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EFFECT OF LEAD, SILVER AND MOLYBDENUM ON STEROIDOGENESIS IN PORCINE OVARIAN GRANULOSA CELLS *IN VITRO*

WPŁYW OŁOWIU, SREBRA I MOLIBDENU NA STEROIDOGENEZĘ *IN VITRO* W KOMÓRKACH ZIARNISTYCH JAJNIKÓW ŚWINI

Abstract: The present study was carried out to investigate possible effects of lead (Pb), silver (Ag) and molybdenum (Mo) administrations on porcine ovarian granulosa cells in relation to progesterone (P_4) release. Ovarian granulosa cells were incubated with/without lead acetate, silver nitrate and ammonium molybdate for 18 hours: 1.0 mg/cm^3; 0.5 mg/cm^3; 0.33 mg/cm^3; 0.17 mg/cm^3; 0.09 mg/cm^3 and the control group without metal addition. The release of progesterone by granulosa cells was assessed by RIA. The release of steroid hormone P_4 was significantly ($p < 0.05$) inhibited after Pb administration at the dose 1.0 mg/cm^3. Secretion of P_4 by granulosa cells was decreased by Ag addition at the doses 0.5 mg/cm^3; 0.33 mg/cm^3; 0.17 mg/cm^3 and 0.09 mg/cm^3. Significant ($p < 0.05$) increase of P_4 release after Mo addition was found. Data obtained from these *in vitro* studies indicate new knowledge that release of steroid hormone progesterone by porcine granulosa cells is associated with doses and variety of chemical treatments (Pb, Ag, Mo). Obtained data indicate the interference of these endocrine disruptors in the pathways of steroidogenesis of porcine ovarian granulosa cells.

Keywords: lead, silver, molybdenum, progesterone, steroidogenesis, granulosa cells

Environmental pollution is one of the major issues of today's world [1]. 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 [2].

Lead is a ubiquitous environmental and industrial pollutant [3–6] found in air, water, brass plumbing fixtures, soil [7, 8], and some foods, vegetables and rice [7]. An

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accumulation of Pb in granulosa cells of the rat ovaries [9], sheep ovaries [10], the liver and kidney of brown hares was reported [11]. Lead can induce ovarian changes in sheep [10] and ovarian granulosa cell toxicities [9].

The primary use of **silver** (Ag) is in industrial applications, including use as conductors, switches, and contacts; plating applications; silver brazing or soldering; and miscellaneous uses such as mirrors, batteries, and catalysts. The second most important use is the production of coins, jewellery, and tableware [12]. Silver salts are also used as disinfectants. Recently, production of silver nanoparticles has been used in various nanotechnologies [13] and suggested as antibacterial agent [14]. The highest concentrations of Ag are usually found in the liver and spleen, and to some extent, in muscles, skin, and brain after ingestion. Water-soluble Ag compounds such as silver nitrate have a local corrosive effect. Repeated exposure in animals causes growth retardation and degenerative changes in the liver. The metal Ag preferentially accumulated in the kidney of scallop, with much lower concentrations in the other organs [15].

Molybdenum (Mo) is an essential trace element [16]. It is of considerable industrial importance, especially in the production of strong, high quality steels for making heavy machines and industrial hardware [17]. Mo is found in all foods and beverages [18], usually at low levels of less than 1 mg/kg. Animal offal and nuts appear to be the only foodstuffs that contain its relatively high levels. In certain conditions, such as when soil is either naturally rich in Mo or has been contaminated by industrial activity, certain food crops and other plants may accumulate unusually high levels of the metal. High levels of Mo have also been detected in plants grown on soil which has been treated with sewage sludge and certain fertilisers [17]. Mo is an essential component of several enzymes [19, 20], including xanthine oxidase and xanthine dehydrogenase [20]. It fulfils important cell functions [21]. Polyoxomolybdates as discrete molybdenum--oxide cluster anions have been investigated in the course of study of their medical applications [22]. Potential anticancer cytostatic and cytotoxic effects of piroxicam complexes with MoO_2^{2+} on human promyelocytic leukemia HL-60 cells have been investigated [23]. Polyoxomolybdates provide promising, novel anti-tumor agents, especially for cancers that are difficult to treat [24]. The effects of tetrathiomolybdate analogue (ATN-224) on endothelial and tumor cell growth were evaluated in cell culture experiments *in vitro*. ATN-224 inhibits superoxide dismutase 1 (SOD1) in tumor and endothelial cells [25].

