Influence of chronic administration of zearalenone on the processes of apoptosis in the porcine ovary

K. Wasowicz[1], M. Gajecka[2], J. Calka[1], E. Jakimiuk[2], M. Gajecki[2]

[1]Division of Animal Anatomy, Department of Functional Morphology, [2]Division of Feed Hygiene and Veterinary Prophylactics, Department of Veterinary Protection of Public Health, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn-Kortowo, Poland

ABSTRACT: Zearalenone (ZEA), a micotoxin produced by *Fusarium* sp. is regarded as a phytoestrogen. Although cytotoxic and genotoxic activity of ZEA was detected, the majority of its toxic influence is related to the ability of binding to estrogen receptors and disrupting the endocrine regulation of the reproductory functions in females. It was previously found that ZEA inhibits proliferation of cells in porcine ovaries, as detected with immunostaining for proliferating cells nuclear antigen (PCNA). The number of PCNA-positive cells was inversely proportional to the dose of ZEA. We decided to answer the question of whether ZEA induces apoptosis in porcine ovaries. Experimental gilts (before first estrus) were fed ZEA in a dosage of 20 (group II) or 40 (group III) µg/kg of body weight/day for 63 days. Control animals (group I) were fed a placebo. After that period animals were sacrificed, ovaries were extirpated, fixed in paraformaldehyde solution, cut into sections with a cryostat and studied for apoptosis with TUNEL kit, and for the presence of apoptosis-promoting protein Bax with immunohistochemistry. It was found that apoptosis was detected with TUNEL only in medium-sized antral ovarian follicles in animals of groups I and II. No apoptosis signal was found in the ovaries of animals in group III. No differences in the distribution and intensity of staining for Bax were found between animals of all investigated groups. The results indicate that ZEA do not induce apoptosis in porcine ovaries, and the inhibition of proliferation must be associated with other mechanisms.

Keywords: *Fusarium* sp.; mycotoxin; TUNEL; Bax; pig

Zearalenone (ZEA) is a lactone mycotoxin produced by moulds of *Fusarium* species. It is widely present in different grains, such as wheat, rye, barley, maize and in fodders stored in a warm and humid environment (Price and Fenwick, 1985). It was found that this toxin affects many processes within the cell. It induces oxidative stress, inhibits DNA replication and protein biosynthesis, as well as exerts genotoxic and cytotoxic effects (Abid-Essefi et al., 2004). However, it seems that the main effect of zearalenone comes from its oestrogen-like activity (Katzenellenbogen, 1996), due to the ability to bind to oestrogen receptor (Lopez et al., 1988). This mycotoxin stimulates the protein biosynthesis and proliferation of cells of the reproductive organs (Ueno and Yagasaki, 1975) and blocks the

activity of certain enzymes involved in steroid biosynthesis (Inaba et al., 2002). Many animal species, mainly monogastric animals, are sensitive to the oestrogenic effect of ZEA, however, prepubertal gilts appear to be the most sensitive. ZEA intoxication evokes in these animals vulva swelling and reddening, vaginal discharge, prolapse of the vagina and anus, augmentation of the mammary gland, infertility and decreased libido (Farnworth and Trenholm, 1981; Etienne and Jemmali, 1982; Balney et al., 1984). Prolonged administration of ZEA to gilts induces clear morphological changes in the reproductive organs – swelling and hypertrophy of the uterus, uterine and vaginal epithelium metaplasia, underdevelopment of ovaries associated with the atresia of ovarian follicles and apoptosis-like

