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Original article

Immunohistochemical evaluation of apoptosis and proliferation in the mucous membrane of selected uterine regions in pre-pubertal bitches exposed to low doses of zearalenone

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Abstract

Mycotoxins are toxic secondary metabolites produced by fungi. Those biologically active compounds occur naturally and they include zearalenone (ZEN), a mycotoxin that contaminates plant material, including the ingredients used in the production of commercial dog food. The influence of monotonic, low-dose and long-term exposure to ZEN on pre-pubertal bitches has not been fully explored to date. This paper describes a 42-day experiment performed on clinically healthy female dogs aged approximately 70 days, with estimated body weight of 8 kg. The animals were randomly divided into two experimental groups (EI and EII) and a control group (C) of 10 animals each. Group EI received 50 µg ZEN/kg (of body weight) *per os*, group EII received 75 µg ZEN/kg BW *per os*, and the control group was administered placebo. The bitches were ovariectomized at the end of the experiment (at around 112 days of age), and selected sections of the uterine wall were subjected to immunohistochemical analyses (TUNEL and PCNA). A shift towards higher apoptotic (AI) and proliferative index (PI) was observed, in particular in group EI. Higher AI and PI values were noted in the epithelium of all uterine regions analysed and in the uterine glands in the uterine horn proximal to the body of the uterus.

Key words: zearalenone, immunohistochemistry, apoptosis, proliferation, uterus, endometrium, pre-pubertal bitches

Introduction

Reproductive system dysfunctions, are relatively frequently diagnosed in pre-pubertal bitches (Verweridis et al. 2004, Tsumagari et al. 2005). It is believed that species-specific hormonal regulation of reproductive processes and high sensitivity to oestrogens play an important role in the etiopathogenesis of those disorders. Hormones administered to bitches for therapeutic and breeding (birth control) purposes can also contribute to reproductive disorders (De Bosschere et al. 2002a). It has been suggested that medical errors, mostly inadequate hormonal treatment, often cause pathological states of the reproductive system in bitches (Hoffmann et al. 1996). Mycotoxins, in particular ZEN which is often present in commercial feed (Zwierzchowski et al. 2004, Gajęcka et al. 2013), are also potential causative agents of reproductive disorders in female dogs (Freeman et al. 2013a, b). Preliminary research revealed differences in ZEN concentrations in commercial feeds, some of which contained substantial amounts of the toxin (Zwierzchowski et al. 2004). Feed contamination with low doses of the mycotoxin (near to NOAEL values, i.e. the highest dose that does not induce clinical symptoms) is of particular significance for bitches, which are often fed monodiets for many months (Gajęcka et al. 2013). ZEN doses below NOAEL values are most often found in commercial feeds (Boermans and Leung 2007, Bohm et al. 2010, Schatzmayr and Streit 2013).

Low doses up to the NOAEL value induce completely different local and overall effects than those observed under exposure to higher doses of mycotoxins (Calabrese 2005, Dobrzyński and Fornalski 2011, Gajęcka et al. 2013, 2015). The NOAEL value supports the determination of the dose-response relationship where there is no statistically or biologically significant increase in the frequency or intensity of any harmful effects of the analysed substance is observed relative to the control sample. The NOAEL dose can also be defined as an experimentally established dose at which the frequency or severity of negative effects in the exposed population are not increased relative to the control sample or at which those effects are biologically or statistically insignificant. In toxicology, NOAEL describes a dose or concentration of a substance (e.g. chemical) or factor (e.g. radiation) which does not induce adverse effects in the studied organisms (without clinical symptoms of disease including mycotoxicosis), whereas negative effects are observed at higher doses or concentrations of that substance or factor (Brandon et al. 2013). The cited authors suggested that the NOAEL value can be used to evaluate the risk of exposure to the evaluated mycotoxins.

In recent years, the classical dose-response paradigm was undermined by the low-dose hypothesis. The above particularly applies to hormonally active agents (Vandenberg et al. 2012) which can interfere with the endocrine (or hormone) system in mammals already when applied in small doses, referred to as endocrine disruptors (EDs). This could take place during exposure to low levels of undesirable substances in feedstuffs, and it can produce a U-shaped or a reverse U-shaped non-monotonic dose response (NMDR) (hormesis; Calabrese 2005). In reference to the NMDR hypothesis, an ambiguous correlation between the dose and the response implies that the risks (clinical symptoms or laboratory results) associated with high doses cannot be directly and unambiguously extrapolated to low doses (Grenier and Applegate 2013).

