermic men. Xu et al. (2003) have associated a Pb concentration in human seminal plasma of 10 ng/mL with lower sperm density and higher oxidative DNA damage in spermatozoa. Pb neurotoxicity can also negatively affect the male reproductive function through an imbalance of the hypothalamic-pituitary-gonad axis. Rats exposed to Pb acetate in drinking water (0.3 %–0.6 %) during their development that had a mean blood Pb of 300 ng/mL showed a significant decrease in serum and intratesticular testosterone levels and a decrease in sperm count by a suppression of spermatogenesis (Ronis et al. 1996). However, few studies have been focused on the biochemical effects of Pb after spermatogenesis, and particularly on membrane properties associated with sperm quality (Benoff et al. 2003a). In a previous study we observed an immediate reduction in the acrosome integrity of ram spermatozoa exposed in vitro to 50–500 ng/mL of Pb^{2+} (Castellanos et al. 2008). In the present study, we have assessed in vitro effects of Pb in ram spermatozoa to determine a threshold Pb level appropriate to study the mechanism of action of Pb on spermatozoa when it contacts with seminal plasma or fluids of the female genital tract.

Materials and Methods

Semen was collected by artificial vagina from six healthy rams (*Ovis aries*) of Manchega breed from the Animal Reproduction Centre (CERSYRA, Valdepeñas). Sperm is

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routinely collected in the Manchega males of this Centre for the artificial insemination of the herds of Castilla-La Mancha region. Therefore, these males have been previously evaluated and selected by their quality as donors. We collected two or three ejaculates per animal and the one with the largest volume and best wave motion (above 3) was used for the experiment. The collected volume, sperm concentration, wave motion, individual sperm motility and quality of movement were evaluated. Sperm concentration was determined by transmittance with a spectrophotometer (Type Helios Delta model, Thermo Electron Corporation), diluting 5 μL of ejaculates in 5 mL of sodium citrate 128 mM. Wave motion was subjectively scored from 1 to 5, where 0 is no movement and 5 is strong wave movement, on a wet mount of neat semen using bright field microscopy at ×10 (BH-2 Olympus). Individual sperm motility and quality of movement were also assessed, 5 μL of semen were diluted on 200 μL of phosphate-buffer saline (PBS). After incubation at 37°C for 5 min, 5 μL of sperm diluted were placed between a pre-warmed slide and a 22 mm × 22 mm coverslip and were observed at ×400 under phase-contrast optics (Eclipse 50i Nikon). The percentage of motile sperm was estimated subjectively by three experienced observers after any drifting of the specimen due the placement of the coverslip had stopped. The percentage of motile spermatozoa was determined to the nearest 5 % by analysing four to five fields of view (Evans and Maxwell 1987) under the microscope with values ranging from 0 %, when no motile spermatozoa were observed, to 100 %, when all spermatozoa were moving. Quality of sperm movement was also estimated subjectively on a scale of 0–5, where 0 is no motility and 5 is vigorous progressive movement.

Samples of each ram were diluted in six aliquots of 5 mL to obtain 75 × 10^6 spermatozoa/mL with the modified tyrode medium supplemented with 10 mM sodium pyruvate (Sigma Ultra 99 %) and 6 mg/mL of bovine serum albumin (BSA) (albumin bovine serum, factor V 96 %, Sigma). Pb acetate, (lead II acetate tri-hydrate 99 %, Aldrich) was previously added to the incubation media to obtain six treatments per ram with spiked Pb^{2+} concentrations of 0, 0,5, 5, 50, 500 or 5,000 ng/mL of Pb^{2+} (0, 0.0024, 0.024, 0.24,2.4 or 24 μM). Then, each of the six tubes of each ram was divided in five aliquots of 1 mL for incubation during 15, 30, 60, 120 or 180 min at 37.5°C.