changes in the granular cells (Minervini et al., 2001; Tiemann et al., 2003; Whitehead and Lacey, 2003). The experimental results suggest clearly that ZEA inhibits the proliferation of ovarian cells in a dose-dependent manner, as assessed by staining of the ovarian tissues with antiserum against proliferating cells-associated antigen (PCNA) (Obremski et al., 2003). The inhibition of cell proliferation and the presence of apoptosis-like changes in the cells of ovarian follicles of gilts raised the question about the nature of the process leading to the abnormal structure of the ovaries of animals intoxicated with ZEA. The question is, whether the chronic treatment with ZEA induces the apoptosis in the porcine ovary, leading finally to the reported regressive changes in this organ. Apoptosis is a complex process in which many factors are involved. One of the possible pathways involves pro-apoptotic protein Bcl-2 and anti-apoptotic protein Bax regulating, among others, the release of cytochrome c from the mitochondria (Johnson, 2003). The release of cytochrome c is triggered by the changes in Bax: Bcl-2 ratio in the mitochondrial membrane. The increase in Bax expression promotes formation of Bax homodimers, forming canals in the mitochondrial membrane. The cytochrome in turn activates the set of proteolytic enzymes, called caspases. Processes regulated by caspases lead finally to the DNA fragmentation arising from cleaving DNA molecule between nucleosomes. Apoptosis may manifest itself by increased expression of Bax, the presence of activated caspases and DNA fragmentation. Of these three symptoms, the caspase activation is a comparatively short-time event and detecting the Bax expression and/or DNA fragmentation are most commonly used to study apoptotic changes in tissues.

The present study was designed to investigate whether the chronic administration of ZEA to prepubertal gilts increases the rate of apoptotic processes in the ovary via induction of expression of the pro-apoptotic protein Bax. The presence of apoptotic changes was studied using two methods: immunohistochemical staining for Bax and detection of apoptosis-associated changes in nuclei of ovarian cells with TUNEL method.

MATERIAL AND METHODS

The experimental procedure was accepted by a Local Ethical Committee for Animal Experimentation and was in concord with specific national law on animal welfare.

The experiment was performed on 9 clinically healthy gilts with body weight (b.w.) of ca. 50 kg in prepubertal age. The animals were divided into 3 experimental groups. Animals were given a placebo (group I; $n = 3$), ZEA at a dose of 20 μg/kg b.w. (group II; $n = 3$), or ZEA at a dose of 40 μg/kg b.w. (group III; $n = 3$) in gelatin capsules given with fodder. The capsules were administered once daily for 63 days in a manner ensuring their intake. At the end of the experiment animals were slaughtered in a slaughterhouse and the ovaries were removed. Tissues were immersion-fixed in 4% formaldehyde in a phosphate buffer (PB; 0.1M; pH 7.4) for 48 hours. Then they were transferred into 18% sucrose solution in PB and stored in this solution for 5 days. The fixed ovaries were cut in a cryostat into 12 μm sections which were placed on chrome alum-gelatine-coated slides. In these sections the presence of apoptosis was assayed with TUNEL kit (1 684 809, Roche, USA) using fluoresceine-conjugated 11-dUTP according to the kit manual. In the same sections the expression of Bax was studied with an immunohistochemical staining using polyclonal rabbit anti-Bax antibody (N20, Sc-493, Santa Cruz Biotech, USA) and a secondary goat anti-rabbit antibody conjugated with biotin (E0432, DAKO, Denmark) subsequently detected with Cy3-conjugated streptavidin (016-160-084, Jackson Immunoresearch, USA). Sections were observed and photographed with a fluorescence microscope (Axiophot, Zeiss, Germany), equipped with appropriate filter sets for fluoresceine (TUNEL visualization) and Cy3 (Bax staining visualization). Some sections were stained by haematoxiline-eosine (HE) method in order to visualize the morphological changes. These sections were observed and photographed with the same microscope using bright-field illumination.

RESULTS

Surprisingly, no macroscopic changes could be detected between ovaries from animals of control group (group I) and group receiving 20 μg of ZEA (group II). They were of normal size and numerous small ovarian follicles were seen in their cortex. Ovaries from animals of group III (receiving 40 μg of ZEA) were smaller, smooth and no follicles were visible in their cortex.

