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Original article

The effect of experimental low zearalenone intoxication on ovarian follicles in pre-pubertal bitches

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Abstract

Companion animals, including bitches, may be exposed to zearalenone (ZEN) toxins that are often present in feed, and ZEN intoxication may lead to ovarian dysfunction. This study involved evaluation of the degree of ZEN-induced hypo stimulation of ovary by determination of proliferative and apoptotic indices and description of the ultra-structural organization of ovarian follicles in pre-pubertal bitches subjected to experimental, long-term exposure to low-dose ZEN mycotoxicosis. The experiment involved 30 clinically healthy, immature Beagle bitches aged approximately 70 days with initial average body weight of 8 kg, randomly divided into three groups of 10 animals each: two experimental groups (EI and EII) and a control group (C). Over a period of 42 days, ZEN was administered *per os* to EI animals at a dose of 50 µg/kg BW, and to EII bitches at a dose of 75 µg/kg BW. Control group animals were fed placebo containing no ZEN for 42 days. Analytical samples of the mycotoxin were administered daily in gelatin capsules before morning feeding. All the bitches were subjected to ovario-hysterectomy at the end of the experiment. Proliferation index (PCNA method) and apoptotic index (TUNEL test) values were determined by immune-histological analyses. The median for apoptotic index was determined at 13.45 for group EI, 17.84 for group EII, and 8.59 for group C. The median for proliferation index was determined at 35.25 for group EII, 42.44 for group EI, and 70.60 for group C. The results of ultra-structural examinations of the ovaries revealed that experimental, ZEN-induced hyperestrogenism enhanced apoptosis and lowered the proliferative ability of follicular cells what contributed to organelle destruction in pre-pubertal bitches. The changes observed were particularly advanced in EII animals, which were administered a higher dose of ZEN.

Key words: ZEN, bitch, ovary, PCNA, TUNEL, ultra-structure

Introduction

The number of biologically active cells, such as ovarian cells, must be precisely controlled to support the existence and functioning of multicellular organisms. Four processes occur within a cell to maintain homeostasis: division, differentiation, maturation, and death. Each process may be disturbed or initiated by

non-physiological factors, including mycotoxins, which show a multidirectional pattern of action. Mycotoxins may affect the metabolic pathways of live cells, including those responsible for energy generation, thus initiating the final stage of the cell cycle, namely apoptosis (Moreira et al. 2011).

ZEN is a mycotoxin produced by various fungi of the genus *Fusarium*, in particular *F. graminearum*,

and is commonly found in cereals, mostly maize, as well as in wheat, barley, sorghum, and rye. It particularly affects cereals that were grown and/or harvested in unfavourable conditions and/or were inadequately stored (Gajęcki et al. 2010).

The mammalian gastrointestinal tract quickly and completely absorbs ZEN. Efficient glucuronidation of ZEN in the small intestine and liver significantly reduces the amounts of unconjugated parent (receptor-active) compound that reaches the circulatory system. Enzymatic reductions of ZEN produce alpha-zearalenol, which shows greater affinity for oestrogen receptors than the parent compound (Takemura et al. 2007, Gajęcka et al. 2009). Estrogenic activity thus appears to be the key mechanism behind the pathological effects of ZEN and its main metabolites (Gajęcki et al. 2010). Among farm and companion animals, pre-pubertal gilts and bitches are most sensitive to the presence of ZEN and its metabolites in feed (Döll and Dänicke 2011).

When ingested with commercial feed, ZEN may contribute to reproductive disorders in pre-pubertal bitches (Gajęcka et al. 2004, Boermans and Leung 2007). According to preliminary studies, ZEN concentrations in commercial feeds vary significantly, and some products contain very high levels (Zwierchowski et al. 2004). Bitches are often fed a monodiet for many months. Overdosing of endogenous and exogenous (De Bosschere et al. 2002) oestrogens could lead to different reproductive system disorders associated with hyperestrogenism, and excess oestrogens could distort hormonal regulation of reproductive processes, contributing to numerous health problems during maturation (Gajęcka et al. 2008). During reproductive maturation, ovarian functions are easily deregulated under prolonged exposure to estrogenic substances in feed (Gajęcka et al. 2004, Watson et al. 2007).

The reproductive performance of females, determined by the number of oocytes in the ovaries, is established during foetal development and decreases with every oestrous cycle. According to estimates, more than 99% of ovarian follicles undergo atresia. The results of research studies investigating the developmental potential of female reproductive cells are of growing relevance for animal breeders. The findings support the search for innovative methods to protect oocytes in females subjected to chemotherapy and to prolong the longevity of primordial follicles. In farming, determination of the ideal conditions for *in vitro* and *in vivo* development of preantral follicles will enable breeders to optimize the reproductive performance of females (Brasil et al. 2010). Our published results (Gajęcka and Przybylska-Gornowicz 2012) suggest that zearalenone-induced apoptosis of ovarian

cells in pre-pubertal bitches is an internal process that begins in the mitochondria. The presence of ZEN and its metabolite, α -zearalenol, leads to hyperestrogenism and increased cellular Ca^{2+} levels in mitochondria. The rise in mitochondrial Ca^{2+} levels promotes the production of free radicals (Felty and Roy 2005), an initiating factor for apoptosis (Moreira et al. 2011). In view of the characteristic properties of oestrogen, it could be inferred that the administration of ZEN induced the presence of large calcium deposits in the mitochondria, cellular debris, and residual oocytes in group EII. In another work (Gajęcka 2012) we have found the absence of a positive response to the presence of estrogen receptors $\text{ER}\alpha$ and results obtained in both experimental groups suggested that long-term exposure to low-dose ZEN intoxication decreased the positive response of $\text{ER}\beta$ receptors in the ovaries of experimental bitches, which initiated epigenetic modification mechanisms that inhibited (rather than repressed) the ovarian development.