Progesterone (P_4) is an ovarian steroid [26, 27] produced by porcine ovarian granulosa cells [28, 29] and *corpus luteum* of pigs [30]. It is essential for normal ovarian cycle [26, 27], sexual maturation [31], breast development and embryo development [26, 27]. It is among the intraovarian signals that contribute to regulation of ovarian follicular development and remodeling [30].

The general objective of this *in vitro* study was to examine the secretory activity of porcine ovarian granulosa cells after lead, silver and molybdenum administrations. The study also aimed at examining release of progesterone by porcine ovarian granulosa cells after metal additions.

Material and methods

Preparation, culture and processing of granulosa cells from ovaries

Slovakian White gilts at the ages of 100–120 days were kept under standard conditions at the Experimental Station of the Animal Production Research Centre Nitra. Conditions of their care, manipulations and use corresponded to the instruction of EC no. 178/2002 and related EC documents, and they were approved by local ethics commission. Porcine ovaries at the early and mid-follicular phase of the estrous cycle were obtained from healthy gilts without visible reproductive abnormalities. Ovaries were transported to the laboratory at 4 ^{o}C 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™, 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/cm^3 (determined by haemocytometer). Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1 cm^3 per well) for radioimmunoanalysis (RIA). The plate wells were incubated at 37.5 ^{o}C and 5 % CO_2 in humidified air until a 75 % confluent monolayer was formed (5–7 days). At this point, the medium (1 cm^3 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 chemical substances: lead acetate $Pb(CH_3COO)_2 \cdot 3H_2O$, silver nitrate $AgNO_3$ and ammonium molybdate $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$. The concentrations were diluted as described in Table 1. Further culture was performed for 18 h, and then the culture media from plate wells were aspirated and kept at –20 ^{o}C for further assay.

Table 1

Lead, silver and molybdenum concentrations used in the study

Group	$Pb(CH_3COO)_2 \cdot 3H_2O$, $AgNO_3$, $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$ [mg/cm^3]	Medium [cm^3]	Dilutio rate	Concentrations of $Pb(CH_3COO)_2 \cdot 3H_2O$, $AgNO_3$, $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$ [mg/cm^3]
Control	0	1	0:1	0
Max	1	0	1:0	1.0
A	0.5	0.5	1:1	0.5
B	0.33	0.67	1:2	0.33
C	0.17	0.83	1:5	0.17
D	0.09	0.91	1:10	0.09

Maximum used dose: 1.0 mg $Pb(CH_3COO)_2 \cdot 3H_2O$/cm^3 = 0.546 mg Pb/cm^3; Maximum used dose: 1.0 mg $AgNO_3$/cm^3 = 0.6349 mg Ag/cm^3; Maximum used dose: 1.0 mg $(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$/cm^3 = 0.0776 mg Mo/cm^3.

Immunoassay

Concentrations of P_4 were determined in 25–100 mm^3 incubation medium by RIA. This substance was assayed using RIA kits (Immunotech SAS, Marseille Cedex, France) according to the manufacturer's instructions [3, 31, 32]. All RIA were validated for use in samples of culture medium. RIA assay sensitivity for P_4 was 0.05 ng/cm^3. 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. Assay of substance in incubation medium was 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 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 level $p < 0.05$.

Results

Release of progesterone by porcine ovarian granulosa cells

The reduction of the monolayer of granulosa cells after Pb addition was found by light microscopy (Fig. 1). The release of P_4 by porcine ovarian granulosa cells was 23.37 ± 1.28 ng/cm^3 in the control group. Release of steroid hormone P_4 by granulosa

