Polish Journal of Veterinary Sciences Vol. 15, No. 4 (2012), 711-720

DOI 10.2478/v10181-012-0108-1

Original article

The low doses effect of experimental zearalenone (ZEN) intoxication on the presence of Ca^{2+} in selected ovarian cells from pre-pubertal bitches

M. Gajęcka[1], B. Przybylska-Gornowicz[2]

[1] Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Oczapowskiego 13, 10-718 Olsztyn, Poland
[2] Department of Histology and Embryology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Oczapowskiego 13, 10-718 Olsztyn, Poland

Abstract

The objective of this study was to determine the effect of 42-day ZEN intoxication on the presence of Ca^{2+} in selected ovarian cells from beagle bitches, using the potassium pyroantimonate (PPA) method. Samples were collected from 30 clinically healthy, pre-pubertal, genetically homogeneous animals. The bitches were divided into three groups of 10 animals each: experimental group I – 50 µg ZEN/kg BW (100% NOAEL) administered once daily *per os*; experimental group II – 75 µg ZEN/kg BW (150% NOAEL) administered once daily *per os*; control group – placebo containing no ZEN administered *per os*. An electron microscopic analysis revealed that cells died due to apoptosis, depending on the ZEN dose and the type of cells exposed to intoxication. Lower ZEN doses led to apoptosis-like changes in the cells. Cell death was a consequence of excess Ca^{2+} accumulation in the mitochondria, followed by cell dysfunction and a decrease in or the absence of mitochondrial metabolic activity in oocytes, follicle cells and interstitial cells in experimental bitches.

Key words: zearalenone, bitches, ovary, ultra histology, mitochondrium, Ca^{2+}

Introduction

Among the many diseases that affect livestock and companion animals, increasing attention has been paid recently to mycotoxicoses known for a long time, including zearalenone mycotoxicosis. The symptoms of this disease are observed in tissues that contain high concentrations of estrogen receptors (ERs) in monogastric farm and companion animals (Słomczyńska 2004, Taylor 2010, Bryden 2012).

ZEN is a non-steroidal estrogenic mycotoxin that induces changes in the metabolic profile and the activity of ZEN-sensitive cells, including cells in the ovaries, uterus, mammary glands, liver, bone marrow, brain and epithelium of the small and large intestines (Maresca and Fantini 2010, Gajęcka et al. 2011, 2012a). Research studies investigating ZEN's biotransformation indicate that when administered orally this mycotoxin is nearly completely absorbed. ZEN may be partially metabolized in plant material, the lumen

Correspondence to: M. Gajęcka, e-mail: mgaja@uwm.edu.pl, tel.: +48 89 523 32 37

and walls of the gastrointestinal tract, the liver, and even in granulosa cells of the cumulus oophorus in monogastric animals and humans. This leads to the formation of metabolites, such as α-zearalenol and β-zearalenol or α-zearalanol and β-zearalanol, with varied levels of biological activity (Gajęcka et al. 2009).

The results of our previous work suggest that in dogs and other carnivores ZEN may induce pathological changes in the reproductive system (Gajęcka et al. 2004). At higher doses of the mycotoxin the observed changes are irreversible and they may lead to infertility and absence of ovulation in bitches. Preliminary research points to significant variations in ZEN concentrations in commercial feeds (Zwierzchowski et al. 2004). The phytoestrogen's adverse effects require an in-depth analysis because bitches are often fed a monodiet for many months or even years. Excessive concentrations of endogenous and exogenous estrogens could lead to the development of various diseases associated with hyperestrogenism. Long-term administration of feeds containing ZEN could disrupt hormonal regulation of reproductive processes. The ovaries seem to be particularly sensitive to ZEN. Ovarian functions are easily disregulated under prolonged exposure to exogenous estrogenic substances, particularly as regards oocytes, follicle cells and interstitial cells (Słomczyńska 2004).

The initiation of successive stages of oocyte activation is determined, among others, by Ca^{2+} concentrations in the cytoplasm which have to be raised at a specific time, different for each step (Ozil et al. 2005, Toth et al. 2006).

The mitochondria are one of the organelles controlling the above processes (Gunter and Sheu 2009). The mitochondria produce ATP as a result of oxidative phosphorylation, they regulate intracellular Ca^{2+} homeostasis and the production of reactive oxygen species (ROS) (Gellerich et al. 2010, Moreira et al. 2011). The mitochondria also play a key role in apoptotic processes (Moreira et al. 2011). Changes in the mitochondrial activity potential (defects) could lead to intensified ROS production, activation of mitochondrial mega channels at the point of contact between permeability transition pores (PTP) and the release of apoptotic proteins (Moreira et al. 2011). Changes in the functional and structural properties of mitochondria modulate the key tasks of this organelle, increasing its susceptibility to drugs, including antibiotics, or harmful substances, such as ZEN with toxic and lethal effects (Wallace and Starkov 2002). Various authors have suggested that 17β-estradiol (E_2) (Yang et al. 2004) and other estrogen-like substances, such as phytoestrogens, modify mitochondrial activity. However, mitochondria from different tissues show varied responses to the same stimuli, which could explain differences in mitochondrial activity during intoxication.

In addition, the intrinsic apoptotic pathway may be initiated under the influence of oxidative stress. The death program is irreversibly triggered in the effector phase of apoptosis which groups a number of different pathways characteristic of the excitation phase. The mitochondria play an important role in this process as the pathway is activated by disruption of electron transport or increased Ca^{2+} concentrations, as well as the absence of contact with other cells or DNA damage. The pathway can also be initiated by uncontrolled cell proliferation due to the presence of harmful substances, including mycotoxins (Kiliańska and Miśkiewicz 2003, Maresca and Fantini 2010). The opening of PTP is an early sign of apoptosis and it decreases the transmembrane potential ($\Delta\psi m$), inhibits ATP synthesis, changes thiol compound concentrations and increases Ca^{2+} levels in the mitochondrial matrix. These changes lead to the release of cytochrome c, also referred to as apoptosis protease activating factor-2 (Apaf-2), and other proapoptotic proteins from the mitochondria into the cytosol.