During sexual maturation, ovarian function is determined equally by the intensity of cell proliferation and degeneration. Exposure to ZEN may induce proliferative dysfunction in estrogen-dependent cells, leading to neoplastic changes in the uterus, ovaries (Gajęcka et al. 2011), mammary gland, duodenum, and the large intestine, attributable to the activation of nuclear oestrogen receptors in the presence of ZEN and its metabolites (Chen et al. 2005). The mycotoxin can thus modify the transcription process (Nadal et al. 2005). Endogenous oestrogens generally inhibit proliferation, stimulate differentiation, and promote apoptosis. According to Ranzenigo et al. (2008), phytoestrogens elicit a similar response, whereas small doses of ZEN exert a chemo protective effect, but ZEN in higher doses inhibits the proliferation in specific tissues. Scientific data are scarce in terms of documenting the effect of very low ZEN doses (i.e., No Observed Adverse Effect Level – NOAEL values), and/or less than $<40 \mu\text{g}/\text{kg}$ body weight (BW) (Boermans and Leung 2007). Environmental levels of ZEN are generally low, but in pathological processes, oestrogens and oestrogen-like (i.e., phytoestrogens or/and mycoestrogens) compounds can produce a functional imbalance which stimulates the development of the existing hormonally sensitive neoplasms or can provoke intensive proliferation processes in hormone-reactive cells (Vlata et al. 2006).

Apoptosis is observed at every stage of oogenesis, beginning from primordial follicles, through oogonia, to primary oocytes (Warzych and Lechniak 2005, Concannon 2011). Around two-thirds of primordial follicles degenerate during foetal development or shortly after birth (Brasil et al. 2010) by three possible mechanisms: death by neglect, death by defect, and

altruistic death. Regarding death by neglect, apoptosis is induced because of the low activity of growth factors that operate via specific receptors. Blocking receptor access impairs transfer of intercellular signals, leading to cell death. The second mechanism results from meiotic recombination defects that induce apoptosis. Altruistic death takes place when a group of primordial follicles commits suicide to enhance the survival of healthier cells (Tilly 2001). The diverse aetiology of follicular apoptosis at different stages of development requires further research to investigate the type of processes in ovarian tissues.

Popular detection methods, such as the PCNA (proliferating cell nuclear antigen) test and the TUNEL (*terminal* transferase d-UTP nick end-labelling) assay, are not reliable markers for proliferation and apoptosis because of the possibility of false-positive staining (Skierski 2008). Furthermore, results from these tests are not always correlated with morphological changes characteristic of proliferation or apoptosis. Apoptotic pathways can be highly differentiated; therefore, a comprehensive analysis of these phenomena in oocytes should incorporate various techniques, including morphotic cell evaluations (Motea and Berdis 2010).

This study include the effect of the experimental evaluation, long-term exposure to low-dose of ZEN for determination values of proliferative index (PI), apoptotic index (AI) and investigation of the ultra-structural organization of ovarian follicles in pre-pubertal bitches.

Materials and Methods

All of the experimental procedures involving animals were carried out in compliance with Polish legal regulations determining the terms and methods for performing experiments on animals (opinion of the Local Ethics Committee for Animal Experimentation No. 37/2006 issued on 24 October 2006).

Experimental animals – Thirty immature Beagle bitches aged approximately 70 days, with average body weight of 8 kg, were obtained from local breeders (registered at the Polish Kennel Club) and kept under standard conditions with free access to water. Clinically healthy individuals were divided into three experimental groups (n = 10 each) and fed 50 µg ZEN/kg BW (100% NOAEL), experimental group I (EI); 75 µg ZEN/kg BW (150% NOAEL), experimental group II (EII); or placebo (without ZEN) negative control group (C) as outlined below. All bitches were ovario-hysterectomized at the end of the 42-day treatment period, approximately on day 112 of life. The animals were kept in cages with ad libitum

access to water and fed standard diets tested for the presence of the following mycotoxins: aflatoxin, ochratoxin, ZEN, α -zearalenol, and deoxynivalenol. The estimation of mycotoxins in the diet was carried out according to common separation techniques using immunological affinity columns and high performance liquid chromatography (Hewlett Packard, type 1050 and 1100) (Obremski et al. 2003) with fluorescent and/or UV detection techniques. The values obtained were below the sensitivity of the test.