The concept of the lowest dose, i.e. a dose which induces an opposite effect to that which is normally expected, is becoming increasingly interesting from the point of view of biomedical practice. A thorough understanding of the mechanisms involved in that process and the final result should influence decision-making for a given objective (EFSA 2012). Endocrine disruptors have undermined well-grounded concepts in toxicology, in particular the belief that „the dose makes the poison” because low doses of EDs induce specific changes that are not encountered at higher doses (Vandenberg et al. 2012). Despite the above, numerous research studies have demonstrated that low doses of naturally occurring hormones, including ZEN, produce unspecific responses (Frizzell et al. 2011, 2015).

ZEN is an oestrogenic mycotoxin, and female dogs are particularly sensitive to steroids and steroid-like compounds. Elevated oestrogen levels (total concentrations of endogenous and exogenous oestrogen) can also contribute to systemic disorders (Gajęcki et al. 2010). Oestrogens have a harmful impact on the function of the bone marrow, parenchymal organs and skin. Long-term administration of feeds contaminated with low doses of ZEN, a mycotoxin, can lead to dysfunctions of the uterus (Stopa et al. 2014), in which permanent changes frequently accompany the spontaneous endometritis-pyometra complex (De Bosschere et al. 2002b, Galabova-Kovacs et al. 2004, Freeman et al. 2013a, b).

Recent research into mycotoxicoses has spurred active interest into apoptosis and disorders of the cellular cycle as major contributors to reproductive dysfunctions in bitches (Chu et al. 2006). It is believed that reproductive disorders are caused not only by changes in gene expression that are manifested by an increase in uterine parametric indicators (Stopa et al. 2014). Reduced apoptotic ability, i.e. cell death in re-

sponse to environmental stimuli, is also an important factor in mycotoxin-induced disorders of the reproductive tract. It can increase cell vitality, prolong cell life, reinforce the existing conditions, disrupt the cell cycle and, consequently, the reproductive cycle (Gajęcka et al. 2013).

Apoptosis is an active and genetically conditioned process that is the functional opposite of mitosis and that differs from necrosis. Apoptosis combines a series of morphological, biochemical and molecular changes that lead to cell death. The initiation of apoptosis requires the activation of numerous genes. Apoptosis plays a key role in embryogenesis, organ involution (uterine involution after parturition or oestrus), tissue regeneration and death of specialized cells (endometrial cells at the turn of anoestrus and proestrus in pre-pubertal bitches and during metestrus in mature bitches) (Chu et al. 2006). Apoptosis is a process of programmed cell death that conditions optimal development and physiological function.

The aim of this study was to determine the immunohistochemical degree of apoptosis and proliferation in selected sections of the uterine wall in pre-pubertal bitches exposed to low doses of ZEN.

Materials and Methods

All 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 estimated average body weight (BW) of 8.00 ± 0.95 kg, were obtained from local breeders (registered at the Polish Kennel Club) and kept in standard conditions with free access to water. Clinically healthy female dogs were divided into three groups: experimental group I (EI; $n=10$) was administered ZEN *per os* at 50 $\mu\text{g}/\text{kg}$ BW once daily [100% of the NOAEL – Boermans and Leung (2007) and stimulating/adaptive effects – Calabrese (2005), Vandenberg et al. (2012)], experimental group II (EII; $n=10$) was administered ZEN *per os* at 75 $\mu\text{g}/\text{kg}$ BW once daily (150% of the NOAEL, dose at which clinical symptoms are expected – Vandenberg et al. (2012), and control group (C; $n=10$) was administered placebo without ZEN or other mycotoxins. All

bitches were hysterectomized at the end of the 42-day treatment period, i.e. at the age of approximately 112 days.

Preparation and administration of diets

The animals were kept in cages with *ad libitum* access to water and were fed standard diets tested for the presence of the following mycotoxins: aflatoxin, ochratoxin, ZEN, α -zearalenol (α -ZEL), β -zearalenol (β -ZEL) and deoxynivalenol. Mycotoxin concentrations in the diet were evaluated with the use of common separation techniques involving immunological affinity columns and high-performance liquid chromatography (HPLC) (Hewlett Packard, type 1050 and 1100) (Zwierzchowski et al. 2004) with fluorescent and/or UV detection techniques. The values reported in the analysis of mycotoxin levels in feed were below the sensitivity of the method.