The mitochondria also control steroid synthesis. In addition to the fact that estrogen biosynthesis takes place in the mitochondria, exogenous estrogens are transported specifically to these organelles. In a study where estrogens were administered to oophorectomized rats 75% of the hormone was transported to the mitochondria and not the nuclei of liver, adrenal gland or spleen cells (Felty and Roy 2005). Estrogens have lipophilic properties and therefore they easily diffuse across the lipid bilayer of the cell membrane. The mitochondria are rich in lipids and they act as the cell's estrogen reservoirs (Frizzell et al. 2011).

Both forms of estrogen receptors, ERα and ERβ, have been identified in the mitochondria. Variations have been noted in the affinities of different ligands for ERα and ERβ. E_2, ZEN and ZEN metabolites have varied affinity for the two receptors, depending on the tissue. In addition, the same ligand may display agonistic and antagonistic activity, depending on whether it binds to ERα or ERβ (Gajęcka et al. 2011, 2012a).

The objective of the present study was to determine the effect of low-dose ZEN intoxication on the ultra-structural localization of Ca^{2+} in oocytes, particularly in the mitochondria, from pre-pubertal bitches.

Materials and Methods

The experiment was conducted in accordance with Polish laws that govern the practice of animal testing and experimentation (opinion of the Local Ethics

Committee for Animal Experimentation No. 37/2006 of 24 October 2006).

Thirty immature beagle bitches, 70 days of age, with average body weight of 8 kg, were obtained from local breeders (registered at the Polish Kennel Club), and were kept under standard conditions with free access to water. Clinically healthy bitches were divided into three groups: experimental group I (EI) (n=10) where ZEN (100% NOAEL – no observable adverse effect level – Boermans and Leung 2007) was administered *per os* at 50 µg/kg BW, once daily; experimental group II (EII) (n=10) – ZEN (150% NOAEL) administered *per os* at 75 µg/kg BW, once daily; control group (C) (n=10) – placebo containing no ZEN administered *per os*. ZEN was administered for 42 days. All bitches were ovariectomized at the end of the experiment, i.e. approximately on 90th day of life.

Electron microscopy – The ultra-structural localization of Ca^{2+} in ovarian sections (in particular oocytes, follicle cells and interstitial cells) was determined by the potassium pyroantimonate (PPA) method (Wick and Hepler 1982, Lewczuk et al. 1994). The ovarium cortex and the medulla were dissected, cut into small pieces and fixed in a solution containing 2% glutaraldehyde, 2% potassium pyroantimonate and 0.735% potassium acetate for 2 hours at 4 C (pH 7.5). After the first fixation step, the pieces were rinsed for 10 min three times with 0.735% potassium acetate in distilled water and were incubated for 2 hours in 1% aqueous solution of osmium tetroxide containing 2% potassium pyroantimonate and 0.735% potassium acetate. The pieces were then washed in potassium acetate solution to remove any unreacted potassium pyroantimonate and prevent nonspecific precipitation, they were dehydrated and embedded in Epon 812. Sections contrasted with lead citrate and uranyl acetate as well as uncontrasted ultrathin sections was examined under the FEI Tecnai Spirit G2 12 BioTwin TEM (USA) equipped with the Eagle slow-scan camera (FEI, USA). As a control for the presence of Ca^{2+} in precipitates, thin sections decalcified by incubation in 13.5% EDTA at 60°C for 2 hours were used.

E_2 assay – For the analyses of serum concentrations of E_2 blood samples were collected from each group every seven days at one hour before the morning feeding for the whole time of the experiment. Blood was taken to cold centrifugation tubes with heparin and centrifuged for 20 minutes at 3000 rpm at a temperature of 4°C. The plasma obtained was distributed to plastic "eppendorfs" of 1 ml, frozen and stored at a temperature of -20°C until the analyses were finished. E_2 was extracted from 1 ml of blood serum using ethyl ether (2 ml). The samples were dried under nitrogen and resuspended in 250 µl of PSS. Extraction recovery was 90-95%, and 100 ng/ml E_2 solution in PSS supplemented with 0.1% BSA was the control. E_2 concentrations were determined by EIA using the Multiscan EX plate reader (Labsystem, Finland). Absorbance was measured at 450 nm wavelength. Anti-estradiol serum (donated by Dr G. L. Williams) was used at a final dilution of 1:150 000. The cross-reactivity of the analyzed antibodies was described by Młynarczuk et al. (2005). The calibration curve ranged from 3.125 to 1600 pg/ml and sensitivity was determined at 10-15 pg/ml. Intra-assay and inter-assay coefficients of variation reached 11.0% and 12.5% on average, respectively. The accuracy and reliability of the procedure was expressed by the coefficient of regression, r=0.875. All samples were analyzed in two replications. The results were processed statistically using the STATISTICA StatSoft v.6 application. Correlation analysis was performed between the three studied groups.

Results

Ultra-structural localization of Ca^{2+} by the PPA method.

Group EI:

Oocytes. Ca^{2+} deposits were observed in the mitochondria with well-preserved structure, in the residual endoplasmic reticulum, and between fragments of cellular debris (Fig. 1A).

Follicular cells. Compared with control, Ca^{2+} deposits in the mitochondria were more dispersed in follicle cells (Figs. 1B, 1C). In some follicle cell mitochondria single, large deposits were found, whereas in lysed mitochondria small Ca^{2+} deposits formed clusters. Single deposits were observed in non-coated vesicles. In comparison with the control group, a considerable decrease in the number of Ca^{2+} deposits was noted in the nuclei, whereas large Ca^{2+} deposits were reported in cytoplasmic vacuoles and intercellular spaces. Numerous deposits were observed in the extra-follicular ovarian tissue.