Preparation and administration of diets – Mycotoxin doses were administered daily in gelatin capsules before morning feeding. ZEN samples (Zearalenone Z-0167, Sigma Chemical Co., Steinheim, Germany) were diluted in 300 µl 96% ethyl alcohol (ethyl alcohol, 96% vol., SWW 2442-90; Polskie Odczynniki Chemiczne SA) corresponding to ZEN doses of 50 and 75 µg/kg BW. The resulting solution was introduced into the feed, placed in gelatin capsules, and stored at room temperature for 12 h to evaporate the solvent. After 42 days of oral exposure, all bitches were anesthetized and ovario-hysterectomized.

Immunohistochemical investigations – Samples of ovaries were prepared and processed at the Department of Pathophysiology, Forensic Veterinary Medicine and Administration, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn. Both ovaries were collected from all the animals.

Material sampling and preparation – Three sections of each ovary, sampled for immunohistochemical analyses, were fixed in 10% formalin, neutralized, buffered to pH 7.4 passed through graded alcohols, clarified in xylene and embedded in paraffin blocks. Microtome sections were stained with haematoxylin and eosin (H&E) and PAS according to the method proposed by McManus. We examined cross-sections of the ovary for a minimum of 20 fields of view at 100x magnification. Microscopic images were analysed under a light (halogen lighting at 100 power was used on an Olympus BX50 microscope) microscope at 400x magnification.

TUNEL test – The AI was determined in 30 ovaries obtained during ovario-hysterectomy. The induction of apoptosis in cultured granulosa cells was investigated with the ApoAlert DNA Fragmentation Assay Kit (Clontech). Tissue sections were deparaffinised in xylene and rehydrated by graduated ethanol washes. After 48 h culturing with the studied reagents, the medium was removed, and the cells were rinsed twice with 200 µl PBS (pH 7.4, room temperature). The cells were immersed in 4% formaldehyde and fixed for 25 min and again rinsed twice with PBS. To permeabilize cell membranes, the cells were incubated with 0.2% Triton X-100 (6 min, ice) and rinsed with

PBS (5 min, room temperature). The growth chamber was removed from the slide and the cells covered with equilibration buffer (100 μ l/well). A cover glass was placed on the slide to ensure even distribution of the buffer. The specimens were equilibrated for 10 min. Incubation buffer containing terminal deoxynucleotidyl transferase was prepared. The buffer for every reaction comprised: 45 μ l equilibration buffer, 5 μ l nucleotide mix, and 1 μ l terminal deoxynucleotidyl transferase. After 10 min of equilibration, the cover glass was removed and excess buffer drained off. The cells were covered with the incubation buffer (50 μ l/well), and a cover glass was placed on the slide. The specimens were incubated in darkness (at 37°C, Petri dish lined with wet filter paper, 75 min). After incubation with terminal deoxynucleotidyl transferase, the cells were covered with 2x SSC buffer to inhibit enzyme activity (15 min, room temperature, darkness). The slides were rinsed with PBS (5 min, room temperature), and propidium iodide was applied (propidium iodide/PBS, 10 μ g/ml, 6 min, room temperature, darkness). To remove excess staining solution, the specimens were rinsed three times with distilled water and incubated for 5 min at room temperature. Every experiment was performed in four replications. The cells were observed under a fluorescent microscope (520 nm wavelength) directly after the last rinsing. Six TUNEL images were obtained for every specimen, one for each studied factor. The total number of granulosa cells and the number of apoptotic cells was counted in each image. The results were presented in the form of the AI, i.e., the percentage of apoptotic cells. Prior to statistical analysis, the results were arcsine transformed. To verify the quality of the TUNEL assay, the cells were treated with DNase as positive control (two doses of 25 U/ml and 50 U/ml). To produce a positive biological control, cells were cultured on a medium with the addition of popular apoptosis inducers: tumor necrosis factor- α (10 ng/ml, 100 ng/ml, 1 μ g/ml), staurosporine (0.1 nM, 1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μ M), and actinomycin D (act. D: 500 ng/ml, 1 μ g/ml). Negative control comprised cells incubated in buffer without terminal deoxynucleotidyl transferase. Three independent observers evaluated apoptotic cells. The number of apoptotic cells was determined in 10 fields of view covering a total of around 1000 cells and the number in each field summed to obtain the AI. In the event of differences in the AI values given by independent observers, the reported results were averaged.

PCNA method – The presence of PCNA was determined immunohistochemically with anti-PCNA mouse monoclonal antibodies (DAKO, clone PC-10, IgG2 kappa) diluted 1:150. Immunohistochemical reactions were identical for all preparations. The same