Mycotoxin doses were administered daily in gelatine capsules before morning feeding for 42 days. ZEN (Zearalenone Z-0167, Sigma Chemical, Steinheim, Germany) was diluted in 300 μl of 96% ethyl alcohol (ethyl alcohol, 96% vol., SWW 2442-90; Polskie Odczynniki Chemiczne, Poland) corresponding to ZEN doses of 50 and 75 $\mu\text{g}/\text{kg}$ BW. The resulting solution was introduced into the feed, placed in gelatine capsules and stored at room temperature for 12 h to evaporate the solvent.

Material sampling

After 42 days of oral exposure, all female dogs were anesthetized and hysterectomized. A complete morphometric evaluation of the uterus was performed (left and right horn of the uterus, in the region proximal to the ovaries and to the body of the uterus). Samples of the uterine wall were then collected for histological and immunohistochemical analyses. The samples were prepared and processed at the Department of Pathophysiology, Forensic Veterinary Medicine and Administration, Faculty of Veterinary Medicine of the University of Warmia and Mazury in Olsztyn, Poland.

Histological examination

Samples of the uterine wall, divided into the region proximal to the ovaries and to the body of the uterus, were fixed in 10% formalin, neutralized and buffered to pH 7.4, passed through a series of graded alcohols, purified in xylene and embedded in paraffin

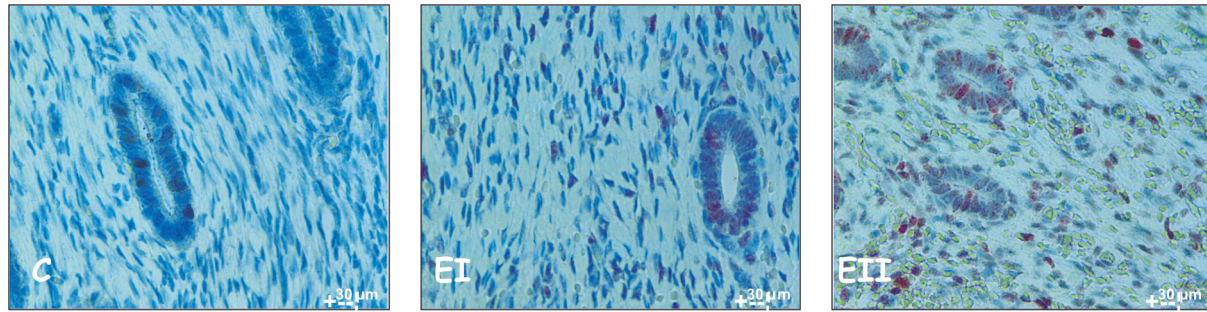


Fig. 1. Positive apoptotic staining; C, EI and EII – group labellings.

blocks. Microtome sections, 5 µm thick, were stained with hematoxylin and eosin (H & E) (McManus 1999). Cross-sections of uterine tissue specimens were examined in minimum 20 fields of view at 100x magnification. The microscopic evaluation was performed under a light microscope (Nikon Eclipse 80i).

Immunohistochemical investigations

For immunolabelling, microtome sections 5 µm thick were mounted onto silane-coated slides. Immunohistochemical reactions were identical for all the specimens. The same reagents, time, temperature and moisture conditions were used for all tissue sections. AI and PI in the luminal and glandular epithelium, lamina propria and myometrium were determined as the number of positive cells (TUNEL+ or PCNA+) per 100 cells (%), in 5 randomly selected fields of view, at 100x magnification (Falco 2009). The results were processed statistically, and the values for uterine structures in the examined animals were presented in graphic form.

TUNEL test. DNA fragmentation was detected with the use of the TACS™ 2 TdT DAB In Situ Apoptosis Detection Kit (TREVIGEN, USA). Deparaffinised and rehydrated sections were treated with proteinase-K (20 µg/ml) for 15 minutes at room temperature, they were incubated with quenching solution for 5 minutes and in a humidity chamber with a working-strength TdT enzyme solution at 37°C for 60 minutes. The reaction was terminated by incubation in working-strength stop/wash buffer for 5 minutes at room temperature. The sections were incubated with streptavidin-horseradish peroxidase solution for 10 minutes at 37°C (humidity chamber) and with diaminobenzidine (DAB) for 5 minutes at room temperature. They were counterstained with 1% methyl green for 1 minute, dehydrated in a graded ethanol series, cleared in xylene, mounted with DPX and coverslipped. Germinal centres of hyperplastic lymph nodes served as positive control. Negative controls

were processed in the absence of the TdT enzyme and showed no staining.