Interstitial cells. Different amounts of Ca^{2+} deposits were observed in the mitochondria of interstitial cells (Fig. 1D). Compared with the control group, a lower number of Ca^{2+} deposits was found in the endoplasmic reticulum and the nuclei. Cytoplasmic vacuoles in the interstitial cells contained Ca^{2+} deposits which formed clusters.

Group EII:

Oocytes. Large Ca^{2+} deposits were randomly distributed in the cellular debris and residues of the oocytes (Fig. 2A).

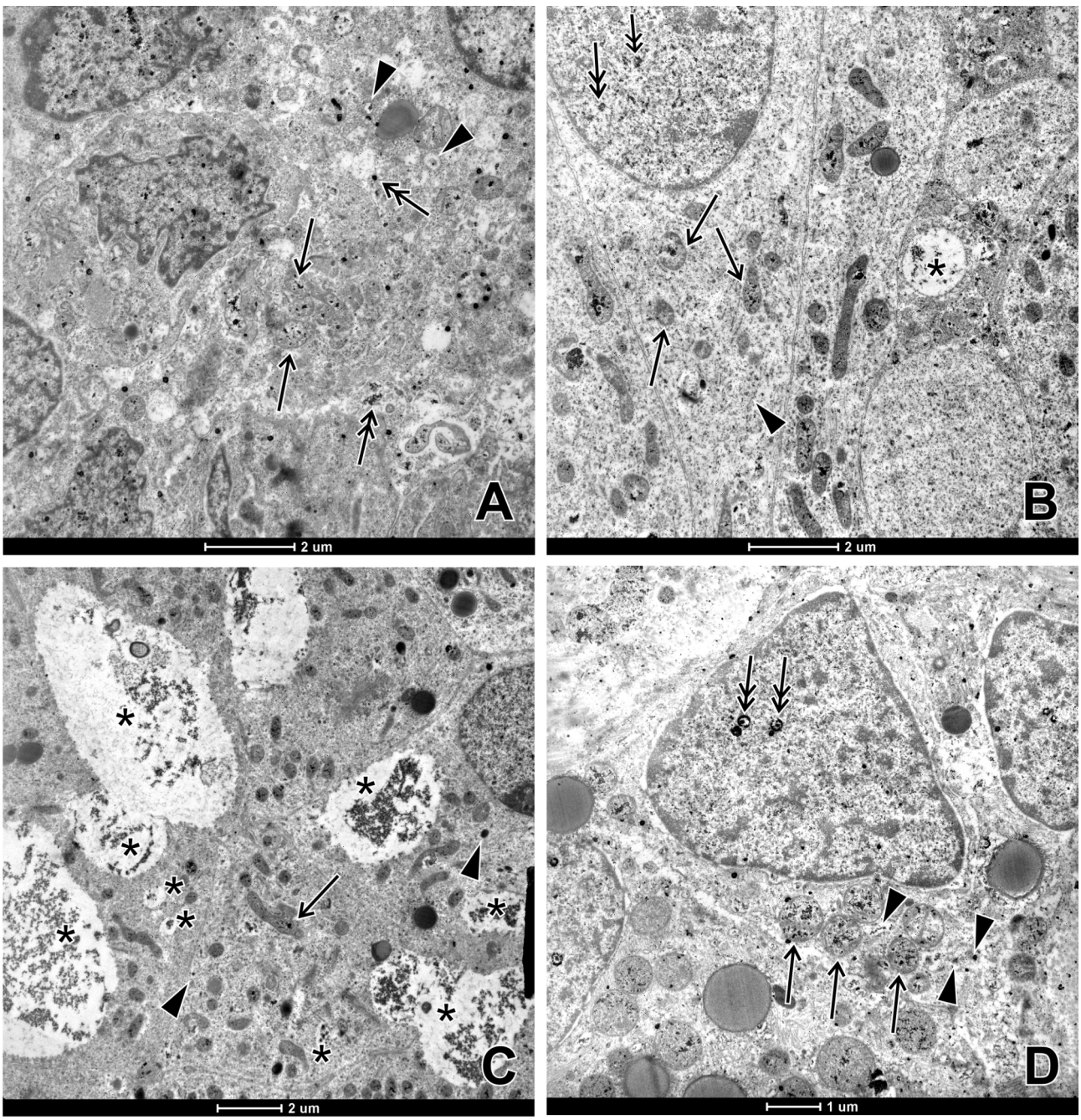

Fig. 1. Electronogram showing Ca^{2+} distribution in the ovaries of **group EI** bitches: **A** – Ovum residue in the primary follicle – calcium deposits in the mitochondria (arrows), small vacuoles (arrow heads) and residual basal cytoplasm (double arrows). **B** – Follicular cells – calcium deposits in the mitochondria (arrows), rough endoplasmic reticulum (arrow heads), nuclei (double arrows), vacuoles (stars) and intercellular spaces. **C** – Follicle with adhesion molecules showing different-sized vacuoles filled with clusters of small calcium deposits (stars), few deposits in the mitochondria (arrows), few deposits of various sizes in the basal cytoplasm (arrow heads). **D** – Interstitial cells with numerous deposits in the mitochondria (arrows), basal cytoplasm (arrow heads) and nuclei (double arrows).

Follicular cells. Deposits of different size, usually large, were scattered throughout the cross-sections of mitochondria or were located at their periphery in follicular cells (Fig. 2B). In the nuclei, a few single different-sized deposits were noted. Dispersed deposits were also observed in the intercellular spaces and in the basal portion of the cells. In the cytoplasm of the follicular cells above the nucleus small clusters of small deposits were found and larger clusters of small deposits were observed in cytoplasmic vacuoles.