Sperm quality parameters were measured in 30 μL of each aliquot at the corresponding incubation time at 37°C. Individual sperm motility and quality of movement was evaluated as above mentioned. Plasma membrane functionality was assessed by means of the hypo-osmotic swelling test as described by Garde et al. (1998). Briefly, 10 μL of diluted sperm sample was mixed with 0.1 mL of hypo-osmotic solution (sodium citrate, 100 mOsmol/kg) and incubating the mixture at room temperature (≈23°C) for 30 min. The samples were then fixed in 2 % glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3) and evaluated at ×400 under phase-contrast optics. The sperm membrane was considered intact if the sperm tail was coiled at the end of the assay and the result was expressed as % positive endosmosis. Sperm viability was evaluated by means of a nigrosin–eosin stain (NE). The NE stain was prepared as described by Tamuli and Watson (1994). The diluted sperm (5 μL) was mixed with the NE stain (10 μL) at 37°C, incubated for 30 s, smeared and dried on a warm plate at 37°C. The samples were evaluated using bright field microscopy at ×400. Live spermatozoa remained unstained, while dead cells were dull pink. The % of live spermatozoa was expressed as viability. Acrosomal integrity was evaluated after a 1:10 dilution in 2 % glutaraldehyde/cacodylate, as above. The % of spermatozoa with intact acrosomes (i.e., with normal apical ridges) was assessed at ×400 under phase-contrast optics. Membrane functionality, viability and acrosomal integrity were studied in 100 spermatozoa per sample.

The remaining 0.97 mL of six Pb treatment samples of each animal incubated during 180 min were centrifuged at 940 rcf for 10 min, the supernatant removed and the remaining pellet washed with 1 mL of cold saline solution. After a second centrifugation the pellet was kept frozen at −80°C for Pb analysis. Before Pb analysis, the pellet was mixed with 200 μL of deionized water and transferred to quartz tubes. Then, 2 mL of HNO_3 (nitric acid suprapur 70 %) were added and left at room temperature during 12 h. After this, 2 mL of H_2O_2 (30 % hydrogen peroxide) were added and the tubes were gradually heated during 1 h in a standard heatblock (VWR) up to 150°C. Then, this temperature was maintained during 3 h. Finally, the digested sample was brought to a final volume of 5 mL with deionized water. Pb analysis was performed by graphite furnace atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer) using 50 μg of $NH_4H_2PO_4$ and 3 μg of Mg $(NO_3)_2$ as matrix modifiers in each atomization. Solutions used for calibration were prepared from a commercial standard with 1 g/L of Pb. The limit of detection calculated as three times the standard deviation of blanks of digestion was 0.59 ng/mL. A reference material of blood (Community Bureau of Reference-BCR 190 with certified value of 772 ng/mL) was also analyzed and the recovery obtained for Pb was 104.3 %.

The effects of Pb concentrations on sperm motility, viability and acrosomal integrity were studied with General Linear Models (GLM), including Pb concentration, incubation time and individual as factors and including their first-order interactions. Least Significant Difference (LSD) test was used to establish post hoc differences between the values found at the different Pb concentrations and controls. Sperm Pb levels at the end of the incubation

experiment were log-transformed to attain a normal distribution and these values were compared between Pb-exposure levels with a One-way Analysis of Variance (ANOVA) with a post hoc LSD test. The level of significance was set at $p < 0.05$. These analyses were performed with IBM SPSS Statistics v. 19.

Results and Discussion

The initial values of sperm parameters from rams #1 to #6 before incubation with Pb were as follows; wave motion: 4, 3.5, 3, 3, 3.5 and 3; individual motility: 80 %, 75 %, 75 %, 75 %, 50 % and 50 %; quality of movement: 4, 3.5, 3, 3.5, 2 and 2.5; and spermatozoa concentrations were 4,946, 3,961, 3,814, 4,304, 4,030, 2,887 $\times$ 10^6 spermatozoa/mL, respectively.

Several parameters of sperm quality have been negatively affected by the incubation time and the presence of Pb in the medium, but the interactions of these two factors were not significant (Table 1; Fig. 1). Moreover, there was a significant effect of the individual on the sperm quality parameters, and the effect of incubation time on sperm quality parameters differed among individuals as the interaction between these two factors reflects (p values between <0.001 and 0.034). On the contrary, the interaction between the individual and Pb was not significant in any of the studied parameters.