reagents, time, temperature, and moisture conditions were used for all tissue sections and were performed on the same day by three persons with the DAKO ARK™ Animal Research Kit for immunohistochemical staining of paraffin sections with mouse primary antibodies. The immunoperoxidase staining method was applied, and the streptavidin-biotin-peroxidase complex solution was used to eliminate non-specific reactions. Following paraffin removal and hydration, the prepared material for PCNA analysis was placed in a citrate buffer with a pH of 6.0 and boiled twice in a 650 MW microwave oven for 5 min each to expose the antigens. To inactivate endogenous peroxidase, the materials were incubated for 5 min in the presence of a peroxidase blocker provided in the kit. Prior to staining, anti-PCNA antibodies were mixed separately with biotinylated anti-mouse immunoglobulin (secondary antibody) at the calculated rates. Independent calculations were made by three persons for the same preparation, and the average result was used. A biotinylated secondary antibody conjugate against the primary antibody was obtained, and a mouse serum blocking reagent was added to fix the biotinylated anti-mouse immunoglobulin that was not bound in previous reactions to minimize potential interactions with endogenous tissue immunoglobulin. The resulting biotinylated primary antibodies were added to the samples and incubated for 15 \pm 5 min. The slides were covered with peroxidase-labeled streptavidin and 3,3'-diaminobenzidine as the chromogen to obtain a stained reaction product. The materials were additionally stained with hematoxylin, dried, and fixed in Canadian balm. Negative controls comprised only IgG2 kappa antibodies, while positive controls were commercially available sections (DAKO). The materials stained by the McManus method (PAS) were viewed under X 480 magnification to determine the number of cells demonstrating traces of the antigen (PCNA+) and the number of cells showing no antigen (PCNA-). The results were processed statistically, and PI values for ovarian and uterine structures of the studied bitches were presented in tabular form. The PI is the percentage ratio of PCNA+ cells to total PCNA- cells in each structure (Falco 2009). To measure PCNA values, 1000 PCNA+ and PCNA- cells were counted in randomly selected fields under X 100 magnification.

Ultra-structural investigation – Investigations of 30 ovaries were performed at the Chair of Histology and Embryology, Chair of Functional Morphology, Faculty of Veterinary Medicine at the University of Warmia and Mazury in Olsztyn. Ovarian specimens were collected directly after ovario-hysterectomy within 3 min after extraction of the reproductive tract. The specimens were fixed in a mixture of 2.5% glutaral-

Table 1. Average apoptotic index values in selected ovarian structures in the control and ZEN-treated bitches (mean \pm SD).

Groups	Oocytes	Primordial follicles	Primary follicles	Atretic follicles
EI	25.54 \pm 7.12 ^{bc}	4.84 \pm 0.56 ^b	3.30 \pm 1.82 ^a	20.12 \pm 0.85
EII	44.07 \pm 12.03 ^b	4.66 \pm 0.47 ^b	2.52 \pm 1.26 ^a	20.13 \pm 1.17
C	11.56 \pm 4.01	0.90 \pm 0.19	1.37 \pm 0.52	20.52 \pm 0.60

^{a, b} – significant ($P \leq 0.05$) or highly significant ($P \leq 0.01$) differences, respectively, between groups EI and EII and group C

^c – highly significant ($P \leq 0.01$) differences between group EI and group EII

Table 2. Average proliferation index values in selected ovarian structures in the control and ZEN-treated bitches (mean \pm SD)

Groups	Oocytes	Primordial follicles	Primary follicles	Atretic follicles
EI	42.44 \pm 27.35 ^a	11.11 \pm 5.04 ^a	23.39 \pm 13.82 ^a	14.24 \pm 2.18 ^a
EII	35.25 \pm 3.14 ^b	8.15 \pm 7.99 ^a	17.23 \pm 16.01 ^a	12.16 \pm 0.75
C	70.60 \pm 3.51	17.82 \pm 3.96	41.67 \pm 27.16	11.87 \pm 1.66

^{a, b} – significant ($P \leq 0.05$) or highly significant ($P \leq 0.01$) differences, respectively, between groups EI and EII and group C

dehyde and 2% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2) at room temperature for 2 h and post-fixed in 2% osmium tetroxide. Dehydration was performed in a graded series of ethanol solutions and propylene oxide. The preparations were embedded in Epon 812 resin. Semithin sections were cut from each block of tissues and stained with toluidine blue in order to choose the places containing developing and atretic ovarian follicles. Ultrathin section prepared from these places were contrasted and examined with Tesla BS 500 electron microscope.

Statistical analyses – The PI and AI values are presented as the arithmetic mean \pm SD of the mean. Data were processed with the use of Statistica software (StatSoft Inc., USA). The resulting means were compared by one-way repeated measures ANOVA and Fisher LSD post-hoc test ($P \leq 0.05$).

Results

AI values of selected ovarian structures – In the oocytes, differences ($p \leq 0.01$) were found among groups EI, EII, and group C. The difference between group EI and group EII was highly significant ($p \leq 0.01$). Regarding primordial follicles (Fig. 1A), highly significant differences ($p \leq 0.01$) were found between both experimental groups and the group C. In primary ovarian follicles (Fig. 1B, C), the differences between both experimental groups and control were significant ($p \leq 0.05$). The AI values were not evenly distributed, and the median for all structures and groups was determined at 13.29 AI, where the lower and upper quartiles of the analyzed structures reached 0.90 and 44.07 AI, respectively (Table 1). The median was determined at 13.45 for EI, 17.84 for EII,

and 8.59 for C. Based on median values for all groups and the values of the lower and upper AI quartiles, the results were divided into four types characterized by different apoptosis intensity: A, very low AI (AI < 5); B, low AI ($5 \leq \text{AI} \leq 13$); C, high AI ($13 \leq \text{AI} \leq 21$); and D, very high AI (AI > 21). The AI values demonstrating type A activity (very low) were determined in primordial and primary ovarian follicles (Fig. 1B, C) in all experimental groups, and the lowest AI values were observed in control. The AI values indicative of type B activity (low) were found only in group C oocytes. Type C (high) activity was characteristic of atretic ovarian follicles in all the groups. The highest level of activity (D) was reported in oocytes in both experimental groups (EI and EII).

