Original article

The effects of experimental administration of low doses of zearalenone on the histology of ovaries in pre-pubertal bitches

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Abstract

The experiment involved 30 clinically healthy Beagle bitches aged approximately 70 days with an initial body weight of approximately 8 kg. The animals were randomly divided into two experimental groups (EI and EII) and a control group of 10 animals each. Group EI was administered 50 μg of body weight zearalenone/kg *per os* for 42 days, group EII received 75 μg of body weight zearalenone/kg *per os* for 42 days, and the control group was administered placebo *per os* for 42 days. The bitches were ovariectomized at the end of the treatment period for anatomopathological examination. At the same time, peripheral blood samples were collected for endocrinological analyses (17β-estradiol and progesterone). Administartion of zearalenone particularly higher doses, resulted in the hyperestrogenism degeneration and atrophy of ovarian cells and tissues with accompanying edema and blood extravasation, leading to increased 17β-estradiol concentrations and an insignificant decrease in progesterone levels.

Key words: zearalenone, low dose, bitches, ovary, histology

Introduction

Fungi of the genus *Fusarium* are the most predominant fungal pathogens in all climate zones. *Fusarium* fungi produce mycotoxins, including zearalenone (ZEN), a widely distributed substance. ZEN is a non-steroidal estrogenic mycotoxin that regulates the sexual reproduction of *Fusarium* fungi (sexual stage: *Gibberella zeae*) (Suchorzyńska and Misiewicz 2009, Panini et al. 2011).

Mycotoxicosis has long been studied in humans and animals. ZEN-induced mycotoxicosis represents a growing problem in farm and companion animals. Dogs, one of the oldest companion animal species, have a monoestral reproductive pattern (Walter et al. 2011). Bitches are often affected by reproductive system dysfunction, such as ovarian cysts, prolonged estrus, or absence of estrus. The species-specific hormonal regulation of reproductive processes (Queiroga et al. 2009), including prolonged proestrus and estrus stages, prolonged progesterone (P_4) and prolactin secretion, and high sensitivity to endogenous and exogenous estrogens, may play an important role in the etiopathogenesis of these dysfunctions (Concan-

non 2011). The use of hormones for therapeutic or biotechnological (contraception) purposes is also a significant consideration.

The potential effects of mycotoxins (mycoestrogens) (Giammarino et al. 2008) found in commercially-available animal feeds have never been investigated (Boermans and Leung 2007). The results of preliminary studies revealed varied concentrations of ZEN in commercial feeds, and relatively high levels of the mycotoxin were observd in selected products. ZEN was present in 42 out of 45 dog feed samples, in concentrations of 5.0 to 299.5 μg/kg of feed (Zwierzchowski et al. 2004). The presence of ZEN in commercially available feeds is an important consideration because bitches are fed mono-diets over long periods of time, and they are particularly sensitive to estrogenic-like substances. Elevated concentrations of endogenous and exogenous estrogens (for example, their total) are a potential cause of other dysfunctions. Long-term administration of feed containing ZEN could disrupt the hormonal regulation (estrogenic hormones) of reproductive processes and leads to ovarian dysfunction (Hatoya et al. 2009). Ovaries are particularly sensitive to estrogenic substances, which are responsible for permanent changes that accompany ovarian cysts (Włodarczyk et al. 2009).

This study was prompted by the results of our previous work into ZEN mycotoxicosis in bitches (Gajęcka et al. 2004). A review of the literature indicates that ZEN mycotoxicosis with low doses has not been investigated in dogs to date.

The objective of this study was to determine the effect of long-term exposure to low doses of ZEN on the histological condition of ovaries in pre-pubertal, genetically homogenous 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 an 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 of body weight (100% NOAEL – no observable adverse effect level (Boermans and Leung 2007), experimental group I (EI)), 75 μg ZEN/kg BW (150% NOAEL, experimental group II (EII)), or placebo (without ZEN, negative control group) as outlined below. All the bitches were ovariectomized at the end of the 42-day treatment period, i.e. approximately the 112th day 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 (α-ZOL) 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 (HPLC) (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. ZEA samples (Zearalenone Z-0167, Sigma Chemical Co., Steinheim, Germany) were diluted μl 96% ethyl alcohol (ethyl alcohol, 96% vol., SWW 2442-90; Polskie Odczynniki Chemiczne SA) corresponding to ZEA 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.