In atretic follicles containing the residues of degenerated oocytes, large Ca^{2+} deposits were observed in follicle cells with damaged internal structure and in the debris left from degenerated oocytes. In follicular

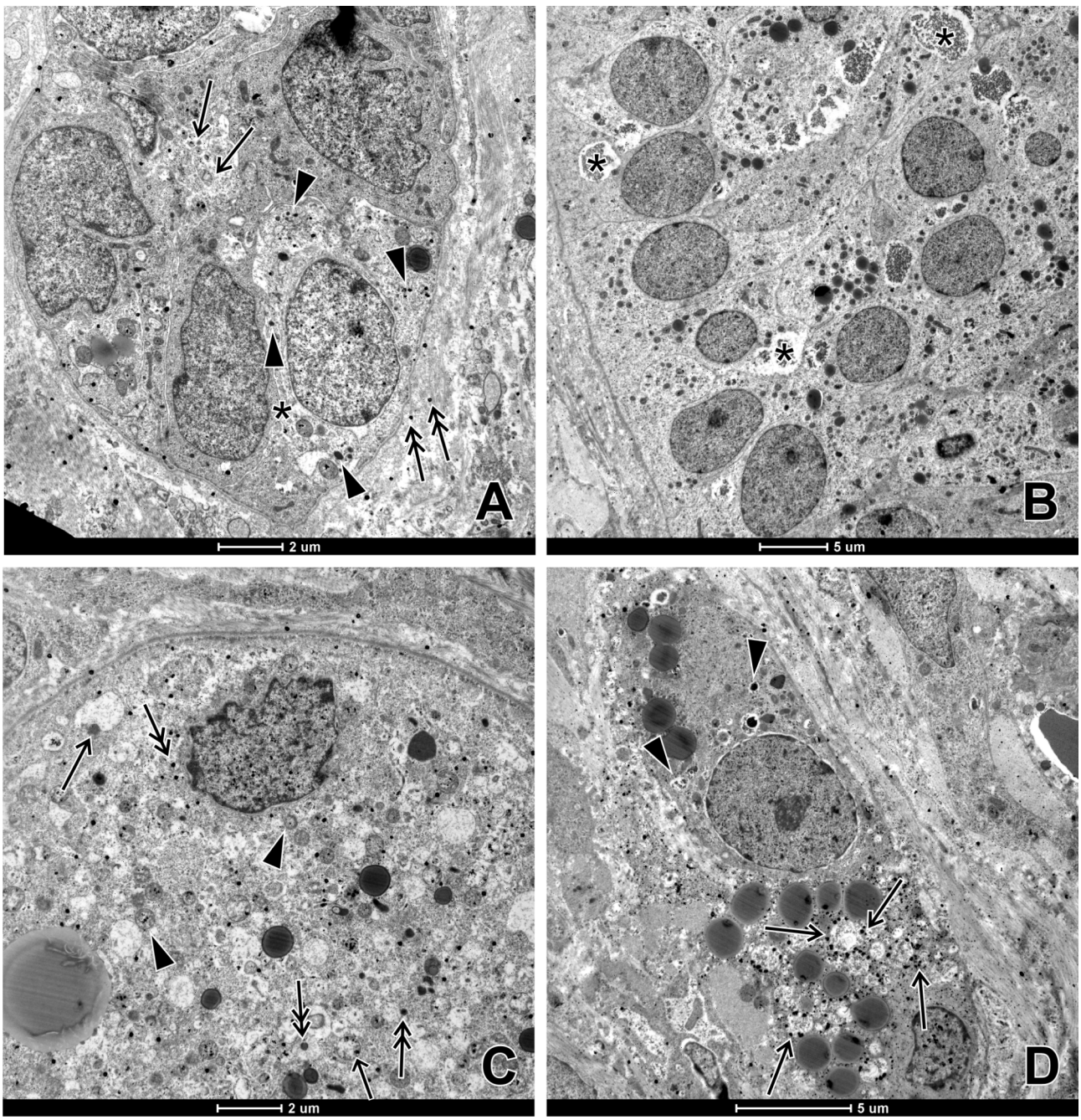

Fig. 2. Electronogram showing Ca^{2+} distribution in the ovaries of **group EII** bitches: **A** – Primary follicle – regularly-shaped deposits in residual first-order oocytes (arrows), follicular cytoplasm (arrow heads) and mitochondria (stars). Individual deposits were observed in follicular stroma (double arrows). **B** – Vesicular follicles – granulosa cells – large, numerous cytoplasmic vacuoles filled with small calcium deposits (stars), few small deposits in the cytoplasm and mitochondria. **C** – Follicle filled with cellular debris showing deposits in the residual mitochondria (arrows), follicles (arrow heads), basal cytoplasm (double arrows) and nuclei and on the follicle circumference by the basal cytoplasm. **D** – Interstitial cells with numerous, regular, medium-sized deposits on the circumference of damaged mitochondria (arrows) and small deposits in reticulum cisterns (arrow heads).

cells with electron dense cytoplasm, aggregates of very small deposits were found. Aggregates were sparse in the nuclei and damaged mitochondria and more numerous in the mitochondria with well-preserved structure.

In the debris of cells (Fig. 2C) different-sized Ca^{2+} deposits were observed in both intact and damaged mitochondria, as well as in a few small vesicles. Large Ca^{2+} deposits were noted in the nuclei within follicular cells debris.

