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Insulin-Like Growth Factor-I and Progesterone Release by Ovarian Granulosa Cells of Hens after Experimental Lead and Molybdenum Administrations *in vitro*

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Abstract: The general objective of this *in vitro* study was to examine the secretory activity of ovarian granulosa cells of hens after lead (Pb) and molybdenum (Mo) administrations. In this study the effect of various doses of Pb and Mo on the insulin-like growth factor-I (IGF-I) and progesterone (P_4) release by ovarian granulosa cells was demonstrated. Ovarian granulosa cells were incubated with lead acetate/ammonium molybdate-group A 0.09 mg/mL, group B 0.17 mg/mL, group C 0.33 mg/mL and the control group without Pb and Mo administrations for 18 h. The IGF-I and progesterone release by granulosa cells of hens was assessed by RIA. It was demonstrated that isolated ovarian cells were able to survive in culture and release hormones IGF-I and P_4 after experimental Pb and Mo administrations. Our observations demonstrated that the secretory activities of granulosa cells of hens were dependent on Pb and Mo doses. Lower Pb concentrations (0.09 mg/mL and 0.17 mg/mL) induced IGF-I release but higher dose (0.33 mg/mL) stimulated P_4 release by ovarian granulosa cells of hens. Higher Mo concentration (0.33 mg/mL) reduced of IGF-I release while doses 0.17 mg/mL and 0.33 mg/mL stimulated P_4 release by ovarian cells of hens. These findings contribute to our knowledge of the effect of lead and molybdenum on ovarian function of hens.

Key words: Lead, molybdenum, granulosa cells, ovary, hens

INTRODUCTION

Environmental pollution is one of the burning issues of the world (Ishaq *et al*., 2009). Heavy metals have been used by humans for thousands of years. Although several adverse health effects of heavy metals have been known for a long time, exposure to heavy metals continues (Jarüp, 2003). As it was published previously, the exposure of hens and chickens to heavy metals caused various alternations of zootechnical parameters (Arpasova *et al*., 2007; 2009) as well as inbalance in internal milieu (Capcarova *et al*., 2008; Kolesarova *et al*., 2008a).

The reproductive health of female could be affected by a number of endogenous as well as exogenous factors, such as exposure to heavy metals. Female reproductive functions can be compromised by exposure to toxic chemicals at a variety of sites, including ovary or reproductive tract (Mlynarcikova *et al*., 2005). Endocrine disruptors represent one class of environmental agent that can impact female fertility by altering ovarian development and function, purportedly through estrogenic, anti-estrogenic and/or anti-androgenic effects (Uzumcu and Zachow, 2007). In ovary of various animals can accumulate some heavy metals such as lead, cadmium (Nampoothiri *et al*., 2007), zinc and copper (Riggio *et al*., 2003). These metals has been shown to exert significant effects on ovarian and reproductive tract morphology, with extremely low dosages reported to stimulate ovarian luteal progesterone biosynthesis and high dosages inhibiting it (Henson and Chedrese, 2004). Lead and cadmium can alter steroid production *in vitro* and exerts a direct influence on granulosa cell function (Priya *et al*., 2004). According to Grasselli *et al*. (2005) cobalt chloride had no effect on progesterone production, although it significantly reduced oestradiol synthesis in swine granulosa cells.

Lead (Pb) is a ubiquitous environmental and industrial pollutant (Wang *et al*., 2006; Liu *et al*., 2001) which the concentrations have increased in the atmosphere as a consequence of extensive industrialization and environmental pollution (Fortoul *et al*., 1999). An accumulation of Pb in granulosa cells of the rat ovaries (Nampoothiri and Gupta, 2006), sheep ovaries (Bires *et al*., 1995), the liver and kidney of brown hares was reported (Kolesarova *et al*., 2008b).

Molybdenum (Mo) is an essential element (Mendel, 2009) for the function of nitrogenase in plants and as a cofactor for enzymes including xanthine oxidoreductase, aldehyde oxidase and sulfide oxidase in animals (National Toxicology Program, 1997). Mo is needed as catalytically active metal during enzyme catalysis. In humans four enzymes depend on Mo: sulfite oxidase, xanthine oxidoreductase, aldehyde oxidase and mitochondrial amidoxime reductase (Mendel, 2009).

Insulin-like growth factors I (IGF-I) is part of a family of peptides, structurally related to proinsulin, that stimulate

cell division and differentiation *in vitro* (Rotwein, 1991). IGF-I was measured in theca and granulosa cells from the ovary of the laying hens (Armstrong and Hogg, 1996). The ovary is a major site of hormonally regulated production of IGF-I (Giudice, 1992). IGF-I in conjunction with gonadotropins are important stimulators of mitosis and ovarian steroid production by granulosa and theca cells, which are required for normal oocyte development and hormonal feedback signalling to the hypothalamus and pituitary (Grado-Ahuir *et al*., 2009). IGF-I plays a key role in the proliferation and differentiation of granulosa cells (Tosca *et al*., 2008).