Material sampling and preparation

After 42 days of oral exposure, all the bitches were anesthetized and ovariectomized. Samples of both ovaries from every bitch 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, Poland. Fragments of ovaries, sampled for histopathological analysis were fixed in 10% formalin, neutralized and buffered to pH 7.4, passed through graded alcohols, clarified in xylene and embedded in paraffin blocks. Microtome sections were stained with hematoxylin and eosin (HE) and PAS according to the method proposed by McManus (1999). The ovary-cross sections were examined for a minimum of 20 fields of view at 100x magnification. Microscopic images were analyzed under a halogen light at 100
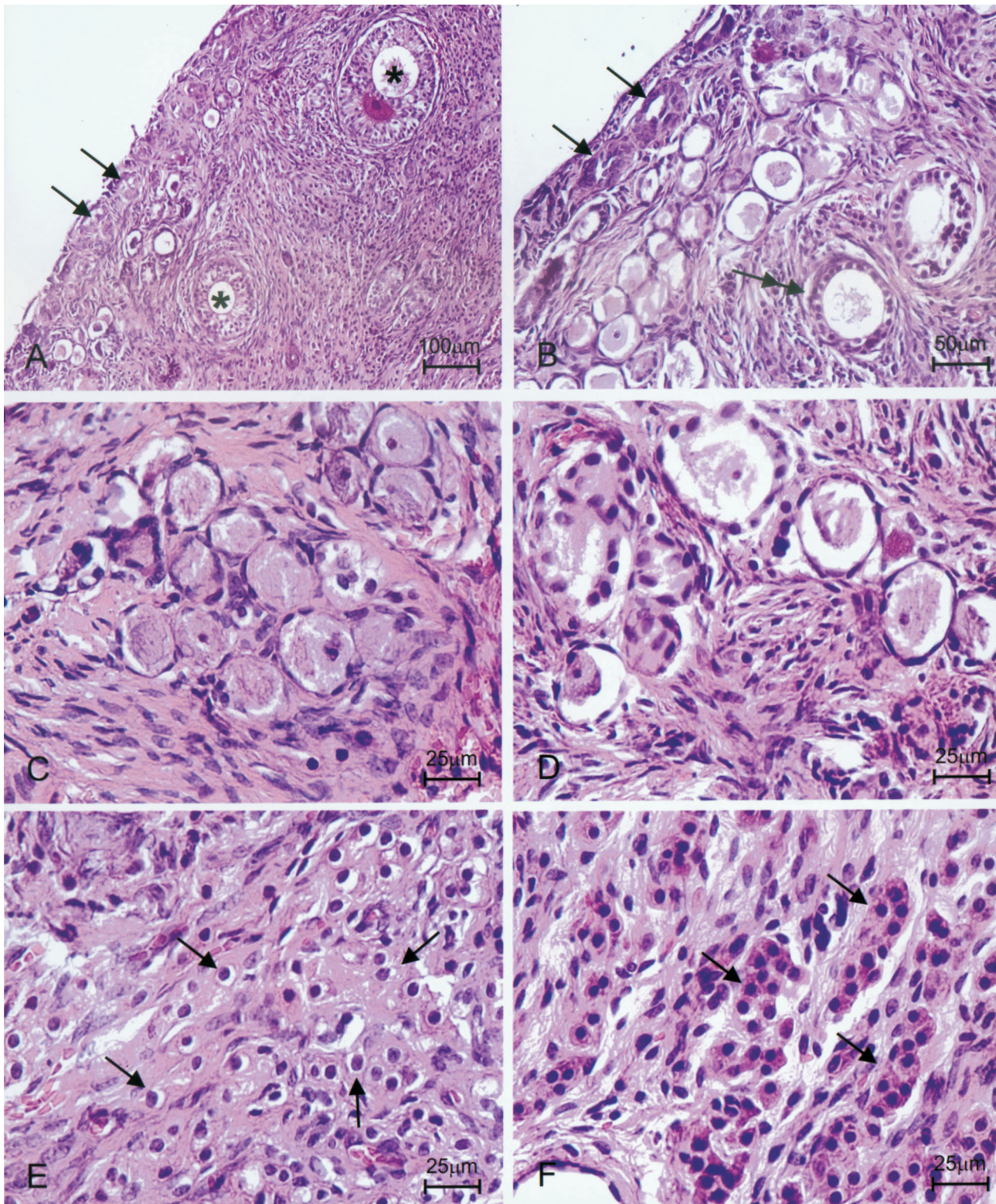
Fig. 1. Histological structure of the ovaries in the control group. A, B: Sections though the cortical part of the ovariy. Small epithelial inclusion glands occurring just beneath the tunica albuginea (arrows). Numerous primordial and some primary follicles (double arrow in Fig. 1A) are present in the outer part of the ovarian cortex. Secondary follicles were also visible (asterisks in Fig. 1A). C: A group of primordial follicles in the ovarian cortex surrounded by the connective tissue. D: Atretic follicles in the ovarian cortex. E: Interstitial gland cells (arrows) in the connective tissue stroma. F: Hilus cells (arrows) in the ovarian medulla.

power with an Olympus BX50 microscope at 400x magnification.

Blood was sampled on the last day of the experiment, one hour after mycotoxin administration. The samples were transferred to chilled centrifuge tubes without heparin and centrifuged at 3000 r.p.m. for 20 minutes at the temperature of 4ºC. The resulting plasma was transferred to plastic Eppendorf tubes, frozen and stored at -18ºC until analysis of selected steroid hormone levels.

