POSSIBLE SIGNALLING PATHWAY OF LEAD THROUGH CASPASE–3 IN OVARIAN GRANULOSA CELLS OF PREGNANT GILTS

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ABSTRACT

Changes in the endocrine system might be a useful indicator of lead (Pb) exposure and its potential toxicity in animals. The study aimed at examining expression of apoptosis-related (caspase-3) peptide in porcine ovarian granulosa cells from pregnant gilts after lead addition. Ovarian granulosa cells were incubated with/without lead acetate for 18 hours: group Max - 0.5 $mg.ml^{-1}$ of lead acetate, group A - 0.25 $mg.ml^{-1}$, group B - 0.083 $mg.ml^{-1}$, group C - 0.063 $mg.ml^{-1}$, group D - 0.046 $mg.ml^{-1}$ and the control group - without Pb addition. Expression of caspase-3 was detected by immunocytochemistry. The expression of caspase-3 was influenced by Pb treatment. In our study, isolated ovarian granulosa cells were able to induce expression of apoptosis-related peptide after Pb administration at the doses of 0.5 $mg.ml^{-1}$; 0.25 $mg.ml^{-1}$; 0.083 $mg.ml^{-1}$; and 0.046 $mg.ml^{-1}$. Our results contribute to a knowledge of mechanisms of lead effect on ovarian granulosa cells of pregnant gilts. Obtained data indicate the interference of Pb in the pathway of apoptosis of porcine ovarian granulosa cells from pregnant gilts through intracellular substances such as caspase–3. This result contributes towards the understanding of mechanisms related to lead-induced alterations in porcine ovarian granulosa cells from pregnant gilts. This heavy metal (Pb) can be a potential risk factor during pregnancy of pigs.

Key words: porcine granulosa cells, pregnant gilts, lead, caspase-3

INTRODUCTION

Lead is a multimedia pollutant **(Kolesarova et al., 2009a,b,c,d; 2010; Shan et al., 2009)**, with multiple sources **(Rosner et al., 2005)**. Exposure through ambient air largely depends on the use of leaded gasoline. Lead exposure through inhalation, and ingestion, is increased near lead–emitting industries **(Skerfving and Bergdahl, 2007)**. Additional inhalation exposure occurs through cigarette smoking **(Skwarzec et al., 2001)**. At one time, lead acetate was used as a sweetener in wines. Furthermore, herbal medicinal products may contain lead **(Saper et al., 2004; Sjöstrand et al., 2007;)**. In particular, vegetables, fruit juice **(Tahiri et al., 2002)**, in specific foodstuffs and in glazed cookware **(Villalobos et al., 2009)** may lead to a large lead intake. Alcoholic beverages can also be a source of lead exposure **(Grandjean et al., 1981)**. Pb is likely to be easily absorbed in the lungs and gastrointestinal tract **(van Alphen, 1999)**. In the blood, most Pb is present in the erythrocytes, leaving less than 1% in plasma. Pb in erythrocytes is mainly bound to haemoglobin **(Simons, 1986; Bergdahl et al., 1997)**. Pb binding to haemoglobin is unaffected by oxygenation. Binding to albumin is quantitatively similar to binding to haemoglobin **(Simons, 1986)**. From the blood plasma, the absorbed Pb is distributed to other organs.

Accumulation of Pb in granulosa cells of the rat ovaries **(Nampoothiri and Gupta, 2006)**, sheep ovaries **(Bires et al., 1995)** and the liver and kidney of brown hares has been reported **(Kolesarova et al., 2008b)**. A number of studies showed a higher accumulation of Pb in the kidneys of animals and humans **(Massanyi et al., 2003; Kramarova et al., 2005; Celechovska et al., 2006; Jancova et al., 2006; López–Alonso et al., 2007)** and the liver of wild animals **(Kramarova et al., 2005; Kolesarova et al., 2008b)**. Pb is excreted from the body mainly through the urine **(Chiang et al., 2008)** and feces **(Claeys–Thoreau et al., 1987)**. Pb is also excreted through bile and pancreatic juice into duodenum and the feces **(Ishihara et al., 1987)**. Pb is also, to some extent, excreted in saliva **(Koh et al., 2003)** and

sweat **(Omokhodion and Cockford, 1991)**. Pb is also incorporated into the semen **(Esteban and Castaño, 2009)** and the placenta **(Skerfving and Bergdahl, 2007)**. Pb can accumulate in the fetus from the second trimester onwards **(Battacharayya, 1983; Borella et al., 1986)**. During lactation, Pb is excreted into the milk (**Namihira et al., 1993; Hallen et al., 1996)**.
The general objective of this *in vitro* study was to examine dose–dependent changes in the expression of apoptosis-related peptide caspase-3 in porcine ovarian granulosa cells from pregnant gilts after Pb experimental administration and to outline the potential intracellular mediator of its effect.