Progesterone (P_4) is an ovarian steroid (Hagan *et al*., 2008; Arnhold *et al*., 2009) produced by chicken ovarian cells (Sirotkin and Grossmann, 2007; Sirotkin *et al*., 2006). It is essential for normal ovarian cycle (Hagan *et al*., 2008; Arnhold *et al*., 2009). The effect of Pb and Mo in connection with IGF-I and progesterone release by ovarian granulosa cells of hens has not been examined previously. The general objective of this *in vitro* study was to examine the secretory activity of ovarian granulosa cells of hens after Pb and Mo administrations. In this study the effect of various doses of Pb and Mo on the IGF–I and P_4 release by ovarian cells of hens was demonstrated.

MATERIALS AND METHODS

Preparation, culture and processing of granulosa cells from ovaries: White Leghorn hens (n=12) about 500 days old, with an egg laying rate of more than 75%, were held under standard conditions at the Experimental Station of the Slovak Agricultural University in Nitra. Conditions of their care, manipulations and use did correspond the instruction of EC no. 178/2002 and related EC documents, and they were approved by local ethic commission. Birds were decapitated between 9:00 and 11:00 and the largest (F1-F2) follicles were isolated from the ovary. The stage of folliculogenesis was determined by recording the time of the last oviposit and by the size and position of the next ovarian follicle. Granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, Mo, USA) at a final concentration of 10^6 cells/mL of medium (determined by haemocytometer). Portions of the cell suspension were dispensed to 24-well culture plates (Nunc™, Roskilde, Denmark, 1 mL/well). The plate wells were incubated at 38.5°C and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (4 days). At this point the medium (1 mL/well) was renewed and ovarian granulosa cells were incubated with the 1% antibiotic-antimycotic solution and with lead acetate/ammonium molybdate administrations as follows: group A (0.09 mg/mL), group B (0.17 mg/mL), group C (0.33 mg/mL) and the control group without Pb and Mo additions for 18 h. The culture media from plate wells were aspirated and kept at -20°C to await further RIA.

Immunoassay: Concentrations of IGF-I and P_4 were determined in 25-100 µL incubation medium by RIA. These substances were assayed using RIA kits (Immunotech SAS, Marseille Cedex, France) according to the manufacturer's instructions (Makarevich and Sirotkin, 1999). All RIA were validated for use in samples of culture medium. RIA assay for IGF-I: the sensitivity was 2 ng/mL. Inter- and intra-assay coefficients of variation did not exceed 6.8% and -6.3%, respectively. RIA assay for P_4: the sensitivity was 0,05 ng/mL. Inter- and intra-assay coefficients of variation did not exceed 9.0% and 5.8%, respectively.

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. Significant differences between the control (without administration of lead acetate/ ammonium molybdate) and experimental groups (with lead acetate/molybdenum administrations -A, B, C) were evaluated by paired t-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 levels $p<0.05$.

RESULTS

Release of IGF-I by ovarian granulosa cells of hens after the lead addition: The IGF-I release by the ovarian granulosa cells in the control group without lead administration and in the experimental groups with lead addition are shown in Fig. 1. Granulosa cells of group A (1.09±0.08 ng/mL) and B (0.77±0.16 ng/mL) released significantly ($p<0.05$) higher concentrations of IGF-I than the control group (0.28±0.19 ng/mL). No significant ($p<0.05$) changes in IGF-I release by ovarian cells of group C (0.30±0.12 ng/mL) after lead administration was found in comparison with the control group.

Release of IGF-I by ovarian granulosa cells of hens after the molybdenum addition: No significant differences ($p>0.05$) in release of IGF-I by ovarian granulosa cells of hens in groups A (0.1±0.07 ng/mL) and B (0.31±0.18 ng/mL) after comparison with the control group were noticed (Fig. 2). Granulosa cells of group C with the highest experimental Mo administration released significantly ($p<0.05$) lower concentrations of IGF-I (0.0 ng/mL) versus the control group (0.28±0.19 ng/mL) (Fig. 2).

