

# Induction of Hsp70 and Antioxidant Status in Porcine Granulosa Cells in Response to Deoxynivalenol and Zearalenone Exposure *in vitro*

Marcela Capcarova, Adriana Kolesarova, Marina Medvedova, Peter Petruska, Alexander V. Sirotkin

**Abstract**—The aim of this study was to determine the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), total antioxidant status (TAS) and accumulation of Hsp70 in porcine ovarian granulosa cells after deoxynivalenol (DON) and zearalenone (ZEA) exposure *in vitro*. Porcine ovarian granulosa cells were incubated with DON/ZEA administrations as follows: group A (10/10 ng/mL), group B (100/100 ng/mL), group C (1000/1000 ng/mL), and the control group without any additions for 24h. In this study mycotoxins developed stress reaction of porcine ovarian granulosa cells and increased accumulation of Hsp70 what resulted in increasing activities of SOD and GPx in groups with lower doses of mycotoxins. High dose of DON and ZEA had opposite effect on GPx activity than the lower doses. Slight increase in TAS of porcine granulosa cells was observed after mycotoxins exposure. These results contribute towards the understanding of cellular stress and its response.

**Keywords**—Deoxynivalenol, zearalenone, antioxidants, Hsp70, granulosa cells.

## I. INTRODUCTION

**M**YCOTOXINS are fungal secondary metabolites, commonly found in food crops and considered to be unavoidable contaminants worldwide due to the widespread nature of fungi in the environment [1]. They are a family of toxic sesquiterpenoids produced by foodborne and environmental fungi that are of concern to human and animal health [2]. Once trichotecenes cross the plasma membrane barrier, they enter the cell, where they can interact with a number of targets, including ribosomes and mitochondria [3]. It was published that trichotecenes elicit a complex spectrum of toxic effects as diarrhea, vomiting, leukocytosis, haemorrhage, anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes and immune modulations and dysfunction [4], [5]. There is a need to rightly evaluate the toxic potential of a toxin [6]. Mycotoxins have direct dose-dependent effects on granulosa cells proliferation, steroidogenesis and gene expression [7]. Deoxyvalenol (DON) is one of the primary trichotecene metabolites found in wheat and is commonly produced by

several phytopathogenic *Fusarium* species [8], cytotoxic and genotoxic to mammalian cells [9].

Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* species, frequent contaminants of maize, wheat, oats and barley [10]. It is non-steroidal mycoestrogen that activates estrogen receptors where it acts as an agonist and partial antagonist to estradiol [11]. In human, ZEA is rapidly absorbed after oral administration. Its derivatives are detected in blood about 30min after oral administration bound to human globulins, as reproductive hormones [12]-[14]. The major toxicity of ZEA and its metabolites is attributed to their estrogenic effects on the genital organs and reproduction [15]. Both mycotoxins may co-exist together, as the same fungi may produce them [10]. Of all domestic species, pigs are the most sensitive species and have been implicated in poor reproductive performance [16].

Antioxidant enzymes in cells of the body are the major defense system to prevent organ injuries from the excessive quantity of reactive oxygen species (ROS) that cause cellular lipid peroxidation [17], [18]. To cope with increased ROS level, cells evolved various means to detoxify ROS including antioxidant enzymes [19]. Superoxidismutase (SOD) catalyses dismutation of superoxide to H<sub>2</sub>O<sub>2</sub> [20] and glutathionperoxidase (GPx) is considered as a major defense against peroxides, superoxide anion, and hydrogen peroxide and assumes an important role in detoxifying lipid and hydrogen peroxide with the concomitant oxidation of glutathione [21]. There are some studies suggesting oxidative stress-induced changes in animal cells after mycotoxin exposure [22], [23].

A variety of stressful situations including environmental stimuli induce a marked increase in heat shock protein (Hsp) synthesis, known as the stress response [24]. The most pronounced stress-related changes and involvement in different cell functions have been demonstrated for Hsp70 [25]. ZEA and T-2 toxin increased ROS production and Hsp70 expression in cultured renal cells [26]. Although both mycotoxins has been shown to cause impairment of reproductive function, disrupted secretion activity of granulosa cells, and apoptosis [27], [28], there is no evidence regarding the effect of DON and ZEA on ovarian granulosa cells in connection with the antioxidative parameters and Hsp70 induction. Therefore, the present study is aimed at evaluating the activity of SOD, GPx, total antioxidant status (TAS) and induction of Hsp70 in porcine granulosa cells exposed to DON and ZEA *in vitro*.

