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REGULAR ARTICLE

DEOXYNIVALENOL-INDUCED ANIMAL OVARIAN SIGNALING: PROLIFERATION AND APOPTOSIS

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ABSTRACT

Fusarium toxins stable natural toxins produced by *Fusarium* species can appear in the food chain. The aim of this *in vitro* study was to examine: 1. the effect of deoxynivalenol (DON) treatment on the ability of rat ovarian cells to release insulin-like growth factor I (IGF-I) and 2. a possible signaling pathway of cell apoptosis through selected markers (Bcl-2, Bax and caspase-3) induced by this natural toxin. Rat ovarian fragments were incubated with DON for 24h: 10, 100 and 1000 ng.ml^{-1}, while the control group received no DON. The secretion of IGF–I was determined by RIA and expression of antiapoptotic (Bcl-2) and proapoptotic (Bax and caspase-3) proteins by Western-blotting analysis. IGF-I release by rat ovarian cells after

DON addition was not significantly (P>0.05) stimulated (at the dose 10 ng.ml^{-1}) and inhibited (at the doses 100 and 1000 ng.ml^{-1}) by the toxin. The impact of DON on the expression of Bcl-2 (26 kDa), Bax (23 kDa) and caspase-3 (34 kDa) was found. Bcl-2 expression was decreased and Bax and caspase-3 increased by DON treatment. In conclusion, our results suggest a direct effect of DON on (1) ovarian functions, (2) mechanisms of proliferation and apoptosis in rat ovarian cells through intracellular regulators: Bcl-2, Bax and caspase-3.

Keywords: Deoxynivalenol, IGF–I, apoptosis, ovary, rat.

INTRODUCTION

Mycotoxins as secondary metabolites produced by microfungi **(Vasatkova *et al.*, 2009)** are natural and very stable toxins **(Schollenberger *et al.*, 2007; Ranzenigo *et al.*, 2008),** which can contaminate grains **(Schollenberger *et al.*, 2007; Ranzenigo *et al.*, 2008),** grain cereals **(Thammawong *et al.*, 2011)** and foodstuffs worldwide **(Schollenberger *et al.*, 2007; Ranzenigo *et al.*, 2008).** Application of biological and biochemical silage additives **(Bíro *et al.*, 2009a; Gálik *et al.*, 2008)** and chemical additives containing organic acids, organic salts and inorganic salt was sufficient to inhibit mycotoxins formation **(Biro *et al.*, 2009b).** Mycotoxins produced by the *Fusarium* molds can cause a variety of human diseases and economic losses in livestock **(Bouaziz *et al.*, 2009).** Trichothecenes, such as deoxynivalenol (DON), are the major mycotoxins of *Fusarium species* **(D'Mello *et al.*, 1999; Larsen *et al.*, 2004).** The effect of fusarium toxins on reproductive functions was partly reported in previous studies **(Alm *et al.*, 2002, 2006; Ranzenigo *et al.*, 2008; Medvedova *et al.*, 2011)**. They inhibit oocyte maturation **(Alm *et al.*, 2002; 2006)**, protein synthesis **(Rotter *et al.*, 1994),** secretion activity and cell proliferation of porcine ovarian granulosa cells **(Medvedova *et al.*, 2011).** It is known that ovarian functions are regulated by some hormones and growth factors such as insulin-like growth factor I (IGF-I), which through intracellular regulators, affect secretion activity, proliferation and apoptosis **(Kolesarova *et al.*, 2010 a,b; Kolesarova *et al.*, 2011)**.

The aim of this *in vitro* study was to examine: 1. the effect of DON treatment on the ability of rat ovarian cells to release growth factor IGF-I and 2. a possible signaling pathway of cell apoptosis through selected markers (Bcl-2, Bax and caspase-3) induced by this natural toxin.

