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COBALT-INDUCED ACTIVATION OF HEAT SHOCK PROTEIN 70 AND ANTIOXIDANT STATUS IN OVARIAN FRAGMENTS OF RATS

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Abstract
The aim of this study was to determine the expression of heat shock protein 70 (Hsp70), activity of superoxide dismutase (SOD) and total antioxidant status (TAS) of rat ovarian fragments exposed to cobalt sulphate (Co) *in vitro*. Fragments were incubated with Co administrations as follows: group E1 (0.09 mg.ml^{-1}), group E2 (0.17 mg.ml^{-1}), group E3 (0.33 mg.ml^{-1}), group E4 (0.5 mg.ml^{-1}), group E5 (1.0 mg.ml^{-1}) and the control group without any additions for 18 h. Co administration increase stress reaction by accumulation of Hsp70 what resulted in increasing activity of SOD. TAS of ovary fragments increased with higher doses of Co whereas low doses had no effect. Trace elements can adversely affect animal female reproductive system and its functions, through either direct or indirect effects on oxidative stress induction

Key words: cobalt, ovary, rats, antioxidants, HSP70

Introduction

Cobalt (Co) has important role in many processes, including reproduction (*Ashmead*, 1993), it is an essential element, but at high concentrations is toxic (*Kubrak et al*, 2011). However, Co can be also acutely toxic in larger doses, cytotoxic and induce apoptosis and at higher concentrations necrosis with inflammatory response. Cobalt metal and salts are also genotoxic, mainly cause oxidative DNA damage by reactive oxygen species, perhaps combined with inhibition of DNA repair (*Simonses et al,* 2012). Chronic overexposure to Co may result in neurotoxic effects and exposure of pregnant and lactating rats resulted in the development of oxidative stress and the impairment of defence systems (*Garoui et al*, 2013). Exposure of rats to Co during late pregnancy and early postnatal period affected antioxidant enzyme activities and lipid peroxidation (*Garoui et al*, 2011). Co exposure of animal organism usually causes the activation of defence systems against reactive oxygen species (ROS) (*Kubrak et al*, 2012). In addition to its well-known function as an integral part of cobalamin (vitamin B_{12}), Co has recently been shown to be a mimetic of hypoxia and a stimulator of the production of ROS (*Kubrak et al*, 2011). In recent years, there has been growing interest in the roles of ROS in female reproduction. ROS are key signals in the initiation of apoptosis in antral follicles and granulosa cells of antral follicles by diverse stimuli, such as exposure to exogenous toxicants, and that antioxidants protect against these stimuli (*Devine et al*, 2012). When ROS are overproduced, oxidative stress may develop in the body (*Jones*, 2008). Superoxide dismutase (SOD) serves as front-line antioxidant defence (*Scandalios*, 2005), reacts with superoxide anion radicals to form oxygen and H_2O_2 (*Ho et al*, 1998). 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). 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).
The aim of present study was to determinate dose-dependent changes in activity of SOD, TAS and expression of Hsp70 in ovarian fragments of rats exposed to Co *in vitro.*

Material and Methods

Preparation, culture and processing of ovary

Rats (Wister rats, Slovak University of Agriculture in Nitra, Slovak Republic) in age 150 days were kept under standard conditions at Slovak University of Agriculture in Nitra. Isolated ovaries were washed in a sterile physiological solution. Ovaries were cut by razor blade into fragments (totally 72 pieces) approximately 2 mm in size. Fragments of ovaries were washed in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and incubated for 24 h in culture plates (Nunc™, Roskilde, Denmark, 1 ml/well) in the same medium with 10% fetal calf serum (BioWhittaker™, Verviers, Belgium), 1% antibiotic-antimycotic solution (Sigma, St. Louis, Mo, USA), with or without cobalt sulphate CoSO4.7H2O (Co) addition in various doses (Table 1). 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/mlphenylmethylsuphonyl fluoride, 10 µg/ml aprotonin, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 10mM sodium orthovanadate in phosphate-buffered saline, pH 7.5, all from Sigma, 50µg/well) **(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 SOD and TAS of ovarian fragments of rats 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 ovary fragments. 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±SD. Differences were compared for statistical significance at the level $P<0.05$.

Results and Discussion

Expression of HSP70 in ovary fragments of rats

Single fraction of HSP70 with approximately 70 kDa was spotted in lysates of all groups (control and experimental groups). Addition of Co to the ovary fragments modified the expression of HSP70. Increasing dose of Co administration subsequently elevated the accumulation of HSP (Figure 1) and acted as stress factor. Similarly results were reported by *Shukla et al.* (2009) 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 (*Guo et al*, 2007). The data of this study confirmed our previous results on porcine granulosa cells (*Capcarova et al*, 2013).