PCNA method. The analyses were performed on the same day with the use of the streptavidin-biotin immunohistochemical technique specific for PCNA antibodies (DAKO, Denmark, Clone PC-10, IgG2 kappa), diluted 1:150. All steps were carried out in 1M phosphate buffered saline (PBS; 120 mM/L NaCl, 11.5 mM/L NaH₂PO₄, 31.3 mM/L KH₂PO₄, pH 7.4) at 37°C. The sections were deparaffinised and rehydrated in a graded ethanol series, and they were treated with 10mM citric acid (pH 6.0) in a microwave oven (750 W) for four 2.5-minute periods. After 15 minutes of incubation in the dark in 1% hydrogen peroxide (H₂O₂) in methanol, the sections were rinsed in tap water and, subsequently, in PBS solution to deactivate endogenous peroxidase. The specimens were incubated for 15 minutes in normal horse serum (NHS; DAKO, Denmark) in PBS. They were incubated for 1 h at 37°C with the primary antibodies diluted in 1M PBS-containing NHS. The sections were thoroughly rinsed with PBS, incubated with secondary biotinylated antibodies and diluted in PBS for 30 minutes at 37°C. They were rinsed in PBS and incubated with the streptavidin-biotin peroxidase complex for 10 minutes at room temperature. After subsequent rinses in PBS, the sections were incubated for 1 minute with 0.1% Triton solution and for 5-10 minutes with a solution of 3,3-diaminobenzidine tetrahydrochloride (DAB) in distilled water containing H₂O₂. The specimens were rinsed in tap water, counterstained with hematoxylin for 45 s, dehydrated in a graded ethanol series, cleared in xylene, mounted with DPX and coverslipped. Negative control was performed by substituting the primary antibody with PBS, while commercially available human colon cancer sections (DAKO, Denmark) constituted positive controls.

Statistical analyses

The mean PI values were processed statistically by analysis of variance (ANOVA). Where the null