MATERIAL AND METHODS

Preparation, culture and processing of granulosa cells from ovaries

Slovakian White gilts at the age of 6 months were reared under standard conditions at the Experimental Station of the Animal Production Research Centre Nitra, Slovak Republic. Slovakian White gilts at the age of 6 months were hormonally synchronized using PMSG (1500 m.j., Sergón, Bioveta, Czech Republic) followed by HCG injection (1000 m.j., Werfachor, Austria) 72 h after PMSG and Supergestran injection (1 ml, Lecirelinum 25 $\mu g.ml^{-1}$, Ferring-Léčiva, Czech Republic). Artificial insemination was carried out 24-36 h after the HCG injection. The gilts were slaughtered at fourth days of pregnancy. Animal care, manipulations and use did correspond with the EC directive no. 178/2002 and were approved by local ethics committee. Porcine ovaries from pregnant gilts at the early and mid–follicular phase of the estrous cycle were obtained at slaughter house from healthy gilts without visible reproductive abnormalities. Ovaries were transported to the laboratory at 4ºC and washed in sterile physiological solution. Follicular fluid was aspirated from 3–5 mm follicles. Granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Belgium) and resuspended in the same medium supplemented with 10 % fetal calf serum (BioWhittaker™, Belgium) and 1 % antibiotic–antimycotic solution (Sigma, USA) at a final concentration of 10^6 $cells.ml^{-1}$ of medium (counted by haemocytometer). Portions of the cell suspension were cultured in Lab-Tek 16-welled chamber slides (Nunc Inc., USA, 100 µl per well; for immunocytochemistry) **(Kolesarova et al., 2008a; Kolesarova et al., 2010)**. The plate wells were incubated at 37.5 ºC and 5 % CO_2 in humidified air until a 75 % confluent monolayer was formed (5–7 days). At this point the medium (1 ml per well) was renewed and ovarian granulosa cells were incubated with the same supplements (10 % fetal calf serum, 1 % antibiotic–antimycotic solution) and with or without lead acetate administration – lead (II) acetate trihydrate [$Pb(CH_3COO)_2.3H_2O$] as follows: group Max (0.5 mg Pb. ml^{-1}), group A (1:1 dilution), group B (1:5 dilution), group C (1:7 dilution), group D (1:10 dilution) and the control group without administration of Pb. Maximum used dose was 0.5 $mg.ml^{-1}$ of lead acetate (0.273 $mg.ml^{-1}$ of Pb), group A 0.25 $mg.ml^{-1}$, group B 0.083 $mg.ml^{-1}$, group C 0.063 $mg.ml^{-1}$, group D 0.046 $mg.ml^{-1}$. Further culture of cells was done for 18 h, and then the culture media from plate wells were aspirated and stored at –20 ºC for further assay.

Immunocytochemistry

Signaling substance within granulosa cells plated on chamber slides were detected using immunocytochemistry based on a previous study **(Osborn and Isenberg, 1994)**. The ImmunoCruz Staining System and primary mouse monoclonal antibodies against caspase–3 (Santa Cruz Biotechnology Inc., USA) were used as directed by the manufacturer at a dilution of 1:500. Visualisation of the primary antibody binding sites was achieved with a secondary rabbit polyclonal antibody against mouse IGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:1000) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, USA, 10 %). Chamber slides stained with peroxidase/DAB reagent

were mounted with Glycergel (DAKO, USA) mounting medium. The general cell morphology, presence and localization of specific immunoreactivity in cells, as well as the counting of the percentage of cells containing specific immunoreactivity were performed by light microscopy **(Massanyi et al., 2007; Kolesarova et al., 2010)**.

Statistical analysis

Each experimental group was represented by four culture wells of cultured granulosa cells. Assays of substances in incubation medium were performed in duplicate. The data presented are means of values obtained in three separate experiments performed on separate days using separate pools of ovaries from 10 – 12 animals. Significant differences between the control and experimental groups were evaluated by paired chi–square (χ^2) test using statistical software Sigma Plot 11.0 (Jandel, Corte Madera, USA). The data are expressed as means ± SEM. Differences were compared for statistical significance at the level $P<0.05$.