Measurement of hormone concentrations in blood plasma

For the 17β-estradiol (E_2) assay, samples were prepared and processed, and E_2 was extracted from 1 ml of blood serum using ethyl ether (2 ml). The extracts were dried under nitrogen and re-suspended in 250 μl of PSS. The recovery of extraction was 90-95% (100 ng/ml E_2 solution in PSS supplemented with 0.1% BSA was used as an extraction control). The concentration of E_2 was determined by EIA using the Multiscan EX reader plate (Labsystem, Finland). The absorbance was measured at 450 nm wavelength. Anti-estradiol serum (a gift from Dr. G.L. Williams at A M Texas University, College Station, USA) was used at a final dilution of 1:150 000. The cross-reaction of this antibody was described by Mlynarczuk et al. (2005). The range of the curve was 3.125-1600 pg/ml, and the sensitivity of this method was 10-15 pg/ml. The average intra- and inter-assay coefficients of variation were 11.0% and 12.5%, respectively. The accuracy and reliability of the procedure were expressed by the coefficient of regression, r=0.875. All samples were measured in two replicates.

P_4 was extracted from serum using petroleum ether (PoCH, Poland). One part of serum (no less than 1 ml) and four parts of ether were shaken for 15 min and stored at -20ºC overnight. The supernatant was dried under nitrogen and the samples dissolved in 250 μl of PBS. P_4 concentrations were determined by EIA (Multiscan EX, Lab Systems, Finland) as described by Prakash et al. (1987). P_4 labeled with horseradish peroxidase was used at a final dilution of 1:8000. The antiserum (IFP4) used at a final dilution of 1:100 000 was characterized in earlier studies (Kotwica et al. 1994). The range of the standard curve was 0.05-25 ng/ml. The sensitivity of the procedure was 0.15 ng/ml. The intra-assay coefficient was 10.8%.

Statistical analysis

The results of the experiment are presented using mean values (*x*) and standard deviation (±SD), and were analysed using analysis of variance (ANOVA). If the null hypothesis was rejected, the differences were verified by the Student's *t*-test. The above analyses were carried out using STATISTICA® software (Statsoft). The analysis of correlation was performed between the groups studied (EI to EII, EI to C and EII to C).

Results

Histological structure of the ovaries in the control group

The outer surface of the ovary was covered by a single layer of epithelial cells, varying in shape from flat to cuboidal. The tunica albuginea was thin, but clearly visible (Fig. 1A,B). A few, small epithelial inclusion glands were observed just beneath the tunica albuginea (Fig. 1A,B). Numerous primordial and primary follicles were present in the outer part of the ovarian cortex (Fig. 1A,B,C). They formed small groups separated by wide strips of the connective tissue (Fig. 1C). Single secondary follicles were dispersed through the ovarian cortex (Fig. 1A). They have the diameter ranging from 60 to 180 μm and comprised 2-10 layers of cuboidal granulosa cells. The moderate number of small, round or oval, atretic follicles was observed in the vicinity of primordial and primary follicles (Fig. 1B,D). In the cortical region close to the medulla, atretic follicles were larger and variously shaped. Interstitial gland cells formed a few small clusters in the connective tissue stroma (Fig. 1E). The ovarian medulla was composed by the dense connective tissue and contained numerous large blood vessels, lymphatic vessels and bundles of nerve fibres. The rete ovary and hilus cells (Fig. 1F) were observed close the hilus.

