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REGULAR ARTICLE

ANTIOXIDANT STATUS AND EXPRESSION OF HEAT SHOCK PROTEIN OF COBALT-TREATED PORCINE OVARIAN GRANULOSA CELLS

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ABSTRACT

The aim of this study was to determine the activity of superoxide dismutase (SOD), total antioxidant status (TAS) and expression of heat shock protein 70 (Hsp70) of porcine ovarian granulosa cells cultured *in vitro* after cobalt (Co) administrations. Ovarian granulosa cells were incubated with cobalt sulphate administrations as follows: group E1 (0.09 $mg.ml^{-1}$), group E2 (0.13 $mg.ml^{-1}$), group E3 (0.17 $mg.ml^{-1}$), group E4 (0.33 $mg.ml^{-1}$), group E5 (0.5 $mg.ml^{-1}$) and the control group without any additions for 18 h. Co administration developed stress reaction and promoted accumulation of Hsp70 what resulted in increasing activity of SOD. TAS of granulosa cells increased with higher doses of Co whereas low doses had no effect on this parameter. Trace elements can adversely affect animal female reproductive system and its functions, through either direct or indirect effects on oxidative stress induction.

Keywords: Granulosa cells, cobalt, SOD, TAS, Hsp70

INTRODUCTION

Cobalt (Co) is an essential element; it is a constituent of vitamin B12 **(Neve, 1991).** Inhalation of Co is associated with generation of reactive oxygen species (ROS) **(Stefaniak *et al.*, 2009).**

ROS are the by-products of normal cell metabolism during enzymatic electron-transporting processes such as mitochondrial respiration, and there is an array of antioxidant systems to maintain the redox balance **(Kakkar and Singh, 2007).** Under normal conditions, it is unavoidable for oxygen to be transformed into ROS, such as hydrogen peroxide, superoxide anions and hydroxyl radicals **(Andreyev *et al.,* 2005).** Excessive accumulation of ROS can result in the development of oxidative stress **(Clarkson and Thompson, 2000)**. Small physiological amounts of ROS are required in signalling pathways for cellular activation **(Hansen *et al.*, 2006).** Total antioxidant status (TAS) represents the level of cumulative antioxidant reserve of the body and enables evaluation of the average antioxidant potential. Thus, the overall antioxidant status will give more relevant biological information compared to that obtained by the measurement of individual components **(Millet *et al.*, 1993)**. Heat shock proteins (Hsps) belong to a large and diverse group of unrelated proteins known as chaperones that assist in correct non-covalent assembly and/or disassembly of other polypeptide-containing structure **(Ellis, 1997)** and are found in eukaryotes and prokaryotes **(Tsan and Gao, 2004; Luo *et al.*, 2008).** The most pronounced stress-related changes and involvement in different cell functions have been demonstrated for HSP70 **(Beere, 2004; Jego *et al.*, 2010; Sirotkin and Bauer, 2011)**. It has been shown that Hsp70 is released from various cells (glial cells, peripheral blood mononuclear cells, human macrophages, human epithelial cells, granulosa cells) in response to cellular stress **(Swenson *et al.*, 2006; Luo *et al.*, 2008; Sirotkin and Bauer, 2011).** A variety of stressful situations including environmental stimuli (heavy metals) induce a marked increase in Hsp synthesis, known as the stress response **(Jaattela, 1999; Tsan and Gao, 2004).**

HSPs have been reported that may play a critical role in decreasing the accumulation of ROS. Hsp70 contributes to decrease ROS accumulation by increasing glutathione peroxidase (GPx) and glutathione reductase (GR) activities during ischemic stress **(Guo *et al.*, 2007).**

The aim of present study was to determinate dose-dependent changes in activity of SOD, TAS and expression of Hsp70 in porcine ovarian granulosa cells *in vitro*.

MATERIAL AND METHODS

Preparation, culture and processing of granulosa cells from ovaries

Slovakian White gilts were kept under standard conditions at the Experimental Station of the Animal Production Research Centre Nitra, Slovak Republic.

Porcine ovaries at the early and mid–follicular phase of the estrous cycle were obtained at slaughter house from healthy Slovakian White gilts without visible reproductive abnormalities. 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 dispenswed to 24-cultured plates (Nunc Inc., USA, 1 ml per well). 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 cobalt sulphate $CoSO_4.7H_2O$ (Co) in various concentrations (Table 1) and the control group without administration of Co. 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. Cells intended for SOD activity analyse and Western immunoblotting were lyzed in ice-cold lysis buffer (1% Triton X-100, 0.5% Igepal NP-40, 5mM EDTA, 20µg.ml^{-1}phenylmethylsuphonyl fluoride, 10 µg.ml^{-1} aprotonin, 10 µg.ml^{-1} leupeptin, 5 µg.ml^{-1} pepstatin, 10mM sodium orthovanadate in phosphate-buffered saline, pH 7.5, all from Sigma, 50µg.well^{-1}) **(Sirotkin and Bauer, 2011)**.