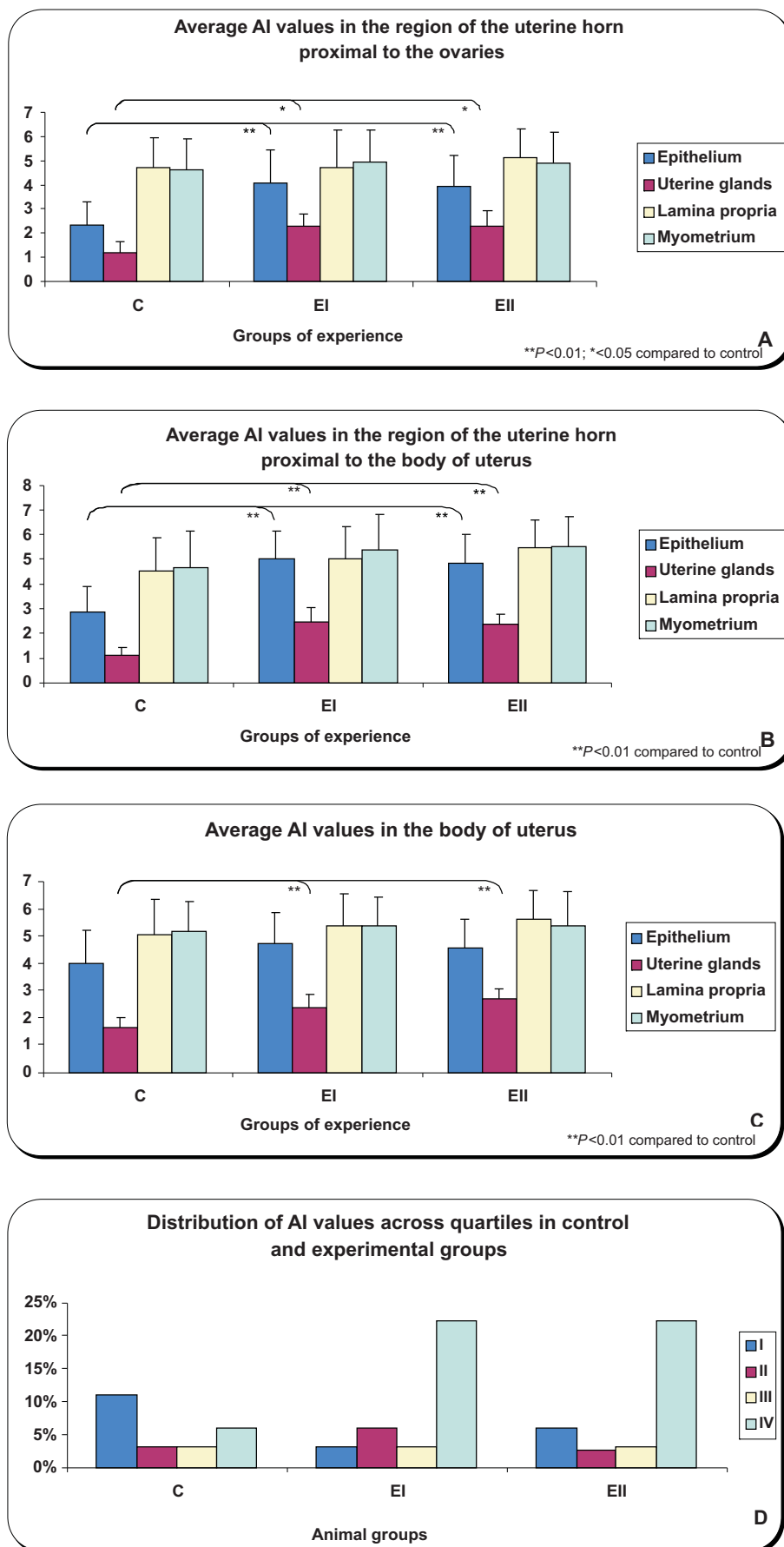


Fig. 2. Apoptotic index values of selected uterine regions in pre-pubertal bitches.

hypothesis was rejected, the differences were verified by the Student's t-test. The analyses were performed using STATISTICA® software (Statsoft). Correlations were determined between the evaluated groups of animals and selected endometrial structures (EI and EII, EI and C, EII and C).

Results

Apoptotic index (AI) of selected uterine structures in bitches

The results point to significant ($p \leq 0.05$) or highly significant ($p \leq 0.01$) differences in the rate of apoptosis between group C (control group) vs. groups EI (experimental group I) and EII (experimental group II) (Fig. 1, 2A, 2B and 2C).

In the region of the uterine horn proximal to the ovaries (UO) (Fig. 2A), significant differences ($p \leq 0.05$) in AI values were observed in the uterine glands between group C vs. groups EI and EII. In epithelial cells of the same uterine region, highly significant differences ($p \leq 0.01$) were found between group C vs. groups EI and EII.

In the region of the uterine horn proximal to the body of the uterus (UBU), highly significant differences ($p \leq 0.01$) in AI values and identical correlations were observed between group C vs. groups EI and EII in the epithelium and uterine glands (Fig. 2B).

In the body of the uterus (BU) (Fig. 2C), highly significant differences ($p \leq 0.01$) in the AI values of uterine glands were determined between group C and both experimental groups.

An analysis of the average AI values in the examined uterine structures revealed statistically significant differences in each group.

In group C significant differences ($p \leq 0.05$) in AI values were found between epithelial cells in UBU and BU regions. Highly significant differences in the epithelium and uterine glands were observed between UO and BU regions, and in the uterine glands between UBU and BU regions.

No significant differences in AI values were observed in group EI or group EII.

AI values were not evenly distributed. The median of all uterine regions, structures and groups was determined at 4.065, where the lower and upper quartiles for the examined structures reached 1.329 and 5.399 AI, respectively. The values of the median in control and experimental groups were as follows: group C – 3.493, group EI – 4.313, group EII – 4.390. Subject to the value of the median for all the groups and the lower and upper quartile of AI data, the results were divided into four activity levels: I – very low AI

($AI \leq 2.35$), II – low AI ($2.35 \leq AI \leq 3.40$), III – high AI ($3.40 \leq AI \leq 4.45$), IV – very high AI ($AI \geq 4.45$). Level I (very low) activity was observed in the uterine glands in nearly all groups, and the lowest AI values were determined in group C. In group EI, activity level I was found only in the UO region in the uterine glands, and in group EII – in UO and UBU regions in the uterine glands. Low AI values corresponding to activity level II were observed in group C in the UO region in the epithelium, in group EI – in UBU and BU regions in the uterine glands, and in group EII – in the BU region in the uterine glands. High AI values corresponding to activity level III were observed in group C in the BU region in the epithelium and in both experimental groups in the UO region in the epithelium. The remaining AI values (61%, including 17% in group C, in the lamina propria and the myometrium in all analysed regions) corresponded to the highest activity level IV.