Sperm motility was significantly reduced by increasing Pb concentration in the incubation medium ($p = 0.023$), with incubation time ($p < 0.001$) and by individual ($p < 0.001$). The quality of motility was also affected by incubation time ($p < 0.001$) and individual ($p < 0.001$), but not by Pb concentration. Viability was markedly reduced by incubation time ($p < 0.001$), individual ($p < 0.001$) and Pb concentration ($p = 0.003$). Similarly, membrane functionality was affected by incubation time ($p < 0.001$), individual ($p < 0.001$) and Pb concentration ($p = 0.004$). Acrosomal integrity was also affected by incubation time ($p < 0.001$) and Pb concentration ($p < 0.001$), but not by individual ($p = 0.081$) (Table 1). Acrosomal integrity was the parameter that showed a better dose–response in relation to Pb concentration in the incubation medium, especially at 30 min of incubation (Fig. 1). Acrosomal integrity was significantly lower than in the control at Pb concentrations of 50 and 5,000 ng/mL. This parameter was also significantly reduced respect to the control at 120 min of incubation with 500 and 5,000 ng/mL of Pb and at 180 min with 5, 500 and 5,000 ng/mL of Pb (Fig. 1).

The presence of Pb in the incubation medium was associated with elevated Pb levels in spermatozoa after incubation during 180 min, especially at Pb concentrations in the medium ≥50 ng/mL ($p < 0.001$; Fig. 2). Pb present in the spermatozoa pellet was (mean ± SE) 6.3 % ± 1.3 % of the total Pb added to the incubation medium with ≥50 ng/mL. The mass of the spermatozoa pellet was 103 ± 13 mg. In terms of ng Pb/10^6 spermatozoa, the detected values for samples incubated with 0, 0.5, 5, 50, 500 and 5,000 ng/mL were <0.002, 0.008 ± 0.005, 0.005 ± 0.001, 0.026 ± 0.010, 0.129 ± 0.051 and 3.157 ± 0.768, respectively.

Although most of the experimental work in rodents have associated blood Pb levels of >300–400 ng/mL with impairment of spermatogenesis (Apostoli et al. 1998), blood Pb levels as low as 49 ng/mL have been related in non-occupationally exposed men with an increased presence in semen of immature sperm cells and with abnormal morphology, a decrease of Zn in seminal plasma, an increase of serum levels of testosterone and estradiol, and a decrease in serum level of prolactin (Telisman et al. 2007). Lead levels in sperm and seminal plasma are markedly lower than in blood. Hernández-Ochoa et al. (2005) have found in urban men a

Table 1 Results of the general linear models used to test the effects of the lead exposure level, incubation time, individual, and their first order interactions (independent variables) on the sperm quality parameters (dependent variables) of ram spermatozoa treated with lead acetate

Independent variables	Dependent variables				
	Motility	Quality of motility	Sperm viability	Membrane functionality	Acrosomal status
Time	$F_{4,20} = 20.85$	$F_{4,20} = 20.6$	$F_{4,20} = 84.26$	$F_{4,20} = 20.97$	$F_{4,20} = 80.28$
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Pb	$F_{5,25} = 3.21$	NS	$F_{5,25} = 4.77$	$F_{5,25} = 4.63$	$F_{5,25} = 12.85$
	$p = 0.023$		$p = 0.003$	$p = 0.004$	$p < 0.001$
Ram #	$F_{5,22} = 98.26$	$F_{5,20} = 71.3$	$F_{5,20} = 30.89$	$F_{5,20} = 16.23$	NS
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	
Time × Pb	NS	NS	NS	NS	NS
Time × Ram #	$F_{20,100} = 7.26$	$F_{20,100} = 5.92$	$F_{20,100} = 1.77$	$F_{20,100} = 4.32$	$F_{20,100} = 5.02$
	$p < 0.001$	$p < 0.001$	$p = 0.034$	$p < 0.001$	$p < 0.001$
Pb × Ram #	NS	NS	NS	NS	NS