Table 1 Cobalt concentrations used in the study

Group	$CoSO_4.7H_2O$ $mg.ml^{-1}$	Medium ml	Dilution rate	Concentration of $CoSO_4.7H_2O$ $(mg.ml^{-1})$
Control	0	1	0:1	0
E1	0.09	0.91	1:10	0.09
E2	0.17	0.83	1:5	0.17
E3	0.33	0.67	1:2	0.33
E4	0.5	0.5	1:1	0.5
E5	1	0	1:0	1.0

SOD and TAS analysis

The activity of antioxidant enzyme superoxide dismutase (SOD) and the total antioxidant status (TAS) of ovarian granulosa cells was assayed by spectrophotometer Genesys 10 (using antioxidant RANDOX kits (Randox Labs., Crumlin, UK) according to the manufacturer's instructions.

Western blotting

The separation of HSP70 performed using SDS-PAGE and its subsequent visualization by Western immunoblotting using mouse monoclonal antibody against HSP70 and housekeeping protein GAPDH (1:250 dilution; all from Santa Cruz, Santa Cruz, CA, USA), secondary HRP-conjugated anti-mouse IG antibodies (Sevac. Prague, Czech Republic), ECL detection reagents and ECL Hyper-film (Amersham International) was performed as described previously (**Sirotkin and Makarevich 1999; Sirotkin and Bauer, 2011**). The primary antisera against HSP70 and GAPDH were specific for mouse, rat, human, porcine and bovine cells. Incubation medium without cells, or samples processed in the absence of primary antibody, were used as negative controls. The molecular weights of fractions were evaluated using a molecular weight calibration set (18, 24, 45 and 67 kDa; ICN Biomedicals, Inc., Irvine, CA, USA). Band intensity was evaluated by densitometry analysis (not shown here).

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 one-way ANOVA 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

Under normal physiological conditions, cells interact with each other to synchronize their metabolic activity, gene expression, and other basic cellular processes. Our previous study revealed that Co was able to induced changes in porcine ovarian granulosa cells. IGF-I release by granulosa cells was stopped by Co administration at the concentration 1 $mg.ml^{-1}$. Changes of the expression of proliferation related peptide cyclin B1 and apoptosis related peptide caspase-3 were also observed **(Kolesárová *et al.*, 2010).**

Activity of SOD in porcine granulosa cells

The results of the activity of SOD in porcine granulosa cells are presented in Figure1. Activity of investigated antioxidant enzyme was elevated in all experimental groups when compared to the control. The highest activity was determined in the group with the highest dose of cobalt (E5, 1 $mg.ml^{-1}$). Statistical analysis revealed significant differences ($P<0.05$) between the control group and E2 group, and between the control and E5 group. Differences among other groups remained insignificant ($P>0.05$).

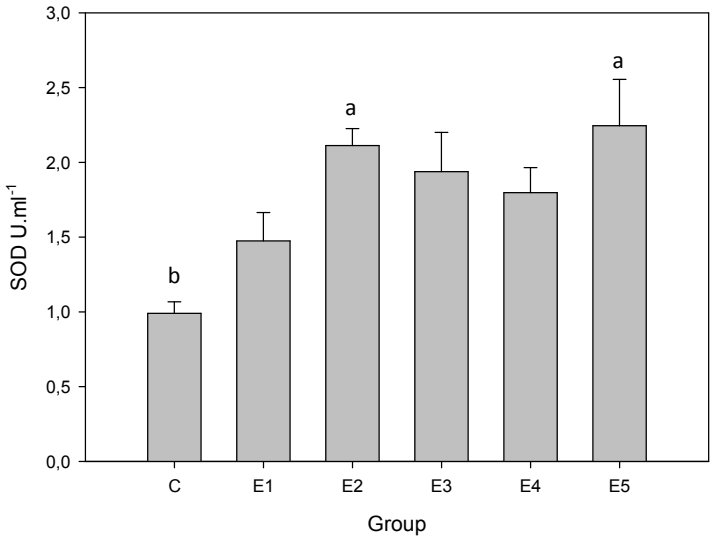

Figure 1 Effect of cobalt on SOD activity in porcine ovarian granulosa cells

Control represents culture medium without cobalt addition. Groups E1-E5 received cobalt sulphate at various doses Values are means ±SEM. *a,b* denote values significantly different ($P< 0.05$).