Interstitial cells. A variable number of Ca^{2+} deposits were observed at the periphery of damaged mitochondria in interstitial cells (Fig. 2D). Compared with the control group, the number of Ca^{2+} deposits

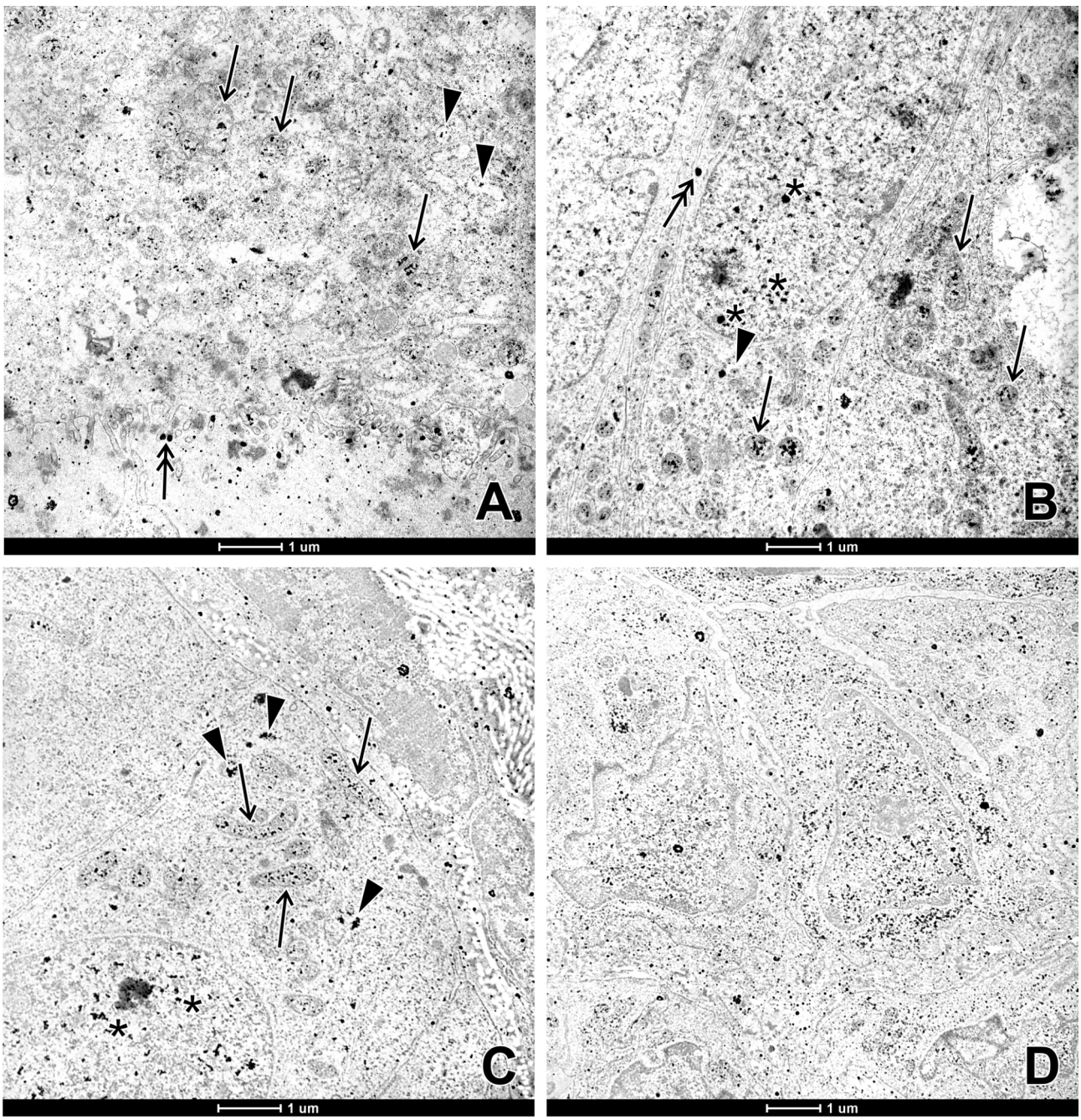

Fig. 3. Electronogram showing Ca^{2+} distribution in the ovaries of **group C** bitches: **A** – Oocytes cytoplasm with numerous small deposits in the mitochondria (arrows), reticulum cisterns (arrow heads) and cytoplasm. Regular, medium-sized deposits in microvilli are protruding into the oocyte space (double arrows). **B** – Follicular cells with deposits in the mitochondria (arrows), Golgi apparatus follicles (arrow heads), cytoplasm (double arrows) and nuclei (stars). **C** – Basal follicle circumference exhibits numerous deposits in the mitochondria (arrows), follicles and small vacuoles (arrow heads) and the nucleus (stars), and numerous deposits in connective tissue stroma. **D** – Interstitial cells with calcium deposits evenly distributed in the nucleus and cytoplasm.

was smaller in the endoplasmic reticulum of interstitial cells. In the cell nuclei, mostly large, single Ca^{2+} deposits were noted.

Group C:

Oocytes. Ca^{2+} deposits were present in the mitochondria, smooth and rough endoplasmic reticulum cisterns, Golgi apparatus, cytoplasm, multivesicular bodies, and surface microvilli of oocytes (Fig. 3A). Small deposits were randomly distributed in the mitochondrial matrix. The number of deposits varied considerably between mitochondria in the same oocytes. Small, single deposits were observed in the cytoplasm. The deposits found in the spaces

Table 1. Average plasma concentrations of E_2 (pg/ml) in bitches on the last day of the experiment (X, SD).

Specification / Group	C	EI	EII
E_2	4.6 ± 0.15	14.66 ± 6.92*	21.58 ± 4.15**#

*, ** – indicate significant differences (P<0.05, P<0.01) between two experimental groups (El, EII) and control group (C);
– indicates significant difference (P<0.05) between both experimental groups.

separating oocytes and follicular cells were generally larger than those observed inside the oocytes.

Follicular cells. Ca^{2+} deposits were found in the mitochondria and the nuclei (Figs. 3B, 3C), and their numbers differed considerably between mitochondria. In cell nuclei, variously-sized deposits were dispersed throughout the karyoplasm. Individual deposits were observed in intercellular spaces, Golgi apparatus, multivesicular bodies, and small non-coated vesicles.