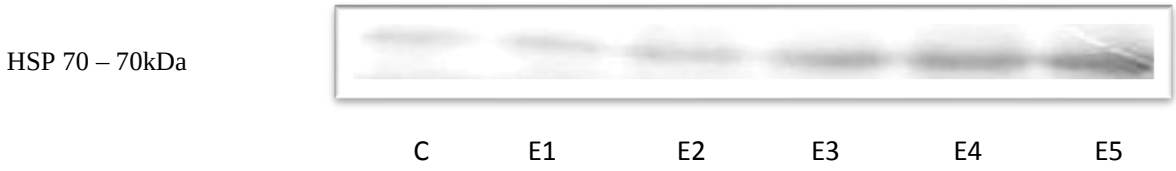

Figure 1.

Accumulation of Hsp 70 in ovary fragments of rats. Control represents culture medium without cobalt addition.

Groups E1-E5 - cobalt sulphate at various doses.

Activity of SOD in ovary fragments of rats

The results of the activity of SOD in ovary fragments of rats are shown in Figure 2. Activity of SOD was elevated in all experimental groups when compared to the control. The highest activity was determined in the groups with the highest doses of cobalt (E4 and E5). Statistical analysis revealed significant differences ($P<0.05$) between the control group and E4 group, and between the control and E5 group. Differences among other groups remained insignificant ($P>0.05$).

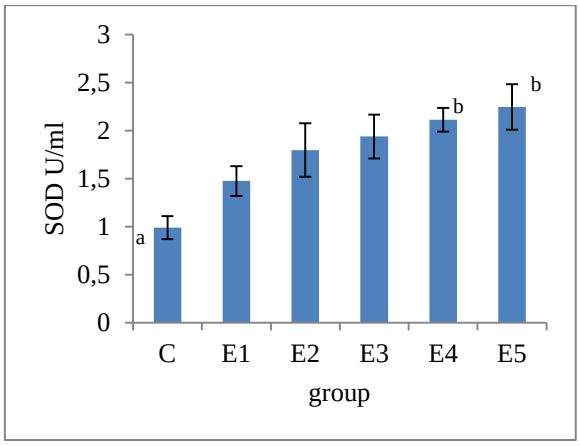

Figure 2.

Effect of cobalt on SOD activity in rat's ovarian fragments. Control represents culture medium without cobalt addition.

Groups E1-E5 - cobalt sulphate at various doses. Values are means±SD. *a,b* denotes values significantly different (*P*<0.05)

Similar results were achieved in our previous study with porcine granulosa cells (*Capcarova et al*, 2013). Likewise, exposure of goldfish to 50, 100 and 150 mg/l of Co for 96 h enhanced lipid peroxide levels and activities of SOD (*Kubrak et al*, 2012). Cobalt ions can enhance the generation of reactive oxygen species (ROS), which may be the reason for cobalt toxicity (*Kubrak et al,* 2012). The Co-induced stimulatory effect on SOD apparently resulted in H_2O_2 accumulation, overwhelming cell antioxidant defence (*Chandel et al*, 1998).

TAS of ovary fragments of rats

The results are presented in Figure 3. TAS of ovarian fragments of rats exposed to Co *in vitro* was insignificantly (P>0.05) reduced against the control in the groups with lower Co doses (E1, E2, and E3). Higher doses of Co (E4, and E5 groups) caused significant increase (P<0.05) of TAS in granulosa cells when compared to the control and E1, E2 and E3 groups. Similar results were found in our previous study with porcine granulosa (*Capcarova et al*, 2013) that could indicate the presence of oxidant/antioxidant imbalance due to various doses of Co addition in animal cells and involvement of antioxidant mechanisms.

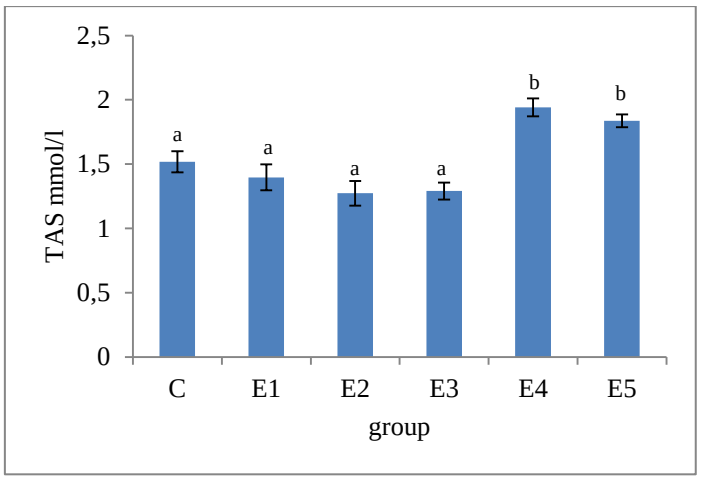

Figure 3.

Effect of cobalt on TAS in rat's ovarian fragments. Control represents culture medium without cobalt addition.

Groups E1-E5 - cobalt sulphate at various doses. Values are means±SD. *a,b* denotes values significantly different (*P*<0.05)

Conclusion

Our results demonstrated that Co administration developed stress reaction of rat ovarian fragments and promoted accumulation of HSP70 what resulted in increasing activity of SOD. TAS increased with higher doses of Co whereas low doses had no effect on this parameter. These results contribute towards the understanding of cellular stress and its response.

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