NS not significant

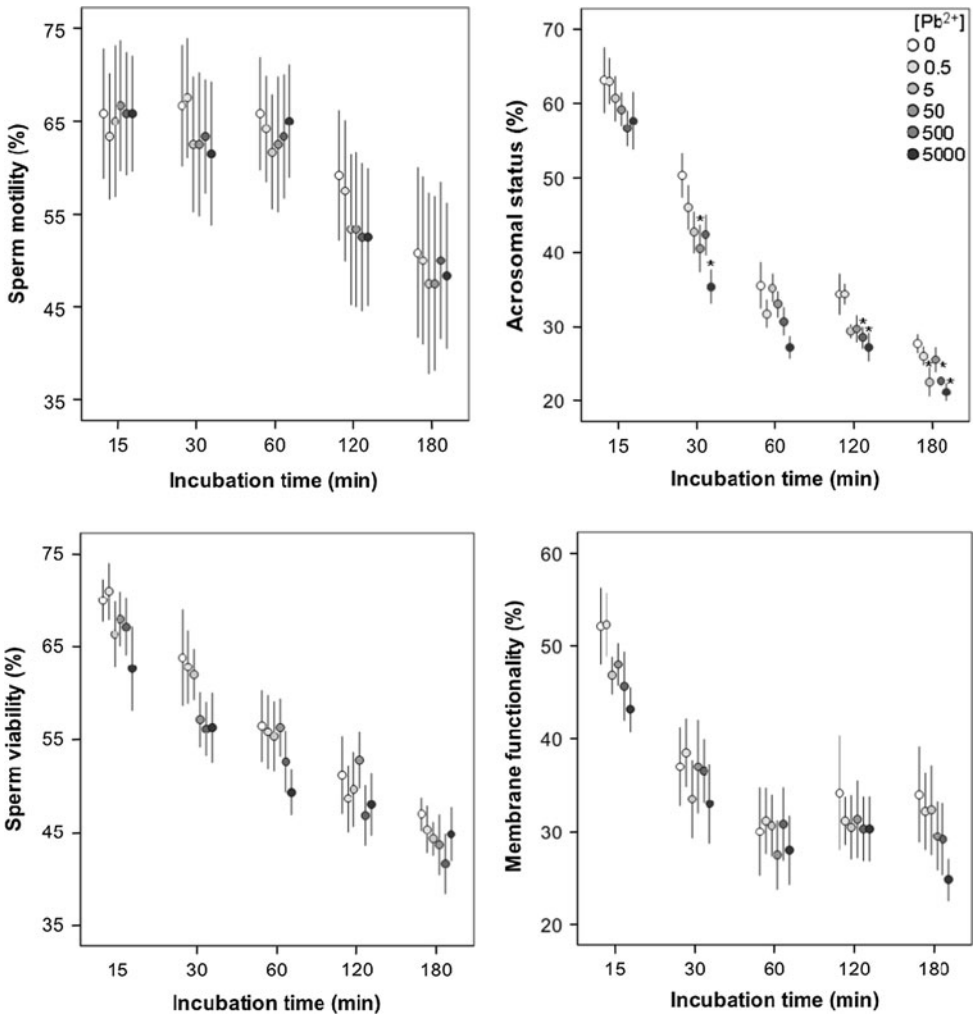

Fig. 1 Effect of Pb concentration and incubation time upon different spermatozoa parameters (mean ± SE of six rams). Acrosomal status was affected by Pb concentrations at incubation times of 30 min ($F_{5,30} = 3.173$, $p = 0.020$), 120 min ($F_{5,30} = 2.88$, $p = 0.031$) and 180 min ($F_{5,30} = 2.661$, $p = 0.042$). *Asterisks* denote differences respect to the control at the corresponding incubation time

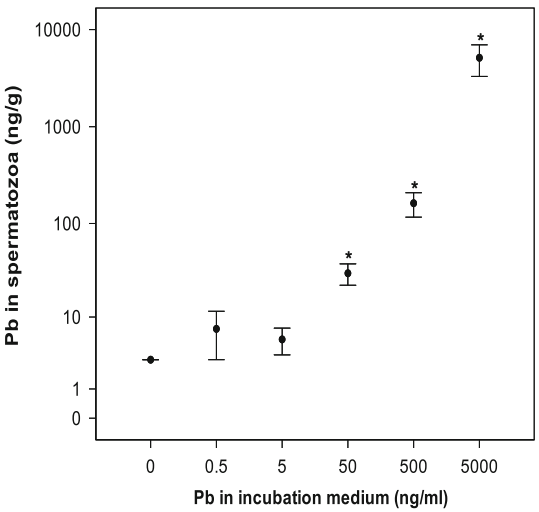

Fig. 2 Lead concentration in spermatozoa (mean ± SE of six rams) after incubation with different Pb concentrations ($F_{5,30} = 72.74$, $p < 0.001$). *Asterisks* denote differences respect to the control