Similar results were found by **Grasseli *et al.* (2005)** in cultured swine granulosa cells exposed to cobalt chloride. The Co-induced stimulatory effect on SOD apparently results in H_2O_2 accumulation, overwhelming cell antioxidant defence **(Chandel *et al.*, 1998).** Our previous results revealed that porcine granulosa cells were capable of maintaining a stable SOD activity despite various doses of lead (Pb) used in our experiment *in vitro*, even though all values in Pb treated groups were increased against the control (**Capcarová *et al.*, 2009).**

TAS of porcine granulosa cells

The results are shown in Figure 2. TAS of porcine granulosa cells exposed to cobalt *in vitro* was reduced against the control in the groups with lower cobalt dose (E1, and E2). Higher doses of cobalt (E3, E4, and E5 groups) caused increase of TAS in granulosa cells when compared to the control and E1-E2 groups. TAS values of E3 and E4 groups significantly differed ($P<0.05$) from those measured in the control, E1 and E2 groups. Value of TAS in E5 group was increased in comparison with the control, E1 and E2 group, however without significant difference ($P>0.05$). The results could indicate the presence of oxidant/antioxidant imbalance due to various doses of Co addition in porcine ovarian granulosa cells and involvement of antioxidant mechanisms. In our previous study the activity

of SOD in porcine granulosa cells insignificantly increased and TAS was decreased by virtue of lead treatment **(Capcarova et al., 2009)**.

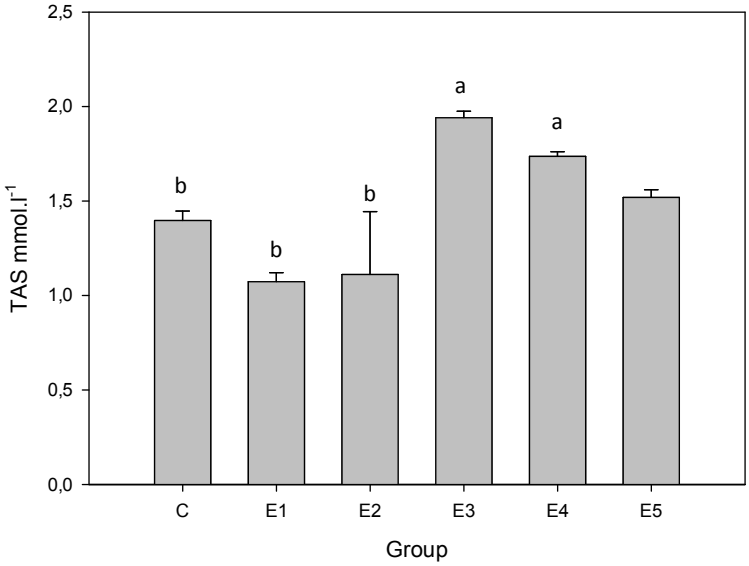

Figure 2 Effect of cobalt on TAS in porcine ovarian granulosa cells

Control represents culture medium without cobalt addition. Groups E1-E5 received cobalt sulphate at various doses Values are means ±SEM. *a,b* denote values significantly different ($P< 0.05$).

Expression of HSP70 in porcine granulosa cells

Single fraction of HSP70 with approximately 70 kDa was spotted in lysates of all groups (control and experimental groups). Addition of Co affected the expression of HSP70. With increasing dose of Co administration the accumulation of HSP increased (Figure 3).

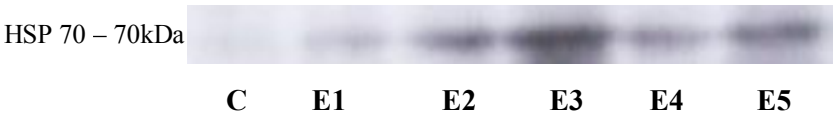

Figure 3. Accumulation of Hsp70 in porcine ovarian granulosa cells. Control represents culture medium without cobalt addition. Groups E1-E5 received cobalt sulphate at various doses.

Some of the stress signals released by cells correspond to the Hsp, which are expressed in response to the insult **(De Maio, 2011).** In our study Co exposure acted as stressful factor in granulosa cells. Similarly to our results, **Shukla *et al.* (2009)** reported marked increase in Hsp70 in rat lungs after cobalt treatment. It was reported that there exists an interrelationship between Hsp70 and redox status. Oxidative stress and antioxidants seems

to regulate Hsp70 expression **(McLaughlin *et al.*, 2003; Guo *et al.*, 2007). Guo *et al.* (2007)** reported that Hsp70 significantly increased activities of GPx.

CONCLUSION

Our results demonstrated that Co administration developed stress reaction of porcine granulosa cells and promoted accumulation of Hsp70 what resulted in increasing activity of SOD. TAS of granulosa cells increased with higher doses of Co whereas low doses had no effect on this parameter. Results of this study provide a foundation for further analysis and researches of heavy metals impact on living cells and the system of possible protection against its effects as well as evaluation of various dose dependencies on antioxidant status of cells. These results contribute towards the understanding of cellular stress and its response.

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