Histological structure of the ovaries in the group EI

The surface epithelium formed a single layer covering the tunica albuginea. Small epithelial inclusion glands composed of cuboidal cells were observed under the tunica albuginea (Fig. 2A). Clusters of primordial and primary follicles were regularly distributed in the outer part of the cortex and separated by thick strips of the connective tissue (Fig. 2B,C). Each cluster contained from few to more than ten follicles. The secondary follicles were infrequently observed. Atretic follicles of various shape, from round to elongated, were much more numerous than those in the control group (Fig. 2A,B,D,E). They occurred individually or in clusters and their size increased towards the medulla. The connective tissue stroma contained
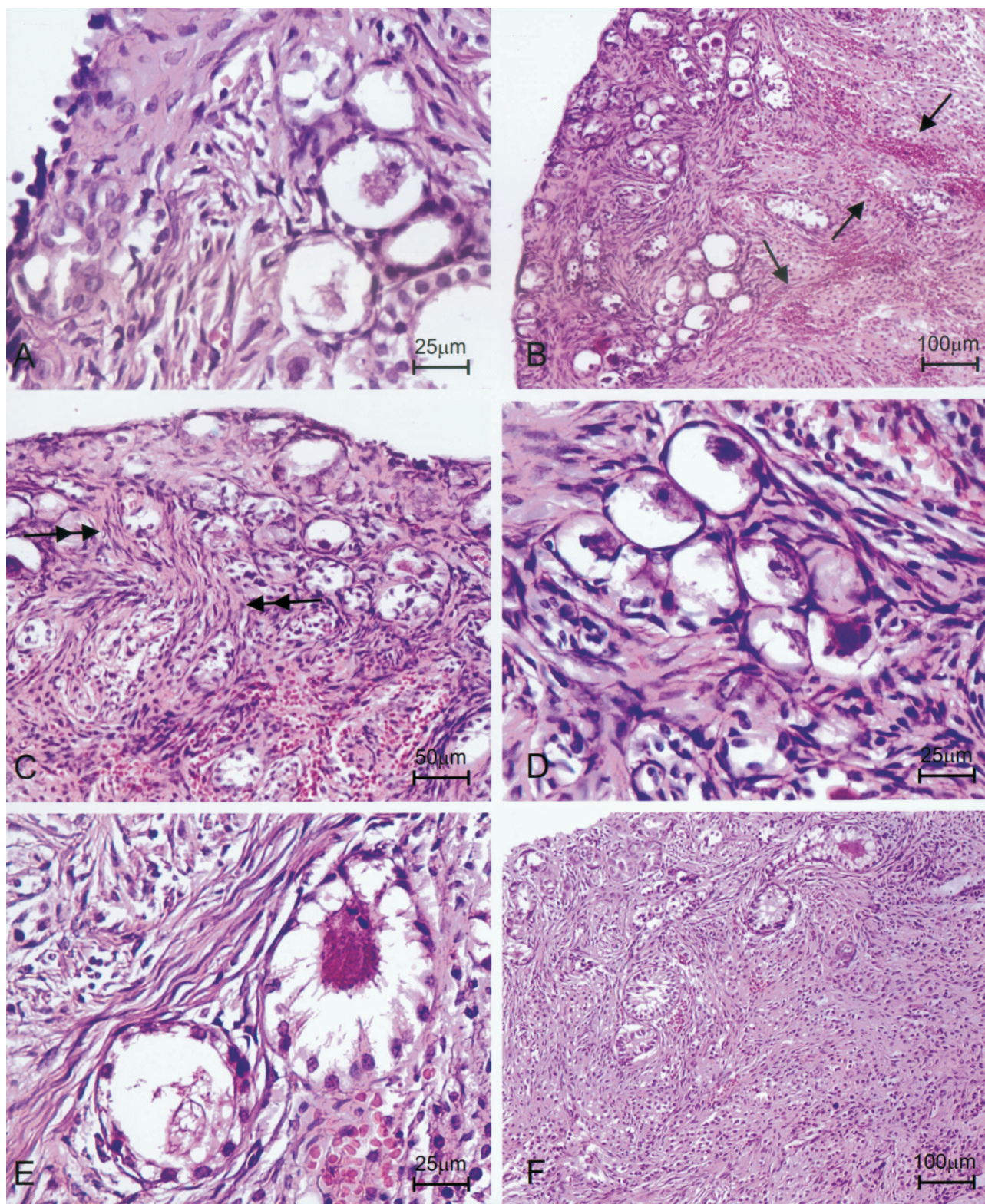
Fig. 2. Histological structure of the ovaries in the group EI. A, B, C: Sections though the cortical part of ovary. Numerous capillary vessels with considerably dilated lumen (Fig. 2B arrows) and thick strips of the connective tissue (Fig. 2C double arrows). D, E: Atretic follicles of various size and internal structure. F: Large areas of the connective tissue stroma filled by interstitial gland cells.
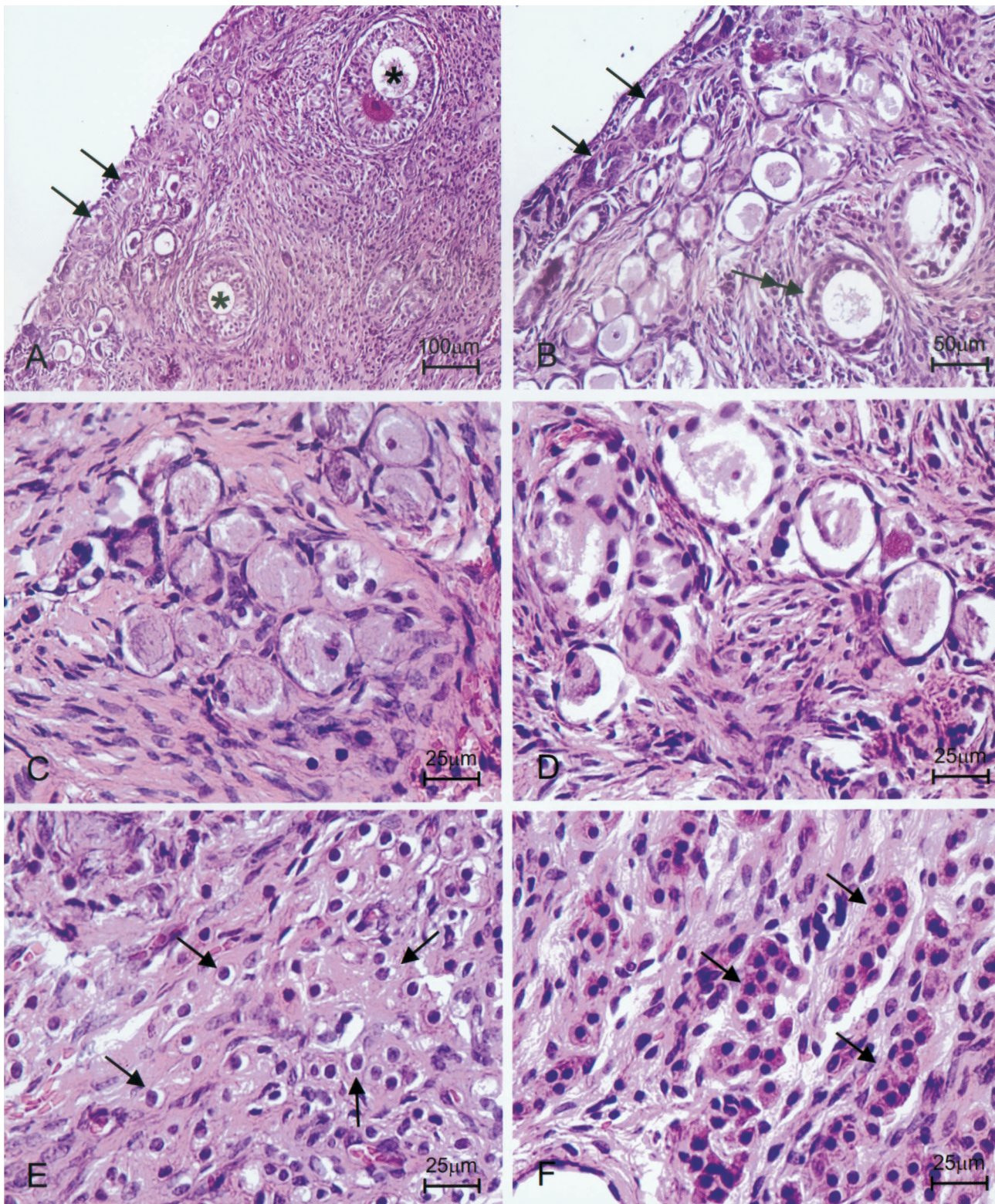
Fig. 3. Histological structure of the ovary in the group EII. A, B: Numerous small epithelial inclusions glands (arrows) as well as some invaginations of the surface epithelium into the connective tissue (asterisks). Only a few (Fig. 3B) or lack in Fig. 3A of primordial follicles were found. C: Numerous small atretic follicles (arrows) in the ovarian cortex. D: Large atretic vesicles in the part of cortex localized close to the medulla (arrows). E: Numerous interstitial glandular cells forming strips and clusters in the connective tissue stroma. The presence of dilated blood vessels filled with erythrocytes was found. F: Pronounced congestion and considerable fibrosis in the ovarian cortex.