Interstitial cells. Numerous different-sized Ca^{2+} deposits were evenly distributed in the cytoplasm and the cell nuclei of interstitial cells (Fig. 3D). In the cytoplasm deposits were found in the mitochondria and the smooth endoplasmic reticulum.

Hormone assay. Hormone assays revealed elevated concentrations of E_2 in both experimental groups (Table 1). The levels of E_2 in blood of the bitches of EI (P<0.05) and Ell (P<0.01) groups were higher than in the control group. However, the E_2 level in EII group was higher (P<0.05) compared to EI group.

Discussion

Physiological effect of estrogens, including ovulation and estrus, are attributed to the hormones' physiological functions, and their intensity varies with estrogen concentrations. The results of our previous work indicated that acute, persistent, ZEN-induced hyperestrogenism in multiparous bitches is not accompanied by an increase in endogenous estrogen levels. Low or average concentrations of endogenous estrogens, including E_2, were noted in the range of 20-40 pg/ml (Gajęcka 2006). A ZEN-induced decrease in estrogen concentrations in other animal species was also described by other authors (Turcotte et al. 2005). Clinical and behavioral symptoms of estrus were accompanied by low estrogenic secretion in both pre-pubertal gilts and mature pigs. The noted concentrations were substantially lower than the physiological levels noted in estrus (up to 100 pg/ml). Similar observations were made in sexually mature pigs (Gajęcka et al. 2011).

The above – mentioned dysfunction can be attributed to ZEN's various negative effects on the neurohormonal system. The mycotoxin inhibits the secretion of gonadotropins (follicle-stimulating hormone and luteinizing hormone) which control estrogen production and it could damage follicular cells where estrogens are synthesized (Malekinejad et al. 2006a,b). ZEN could also exert a negative influence on the activity of enzymes controlling steroid homeostasis (Ververidis et al. 2004, Malekinejad et al. 2006a,b, Yang et al. 2007). Research has demonstrated that ZEN and its metabolites are capable of binding to ER, causing characteristic estrogen effects in various organs. Although ZEN's most biologically active metabolite, α-zearalenol, demonstrates several-fold lower affinity for ER than E_2 (Słomczyńska 2004), the above mentioned compounds may exert their agonistic or synergistic activities when endogenous estrogens are secreted at lower levels (Gajęcka et al. 2012a).

A different situation was encountered in our experiment where E_2 values were several-fold higher in both experimental groups than in control, although the noted values were generally low (Table 1). However, asymptomatic hyperestrogenism was induced in pre-pubertal bitches due to the administered NOAEL doses. The intoxication state was not accompanied by clinical symptoms, but it was powerful enough to induce apoptotic-like changes in estrogen-sensitive cells (oocytes, follicular cells, interstitial cells) and tissues (ovaries) (Gajęcka et al. 2004). At all stages of apoptosis, a common symptom is an increase in mitochondrial membrane permeability. Cell death resulting from mitochondrial membrane permeability can be divided into three phases: initiation, decision and degradation. The degradation phase involves the release of apoptogenic molecules that normally reside in the mitochondria, leading to catabolic changes and cell degradation.

In a study on pigs administered ZEN at NOAEL doses hyperestrogenism was observed in pre-pubertal gilts at the level of the ovaries without any clinical symptoms (Gajęcka et al. 2011). Mycotoxicosis induced by ZEN administered at NOAEL doses can be very misleading because it does not produce any symptoms indicative of the disease. The progression of the pathological state to the verge of apoptosis can only be determined at the cellular level (Gajęcka et al. 2012b).

Other authors (Słomczyńska 2004, Turcotte et al. 2005) have observed that the clinical symptoms of hy-

perestrogenism can be induced by mycotoxins in bitches and other species. It should be noted that the nature of those symptoms somewhat differs in dogs owing to the specificity of the canine reproductive cycle.

The results of our previous studies investigating pigs and bitches intoxicated with ZEN and its metabolites revealed advanced regressive metamorphoses such as degeneration and atrophy of ovarian cells and tissues, mostly granulosa cells (Gajęcka et al. 2004). Numerous edemas and blood extravasations were also found as characteristic estrogenic symptoms (Gajęcka et al. 2004, 2011). Our findings have been validated by other authors as regards nearly all ovarian structures. Considerable differences were noted in the severity of regressive changes and blood flow dysfunction in animals administered various doses of ZEN. The characteristic, damaging impact on ovarian tissue noted in this study could be attributed to ZEN's long-term effects, regardless of the phytoestrogen's dose.

At the ultra-structural level estrogens and estrogen-like substances increase Ca^{2+} concentrations in the mitochondria. The estrogen-stimulated increase in cellular Ca^{2+} levels has been found capable of activating mitochondrial protein phosphatase which leads to the dephosphorylation of cytochrome c oxidase. Active protein increases mitochondrial membrane potential and increases Ca^{2+} concentrations in the mitochondria. The mechanism of increase of Ca^{2+} levels has not been fully elucidated (Gellerich et al. 2010), but it could be induced by the estrogen-inhibited release of Ca^{2+} from the mitochondria, subject to the presence of Na^{+} ions. The increase in mitochondrial Ca^{2+} levels promotes ROS production.

In view of estrogen's characteristic properties, the presence of large and dispersed Ca^{2+} deposits in the mitochondria, cellular debris and residual oocytes in group EII can be assumed to be induced by the administration of ZEN.