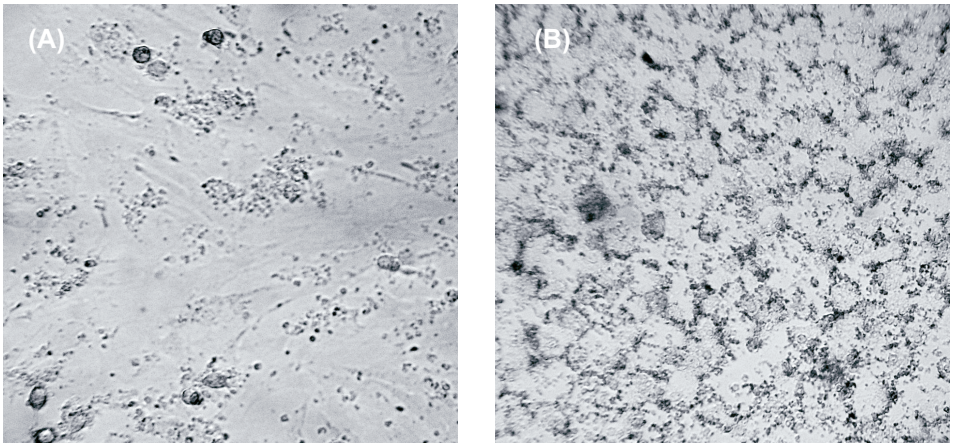

Fig. 1. Effect of lead on monolayer of ovarian granulosa cells. A – Control represents culture medium without lead addition. B – Group Max received lead acetate at 1.0 mg/cm^3. Light microscopy (Magnification 45x)

cells in the group Max (6.95 ± 0.84 ng/cm^3) was significantly ($p < 0.05$) inhibited after Pb administration. Groups A (21.07 ± 3.23 ng/cm^3), B (27.72 ± 3.33 ng/cm^3), C (28.03 ± 3.02 ng/cm^3) and D (25.98 ± 0.94 ng/cm^3) showed no significant ($p > 0.05$) differences in comparison with control group (Fig. 2). The lowest amount of P_4 was released by ovarian cells in the experimental group Max with the highest Pb administration used in this study.

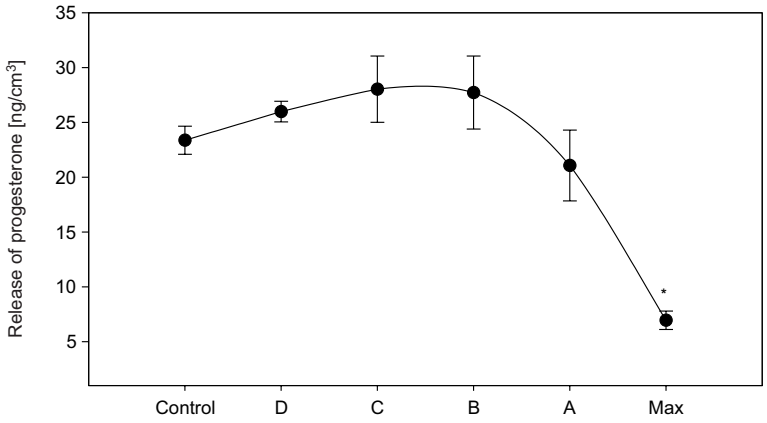

Fig. 2. Effect of lead on progesterone release by porcine ovarian granulosa cells. Control represents culture medium without lead addition. Group Max received lead acetate at 1.0 mg/cm^3; group A 0.5 mg/cm^3; group B 0.33 mg/cm^3; group C 0.17 mg/cm^3; and group D 0.09 mg/cm^3. Values are means ± SEM. * Significant differences in comparison with control $p < 0.05$ were evaluated by paired t-test. RIA

The reduction of the monolayer of granulosa cells after Ag addition by light microscopy (Fig. 3) was found, too. The release of P_4 by ovarian granulosa cells was 24.72 ± 1.56 ng/cm^3 in the control group (Fig. 4). Release of P_4 by granulosa cells of