a relatively large number of interstitial gland cells in a form of small clusters (Fig. 2F). The characteristic feature of the ovarian cortex in this group of animals was by the presence of numerous capillary vessels with considerably dilated lumen (Fig. 2A). The structure of the ovarian medulla was similar to that observed in the control group.

Histological structure of the ovaries in the group EII

The surface epithelium was formed by cuboidal or squamous cells. Numerous small epithelial inclusion glands as well as some invaginations of the surface epithelium into the connective tissue were observed in the most outer part of the cortex (Fig. 3A,B). As a consequence, the tunica albuginea did not form a single distinctive layer. The primordial and primary follicles were infrequently observed (Fig. 3B,D). The secondary follicles, usually containing only two layers of granulosa cells, were more numerous than those found in the two other groups investigated. An abundance of atretic follicles, with variable size and internal structure, was observed throughout the cortex (Fig. 3A,C,D,E). The largest, variously shaped follicles were observed in the part of the cortex localized close to the medulla (Fig. 3D). Numerous interstitial glandular cells formed strips and clusters in the connective tissue stroma (Fig. 3E). The ovarian cortex was characterized by a pronounced congestion and a considerable fibrosis (Fig. 3E,F).

Hormone tests

The results of the hormone tests are given in Table 1. Significant differences in E_2 concentrations were measured between group EI and the control group ($P \leq .05$), between groups EI and EII ($P \leq .05$), and between group EII and the control group ($P \leq .01$). In addition, significant differences in P_4 concentrations were measured between group EII and the control group ($P \leq .05$). The results of endocrinological analyses of ovaries of pre-pubertal bitches indicate that experimental hyperestrogenism increased E_2 concentrations and decreased P_4 levels.

Discussion

In dogs and other animal species, ZEN induces clinical symptoms of hyperestrogenism (Gajęcka et al. 2004, 2008b, 2011b, Słomczyńska 2004, Turcotte et al. 2005, Fink-Gremmels and Malekinejad 2007). Due to the high specificity of reproductive cycles, the reported symptoms have been somewhat different for various species investigated (Grundy et al. 2002, Songsasen and Wildt 2007, Zanghf et al. 2007).