MATERIAL AND METHODS

Preparation, culture and processing of rat ovarian fragments

Ovaries were obtained from adult rats 4 months of age slaughtered by decapitation at follicular stage of the ovarian cycle (determined by visual inspection of the ovaries) without visible reproductive abnormalities. Decapitation was performed under ether anesthesia according to EU and Slovak guidelines of performance animal experiments. Isolated ovaries were transported to the laboratory in containers at 4ºC and washed in sterile physiological solution. Thereafter ovaries were cut by razor blade into fragments approx. 2 mm size. Ovarian fragments (n = 48) were washed in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and incubated for 24h 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 DON (Romer Labs Division Holding GmbH, Tulln, Austria) at the doses 10, 100 and 1000 $ng.ml^{-1}$. After 24h of culture the media from wells were aspirated and kept at -70ºC for RIA assay. Ovarian fragments were lysed in sample buffer (2.5 ml 4x stacking gel buffer: 3.0 g Trisbase, 50 ml ddH2O, pH 6.8 + 4.0 ml 10% SDS, 2.0 ml glycerol, 2.0 mg bromophenol blue, ddH2O to 10 ml) and subjected to 3 cycles of freezing (-18ºC) and thawing and 10 min of centrifugation at 600xg in Eppendorf tubes to achieve cell lysis prior to electrophoresis and Western blotting.

Immunoassay

Concentrations of IGF–I were determined in 25–100 µl incubation medium by RIA using RIA kits (Immunotech SAS, Marseille Cedex, France) according to the manufacturer's instructions. RIA assay sensitivity for IGF-I was 2 $ng.ml^{-1}$. Inter- and intra-assay coefficients for variation did not exceed 6.8% and 6.3%, respectively.

Protein gel electrophoresis and immunoblotting

The expression of Bcl-2, Bax and caspase-3 in rat ovarian cells was evaluated by using SDS-PAGE and Western immunoblotting, as described previously **(Laemmli, 1970; Sirotkin and Makarevich, 1999).** Following electrophoresis, the samples were transferred to

nitrocellulose membrane parablot NCP (Macherey–Nagel, Düren, Germany) for 1 h at 100V using a mini trans-blot system (Bio-Rad Labs, Richmond, CA, USA). Endogenous peroxidase in samples was quenched by incubation in 3% H_2O_2 for 15 min. Thereafter the membranes were probed for 1 h with mouse monoclonal antibodies against Bcl-2, Bax and caspase-3 (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and housekeeping protein GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (dilution 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA; not shown). These antisera were able to bind to corresponding antigens of mouse, rat, human and Xenopus origin. Thereafter membranes were washed for 15 min each in TTBS, incubated with secondary polyclonal goat antibodies against mouse IgGs, labeled with horseradish peroxidase (dilution 1:1000; Sevac, Prague, Czech Republic). The antibody was then washed off in TTBS and the immunoreactive bands were visualized using the detection reagents Upstate Visualizer™ Western Blot (Temecula, CA, USA) and exposed on ECL Hyper-film (Amersham International plc, Little Chalfont, Bucks, UK). Molecular weight standards (molecular weight calibration kit 14.4 to 94 kDa, Serva, Heidelberg, Germany) were run under the same conditions to identify the molecular weight of detected protein bands. Incubation medium without cells with 10% bovine calf serum was used as negative control.

Statistical Analysis

Each experimental group was represented by six culture wells of ovarian fragments (n=48). Assay of the substances in incubation medium were performed in duplicate. Significance of differences between the groups was evaluated by one-way ANOVA using statistical software Sigma Plot 11.0 (Jandel, Corte Madera, USA). The data are expressed as means ± SD. The data are expressed as means ± SEM. Differences were compared for statistical significance at the P - level less than 0.05 ($P<0.05$).

RESULTS

IGF-I release by rat ovarian cells after DON addition was not significantly ($P>0.05$) stimulated (at the dose 10 $ng.ml^{-1}$) and inhibited (at the doses 100 and 1000 $ng.ml^{-1}$) (Fig.1). Ovarian fragments at the lowest dose of DON (10 $ng.ml^{-1}$) released the highest concentration of IGF-I in comparison to control. On the other hand, the highest dose of DON release of the lowest concentration of IGF-I. Expression of anti-apoptotic (Bcl-2) and apoptotic (Bax and

caspase-3) proteins were influenced by DON treatment (Fig.2). Bcl-2 expression was decreased and expression of Bax and caspase-3 was incresed by DON treatment at all doses (10, 100 and 1000 ng.ml^{-1}).