geometric mean of Pb levels in blood of 93.1 ng/mL that corresponded to 0.047 ng/10^6 spermatozoa and 2.02 ng/mL of seminal fluid. In fact, seminal plasma and spermatozoa Pb levels may be better determinants of sperm quality than blood Pb levels. The minimum concentrations of Pb in human spermatozoa associated with abnormal semen quality were 0.07 ng/10^6 spermatozoa for a negative effect on sperm motility and 0.24 ng/10^6 spermatozoa for reduced sperm viability (Hernández-Ochoa et al. 2005). In our experiment, final Pb concentrations in spermatozoa incubated in medium with 50 ng/mL of Pb was 0.026 ng/10^6 cells, which is slightly lower than the level observed in vivo. Nevertheless, the concentration of Pb in the incubation medium was much higher than the values detected in seminal plasma, probably because Pb uptake in spermatozoa was more effective in vivo than in our in vitro model.

Other in vitro studies have evaluated the effects of Pb on spermatozoa. Rabbit spermatozoa incubated in a medium with 5,170 ng/mL of Pb showed a decrease in the fertilization rate

(Foote 1999). Bull spermatozoa incubated with 2,500 ng/mL of Pb showed a decrease in the sperm motility but no effect was observed at 250 ng/mL (Alexaki et al. 1990). Buffalo spermatozoa incubated with 100 ng/mL for 1 h showed a reduced acrosomal integrity (Selvaraju et al. 2011). Here, we have observed an adverse effect on acrosomal integrity at lower Pb levels than those reported in other studies. On the other hand, Benoff et al. (2003b) exposed human sperm to Pb concentrations similar than in our experiment (51.5–5,180 ng/mL) and observed an increase in the spontaneous acrosome reaction in a dose-dependent manner.

A premature acrosome reaction or a reduced acrosomal integrity have also been reported in spermatozoa of Pb-exposed males of different species. In wild ungulates such as red deer, the percentage of acrosomal integrity in spermatozoa collected from the cauda epididymae was lower in Pb-polluted areas (79 %) than in control areas (90 %) (Reglero et al. 2009). These Pb-exposed deer also had lower Cu levels and higher Se levels in testis, decreased activities of SOD and GPX in testis and spermatozoa, and lower levels of arachidonic acid in testis and spermatozoa (Reglero et al. 2009; Castellanos et al. 2010). Sperm Pb level in deer from these Pb-polluted sites was 0.003 ng/10^6 cells (Reglero et al. 2009), which is lower than the resultant level in ram spermatozoa incubated here with 50 ng/mL. In the case of rodents, Hsu et al. (1998) found that the percentage of acrosome-reacted spermatozoa was significantly higher in rats receiving weekly an intraperitoneal injection of 50 mg/kg of Pb acetate for 6 weeks (7.5 %) than in the controls (1.7 %). Johansson (1989) also observed a premature acrosome reaction in spermatozoa of Pb-exposed mice and this reduced their ability to fertilize oocytes. In humans, Pb seminal plasma levels ranging from 100 to 1,500 ng/mL were negatively correlated with the artificial insemination rate and positively correlated with a premature acrosome reaction (Benoff et al. 2003a).

In conclusion, in vitro Pb-exposure of spermatozoa in an incubation medium with 50 ng/mL of Pb during 30 min can be appropriate for the study of the mechanism of action of extracellular Pb on spermatozoa, especially for a target such as the acrosomal status and its impact on fertility.

Acknowledgments The authors thank P. Camarero, M. Martinez-Haro for their technical assistance. CERSYRA personnel are also acknowledged for providing facilities and animals for the experiment. This study has been funded by the Department of Education and Science of the Junta de Comunidades de Castilla-La Mancha under projects PCC-05-004-2 and PCI08-0096-1295.

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