ZEN, a phytoestrogen, exerts a powerful effect on the ovaries, the main estrogen-producing organ in the body (Alm et al. 2006, Fink-Gremmels and Malekinejad 2007), and its impact is evaluated based on its biological effects on the ovaries (Scippo et al. 2002). In bitches, ovarian pathologies pose a significant health problem. Hormonal dysfunction caused by elevated estrogen levels plays an important role in the etiopathogenesis of ovarian diseases (Whitehead and Rice 2006). In this study, ZEN and its metabolites contributed to numerous regressive changes, including degeneration and atrophy of cells and tissues, mostly granulosa cells. The presence of the numerous dilated blood vessels filled with erythrocytes (Fig. 2B and 3E), characteristic symptoms of estrogenic activity, were also observed in nearly all ovarian structures (Gajęcka et al. 2004). In our previous studies of multiparous bitches (Gajęcka et al. 2008b), ZEN was found to have similar effects. Other research has yielded comparable results with respect to other phytoestrogens (McClain et al. 2005).

Based on our knowledge of the correlations between endogenous steroids and estrogen receptors in animals, the effect of ZEN and its metabolites on ovarian function can be evaluated without analyzing estrogen receptors (Gajęcka 2012). This approach would explain the histological changes in the ovaries of pre-pubertal bitches.

Bitch ovaries are covered with the ovarian surface epithelium (OSE) which plays a variety of physiological roles. Firstly, it acts as a partial barrier for bioactive diffusion between the ovarian stroma and OSE. Secondly, it conditions the periodic release of mature or maturing ovarian follicles. In the control group, OSE had normal histological structure. In experimental groups, the histological structure of OSE indicates

Table 1. Mean (±SD) blood plasma concentrations of 17β-estradiol (E_2) and progesterone (P_4).

Specification/Group	Control	EI	EII
E_2 pg/ml	4.60 ± 0.15	14.66 ± 6.92*	21.58 ± 4.15**[a]
P_4 ng/ml	0.1573 ± 0.065	0.1413 ± 0.030	0.1316 ± 0.024*

*$P \leq .05$ compared to control; **$P \leq .01$ compared to control; [a]$P \leq .05$ compared to group EI.

that these cells are present in other ovarian structures. Small epithelial inclusion glands were observed under the tunica albuginea in group EI (Fig. 2A). In group EII, the glands were more pronounced, and the noted invaginations observed reached the external part of the cortex (Fig. 3A, 3B). It can be assumed that invaginations were produced by ZEN and α-ZOL (unpublished study), whereas the size and frequency of invaginations were directly proportional to ZEN doses in feed. Some researchers (Auersperg et al. 2001) have suggested that invaginations of the type are observed in multiparous bitches as a result of postovulatory proliferation, which is unlikely to take place in pre-pubertal bitches. The above could imply that the changes observed in the studied animals were pathological.

Changes in the histological structure of OSE and the ovarian cortex could also be provoked by greater congestion in the cortex region of group EII dogs (Fig. 3E, 3F) as well as by the presence of numerous capillary vessels with considerably dilated lumina (Fig. 2A) in group EI. Both factors intensify blood flow through ovarian tissues, which implies faster access of ZEN and α-ZOL to estrogen-dependent cells in the ovary, i.e. cells containing ER proteins. In our previous study, the presence of only ERβ was found in pre-pubertal bitches (Gajęcka 2012). ZEN has higher affinity for ERα than to ERβ, and is a powerful antagonist of ERβ (Mueller et al. 2004). ZEN affinity for ERβ of varies concentrations and the phase of the estrous cycle (Hatoya et al. 2009). Locations of ER subtypes in the ovaries of various animal species reported by Korach et al. (2003) and in our previous study (Gajęcka 2012) indicate that the changes observed in ovarian tissues could be induced by ZEN and α-ZOL effects on ERβ in the ovaries. It should also be noted that an increase in ZEN concentrations in feed is accompanied by a decrease in ERβ in ovarian tissues (Gajęcka 2012). The above could imply that congestion in the ovarian cortex is caused by non-genomic effects of estrogens (Bishop and Stormshak 2008). One of such estrogens is ZEN, an exogenous ligand (Barton 2012).

Primordial and primary follicles were found mostly in the external part of the cortex, and their proportion in ovarian tissues was characterized by a decreasing trend in both experimental groups (Fig. 2B, 2C and Fig. 3B, 3D) in comparison with the control (Fig. 1A, 1B, 1C). This trend was more expressed in group EI, implying that it was proportional to the mycotoxin dose in feed (above NOAEL). The above can be partially attributed to the invagination of OSE into the tunica albuginea (group EI; Fig. 2A) and even into the surface of the cortex region (group EII – Fig. 3A,B), i.e. the stroma. This can lead to local thinning

of the tunica albuginea and the underlying stroma. The described condition is observed in sexually mature bitches shortly before ovulation. It is a pathological condition in pre-pubertal females because primordial follicles are produced between the age of 17 and 54 days (Songsasen et al. 2009). Primary follicles are observed in the course of the following 120 days, whereas the experiment was performed on bitches aged between 70 and 112 days (Concannon 2011).