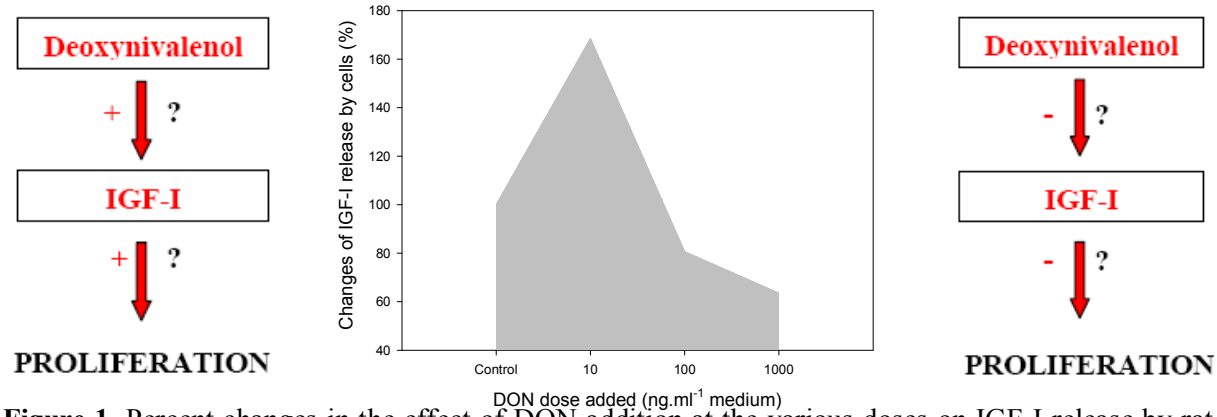

Figure 1. Percent changes in the effect of DON addition at the various doses on IGF-I release by rat ovarian fragments for 24 h and possible signaling pathway of cell proliferation. Control represents culture medium without DON; other groups represent culture medium with DON at the doses of 10, 100 and 1000 ng.ml^{-1} in well plates. Not significant (P>0.05) differences compared to control. RIA.

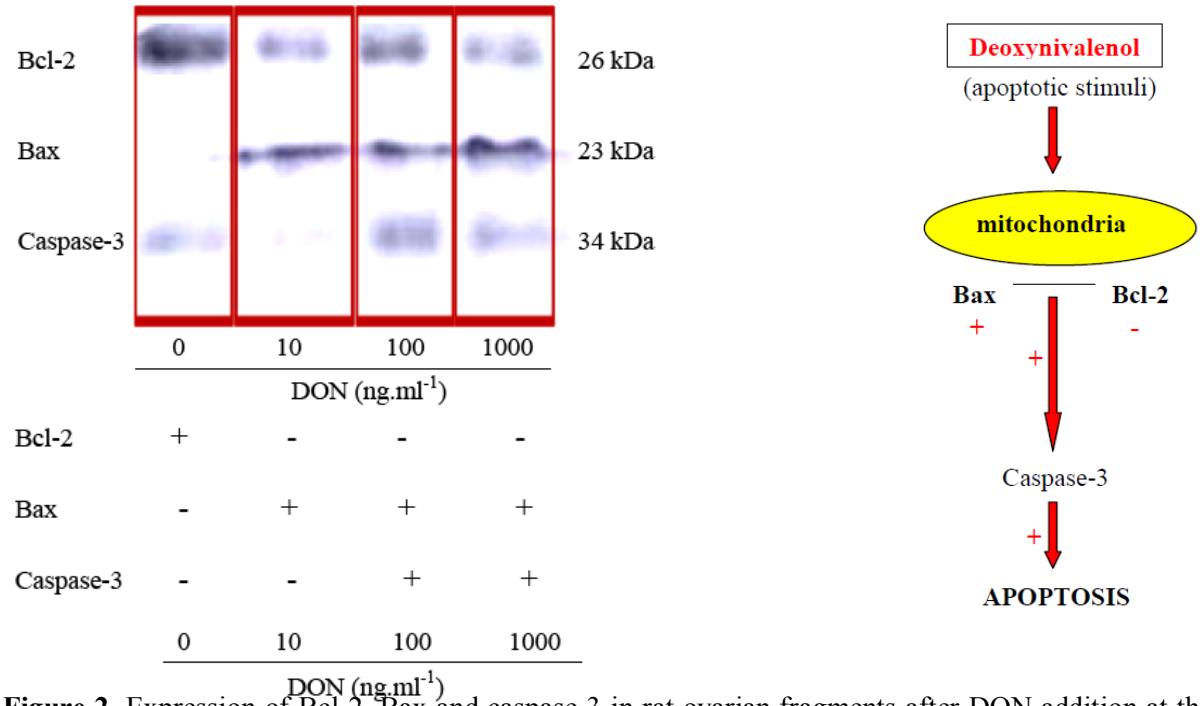

Figure 2. Expression of Bcl-2, Bax and caspase-3 in rat ovarian fragments after DON addition at the various doses for 24 h and possible signalling pathway of cell apoptosis. Western blotting. (+) stimulation, (-) inhibition.