PI of selected ovarian structures in bitches – Investigation of the oocytes revealed differences ($p \leq 0.01$) among groups EI, EII, and group C. Primordial ovarian follicles were characterized by significant differences ($p \leq 0.01$) between EI and EII vs group C. An analysis of primary ovarian follicles showed significant differences ($p \leq 0.01$) between group C (Fig. 2A) and groups EI (Fig. 2B) and EII (Fig. 2C). Regarding atretic ovarian follicles, significant differences ($p \leq 0.01$) were determined between group C and group EI. No differences were found between groups EI and EII. The average PI values (Table 2) were not characterized by normal distribution; the median for all the structures was determined at 25.49, and the lower and upper quartiles of the analyzed structures reached 8.15 and 70.6, respectively. Based on median values for all the groups and the values of the lower and upper PI quartiles, the results were divided into four types characterized by different proliferation intensity: A, very low PI (PI < 15); B, low PI ($15 \leq \text{PI} \leq 25$); C, high PI ($25 \leq \text{PI} \leq 35$); and D, very high PI

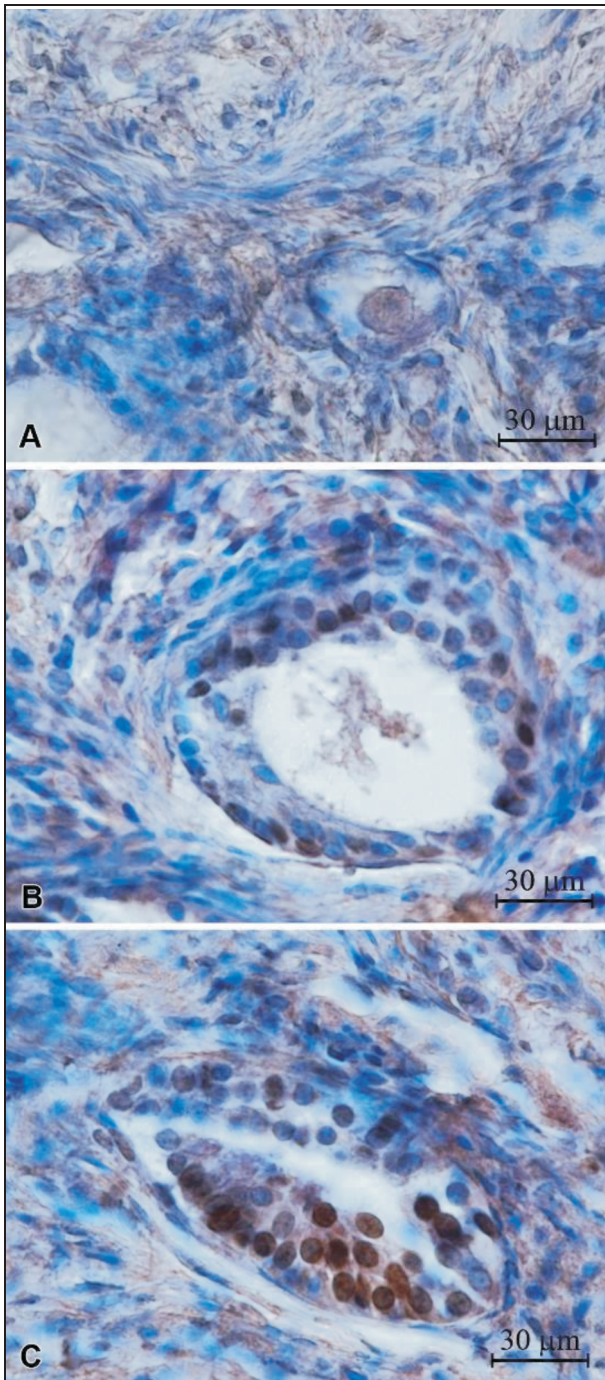


Fig. 1. TUNEL staining of cell nucleus; A – group C. Primordial follicle; B – group EI. Primary follicle with granulosa cells; C – group EII. Primary follicle with granulosa cells.