RESULTS AND DISCUSSION

We observed reduction of the monolayer of granulosa cells after Pb addition by light microscopy (Fig. 1). Imunocytochemistry assay revealed the presence of apoptosis–associated peptide caspase–3 in ovarian granulosa cells of pregnant gilts (Fig. 1). The percentage of ovarian granulosa cells containing caspase-3 was significantly ($P<0.05$) increased by experimental Pb administration (Max 61.30±3.30 %; A 45.70±9.90 %; B 27.84±3.04 %; D 28.80±4.40 %) (Fig. 2). No significantly ($P>0.05$) higher value was demonstrated in the experimental group C (26.50±8.90 %) versus control (9.79±8.29 %) (Fig. 2).

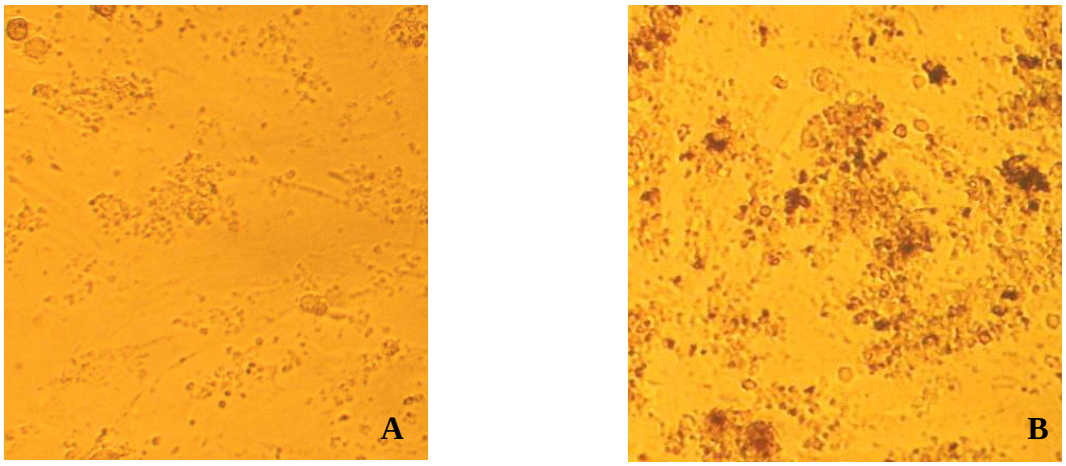

Figure 1 Effect of lead acetate administration on reduction of ovarian granulosa monolayer. A. Control represents culture medium without lead acetate addition. B. Group Max received lead acetate at 0,5 $mg.ml^{-1}$. Light microscopy (Magnification 45x).

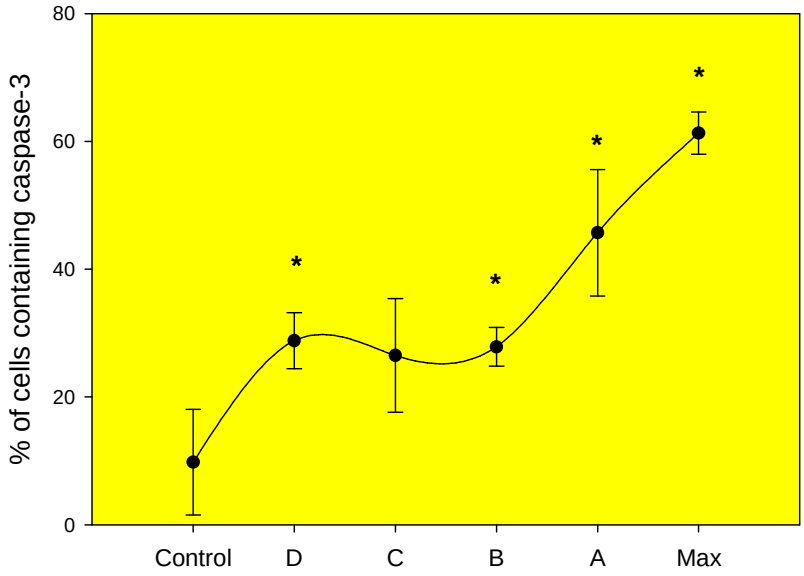

Figure 2 Effect of lead acetate on caspase-3 expression in ovarian granulosa cells from pregnant gilts. Group Max received lead acetate at 0.5 mg.ml^{-1}; group A 0.25 mg.ml^{-1}; group B 0.083 mg.ml^{-1}; group C 0.063 mg.ml^{-1}; and group D 0.046 mg.ml^{-1}. Values are means±SEM. *Significant differences from control P<0.05 were evaluated by chi-square (χ^2) test. Immunocytochemistry.