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## II. MATERIAL AND METHODS

### A. 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 3–5mm follicles. Granulosa cells were isolated by centrifugation for 10min 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 of medium (counted by haemocytometer). Portions of the cell suspension were dispensed to 24-cultured plates (Nunc Inc., USA, 1ml per well). The plate wells were incubated at 37.5°C and 5% CO<sub>2</sub> in humidified air until a 75% confluent monolayer was formed (5–7 days). At this point the medium (1ml 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 DON and ZEA in various doses 10, 100, and 1000 ng/ml. A group without administration of mycotoxins served as the control. Further culture of cells was done for 24h, and then the culture media from well plates were aspirated and stored at –20°C for further assay. Cells intended for SOD and GPx activity analyze and Western immunoblotting were lysed in ice-cold lysis buffer (1% Triton X-100, 0.5% Igepal NP-40, 5mM EDTA, 20μg/ml phenylmethylsulphonyl fluoride, 10μg/ml aprotinin, 10μg/ml leupeptin, 5μg/ml pepstatin, 10mM sodium orthovanadate in phosphate-buffered saline, pH 7.5, all from Sigma, 50μg/well) [25].

### B. Immunocytochemistry

Signaling substances within granulosa cells plated on chamber slides were detected used immunocytochemistry. The ImmunoCruz Staining System and primary mouse monoclonal antibodies against Hsp (Santa Cruz Biotechnology Inc., Santa Cruz, CA, 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 polyclonal antibody against mouse IGS, labeled with horseradish peroxidase (Sevac, Prague, Czech Republic, dilution 1:1000) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, IN, USA, 10%). Chamber slides are stained with peroxidase/DAB reagent were mounted with Glycergel (DAKO, Carpinteria, CA, USA) mounting medium. The counting of the percentage of cells containing specific immunoreactivity was done by light microscopy [29].

### C. SOD and TAS Analysis

The activity of antioxidant enzymes (SOD and GPx), the total antioxidant status (TAS) of ovarian granulosa cells were assayed by spectrophotometer Genesys 10 (using antioxidant

RANDOX kits (Randox Labs., Crumlin, UK) according to the manufacturer's instructions.

### D. 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 [25], [30]. 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).

### E. 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 ± SD. Differences were compared for statistical significance at the level  $P < 0.05$ .

## III. RESULTS AND DISCUSSION

Under normal physiological conditions, cells interact with each other to synchronize their metabolic activity, gene expression, and other basic cellular processes. In this paper the dose-dependent effect of DON and ZEA on porcine granulosa cells was examined. Our previous study indicated a direct effect of DON on porcine ovarian granulosa cells concerning steroidogenesis, proliferation, and apoptosis [27], [28]. Mycotoxins as contaminants of animal food can impair fertility in farm animals [31]. It was published that ZEN and T-2 toxin enhanced ROS levels of porcine [32] and rat granulosa cells [33]. Diets contaminated with DON and ZEA at 8.3mg/kg were able to cause the oxidative stress in fattening chickens [34]. Enhanced ROS level induced by mycotoxins is connected with DNA damage as DNA strand breaks, chromosome damage and related oxidative stress can occur [35].

### A. The Effect of DON and ZEA on SOD Activity of Granulosa Cells

The results of the activity of SOD in porcine granulosa cells are presented in Figs. 1, 2. The activity of SOD was elevated in all experimental groups of DON treatment when compared

to the control. ZEA exposure caused increase of activity of SOD in first two experimental groups (E1 and E2). The highest activity was determined in the group with the lowest dose of mycotoxins (E1, 10 ng/ml) in both treatments (DON, ZEA). Statistical analysis revealed significant differences in SOD activity ( $P < 0.05$ ) between the control and E1 group in DON experiment and between E1/E2 and E3 group in the case of ZEA exposure. Differences among other groups remained insignificant ( $P > 0.05$ ).