Proliferative index (PI) of selected uterine structures in bitches

All specimens used in immunohistochemical studies revealed high levels of PCNA activity (Fig. 3).

The differences in the PI values of the evaluated uterine structures are presented in Fig. 4. In all evaluated uterine regions (Fig. 4A, 4B and 4C), highly significant differences ($p \leq 0.01$) between the PI values of the lamina propria were observed between group C and both experimental groups with a growing trend in the latter. Similar differences were noted in the uterine glands in the UBU region between group C and group EI (Fig. 4B). Significant differences ($p \leq 0.05$) in the PI values of uterine glands were observed in the UBU region between group C and group EII (Fig. 4B). The noted values were lower in group C.

Highly significant differences ($p \leq 0.01$) in the PI values of the lamina propria were found between UO and BU regions (PI values were higher in BU) in group C. Significant differences ($p \leq 0.05$) in the same uterine regions and structures were observed in group EI (Fig. 4D) and group EII (Fig. 4E). The values were higher in the BU region in both experimental groups. In comparison with group C, PI values were characterised by a growing trend in both experimental groups. The PI values of epithelial cells lining the uterine endometrium were higher in group EI. The PI values of the remaining uterine structures were higher in group EII.

PI values were not evenly distributed. The median of all uterine regions, structures and groups was determined at 12.275, where the lower and upper quartiles

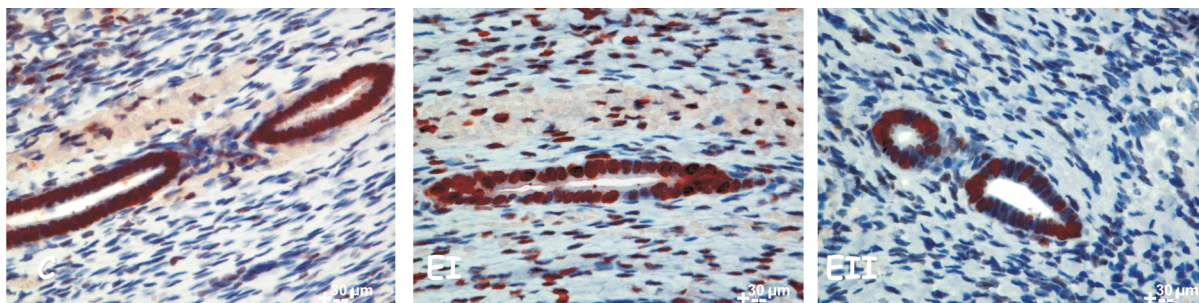


Fig. 3. Positive PCNA protein staining; C, EI and EII name of groups.