According to Auersperg et al. (2001), stromal thinning can induce apoptosis. The above has been confirmed by the results of our unpublished study (Gajęcka 2013) which determined apoptotic and proliferative indices of ovarian follicles in the same bitches. Intensified apoptosis and slower proliferation were observed in primordial and primary follicles in both oocytes and in follicular cells. Our previous findings (Gajęcka and Przybylska-Gornowicz 2012) also indicate that cell death resulted from excessive Ca^{2+} accumulation in 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.

The share of atretic follicles increased in both experimental groups (Fig. 2A, 2B, 2D, 2E and Fig. 3A, 3C, 3D, 3E) in comparison with the control (Fig. 1B, 1D), and it was directly proportional to the ZEN dose in feed. Most follicles were observed in the medulla (Fig. 3D), therefore, it can be assumed that follicular atresia was not spontaneous (the bitches examined were very young) and that it was induced in larger follicles (Doležel et al. 2004). The above hypothesis is supported by the fact that intoxication lasted 42 days, but the available data covered only the last day of the experiment.

In larger follicles, atresia begins in the follicular wall, i.e. from the exterior, as connective tissue enters follicles. During this process, theca cells become similar to paralutein cells, and they become a structural element of the interstitial gland as a source of additional estrogen (Akihara et al. 2007). The above is illustrated by the results in Table 1 which show a significant increase in the concentrations of E_2 in both experimental groups, in particular in group EII. This increase was accompanied by a significant drop in P_4 levels in group EII as compared with the control.

The results of histological analyses of ovaries in the studied bitches indicate that ZEN and its metabolites have an adverse effect on the ovarian morphology. Ovarian structures were significantly damaged in both experimental groups (more profound changes were observed in group EII at 150% NOAEL), whereas no pathological changes were found in the control animals. Those changes involved pathological invaginations of OSE with morphological conse-

quences and the disappearance of ovarian follicles, which was accompanied by numerous extravasations and the presence of vessels with dilated lumina. Our findings suggest that ZEN exerts a negative effect even at very low doses which are encountered in the natural environment. In the studied bitches, long-term (42 days) exposure to ZEN administered at threshold doses contributed to histopathological changes in the ovarian tissue.

The results of endocrinological analyses of ovaries in pre-pubertal bitches indicate that experimental hyperestrogenism increased E_2 concentrations and decreased P_4 levels.

Acknowledgements

References

Akihara Y, Shimoyama Y, Kawasako K, Komine M, Hirayama K, Terasawa A, Ohmachi T, Matsuda K, Okamoto M Taniyama H (**2007**) Histological and immunohistochemical evaluation of canine ovary. Reprod Dom Anim 42: 495-501.

Alm H, Brüssow KP, Torner H, Vanselow J, Tomek W, Dänicke S, Tiemann U (**2006**) Influence of Fusarium-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. Reprod Toxicol 22: 44-50.

Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. (**2001**) Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 22: 255-288.

Barton M (**2012**) Position paper: The membrane estrogen receptor GPER – Clues and questions. Steroids 77: 935-942.

Bishop CV, Stormshak F (**2008**) Non-genomic actions of progesterone and estrogens in regulating reproductive events in domestic animals. Vet J 176: 270-280.

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

Concannon PW (**2011**) Reproductive cycles of the domestic bitch. Anim Reprod Sci 124: 200-210.

Doleżel R, Kyliánková R, Kummer V, Maškova J, Stará P, Vitásek R (**2004**) Follicular population and oestrogen receptor alpha in ovary of the bitch. Acta Vet Brno 73: 37-43.

Fink-Gremmels J, Malekinejad H (**2007**) Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone. Anim Feed Sci Tech 137: 326-341.

Gajęcka M (**2012**) The low doses effect of experimental zearalenone intoxication on the immunoexpression of es-

trogen receptors in the ovaries of pre-pubertal bitches. Pol J Vet Sci 15: 685-691.

Gajęcka M (**2013**) The effect of experimental low zearalenone intoxication on ovarian follicles in pre-pubertal bitches. (in press).

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, Obremski K, Jakimiuk E, Skorska-Wyszyńska E, Zielonka Ł, Gajęcki M (**2008a**) Histopathological examination of ovaries in bitches after experimental zearalenone mycotoxicosis. Pol J Vet Sci 11: 363-366.

Gajęcka M, Przybylska-Gornowicz B (**2012**) The low doses effect of experimental zearalenone (ZEN) intoxication on the presence of Ca^{2+} in selected ovarian cells from pre-pubertal bitches. Pol J Vet Sci 15: 711-720.