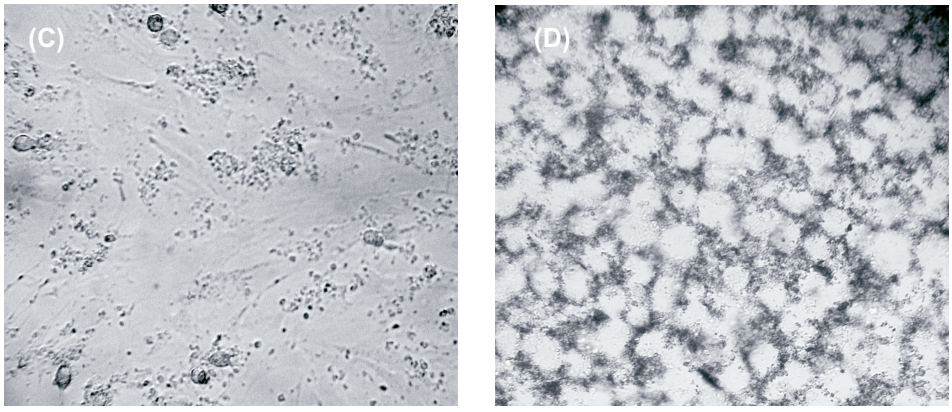

Fig. 3. Effect of silver on monolayer of ovarian granulosa cells. C – Control represents culture medium without silver addition. D – Group Max received silver nitrate at 1.0 mg/cm^3. Light microscopy (Magnification 45x)

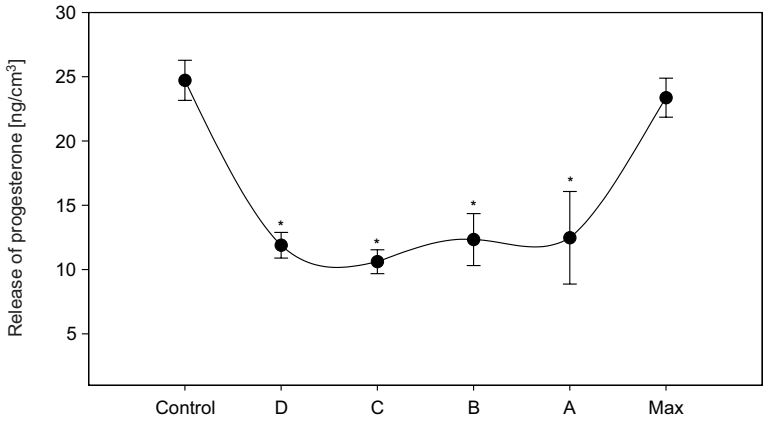

Fig. 4. Effect of silver on progesterone release by porcine ovarian granulosa cells. Control represents culture medium without silver addition. Group Max received silver nitrate at 1.0 mg/cm^3; group A 0.5 mg/cm^3; group B 0.33 mg/cm^3; group C 0.17 mg/cm^3; and group D 0.09 mg/cm^3. Values are means ± SEM. * Significant differences in comparison to control $p < 0.05$ were evaluated by paired t-test. RIA

experimental groups A (12.47 ± 3.60 ng/cm^3), B (12.33 ± 2.02 ng/cm^3), C (10.61 ± 0.93 ng/cm^3) and D (11.89 ± 1.00 ng/cm^3) showed significant ($p < 0.05$) inhibition compared with the control group (Fig. 4). Similar release of P_4 was found in control and Max groups (23.37 ± 1.52 ng/cm^3).

The reduction of the monolayer of granulosa cells after Mo addition was found with light microscopy (Fig. 5). The release of P_4 by porcine ovarian granulosa cells was 17.01 ± 2.53 ng/cm^3 in the control group. P_4 release by granulosa cells of experimental groups A (21.59 ± 1.38 ng/cm^3), B (22.29 ± 2.85 ng/cm^3), C (20.93 ± 0.99 ng/cm^3) and D (16.61 ± 1.60 ng/cm^3) showed no significant ($p > 0.05$) differences in comparison