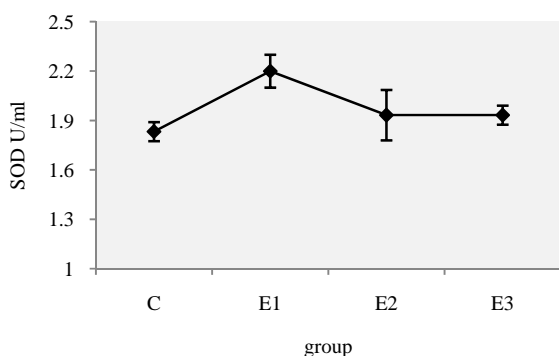


Fig. 1 The effect of DON on SOD activity of granulosa cells C – control group, E1-E3 – experimental groups with various doses of DON, a-b – means significant differences ( $P < 0.05$ ), one-way ANOVA

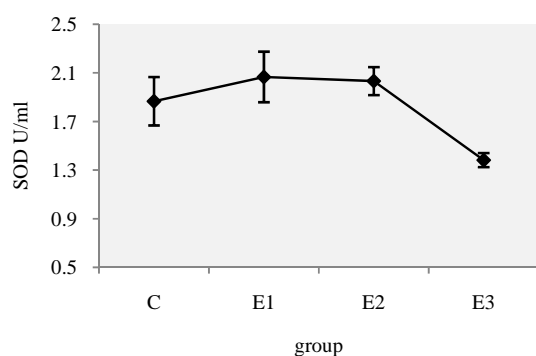


Fig. 2 Effect of ZEA on SOD activity of granulosa cells C – control group, E1-E3 – experimental groups with various doses of ZEA, a-b – means significant differences ( $P < 0.05$ ), one-way ANOVA

Boeira et al. [36] observed increased SOD activity in testes of Swiss albino mice after application of acute high dose of ZEA. Similar results were obtained after T-2 toxin treatment [37]. On the other hand, Placha et al. [38] found reduced SOD activity in DON-treated broilers in comparison to the control. In our study low doses of mycotoxins caused increase in SOD activity however high dose (mainly ZEA) decreased SOD activity. It was published that ZEA induced a decrease of the SOD gene expression in piglets [39].

#### B. The Effect of DON and ZEA on Gpx Activity of Granulosa Cells

GPx activity in the groups E1 and E2 exposed to DON or ZEA showed insignificant ( $P > 0.05$ ) increase in ovarian

granulosa cells compared to the control (Figs. 3, 4). The highest doses of mycotoxins (E3 groups in both treatments) caused decrease in GPx activity when compared to the control, E1 and E2 group. The differences among the groups were insignificant except those found between E2 and E3 group in ZEA treatment (Fig. 4). According to Wu et al. [40] T-2 toxin induced ROS accumulation in granulosa cells of rats accompanying with losses of activities of SOD and GPx. Another report found that the diet containing multiple mycotoxins caused decrease in GPx activity in the serum and liver of mice suggesting that under the oxidant stress mice are able to increase their production of GPx to get rid of the excessive oxygen peroxide ( $H_2O_2$ ) generated from the dismutation of  $O_2^-$  [41]. From our results it is obvious that SOD in granulosa cells catalyzed the dismutation of raising amount of  $O_2^-$  caused by mycotoxins exposure to  $H_2O_2$ , protecting the cells from superoxide damage. GPx catalyzed the reduction of  $H_2O_2$  in water. Similar explanation of mycotoxin-induced oxidative stress and antioxidant metabolism in mouse was given by Hou et al. [41].