Lead is a ubiquitous heavy metal and its toxicity remains an important public health issue **(Tahiri et al., 2002)**. There is some information indicating an effect of Pb on female sexual maturation. Pb can delay sexual maturation; however, the mechanism and critical time of insult are not clearly defined **(Dearth et al., 2002)**. Lead is distributed to the ovary **(Barry, 1975)** and can exert a direct influence on granulosa cell function **(Priya et al., 2004)**. A low Pb concentration in the mouse ovary caused dysfunction of folliculogenesis, with fewer primordial follicles and an increase in atretic antral follicles **(Taupeau et al., 2001)**. Pb administration reduced the rate of reproduction as assessed by number of living viable embryos **(Gupta et al., 1995)**. Pb is embryotoxic and fetotoxic in experimental animals **(Skerfving and Bergdahl, 2007)**. The role of Pb in the control of pregnant ovarian granulosa cell functions related to caspase-3 is not known yet.

The present paper shows an presence of Pb in ovarian granulosa cells of pregnant gilts, as it was previously reported from human follicular fluid **(Silberstein et al., 2006; Al-Saleh et al., 2008)**. Lead was able to induced changes in porcine ovarian granulosa cells as it was published in previous studies in case porcine ovarian granulosa cells **(Kolesarova et al., 2009a,b,c; 2010)** and ovarian granulosa cells of pregnant gilts **(Kolesarova et al., 2009d)**. Our observations represent the influence of Pb on caspase-3 expression in ovarian granulosa cells from pregnant gilts. In our study isolated ovarian granulosa cells were able to induce expression of apoptosis-related peptide after Pb administration at the doses 0.5 mg.ml^{-1}; 0.25 mg.ml^{-1}; 0.083 mg.ml^{-1}; and 0.046 mg.ml^{-1} as it was previously reported in ovarian granulosa cells from non-pregnant gilts after Pb addition at the doses 0.5 mg.ml^{-1}; 0.25 mg.ml^{-1}; and 0.046 mg.ml^{-1} **(Kolesarova et al., 2010)**. Histological changes in the number of ovarian follicles and the increased occurrence of primary atretic follicles indicate alterations in the membrane structures and organelles of oocytes and in the follicular cells of the *stratum*

granulosum in sheep **(Bires et al., 1995)**. Exposure of human granulosa cells to cadmium and morphological alterations in the monolayer depending on dose and exposure time was examined **(Paksy et al., 1997)**. The effect of the Pb proved to be concentration dependent. While 100–400 μM lead had no effect on the integrity of the monolayer, concentrations as high as 800 μM or higher inhibited cell adhesion and induced detachment of cells **(Paksy et al., 2001)**. Similarly in our experiment we observed reduction of the monolayer of granulosa cells after Pb addition by light microscopy (Fig. 1). Besides preconceptional chromosome damage or a direct teratogenic effect on the fetus, interference with the maternal/fetal hormone environment or developmental toxicity to the embryo/fetus is possible. Also, vascular effects on the placenta are plausible; elevated blood pressure during pregnancy means increased risks to both the mother and fetus **(Skerfving and Bergdahl, 2007)**.

CONCLUSION

Obtained data indicate the interference of Pb in the pathway of apoptosis of porcine ovarian granulosa cells from pregnant gilts through intracellular substances such as caspase–3 (Fig. 3). These results contribute towards the understanding of mechanisms relating to lead-induced alterations in porcine ovarian granulosa cells from pregnant gilts (Fig. 3). This heavy metal - Pb, as an environment risk factor, can be a potential risk factor for during pregnancy of pigs.

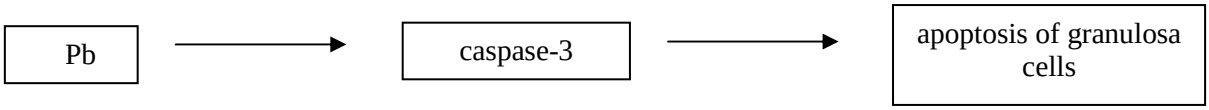

Figure 3 Possible signal pathway through caspase–3 affected by Pb compound.

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Acknowledgment
The authors are thankful to Katarina Tothova and Ing. Zofia Kuklova for skillful technical assistance. This work was financially supported by the Slovak Research and Development Agency project no. 0299–06 and the Ministry of Education of the Slovak Republic project no. 1/0696/08.

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