The transitional accumulation of Ca^{2+} in mitochondria in a state of cell stimulation is one of the mechanisms supporting Ca^{2+} homeostasis and calcium signaling control in the cell. During cell stimulation, Ca^{2+} concentrations in the cytosol increase, but this increase is not identical at all sites and it is particularly visible in the area of calcium channels in the plasma membrane and the endoplasmic reticulum which releases Ca^{2+} (Gellerich et al. 2010) (Figs. 3A, 3B, 3C). Mitochondrial Ca^{2+} buffering not only influences the shape of the calcium signal (amplitude, oscillation frequency), but it also exerts a protective effect on the cells. The above processes could be weakened, thus initiating the process of organelle destruction and, consequently, apoptosis of tissues and cells, as observed in the present study (Figs. 2B, 2D). The described mechanism is particularly important when cell excitation is accompanied by a very high, local increase in Ca^{2+} concentrations (Figs. 2A, 2B, 2C, 2D) or the absence of Ca^{2+} outflow. A local increase in Ca^{2+} levels is required for secretion (Denton 2009) in sexually mature organisms. The presence of Ca^{2+} in the mitochondrial matrix could result from uniport concentration, as well as from the inability of Ca^{2+} to flow out due to the effects exerted by ZEN in experimental groups. Physiological Ca^{2+} concentrations in the mitochondrial matrix should respond to changes in Ca^{2+} levels in the cytosol (Figs. 3A, 3D). This assumption is supported by the fact that Ca^{2+} concentrations in the mitochondria have to be increased to regulate dehydrogenase activity in the mitochondrial matrix and maintain hormonal homeostasis at the prereceptor level. In addition, ZEN acts as a matrix substance for the dehydrogenases (Penning 2003, Gellerich et al. 2010).

If the above arguments were to be applied to the analyzed pre-pubertal bitches whose secretion processes were at the developmental phase, it could be concluded that the physiological processes of granulosa cell excitation were only beginning to take place and that energy levels (ATP) in those cells were low. Our results suggest that ZEN stimulated steroidogenesis in both experimental groups. As an undesirable substance, ZEN exerted its effects based on the hormesis principle (Heberer et al. 2007). In our study, ZEN supported apoptosis in both experimental groups.

Global Ca^{2+} concentrations in the cytosol had to be much higher than normal levels, although it was not always observed, e.g. in group EI (Fig. 1D). The mitochondria probably surrounded the region with higher Ca^{2+} concentrations and by absorbing Ca^{2+} they created a barrier preventing the calcium signal from spilling inside the cell (Gunter and Sheu 2009). ZEN, as an undesirable substance, had a more powerful defragmenting, or even destructive, effect on oocytes and granulosa cells in both experimental groups. Our findings are consistent with the results of other studies where pigs and bitches were administered similar ZEN doses (Gajęcka et al. 2011).

According to Ajduk et al. (2007), in an anomalous state, Ca^{2+} concentrations inside the cell increase abnormally and reach excessive values, possibly due to excessive activation of calcium channels stimulated by glutamate in the central nervous system, for example as a result of an ischemic episode. This causes a number of changes in the cell, including in the mitochondria, and consequently may lead to cell death. The mitochondrial intermembrane space contains proteins which, when released into the cytosol, trigger the intrinsic or mitochondrial apoptotic path-

way. Apoptosis, programmed cell death, is strictly regulated and controlled by biochemical processes that require the expression of various genes (Rizzuto and Pozzan 2006).

Conclusions

It can be assumed that ZEN is able to penetrate the cell membrane by forming lipophilic complexes with ions. The disruption of membrane functions initiates a cascade of changes which upset the ion balance between intracellular and extracellular fluid. Inhibited transport of divalent cations impairs the functioning of the sodium-potassium pump, decreases ATP production and upsets the sodium-calcium balance which increases Ca^{2+} concentrations inside the cell. The intensified inflow of Ca^{2+} forces the mitochondria to store the ions to maintain homeostasis. Mitochondrial Ca^{2+} overload inhibits oxidative phosphorylation and may lead to cell death. The mode of action of ZEN probably supported a fast depletion of energy reserves (ATP) and mitochondrial damage contributed to cell death.

The results of the present work clearly indicate that cell death resulted from excessive Ca^{2+} accumulation in the mitochondria, mitochondrial dysfunctions and the ensuing drop or even loss of mitochondrial metabolic activity in oocytes, follicular cells and interstitial cells from pre-pubertal bitches.

Acknowledgements

This study was financed by research grant N N308 242635 from the Ministry of Science and Information Technology in Poland.

References

Ajduk A (**2007**) The role of calcium ions in activation of the mammalian embryo development. Postępy Biologii Komórki 34: 715-729.

Boermans HJ, Leung MC (**2007**) Mycotoxins and the pet food industry: toxicological evidence and risk assessment. Int J Food Microbiol 119: 95-102.

Bryden WL (**2012**) Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. Anim Feed Sci Tech 173: 134-158.

Denton RM (**2009**) Regulation of mitochondrial dehydrogenases by calcium ions. Biochim Biophys Acta 1787: 1309-1316.

Felty Q, Roy D (**2005**) Estrogen, mitochondria and growth of cancer and non-cancer cells. J Carcinog 4: 1.

Frizzell C, Ndossi D, Verhaegen S, Dahl E, Eriksen G, Srrlie M, Ropstad E, Muller M, Elliott CT, Connolly L (**2011**) Endocrine disrupting effects of zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis. Toxicol Lett 206: 210-217.

Gajęcka M (**2006**) The impact of experimental zearalenone mycotoxicosis on the reproductive tract in bitches. Doctoral thesis. University of Warmia and Mazury in Olsztyn, Poland.

Gajęcka M, Jakimiuk E, Polak M, Otrocka-Domagała I, Janowski T, Zwierzchowski W, Obremski K, Zielonka Ł, Apoznański J, Gajęcki M (**2004**) Zearalenone applied *per os* provides adverse effects in structure of chosen parts of bitch reproductive system. Pol J Vet Sci 7: 59-66.

Gajęcka M, Jakimiuk E, Zielonka Ł, Obremski K, Gajęcki M (**2009**) The biotransformation of chosen mycotoxins. Pol J Vet Sci 12: 293-303.