Histological examination also revealed that there were no clear-cut differences between the ovaries of animals from group I and II. In the ovaries of animals from groups I (Figure 1A) and II (Figure 1B),
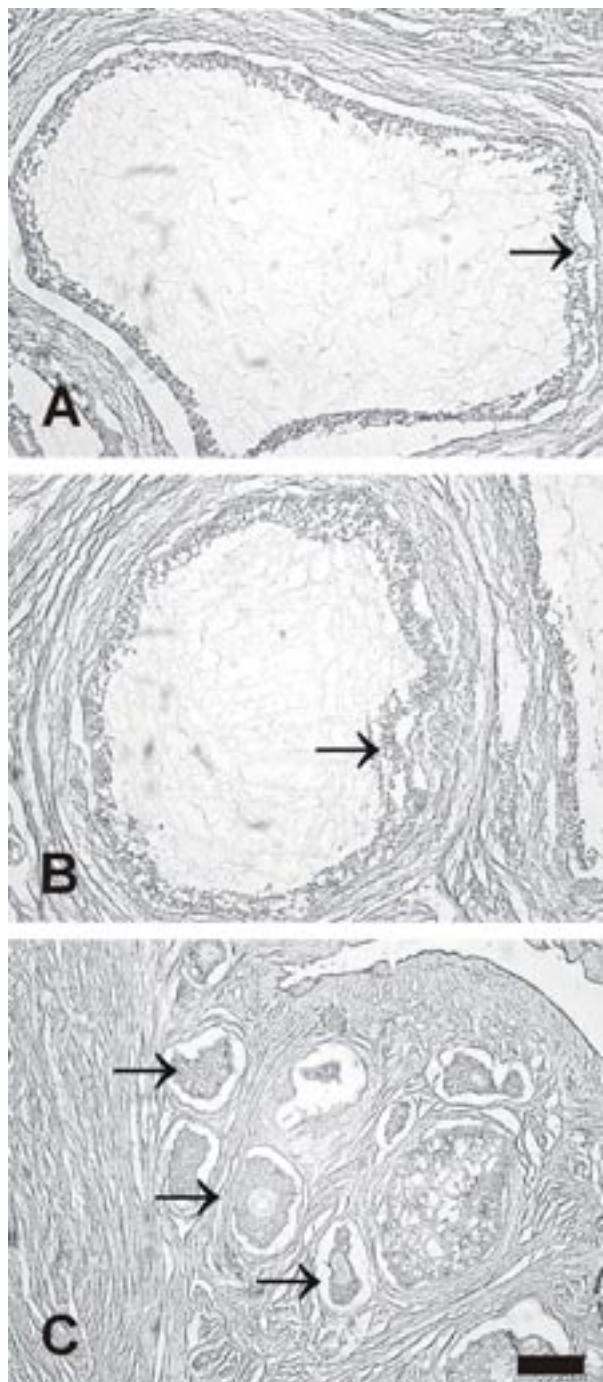

Figure 1. Sections of ovaries from animals of group I (A), II (B) and III (C) stained with haematoxyline-eosine method. In group I (A) and II (B) numerous antral ovarian follicles wers seen (arrows). In group III (C) no antral follicles were visible and only follicles without cavity were present (arrows); bar = 100 μm

the cortex contained ovarian follicles in different stages of development. The follicles visible belonged to the classes of primary, preantral and antral follicles with cavities developed to various extents. No big preovulatory follicles were visible. However, in the ovarian cortex of animals from the group III (Figure 1C), numerous preantral and atretic follicles were visible, but no antral follicles were present.

TUNEL signals were detected in the granular layer of large antral follicles in the ovaries of animals from the group I (Figure 2A) and II (Figure 2B). No TUNEL signal was detected in the ovaries of animals from group III (Figure 2C). No differences in the intensity and distribution of Bax-immunoreactivity were found between ovaries of animals from groups I (Figure 2D), II (Figure 2E) and III (Figure 2F). Immunohistochemical staining for Bax revealed the presence of Bax-immunoreactivity in various cell populations, both in ovarian follicles and in connective tissue.

