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Increased chromatin fragmentation and reduced acrosome integrity in spermatozoa of red deer from lead polluted sites

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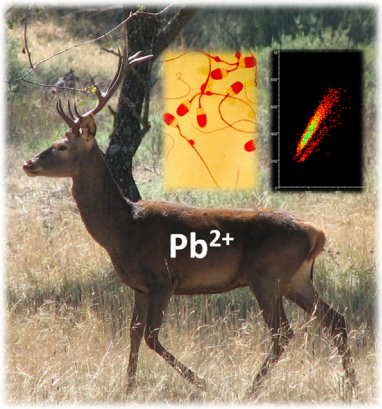
HIGHLIGHTS

- Lead-mining pollution increased testis and sperm Pb levels in red deer.
- Mining pollution reduced the acrosome integrity of red deer spermatozoa.
- Lead-exposed deer showed an increased glutathione peroxidase activity in sperm.
- Lead pollution was associated with higher DNA fragmentation in spermatozoa.

GRAPHICAL ABSTRACT



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ABSTRACT

Vertebrates are constantly exposed to a diffuse pollution of heavy metals existing in the environment, but in some cases, the proximity to emission sources like mining activity increases the risk of developing adverse effects of these pollutants. Here we have studied lead (Pb) levels in spermatozoa and testis, and chromatin damage and levels of endogenous antioxidant activity in spermatozoa of red deer (*Cervus elaphus*) from a Pb mining area (n = 37) and a control area (n = 26). Deer from the Pb-polluted area showed higher Pb levels in testis parenchyma, epididymal cauda and spermatozoa, lower values of acrosome integrity, higher activity of glutathione peroxidase (GPx) and higher values of DNA fragmentation (X-DFI) and stainability (HDS) in sperm than in the control area. These results indicate that mining pollution can produce damage on chromatin and membrane spermatozoa in wildlife. The study of chromatin fragmentation has not been studied before in spermatozoa of wildlife species, and the sperm chromatin structure assay (SCSA) has been revealed as a successful tool for this purpose in species in which the amount of sperm that can be collected is very limited.

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1. Introduction

Lead (Pb) is a heavy metal widely present in the environment because of its use by man for centuries. Mining and smelting areas show elevated levels of Pb pollution during and beyond their production period (Wilson and Pyatt, 2007; Beyer et al., 2007). One of these polluted sites is the Alcudia Valley–Sierra Madrona Pb-Zn mining area, where argentiferous galena was extracted since the Roman expansion in the Iberian Peninsula until the 2nd half of the 20th century, when the last mines closed. Nowadays, water, soil and plants around these mines are polluted with Pb and other elements (Reglero et al., 2008); and this affects wildlife and livestock by altering their health status and biological functions such as reproduction (Reglero et al., 2009a, 2009b; Rodríguez-Estival et al., 2011, 2013). This mining pollution is also relevant in terms of food safety due to the contamination of game meat (Taggart et al., 2011) and livestock (Rodríguez-Estival et al., 2013; Pareja-Carrera et al., 2014).

Heavy metals can produce adverse effects on animals, including the reduction of sperm quality (Lavranos et al., 2012; Marzec-Wróblewska et al., 2012), through several mechanism of action such as competing with other cations, binding with sulfhydryl groups of proteins or depleting glutathione (GSH) levels (Jornova and Valko, 2011). In the case of Pb, changes produced on oxidative stress parameters can vary between tissues, especially between reproductive and others tissues (Reglero et al., 2009a, 2009b; Tvrdá et al., 2013). An example of these differences in the response to oxidative stress among tissues has been observed in red deer (*Cervus elaphus*) from Alcudia Valley–Sierra Madrona mining area. Red deer from the mined area had 39–56% less total GSH (tGSH) in liver than those from control sites (Reglero et al., 2009a; Rodríguez-Estival et al., 2011); and a similar reduction (28–46%) was found in spleen (Rodríguez-Estival et al., 2013). On the contrary, tGSH level in testis was 15% higher in the mining area (Reglero et al., 2009b). Moreover, red deer from the mining area had higher activities of superoxide dismutase (SOD) in liver (98%; Rodríguez-Estival et al., 2011) and glutathione peroxidase (GPX) in spleen (81–130%; Rodríguez-Estival et al., 2012) than in the control sites. The effect of metal pollution was again different in the reproductive system, because lower activities of GPX (−16%) and SOD (−14%) were found in testis, as well as lower activity of SOD in spermatozoa (−42%) (Reglero et al., 2009b). Testis and spermatozoa of red deer from the mining area also showed lower arachidonic acid (20:4n-6) percentages in the fatty acid composition than in the control area (Castellanos et al., 2010). The lower SOD activity found in testis was associated with a reduction in the Cu levels in testis of deer from the mining area (Reglero et al., 2009b). The percentage of 20:4n-6 in spermatozoa was also positively correlated with Cu levels in testis (Castellanos et al., 2010). These results suggested that Pb pollution in the mining area altered Cu homeostasis, and this had consequences on the activity of cytosolic and extracellular Cu/Zn SOD. Arachidonic acid is important in sperm function, because it is released from spermatozoa membranes by phospholipase A to be used by lipoxygenase for producing 15-hydroxyl-5,8,11,13-eicosatetraenoic acid (15-HETE) and prostaglandins (PGE) involved in the process of acrosome reaction and sperm capacitation (Breitbart and Spungin, 1997). These changes may explain the lower acrosome integrity and sperm viability found in red deer from Alcudia Valley–Sierra Madrona Pb mining area (Reglero et al., 2009b).