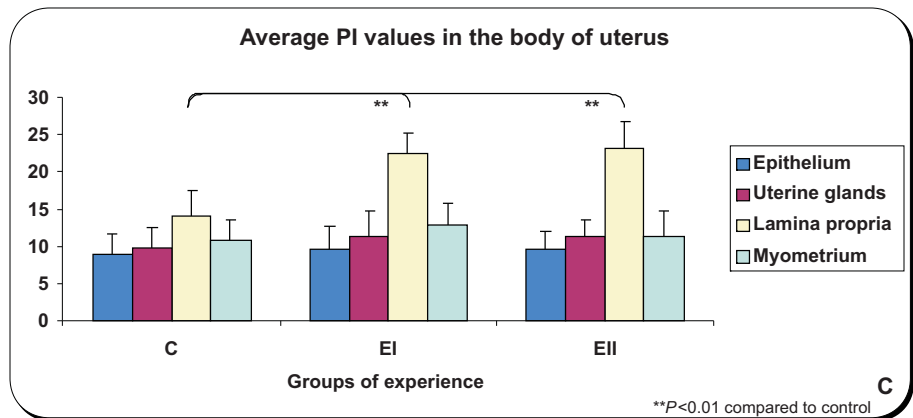
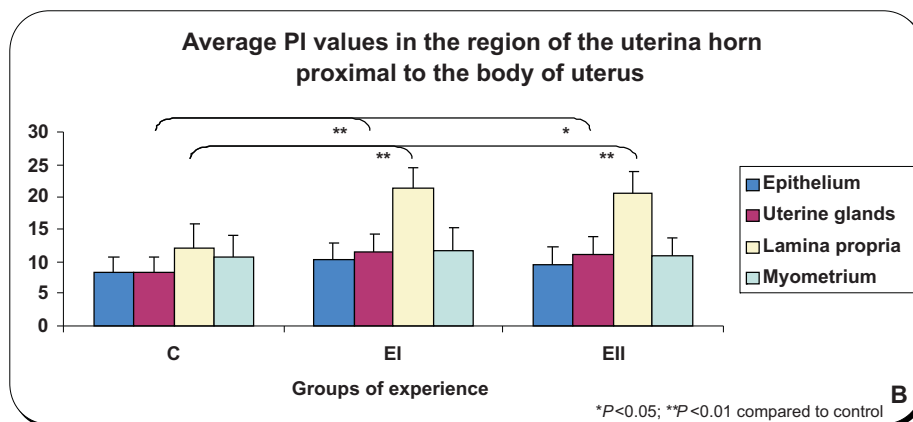
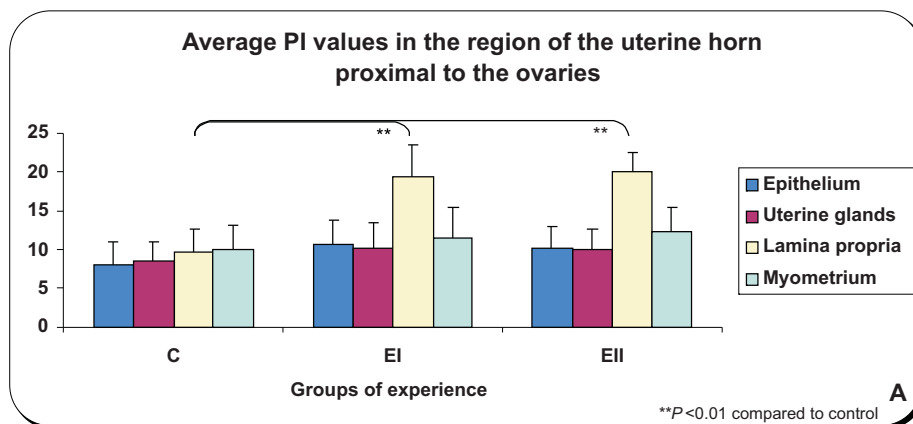


Fig. 4. (A, B, C)