($PI > 35$). The lowest PI value indicative of type A activity was observed in primordial follicles of group EII animals. The highest PI value characteristic of type D activity was found in control group oocytes. In general, the highest PI values were noted in oocytes (type D activity), primordial follicles (type B activity), and primary follicles (type D activity) of control group animals. The lowest PI values were determined in the corresponding ovarian structures in group EII. Atretic

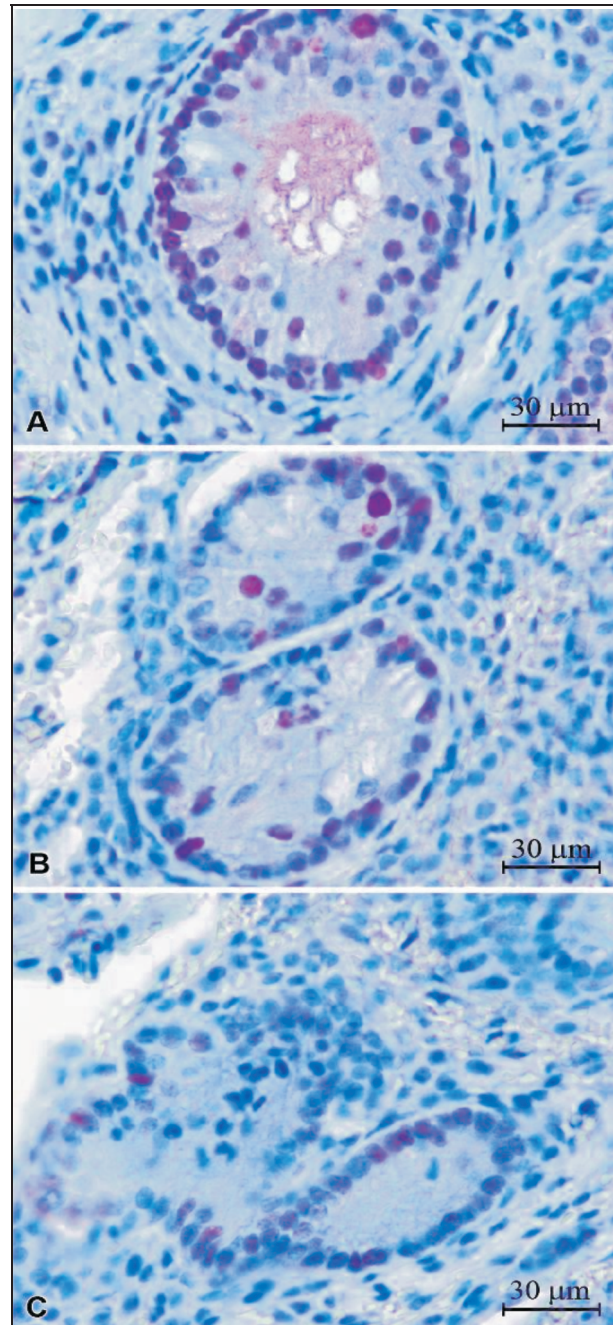


Fig. 2. Positive PCNA protein staining; A – group C. Primary follicle with granulosa cells; B – group EI. Primary follicles with granulosa cells; C – group EII. Primary follicles with granulosa cells.

ovarian follicles were characterized by very low PI values (type A activity) in all the groups.

Statistical results – The AI and PI values were expressed in numerical form, and the corresponding values for selected ovarian structures are presented in Tables 1 and 2.

Ultra structural investigations – *Control group* – In the control group, primordial and primary ovarian follicles revealed the presence of oocytes with an ultra-