Gajęcka M, Przybylska-Gornowicz B, Obremski K, Polak M, Jakimiuk E, Skorska-Wyszyńska E, Zielonka Ł, Gajęcki M (**2008b**) Ultrastructural changes of ovarian follicle and corpus luteum after experimental zearalenone mycotoxicosis in bitch. Pol J Vet Sci 11: 327-337.

Gajęcka M, Rybarczyk L, Zwierzchowski W, Jakimiuk E, Zielonka Ł, Obremski K, Gajęcki M (**2011a**) 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, Woźny M, Brzuzan P, Zielonka Ł, Gajęcki M (**2011b**) Expression of CYPscc and 3β-HSD mRNA in bitch ovary after long-term exposure to zearalenone. Bull Vet Inst Pulawy 55: 777-780.

Giammarino A, Manera M, Robbe D, Perugini M, Minervini F, Amorena M (**2008**) Influence of mycotoxins on spontaneous contraction in myometrial strips of prepubertal lamb. Res Vet Sci 84: 471-476.

Grundy SA, Feldman E, Davidson A (**2002**) Evaluation of infertility in the bitch. Clin Tech Small Anim. Pract. 17: 108-115.

Hatoya S, Sugiyama Y, Nishida H, Okuno T, Torii R, Sugiura K, Kida K, Kawate N, Tamada H, Inaba T (**2009**) Canine oocyte maturation in culture: significance of estrogen and EGF receptor gene expression in cumulus cells. Theriogenology 71: 560-567.

Korach KS, Emmen JM, Walker VR, Hewitt SC, Yates M, Hall JM, Swope DL, Harrell JC, Couse JF (**2003**) Update on animal models developed for analyses of estrogen receptor biological activity. J Steroid Biochem Mol Biol 86: 387-391.

Kotwica J, Skarzynski DJ, Jaroszewski JJ, Bogacki M (**1994**) Noradrenaline affects secretory function of corpus luteum independently on prostaglandins in conscious cattle. Prostaglandins 48: 1-10.

McClain RM, Wolz E, Davidovich A, Pfannkuch F, Bausch J (**2005**) Subchronic and chronic safety studies with genistein in dogs. Food Chem Toxicol 43: 1461-1482.

McManus PM (**1999**) Frequency and severity of mastocytemia in dogs with and without mast cell tumors: 120 cases (1995-1997). J Am Vet Med Assoc 215: 355-357.

Młynarczuk JJ, 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.

Panini NV, Salinas E, Messina GA, Raba J (**2011**) Modified paramagnetic beads in a microfluidic system for the determination of zearalenone in feedstuffs samples. Food Chem 125: 791-796.

Prakash BS, Meyer HH, Schallenberger E, Van de Wiel DF (**1987**) Development of sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. J Steroid Biochem 28: 623-627.

Obremski K, Gajęcki M, Zwierzchowski W, Bakuła T, Apoznański J, Wojciechowski J (**2003**) The level of zearalenone and α-zearalenol in the blood of gilts with clinical symptoms of toxicosis, feed diets with a low zearalenone content. J Anim Feed Sci 12: 529-538.

Queiroga FL, Pérez-Alenza D, Silvan G, Peña L, Illera JC (**2009**) Positive correlation of steroid hormones and EGF in canine mammary cancer. J Steroid Biochem Mol Biol 115: 9-13.

Scippo ML, Van De Weerdt C, Willemsen P, François JM, Rentier-Delrue F, Muller M, Martial JA, Maghuin-Rogister G (**2002**) Detection of illegal growth promoters in biological samples using receptor binding assays. Anal Chim Acta 473: 135-141.

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

Songsasen N, Fickes A, Pukazhenthi BS, Wildt DE (**2009**) Follicular morphology, oocyte diameter and localization of fibroblast growth factors in the domestic dog ovary. Reprod Domest Anim 44 (Suppl 2): 65-70.

Songsasen N, Wildt DE (**2007**) Oocyte biology and challenges in developing in vitro maturation systems in the domestic dog. Anim Reprod Sci 98: 2-22.

Suchorzyńska M, Misiewicz A (**2009**) Mycotoxigenic phytopathogenic fungi of *Fusarium* genus and their identification by PCR techniques. Post. Microbiol 48: 221-230.

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.

Walter B, Otzdorff C, Brugger N, Braun J (**2011**) Estrus induction in Beagle bitches with the GnRH-agonist implant containing 4.7 mg Deslorelin. Theriogenology 75: 1125-1129.

Whitehead SA, Rice S (**2006**) Endocrine-disrupting chemicals as modulators of sex steroid synthesis. Best Pract Res Clin Endocrinol Metab 20: 45-61.

Włodarczyk R, Bukowska D, Jaśkowski JM (**2009**) Oogenesis and ultrastructure of bitch oocytes. Życie Wet 84: 972-977.

Zanghf A, Catone G, Marino G, Quartuccio M, Nicntina PA (**2007**) Endometrial polypoid adenomyomatosis in a bitch with ovarian granulosa cell tumour and pyometra. J Comp Pathol 136: 83-86.

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.