Gajęcka M, Rybarczyk L, Jakimiuk E, Zielonka Ł, Obremski K, Zwierzchowski W, Gajęcki M (**2012a**) The effect of experimental long-term exposure to low-dose zearalenone on uterine histology in sexually immature gilts. Exp Toxicol Pathol 64: 537-542.

Gajęcka M, Rybarczyk L, Zwierzchowski W, Jakimiuk E, Zielonka Ł, Obremski K, Gajęcki M (**2011**) The effect of experimental, long-term exposure to low-dose zearalenone mycotoxicosis on the histological condition of ovaries in sexually immature gilts. Theriogenology 75: 1085-1094.

Gajęcka M, Zielonka Ł, Jakimiuk E, Dąbrowski M, Obremski K, Gorlo G, Mróz M, Gajęcki M (**2012b**) Diagnostic significance of selected analytical indicators of zearalenone mycotoxicosis in animals. Med Weter 68: 566-570.

Gellerich FN, Gizatullina Z, Trumbeckaite S, Nguyen HP, Pallas T, Arandarcikaite O, Vielhaber S, Seppet E, Striggow F (**2010**) The regulation of OXPHOS by extramitochondrial calcium. Biochem Biophys Acta 1797: 1018-1027.

Gunter TE, Sheu S-S (**2009**) Characteristics and possible functions of mitochondrial Ca^{2+} transport mechanisms. Biochem Biophys Acta 1787: 1291-1308.

Heberer T, Lahrssen-Wiederholt M, Schafft H, Abraham K, Pzyrembel H, Henning KJ, Schauzu M, Braeunig J, Goetz M, Niemann L, Gundert-Remy U, Luch A, Appel B, Banasiak U, Bol GF, Lampen A, Wittkowski R, Hensel A (**2007**) Zero tolerances in food and animal feed – are there any scientific alternatives? A European point of view on an international controversy. Toxicol Lett 175: 118-135.

Kiliańska ZM, Miśkiewicz A (**2003**) Caspases of the vertebrates – their role in apoptosis. Postępy Biologii Komórki 30: 129-152.

Lewczuk B, Przybylska B, Wyrzykowski Z (**1994**) Distribution of calcified concretions and calcium ions in the pineal gland. Folia Histochem Cytobiol 32: 243-249.

Malekinejad H, Colenbrander B, Fink-Gremmels J (**2006a**) Hydroxysteroid dehydrogenases in bovine and porcine granulosa cells convert zearalenone into its hydroxylated metabolites α-zearalenol and β-zearalenol. Vet Res Commun 30: 445-453.

Malekinejad H, Van Tol HTA, Colenbrander B, Fink-Gremmels J (**2006b**) Expression of 3α- and 3β-hydroxy steroid dehydrogenase mRNA in COCs and granulosa cells determines zearalenone biotransformation. Toxicol In Vitro 20: 458-463.

Maresca M, Fantini J (**2010**) Some food-associated my-

cotoxins as potential risk factors in humans predisposed to chronic intestinal inflammatory diseases. Toxicon 56: 282-294.

Młynarczuk J, Niewiadowska A, Kotwica J (**2005**) Polychlorinated biphenyls impair FSH-stimulated effect on the secretion of steroids and oxytocin from cultured bovine granulosa cells. Bull Vet Inst Pulawy 49: 411-417.

Moreira PI, Custódio JBA, Nunes E, Oliveira PJ, Moreno A, Seica R, Oliveira CR, Santos MS (**2011**) Mitochondria from distinct tissues are differently affected by 17β-estradiol and tamoxifen. J Steroid Biochem Mol Biol 123: 8-16.

Ozil JP, Markoulaki S, Toth S, Matson S, Banrezes B, Knott JG, Schultz RM, Huneau D, Ducibella T (**2005**) Egg activation events are regulated by the duration of a sustained [Ca^{2+}]cyt signal in the mouse. Dev Biol 282: 39-54.

Penning TM (**2003**) Hydroxysteroid dehydrogenases and pre-receptor regulation of steroid hormone action. Hum Reprod Update 9: 193-205.

Rizzuto R, Pozzan T (**2006**) Microdomains of intracellular Ca^{2+}: molecular determinants and functional consequences. Physiol Rev 86: 369-408.

Słomczyńska M (**2004**) The effect of phytoestrogens on the reproductive tract. Pol J Vet Sci 7: 223-226.

Taylor SE, Martin-Hirsch PL, Martin FL (**2010**) Oestrogen receptor splice variants in the pathogenesis of disease. Cancer Lett 288: 133-148.

Toth S, Huneau D, Banrezes B, Ozil JP (**2006**) Egg activation is the result of calcium signal summation in the mouse. Reproduction 131: 27-34.

Turcotte JC, Hunt PJB, Blaustein JD (**2005**) Estrogenic effects of zearalenone on the expression of progestin receptors and sexual behavior in female rats. Horm Behav 47: 178-184.

Ververidis HN, Boscos CM, Stefanakis A, Saratsis P, Stamou AI, Krambovitis E (**2004**) Serum estradiol-17β, progesterone and respective uterine cytosol receptor concentrations in bitches with spontaneous pyometra. Theriogenology 62: 614-623.

Wallace KB, Starkov AA (**2002**) Mitochondrial targets of drug toxicity. Annu Rev Pharmacol Toxicol 40: 353-388.

Wick SM, Hepler PK (**1982**) Selective localization of intracellular Ca^{2+} with potassium antimonite. J Histochem Cytochem 30: 1190-1204.

Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM Jr, Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J, Koulen P, Simpkins JW (**2004**) Mitochondrial localization of estrogen receptor beta. Proc Natl Acad Sci USA 101: 4130-4135.

Zwierzchowski W, Gajęcki M, Obremski K, Zielonka Ł, Baranowski M (**2004**) The occurrence of zearalenone and its derivatives in standard and therapeutic feeds for companion animals. Pol J Vet Sci 7: 289-293.