Oxidative DNA damage has been associated with lowered fertility in several species (Aitken and Sawyer, 2003; Gillan et al., 2005). Pb exposure in humans has been associated with oxidative damage of DNA and alteration of the chromatin stability in spermatozoa (Quintanilla-Vega et al., 2000; Xu et al., 2003). Both experimental studies with mice and epidemiological studies in humans have revealed negative effects of Pb exposure on chromatin fragmentation (Hernández-Ochoa et al., 2005, 2006). In the present study, we have tested the hypothesis that red deer from Alcudia Valley–Sierra Madrona mining area could have higher levels of DNA damage than deer from control sites. The sperm chromatin structure assay (SCSA) has been used in our study to detect DNA damage and/or alterations in the histones and protamines of nucleosomes (Evenson, 2013), and to analyze the correlation between genotoxic effect of Pb exposure, the quality parameters of sperm and their response to oxidative stress caused by this metal. The study of chromatin fragmentation has not been studied before in spermatozoa of wildlife species. The present study may show the potential of SCSA in wildlife species in which the amount of sperm that can be collected is very limited.

2. Material and methods

2.1. Study area

The study area extends along the Alcudia Valley–Sierra Madrona mining area and a control area at Montes de Ciudad Real region (Central Spain). Further details of these study areas have been described in Reglero et al. (2009a).

2.2. Sampling

Testes of 63 adult (>2 years of age) red deer (*Cervus elaphus*) were obtained from hunters during the autumns of 2008 and 2009 in the mining (n = 37) and the control areas (n = 26). The samples were collected after "monterias" (large driven hunts) in five hunting estates of the mining area and another five estates from the control area (Fig. S1). The testes were transported in a portable cooler to the laboratory within 1–2 h of being removed from the carcasses. Then, spermatozoa were collected from cauda epididymides as described by Soler et al. (2005). Briefly, spermatozoa were obtained by cutting the distal portion of the epididymis with a surgical blade and were placed in 1 ml of phosphate saline buffer (pH 7.2; 8 g/l NaCl, 0.2 g/l KCl, 0.1 g/l $MgCl_2$. $6H_20$, 0.1 g/l $CaCl_2$, 1 g/l Na_2HPO_4, 0.15 g/l NaH_2PO_4, and 0.2 g/l KH_2PO_4) containing bovine serum albumin (at 5 g/l). After sperm collection, motility, viabilility and acrosome integrity were evaluated. Moreover, sperm chromatin stability was assessed. The remaining sperm sample was frozen at −80 °C for the determination of oxidative stress biomarkers and Pb analysis. Testes were then frozen at −80 °C for further analysis of Pb levels in testicular parenchyma and epididymal cauda tissue.

2.3. Sperm quality measurements

After spermatozoa collection, a routine sperm evaluation was carried out. Sperm concentration, sperm viability and acrosome integrity were studied following the protocols described by Soler et al. (2005). Briefly, sperm concentration in the suspension was determined by microscopy in a Bürker counting chamber. The percentage individual motile sperm (motility) was noted, and the quality of motility was assessed using a scale that ranged from 0 (lowest, immobile or dead) to 5 (highest, progressive and vigorous movement). Membrane integrity was evaluated by using 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 sec, 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 in 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes (i.e., with normal apical ridges) was assessed at × 400 under phase-contrast optics.

2.4. Analysis of oxidative stress biomarkers

Sperm suspensions were adjusted to 240×10^6 spermatozoa/ml in 0.5 ml of buffer before antioxidants analysis. Levels of tGSH and the