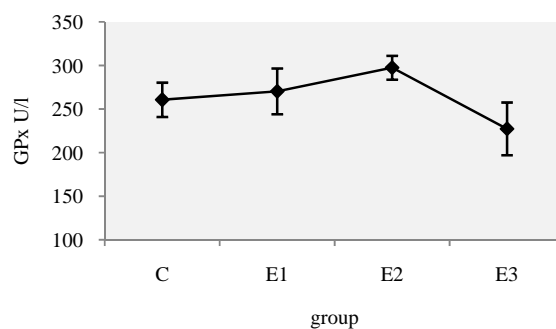


Fig. 3 The effect of DON on GPx activity of granulosa cells C – control group, E1-E3 – experimental groups with various doses of DON, differences were not significant ( $P > 0.05$ ), one-way ANOVA

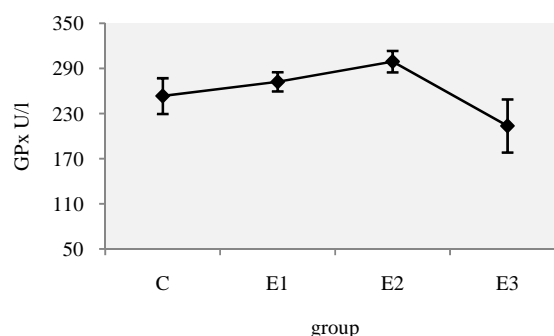


Fig. 4 The effect of ZEA on GPx activity of granulosa cells C – control group, E1-E3 – experimental groups with various doses of ZEA, a-b – means significant differences ( $P < 0.05$ ), one-way ANOVA

#### C. TAS of Porcine Granulosa Cells after DON and ZEA Exposure

The results are shown in Figs. 5, 6. TAS of porcine granulosa cells exposed to mycotoxins *in vitro* was increased against the control, however differences remained

insignificant ( $P>0.05$ ). The results could indicate the presence of oxidant/antioxidant imbalance due to various doses of mycotoxins addition in porcine ovarian granulosa cells and involvement of antioxidant mechanisms. Mycotoxins might induce some alterations in membrane structure, which consequently stimulates lipid peroxidation [42] what causes increased production of ROS [43] and activation of antioxidant systems of cells.

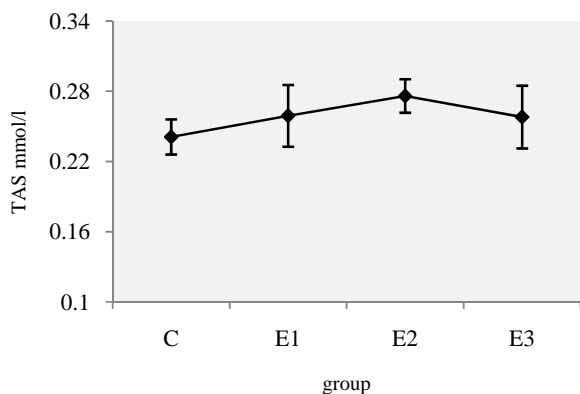


Fig. 5 The effect of DON on TAS of granulosa cells C – control group, E1-E3 – experimental groups with various doses of DON, differences were not significant ( $P>0.05$ ), one-way ANOVA

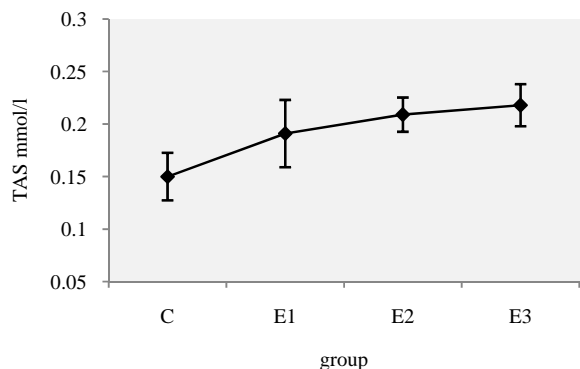


Fig. 6 The effect of ZEA on TAS of granulosa cells C – control group, E1-E3 – experimental groups with various doses of ZEA, differences were not significant ( $P>0.05$ ), one-way ANOVA

#### D. Accumulation and Induction of Hsp70 in Porcine Granulosa Cells after DON and ZEA Treatment

In all groups of both treatments (DON, ZEA) addition of mycotoxins insignificantly ( $P>0.05$ ) increased the percentage of cells containing Hsp70 (Figs. 7, 8) in comparison with cells cultured without exogenous mycotoxins. Significant induction of Hsp 70 in cultured human HepG2 cells was observed [44].