DISCUSSION

The presented results showed that the effect of ZEA administration was dependent on the dose, but the relationship was found to be "non-linear". There were apparently no differences in regard to the gross morphology, histological structure and presence of the TUNEL signal between the ovaries taken from animals of control group (group I) and the group receiving ZEA in the dose of 20 μg/kg. However, the higher of the doses used, 40 μg/kg induced in the ovaries of the animals of group III clear macroscopic, and microscopic changes, regarding both histological structure and the presence of TUNEL signal. However, the presented results suggest that despite the presence of clear changes in ovaries of animals in group III, these changes are not the effect of intensified apoptosis. It was found that ZEA did not cause increase in number of ovarian cells displaying the symptoms of apoptosis, both at the level of Bax protein expression and on the level of DNA (as revealed with TUNEL test). On the contrary, the number of cells displaying the positive TUNEL signal was lower in animals intoxicated with the higher dose of ZEA. Additionally, no differences were found between group I, II and III as regards the immunostaining for Bax. These findings raise questions about the mechanisms of ZEA influence on the ovary. One
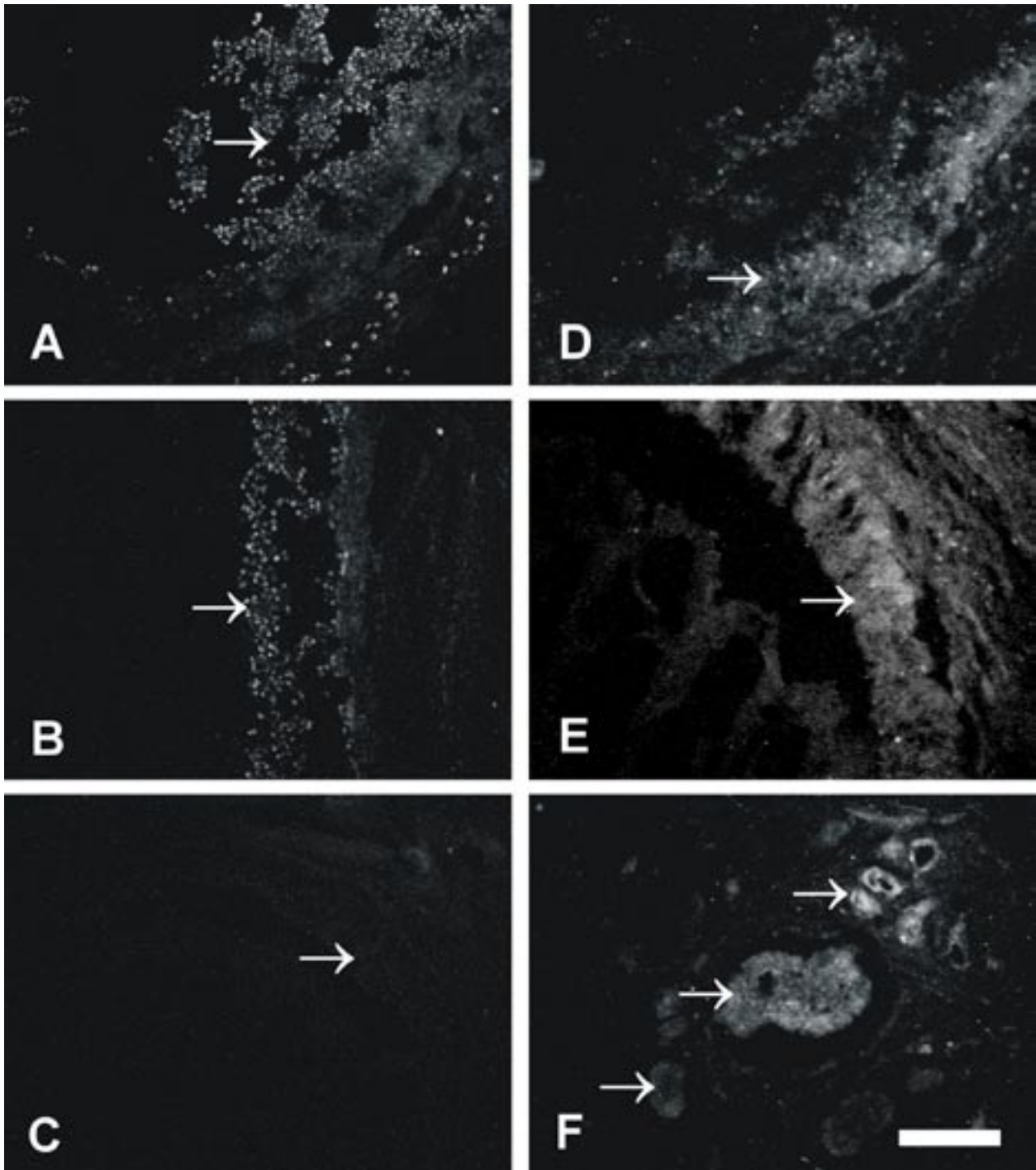
Figure 2. Sections from ovaries of animals from group I (A, D), II (B, E) and III (C, F) in which TUNEL signal (A, B, C) and Bax immunoreactivity (D, E, F) were visualised. TUNEL signal was present only in granulosa cells (arrows) of antral follicles in ovaries from group I (A) and II (B). No TUNEL signal was seen in ovarian follicles (arrow) of animals from group III (C). Immunostaining for Bax was visible in the wall of ovarian follicles (arrows) of groups I (D), II (E) and III (F) and no differences in the distribution of Bax immunostaining was clearly visible; bar = 50 μm