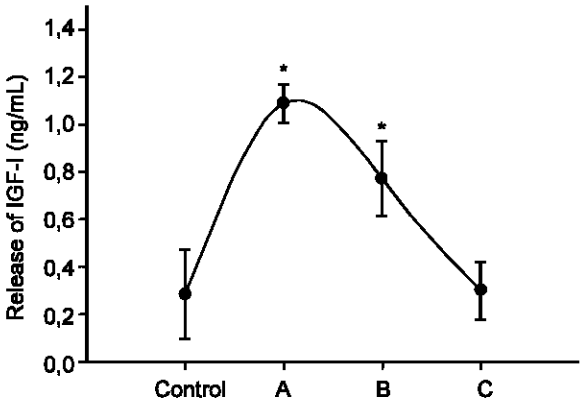

Fig. 1: Effect of lead administration on IGF-I release by ovarian granulosa cells of hens. Control represents culture medium without lead administration. Group A received lead acetate at 0.09 mg/mL, group B 0.17 mg/mL and group C 0.33 mg/mL. Values are means±SEM. *Significant differences from control p<0.05 were evaluated by paired t-test

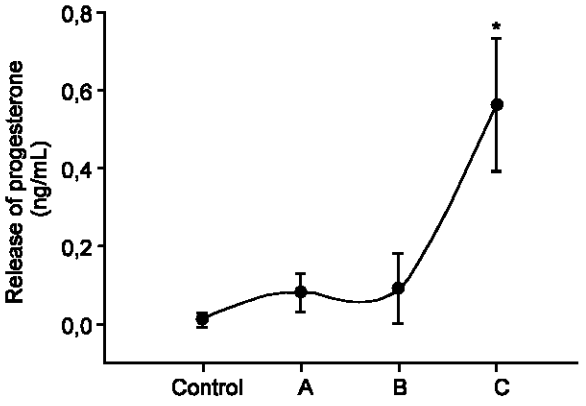

Fig. 3: Effect of lead administration on progesterone release by ovarian granulosa cells of hens. Control represents culture medium without lead administration. Group A received lead acetate at 0.09 mg/mL, group B 0.17 mg/mL and group C 0.33 mg/mL. Values are means±SEM. *Significant differences from control p<0.05 were evaluated by paired t-test

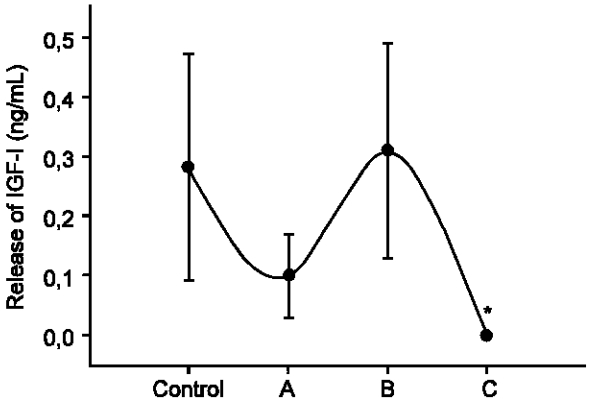

Fig. 2: Effect of molybdenum administration on IGF-I release by ovarian granulosa cells of hens. Control represents culture medium without molybdenum administration. Group A received ammonium molybdate at 0.09 mg/mL, group B 0.17 mg/mL and group C 0.33 mg/mL. Values are means±SEM. *Significant differences from control p<0.05 were evaluated by paired t-test

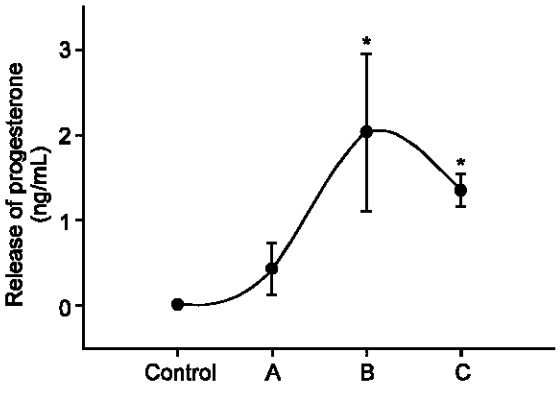

Fig. 4: Effect of molybdenum administration on progesterone release by ovarian granulosa cells of hens. Control represents culture medium without molybdenum administration. Group A received ammonium molybdate at 0.09 mg/mL, group B 0.17 mg/mL and group C 0.33 mg/mL. Values are means±SEM. *Significant differences from control p<0.05 were evaluated by paired t-test

Release of progesterone by ovarian granulosa cells of hens after the lead addition: Release of steroid hormone P_4 by granulosa cells of hens showed no significant (p>0.05) differences in groups A (0.08±0.05 ng/mL) and B (0.09±0.09 ng/mL) in comparison with the control group (Fig. 3). Significantly (p<0.05) the highest amount of P_4 was released by ovarian cells in the group C (0.56±0.17 ng/mL) with the highest Pb administration used in this study (Fig. 3).