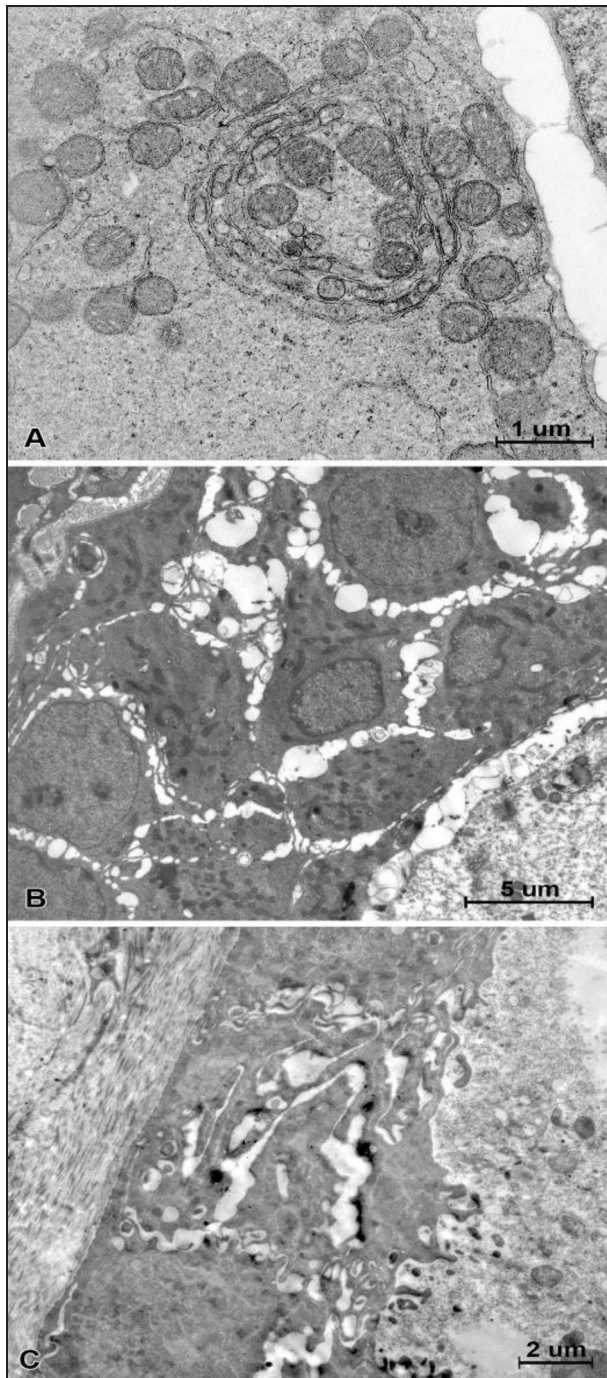


Fig. 3. Ultra structural analysis of ovarian structures in bitches: A – group C. Fragment of an oocyte with a characteristic annular arrangement of the endoplasmic reticulum and numerous mitochondria. Space separating the oocyte from follicle cells; B – group EI. Expansion of intercellular spaces, loss of junctions between follicle cells and the oocyte. Mitochondrial and cytoplasm vacuolization; C – group EII. Atrophy of junctions and vacuolization of intercellular spaces. Atrophy of major organelles and high electron density, hyaline degeneration.

structure characteristic of the development all stage studied. Oocytes were surrounded by a single layer of squamous follicle cells that were separated from the

stroma by the basal lamina resting on an oocyte nucleus containing finely dispersed chromatin. Irregular microvilli projected from the oocyte into the perivitelline space between the oocyte and the surrounding follicle cells. Some were in contact with slender processes of follicle cells projecting toward the oocytes (Fig. 3A). The oocyte cytoplasm was electron-lucent with numerous mitochondria, smooth endoplasmic reticulum cisterns, and few dictyosomes of the Golgi apparatus. The mitochondria were small, round, or oval in shape (Fig. 3A). In primary atretic follicles, the space between the oocyte and follicle cells was expanded. Deposits with average electron density were observed in the intercellular space. The loss of functional complexes and microvilli degeneration was observed. In late primary follicles, primary oocytes were surrounded by a layer of follicle cells giving a stratified epithelium, the membrane granulosa, and the basal lamina separated the outermost layer of the follicle cells and the connective stroma. A well-developed zona pellucida was penetrated by granulosa cell processes. Developing theca folliculi were observed.

Experimental group I – Degeneration processes were observed in oocytes and follicle cells of primary and late primary ovarian follicles (Fig. 3B). Degenerative changes were not found in the remaining follicle interstitial cells. Changes in most primary ovarian follicles were observed in oocytes and in the follicle cell layer. The following changes were found in oocytes (Fig. 3B): mitochondrial vacuolization, loss of mitochondrial cristae, reticular vacuolization, reticular fragmentation, cytoplasmic vacuolization in the peripheral cell layer, and degeneration of microvilli penetrating the space separating oocytes from follicle cells. The following changes were observed in follicle cells (Fig. 3B): loosening of connections between cells and enlargement of intercellular spaces, loss of junctions between follicle cells and oocytes, mitochondrial vacuolization, cytoplasmic vacuolization, the presence of cell debris, and gradual disintegration of theca folliculi.

Experimental group II – Atresia was observed in primary and late primary ovarian follicles (Fig. 3C) and in interstitial cells. Degenerative changes were not found in the remaining follicle structures. In primary follicles most oocytes underwent nearly complete degeneration. Cell debris comprised degenerated remnants of mitochondria, smooth endoplasmic reticulum, vacuolated nuclei, and cytosol. The following changes were observed in follicle cells (Fig. 3C): loss of junctional complexes and vacuolization of intercellular spaces, high electron density combined with degeneration of the cell structure (cytoplasmic hyalinization), and the basement lamina with

variable thickness. All cells had processes extending to the apical surface and lateral surfaces. The majority of late primary oocytes underwent complete degeneration. The following changes were observed in follicle cells: loss of junctional complexes and broadening of intercellular spaces, fragmentation of the apical and lateral parts of cells, and degeneration of internal cellular structures leading to cytoplasmic hyalinization.

Discussion

The effect of low-dose ZEN mycotoxicosis on ovarian structures has been scantily researched to date, and the available studies discuss only selected aspects of the problem (Gajęcka et al. 2008). Because of the absence of the reference data, selected results of this study will be extrapolated to formulate conclusive findings in this field of research.

Fungal metabolites can cause indirect damage to nucleic acids, potentially leading to this final stage. In this study, the degree of apoptosis in ovarian cells was determined by the TUNEL assay, as others have done previously (Motea and Berdis 2010). This method supports the identification of ovarian cells with characteristic symptoms of apoptosis, including those in early stages of suicide when morphological changes are not clearly pronounced (Jiang et al. 2011). In most studies (including the present work), the presence of apoptotic cells is determined with the use of the AI, which indicates the percentage of apoptotic cells in 1000 cells (Dworakowska 2005). In our experiment, the AI median for the entire group of 30 bitches reached 13. The AI median was significantly higher in group EI (13.45), and it was two-fold higher in group EII (17.84) in comparison with control (8.59) and the overall median. Because our results cannot be comprehensively benchmarked with other scientific data, we can only assume that the reported values are high and that they attest to a highly significant increase in apoptosis intensity in the cells of pre-pubertal bitches subjected to mycotoxicosis. The reported values are high in comparison with the oocytes of pre-pubertal cattle in which the median was determined at 7. By contrast, AI medians in the oocytes of adult animals have been as high as 23. Median values increase with age, but in this study, we examined the ovaries of pre-pubertal bitches; therefore, AI values should be physiologically low.