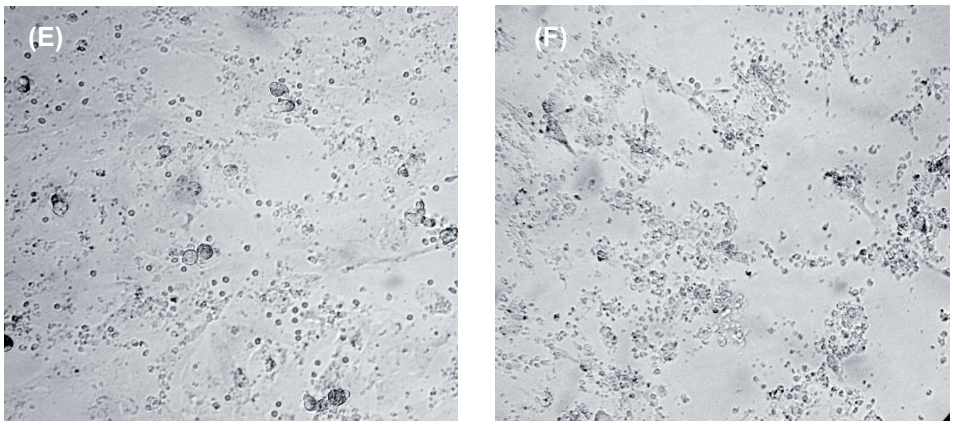

Fig. 5. Effect of molybdenum on monolayer of ovarian granulosa cells. E – Control represents culture medium without molybdenum. F – Group Max received ammonium molybdate at 1.0 mg/cm^3. Light microscopy (Magnification 45x)

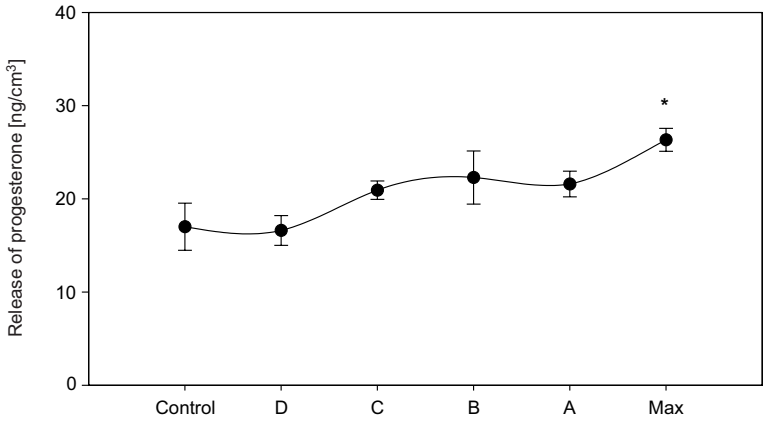

Fig. 6. Effect of molybdenum addition on progesterone release by porcine ovarian granulosa cells. Control represents culture medium without molybdenum addition. Group Max received ammonium molybdate at 1.0 mg/cm^3, group A 0.5 mg/cm^3, group B 0.33 mg/cm^3, group C 0.17 mg/cm^3, group D 0.09 mg/cm^3. Values are means ± SEM. *Significant differences in comparison with control $p < 0.05$ were evaluated by paired t-test. RIA

with control group (Fig 6). Significant ($p < 0.05$) increase comparing with the control group was found in group Max (26.33 ± 1.23 ng/cm^3).

Discussion

Our reports confirm previous data about influence of heavy metals on cellular processes [3, 4, 11, 33]. Neurodevelopmental toxins, such as heavy metals, interrupt growth factor signalling [34]. The effect of metals on organisms can range from acute mortality to chronic effects such as reductions in growth and reproductive output [35]. The levels of elements in follicular fluid (FF) of patients and evaluate the relationship between the concentration of elements in FF, follicular volume, and blood was determined by Silberstein [36].

Lead is the most extensively studied reproductive and developmental toxicant [3, 4, 11]. An accumulation of Pb in granulosa cells of the rat ovaries [9], sheep ovaries [10], chicken granulosa cells [4], human ovarian granulosa cells [37] and porcine granulosa cells [3, 5] was reported. Our observations represent the demonstration of Pb influence on secretory activity of porcine ovarian granulosa. In our study isolated ovarian granulosa cells were able to survive in culture and the release hormones P_4. In our experiments steroid hormone progesterone was released by porcine ovarian granulosa cells. Our observations confirm previous reports on the production of progesterone by porcine ovarian granulosa cells [4, 28, 31]. Pb can cause a reduction in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) binding, which significantly alters steroid production *in vitro* and exerts a direct influence on granulosa cell function [38]. In our study P_4 release by granulosa cells was inhibited after Pb addition at the highest dose 1.00 mg/cm^3. The Pb concentrations (0.5–0.046 mg/cm^3) used in the