Release of progesterone by ovarian granulosa cells of hens after the molybdenum addition: Significant changes in progesterone release by ovarian cells after Mo administration were noticed (Fig. 4). Higher doses of Mo in the experimental groups B (2.03±0.92 ng/mL) and C (1.35±0.19 ng/mL) significantly (p<0.05) stimulated release of P_4 by granulosa cells. No significant (p>0.05) differences in release of P_4 by

ovarian cells after Mo addition in group A (0.43±0.30 ng/mL) when compared with the control group was found.

DISCUSSION

The effect of Pb and Mo on secretory activity of ovarian granulosa cells of hens was demonstrated in this paper. In our study isolated ovarian cells were able to survive in culture and release hormones IGF-I and P_4 after experimental Pb and Mo administrations.

The release of IGF-I by chicken ovarian cells was previously studied (Sirotkin *et al*., 2006; Armstrong and Hogg, 1996). In our experiment granulosa cells of hens with Pb addition (0.09 mg/mL and 0.17 mg/mL) released significantly higher concentrations of IGF-I than the control group without Pb addition. Higher Pb dose (0.33 mg/mL) did not have any effect on the IGF-I release by ovarian cells. The highest production of P_4 by porcine ovarian granulosa cells in case of cadmium treatment was found in the group with addition of 10 ng $CdCl_2$/mL. When the dose of cadmium increased to 20 ng $CdCl_2$/mL its production decreased (Massany *et al*., 2000). Cadmium-induced alterations in the production of progesterone by the human granulosa cells were determined after exposure to concentrations of 8, 16, 32 and 64 μM $CdCl_2$ for 2, 4, 8, 24 and 48 h (Paksy *et al*., 1997). Pre-pubertal female rats maternally exposed to Pb exhibited suppressed serum levels of IGF-I and delayed puberty (Pine *et al*., 2006). The time to puberty onset in mice was markedly influenced by exposure to dietary lead (Iavicoli *et al*., 2004). But our investigation suggests that already low concentrations of lead can affect release of IGF-I by ovarian cells of hens.

It is postulated that Mo administered as thiomolybdate adversely affects the hypothalamo-adenohypophyseal system by interfering with trophic hormone release, leading to the cessation of reproductive activity and ultimately the failure of intermediary metabolism. Whether Mo exerts its effect centrally or directly on the pituitary was not established (Haywood *et al*., 2004). Mo, at high concentrations, induces changes in the epiphyseal growth plate through its effects on copper metabolism. Mo can induce changes in longitudinal bone growth which are distinct from those resulting from copper deficiency (Parry *et al*., 1993). The testes are more sensitive to Mo exposure than the female reproductive organs (Bersényi *et al*., 2008). The highest Mo dose (0.33 mg/mL) used in this study significantly inhibited release of IGF-I by granulosa cells of hens while lower Mo doses did not have the effect on the IGF-I release. The high dietary Mo contents failed to reduce the growth performance of rabbits (Bersényi *et al*., 2008). Our investigation suggests that higher concentrations of Mo can reduce IGF-I release by ovarian cells of hens.

In our experiment steroid hormone P_4 was released by ovarian granulosa cells of hens. The highest Pb dose (0.33 mg/mL) used in this study significantly stimulated release of P_4 while other doses did not influence steroid release. Lead *in vitro* at 1.600 μM (331.5 mg/L) resulted in a significant decrease in progesterone production (Paksy *et al*., 2001).

The higher doses of Mo used in this study (0.17 mg/mL and 0.33 mg/mL) significantly stimulated release of P_4 by granulosa cells. No significant difference in release of P_4 by ovarian cells after Mo addition (0.09 mg/mL) was found. Higher concentrations of Mo can induce P_4 release by ovarian cells of hens. To our knowledge, there are not a lot of similar studies on effect of Mo on IGF-I and P_4 release by granulosa cells of hens.

Conclusion: In conclusion, isolated ovarian cells of hens were able to survive in culture and release hormones IGF-I and P_4 after experimental Pb and Mo administrations. Our observations demonstrated that the secretory activities of granulosa cells of hens were dependent on Pb and Mo doses. Lower Pb concentrations (0.09 mg/mL and 0.17 mg/mL) induced IGF-I release but higher dose (0.33 mg/mL) stimulated P_4 release by ovarian granulosa cells of hens. Higher Mo concentration (0.33 mg/mL) reduced of IGF-I release while doses 0.17 mg/mL and 0.33 mg/mL stimulated P_4 release by ovarian cells of hens. These findings contribute to our knowledge of the effect of lead and molybdenum on ovarian function of hens.

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