DISCUSSION

The impact of DON addition on ovarian functions of rats is suggested in this study. These data confirm previous reports concerning the influence of toxic substances on porcine **(Kolesarova *et al.*, 2010a,b; Medvedova *et al.*, 2011),** rabbit (**Shneidgenová *et al.*, 2007;**

Roychoudhury and Massanyi, 2008), mice **(Pestka, 2003; Amuzie *et al.*, 2010)** and human **(Stawarz *et al.*, 2008; Pestka and Smolinski, 2005)** cellular processes. IGF-I secretion by porcine ovarian granulosa cells after DON treatment has been examined in previous studies **(Ranzenigo *et al.*, 2008; Medvedova *et al.*, 2011).** In our study dose-dependent effect of DON on the IGF-I release by rat ovarian fragments was found. Similar results were also shown in previous study on porcine ovarian granulosa cells **(Medvedova *et al.*, 2011).** On the other hand, DON had inhibitory effects on IGF-I –induced steroid production and decreased cell numbers at the dose of 1000 ng.ml^{-1} **(Ranzenigo *et al.*, 2008).** Similarly IGF–I release by porcine ovarian granulosa cells was inhibited by DON at the dose of 1000 ng.ml^{-1} but not at 10 and 100 ng.ml^{-1}. **Alm *et al.* (2002)** recognized the most potent effect of DON on oocyte maturation, significantly decreasing the proportion of oocytes reaching metaphase II at the concentration of only 1.88 μM. According to our previous study we suggest dose-dependent impact of DON on secretion activity of rat ovarian cells and possible signaling pathway of cell proliferation through growth factor IGF-I (Fig. 1).

The expression of antiapoptotic (Bcl-2) and pro-apoptotic (Bax and caspase-3) proteins were observed by DON addition at all doses (10, 100 and 1000 ng.ml^{-1}). We have found that Bcl-2 expression was reduced by DON treatment. On the other hand expression of Bax and caspase-3 was increased by the toxin treatment. DON could induce apoptosis of human gastric carcinoma cell line SGC-7901, BGC-823 cells *in vitro* in a dose-dependent manner, and possible mechanisms may be increased formation of Bax-Bax homology dimer and decreased formation of Bax-Bcl-2 dimer by up-regulation of the expression of Bax and down-regulation of that Bcl-2 **(Liu *et al.*, 2009).** In previous *in vitro* study RAW 264.7 murine macrophage cells treated with DON addition at the dose 250 ng.ml^{-1} expressed markedly less BAX in cytoplasm than control cells **(Zhou *et al.*, 2005).** In our previous study expression of proapoptotic peptide caspase-3 was not influenced by DON treatment at the doses 10, 100 and 1000 ng.ml^{-1} in porcine ovarian granulosa cells **(Medvedova *et al.*, 2011).** Another study has showed significant increase of caspase-3 in human chondrocytes after T–2 toxin treatment at the doses of 1, 10, 20 ng.ml^{-1} for 5 days **(Chen *et al.*, 2008)**. According to our study apoptosis was significantly increased by DON at the dose of 1 μg.ml^{-1} in lymphocytes **(Malovrh *et al.*, 2010).** Our results suggest possible signalling pathway of cell apoptosis through antiapoptotic (Bcl-2) and proapoptotic (Bax, caspase-3) proteins (Fig. 2).

CONCLUSION

Mycotoxins, which are found in foodstuffs and feedstuffs, belong to natural toxins which can affect the human and animal health. The alterations of endocrine activity might be induced by the cellular stress. In conclusion, our results suggest a direct effect of DON on (1) ovarian functions, (2) mechanisms of proliferation and apoptosis in rat ovarian cells through intracellular regulators: Bcl-2, Bax and caspase-3.

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