present study did not induce the release of P_4 by porcine ovarian granulosa cells [3]. On the contrary, the P_4 release by ovarian granulosa cells of pregnant gilts was significantly stimulated by Pb addition at doses of 0.25 mg/cm^3 and 0.063 mg/cm^3 [3]. Similarly, the P_4 release by granulosa cells of pregnant gilts was significantly stimulated by Hg addition at the doses of 0.25 mg/cm^3 and 0.083 mg/cm^3. P_4 release by ovarian cells of pregnant gilts was not influenced by FSH (1.0 ng/cm^3) + Pb (0.083 mg/cm^3) + Hg (0.083 mg/cm^3) but it was inhibited by the FSH (10 ng/cm^3) + Pb (0.25 mg/cm^3) + Hg (0.25 mg/cm^3) administrations. Further observations suggest possible involvement of the heavy metals Pb and Hg, and pituitary hormone FSH, in the regulation of P_4 release by porcine ovarian granulosa cells of pregnant gilts. Progesterone release by chicken granulosa cells was stimulated after 0.33 mg/cm^3 lead addition [4]. In another report, lead seemed not to exert a specific effect on the steroidogenesis in cultured human granulosa cells, but lead application *in vitro* at 1.600 μM (331.5 mg/dm^3) resulted in a significant decrease in progesterone production. The lead levels measured in the ovarian follicular fluid seemed not to pose a hazard with respect to progesterone secretion by the ovary [37]. The highest production of P_4 by porcine ovarian granulosa cells, in the case cadmium treatment, was found in the group with addition of 10 ng/cm^3 cadmium chloride ($CdCl_2$), and when the dose of cadmium was increased to 20 ng/cm^3 $CdCl_2$ its production decreased [29]. 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 [37]. Data obtained from *in vitro* study indicate that the hormonal release by porcine ovarian granulosa cells is associated with the dose of the metals administration, animal species, and also depends on pregnancy of animals.

The increased use of nano-sized metallic materials is likely to result in the release of these particles into the environment [39]. Silver is not toxic to humans and is not known to cause cancer, reproductive or neurological damage or other chronic adverse effects. Microorganisms could develop resistance to antibacterial silver because an organism would have to undergo simultaneous mutations in every one of its critical functions within a single generation to avoid the effects of silver [40]. This study was conducted to test the effect of Ag on secretory activity of porcine ovarian granulosa cells. In our experiments steroid hormone progesterone was released by porcine ovarian granulosa cells after experimental Ag administration. The release of steroid hormone P_4 by granulosa cells was inhibited by Ag addition at the doses 0.5 mg/cm^3, 0.33 mg/cm^3, 0.17 mg/cm^3 and 0.09 mg/cm^3. Similar release of P_4 was found in control and group with the highest experimental Ag (1.00 mg/cm^3) administration. In other report the progesterone-AAG (α_1-acid glycoprotein, orosomucoid) interaction was inhibited by $Hg^{2+} > Ag^{+} > Cu^{+} > Fe^{2+}$ [41]. The *in vitro* steroid binding process was found to be sensitive to the presence of certain metal ions. The cations seemed to interfere directly with SH groups at the progesterone binding site. The most potent inhibitor of the binding was Hg(II) (50 % decrease in binding in the presence of 8 μmol · dm^{-3}) followed by Cu(II) (10 μmol · dm^{-3}), Ag(I) (13 μmol · dm^{-3}), Zn(II) (17 μmol · dm^{-3}) and Fe(II) (2.5 mmol · dm^{-3}) [42]. The role of Ag in control of porcine ovarian granulosa cells functions related to P_4 is not known yet. The results of our investigation

show that Ag, when given at the highest dose, did not affect P_4 output by porcine ovarian granulosa cells, but Ag at lower doses decreased release of P_4 by granulosa cells. This chemical element can be suppressor of ovarian steroidogenesis and potential risk factor for reproductive functions regulated by steroid hormones.