possibility is the toxic influence of ZEA on the cells of the ovary, leading to its underdevelopment. However, previous studies clearly showed that ZEA induces in other parts of the porcine female reproductive system (uterus) clear proliferative changes (Obremski et al., 2003). It would be rather unlikely

that the same substance shows strong toxic influence on the ovary and strong stimulatory effect in the uterus. So, the other possible mechanism may be associated with the phytoestrogenic activity of ZEA. In this case, ZEA may substitute for endogenous oestrogens and this way disrupt the physiological functions of the hypothalamus-pituitary-ovarian axis (Rainey et al., 1999). This mechanism may inhibit FSH release and, conversely, the oestrogen synthesis in the ovary. However, FSH directly affects the development and functions of the female gonad (Silva et al., 2004). FSH receptors are present on the cellular membrane of the granular cells and influence their development and maturation. FSH up-regulates the expression of its own receptor, stimulates the proliferation of the ovarian cells and induces expression of enzymes responsible for oestrogen synthesis. Oestrogens and inhibine released by the ovarian follicles via a negative feed-back decrease FSH production. ZEA, mimicking the action of oestrogens, may this way inhibit FSH release (Mueller, 2002; Sweeney, 2002). This may explain the absence of antral follicles. So, the effect of ZEA on the ovary would come not from its direct inhibitory effect, but rather from the absence of the stimulation of ovarian development.

On the other hand, oestrogens are known to exert a protective effect on many cells. It is known that oestrogens display a protective effect on the cardiovascular and nervous system in women in the premenopausal period. In the central nervous system oestrogens block the expression of the tumour necrosis factor (TNF) inducing the apoptosis of neurons in the ischemic site (Liao et al., 2002). It may be speculated that ZEA, due to its oestrogen-like activity inhibits apoptotic processes in the ovary of intoxicated gilts. This effect might be compared to the action of phytoestrogens from soybean, or *Cimicifuga racemosa* (Wuttke et al., 2003), showing positive effect in postmenopausal women (Liu et al., 2001). However, it would be not wise to attribute all effects of ZEA to its oestrogen-like activity. As other micotoxins, ZEA is known to exert a direct toxic effect. It may induce the formation of DNA adducts, inhibit DNA replication and protein biosynthesis, as well as impose an oxidative stress (Abid-Essefi et al., 2004). Basically, these processes may finally lead to apoptosis, however, some reports suggest the possible role of necrotic processes. According to some theories the choice between apoptosis and necrosis in the cell depends on the availability of energy carriers (Alonso-Pozos et al., 2003). Since apoptosis is an active, energy-dependent process it requires sufficient amounts of ATP, in absence of which the cell enters the necrosis pathway. It was found that in the equine ovary the process of apoptosis affects mainly follicular cells in small follicles (3–6 mm), while in the big follicles (>6 mm) necrosis is a dominating regressive mechanism. It is suggested that the selection of either process depends on the thickness of the follicular wall, the abundance of blood vessels, oxygen concentration and the amount of ATP generated in granular cells. However, in the animals used in this study the big ovarian follicles were absent, what reduces the possibility of necrotic changes in their ovaries. In conclusion, it may be stated that the most probable mechanism of the morphological changes found in porcine ovaries in this study is based on the oestrogen-like properties of ZEA, but the influence of other toxic mechanisms cannot be excluded.

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Corresponding Author

Krzysztof Wasowicz, University of Warmia and Mazury, Faculty of Veterinary Medicine, Department of Functional Morphology, Division of Animal Anatomy, ul. Oczapowskiego 13, 10-957 Olsztyn-Kortowo, Poland
Tel. +48 89 5233733, fax +48 89 5234986, e-mail: wasowicz@uwm.edu.pl