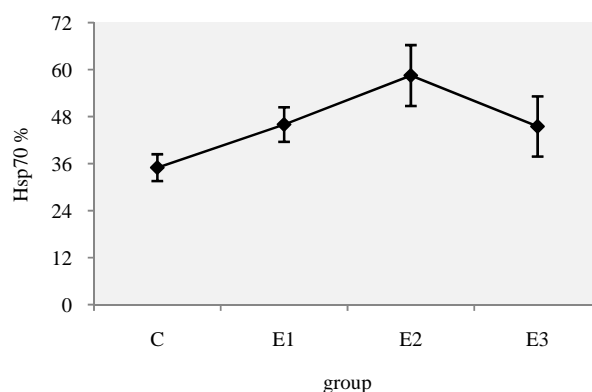


Fig. 7 The effect of DON on percentage of cells containing Hsp70 C – control group, E1-E3 – experimental groups with various doses of DON, differences were not significant ( $P>0.05$ ), one-way ANOVA

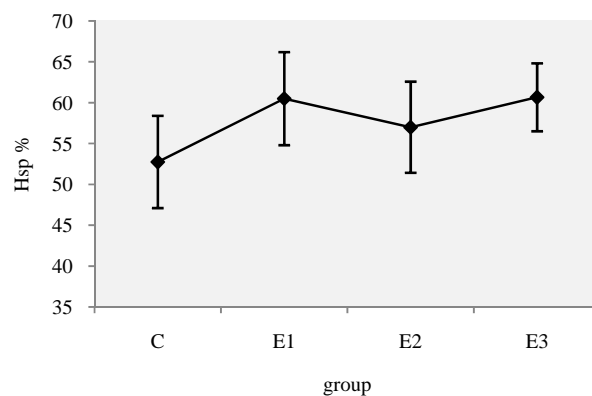


Fig. 8 The effect of ZEA on percentage of cells containing Hsp70 C – control group, E1-E3 – experimental groups with various doses of ZEA, differences were not significant ( $P>0.05$ ), one-way ANOVA

Single fraction of Hsp70 with approximately 70 kDa was spotted in lysates of all groups (control and experimental groups). Addition of mycotoxins affected the accumulation of Hsp70. With increasing dose of mycotoxins administrated the accumulation of HSP increased (Figs. 9, 10).

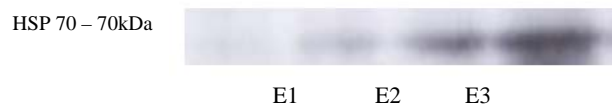


Fig 9 Accumulation of Hsp70 in porcine ovarian granulosa cells. Control represents culture medium without mycotoxins addition. Groups E1-E3 received DON at various doses

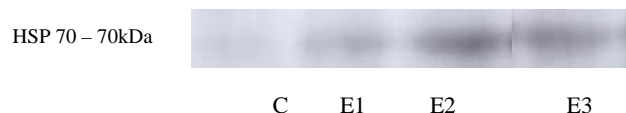


Fig. 10 Accumulation of Hsp70 in porcine ovarian granulosa cells. Control represents culture medium without mycotoxins addition. Groups E1-E3 received ZEA at various doses

Some of the stress signals released by cells correspond to the Hsp, which are expressed in response to the insult [45]. It was reported that there exists an interrelationship between Hsp70 and redox status. Oxidative stress and antioxidants seems to regulate Hsp70 expression [46], [47]. Guo et al. [47] hypothesized that Hsp70 might protect cells from ischemic injuries by regulating cellular redox status. Our previous results revealed higher accumulation of Hsp70 in porcine ovarian granulosa cells after cobalt exposure [48].

#### IV. CONCLUSION

Our results demonstrated that mycotoxins (DON and ZEA) exposure developed stress reaction of porcine ovarian granulosa cells and promoted accumulation of Hsp70 what resulted in increasing activities of SOD and GPx in groups with lower doses of mycotoxins. High dose of DON and ZEA had opposite effect on GPx activity than the lower doses. DON and ZEA caused slight increase in TAS of porcine granulosa cells. These results contribute towards the understanding of cellular stress and its response.

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