The results of ultra-structural tests significantly contributed to our research findings; for example, in group EI, degeneration processes were observed in oocytes, and in primordial and primary follicular

cells. The following changes were observed in oocytes: reticular vacuolization, reticular fragmentation, cytoplasmic vacuolization in the peripheral cell layer, formation of structure less deposits measuring around 1 μm in diameter, and loss of microvilli directed toward the space separating oocytes from follicle cells. The changes observed in follicle cells involved loosening of junctions between cells and expansion of intercellular spaces, loss of connections between follicle cells and oocytes, cytoplasmic vacuolization, and presence of cell debris. Disintegration processes were more pronounced in group EII in which atretic changes were found in primordial and primary follicles, in particular in oocytes, the associated follicles, and interstitial cells. Most oocytes underwent complete degeneration. In the follicular lumen, cellular rubble was observed, consisting of remnants of mitochondria, endoplasmic reticulum, vacuolated nuclei, and cytosol. Follicle cells revealed a loss of intercellular junctions, vacuolization of intercellular spaces, and degeneration of major organelles in the cytoplasm. The internal structure of mitochondria was blurred, and the key organelle responsible for the generation and maintenance of energy had no visible contours. Low ATP levels contribute to the initiation of apoptosis (Moreira et al. 2011). All cells had processes extending to the apical surface and lateral surfaces (Skierski 2008). Some of the changes observed were clearly indicative of apoptosis.

Oocytes remain attached to follicle cells via tight junctions throughout the entire period of growth and maturation. In our study, this role could be attributed ZEN – the loosening of tight junctions and the expansion of intercellular spaces was observed in group EI, and these changes were particularly pronounced in group EII. Other researchers have not observed direct correlations between follicle cell and follicular wall morphology and oocyte quality (Zeuner et al. 2003). According to these authors, a certain number of ovarian follicles undergo apoptotic changes, and the apoptotic signal is transmitted from follicle cells to the oocyte only when a critical activity level is exceeded, e.g., when ATP levels drop as a result of mitochondrial damage (Moreira et al. 2011). In our study, the results of ultra-structural analyses of ovarian specimens collected from group C animals corroborate the above observations. Primordial and primary ovarian follicles revealed the presence of ova with ultra-structures characteristic of the studied development stage and with a low AI of 8.59. In control, the share of adherent follicles was similar to that observed in both experimental groups.

The results of the PCNA test, carried out to measure the intensity of the proliferation process in

the ovary, contributed to our conclusion that a reliable determination of apoptosis requires several indices. The PCNA method is used because in addition to apoptosis, mitotic proliferation is the only mechanism that naturally maintains homeostasis in the organism. PCNA expression determines the percentage of stained cell nuclei. In the PCNA test, a higher number of ovarian cells must be analysed; in our study, 10 fields of view were examined. Multiple fields must be evaluated when positively stained nuclei are not evenly distributed. PCNA expression varies in different sections of the same ovarian tissue; therefore, the final percentage of stained nuclei is strictly determined by the size of the analysed specimen and the number of viewed fields (Dworakowska 2005). The proliferative activity of the ovarian follicles studied was less pronounced in both experimental groups, as demonstrated by the overall PI median of 25.49. The values of the PI median reported in each group (EI = 22.79, EII = 18.20, C = 35.49) also indicate that zearalenone-induced mycotoxicosis slowed proliferation processes in the oocytes of primordial and primary ovarian follicles in comparison with group C in which no such differences were observed in the proliferative activity of atretic ovarian follicles. Oestrogens generally inhibit proliferation (Van Cruchten et al. 2004). In our study, the period of intoxication was relatively long, and the described proliferative changes were characteristic of hyperestrogenism (De Bosschere et al. 2002). Intensified proliferation was observed in group C animals in which normal ovarian structures were maintained. In control group bitches, the levels of PCNA expression point to physiological proliferation and normal oestrogen secretion (at a very low level) by ovarian follicles (Songsasen and Wildt 2007).

Diagnostic value of the analysed indicators – Scientific data that document the combined effect of the analysed indicators (AI, PI, ovarian ultrastructure) on pre-pubertal bitches subjected to long-term ZEN intoxication are scarce. The significance of individual parameters has been studied extensively (Skierski 2008), but most researchers used different animal models, primarily laboratory animals, which were not exposed to ZEN mycotoxicosis. In our work, the reported AI and PI values and the ultra-structural organization of ovarian follicles provided valuable information indicating that the presence of even very low doses of ZEN (NOAEL) in commercial feed intensifies apoptosis, slows proliferation, induces adverse ultra-structural changes in primordial and primary follicles in both oocytes and follicle cells in pre-pubertal bitches. The degree of decrease in the activity and atresia was directly proportional to the administered ZEN dose. This combined analysis of the discussed indicators of ZEN effects in the ovaries of pre-puber-

tal bitches also expands our knowledge of the processes that take place in the ovaries (Motea and Berdis 2010).

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