Exposure to a number of metals can affect neuroendocrine and thyroid signalling, which can result in adverse effects on development, behaviour, metabolism, reproduction, and other functions [43]. The testes are more sensitive to Mo exposure to the female reproductive organs [44]. Our studies are first contribution about the effect of Mo on the ovarian chicken granulosa cells [4] and porcine ovarian granulosa cells. Progesterone release by porcine ovarian granulosa cells was stimulated by the addition of Mo at the dose 1.0 mg/cm^3. Our previous study [4] shows that progesterone release by chicken granulosa cells was stimulated by Mo doses 0.17 mg/cm^3 and 0.33 mg/cm^3. The effect of an induced Cu deficiency on the fertility of South Africa Mutton Merino ewes (*Ovis aries*) was investigated. The incidence of estrus of adult ewes suffering from an induced Cu deficiency by supplementing molybdenum (Mo – 38 mg Mo/kg feed) and sulphur (S – 0.34 %) to their diet was compared with that of a control group (Mo – 1.3 mg/kg; S – 0.22 %). No significant differences in plasma progesterone concentrations were recorded during the estrus cycles. It is suggested that Mo and S induced Cu deficiency inhibits gonadothropin releasing hormone (GnRH) release or the production of FSH and/or LH to such an extent that cyclicity in the ewe is suppressed [45]. Meador et al [46] show that Mo, membrane-permeable form of the Ca^{2+} – chelating agent EGTA, or protease inhibitors substantially increases detectable rat uterine progesterone receptors. Thiomolybdate depressed estradiol production in a dose-dependent manner at doses >1 μg/cm^3 and prevented the characteristic clumped appearance of granulosa cells in this serum-free system. Although the supplementation of copper alone had no effect at physiological doses, the use of the equimolar copper and thiomolybdate media ameliorated the effect of tetrathiomolybdates on both estradiol production and cellular morphology [47]. In our present study the effect of Mo addition on the progesterone release by granulosa cells was examined. The report shows a direct influence of Mo on granulosa cells functions (steroidogenesis).

Data obtained from these *in vitro* studies bring new knowledge that release of steroid hormone progesterone by porcine granulosa cells is associated with doses and variety of chemical treatments (Pb, Ag, Mo). Obtained data indicate the interference of these endocrine disruptors in the pathways of steroidogenesis of porcine ovarian granulosa cells.

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WPŁYW OŁOWIU, SREBRA I MOLIBDENU NA STEROIDOGENEZĘ *IN VITRO* W KOMÓRKACH ZIARNISTYCH JAJNIKÓW ŚWINI

Abstrakt: Zbadano wpływ ołowiu (Pb), srebra (Ag) i molibdenu na wydzielanie progesteronu (P_4) przez komórki ziarniste jajników świni. Komórki ziarniste inkubowano w obecności octanu ołowiu, azotanu srebra i molibdenianu amonu przez 18 godzin: 1.0 mg/cm^3; 0.5 mg/cm^3; 0.33 mg/cm^3; 0.17 mg/cm^3; 0.09 mg/cm^3. Założono również grupę kontrolną komórek ziarnistych, które nie były eksponowane na jony metali. Wydzielanie progesteronu przez komórki ziarniste zostało zbadane metodą RIA. Wydzielanie sterydu P_4 przez komórki ziarniste zostało zahamowane w sposób statystycznie istotny ($p < 0.05$) przez ołów podany w dawce 1 mg/cm^3. Wydzielenie P_4 zmniejszyło się w obecności Ag w stężeniach 0.5 mg/cm^3; 0.33 mg/cm^3; 0.17 mg/cm^3 i 0.09 mg/cm^3. Stwierdzono statystycznie istotny wzrost wydzielania P_4 po podaniu Mo ($p < 0.05$). Wyniki uzyskane w przedstawionym eksperymencie prowadzonym *in vitro* wykazują, że wydzielanie progesteronu przez komórki ziarniste jajników świni może być modyfikowane przez różne czynniki chemiczne (Pb, Ag, Mo) stosowane w zróżnicowanych dawkach. Odnotowane zjawisko jest prawdopodobnie związane z zaburzeniem niektórych szlaków steroidogenezy zachodzącej w komórkach ziarnistych jajników świni.

Słowa kluczowe: ołów, srebro, molibden, progesteron, steroidogeneza, komórki ziarniste