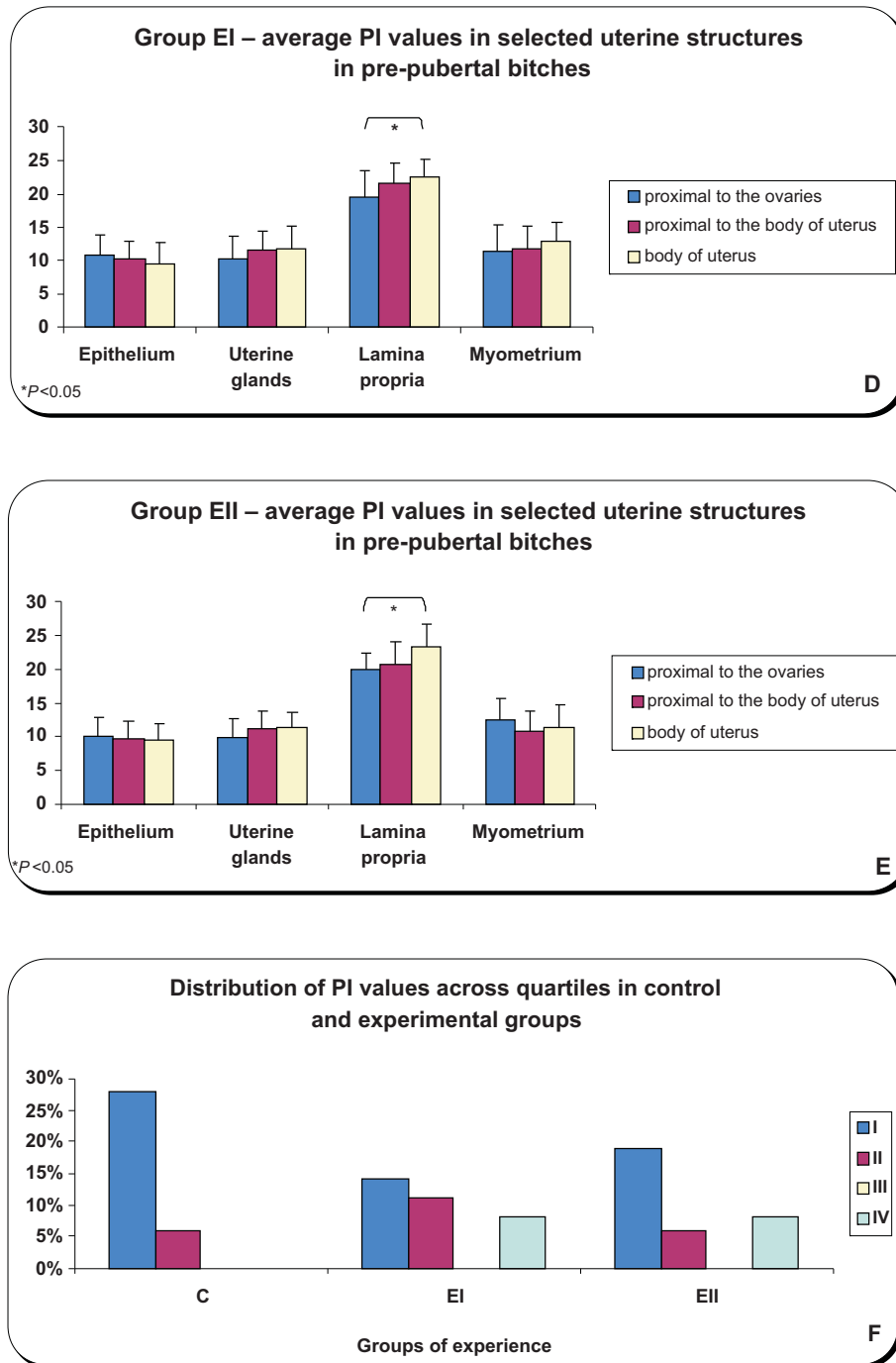


Fig. 4. Proliferative index values of selected uterine regions in pre-pubertal bitches.

for the examined structures reached 8.444 and 21.266 PI, respectively (Fig. 4D and 4E). The values of the median in the control and experimental groups were as follows: group C – 9.898, group EI – 13.622, group EII – 13.306.

Subject to the value of the median for all groups and the lower and upper quartiles of PI data, the results were divided into four activity levels: I – very low AI (AI ≤ 11), II – low AI (11 ≤ AI < 15), III – high AI (15 ≤ AI < 18), IV – very high AI (AI ≥ 18). The highest PI values corresponding to activity level

IV were observed in both experimental groups in the lamina propria in all examined uterine regions (18% of the analysed samples). PI values corresponding to activity level III were not observed. Low PI values corresponding to activity level II were determined in the lamina propria in UBU and BU regions in group C, in the uterine glands and myometrium in UBU and BU regions in group EI, and in the myometrium in UHO and BU regions in group EII (23% of the analysed samples). The remaining PI values (59%) were very low (activity level I), and they were observed

mainly in the epithelial cells lining the uterine endometrium of all regions in groups C and EII (33%), as well as in the myometrium of all regions and of the lamina propria in the UO region in group C.

Discussion

It should be noted that the present experiment was performed on pre-pubertal bitches. The animals used in the study were sexually immature because it is known that young individuals can respond more expressively and unambiguously to external stimuli. In most cases, however, their responses are similar to those observed in older individuals (Vandenberg et al. 2012, Waters 2014).

According to our knowledge, histological and metabolic changes in uterine tissues exposed to low doses of ZEN can lead to potentially harmful side effects that are difficult to anticipate. This uncertainty can result from the dose as well as the period of exposure. Low doses (NOAEL) can provoke surprising responses: (i) the presence of undesirable substances, such as mycotoxins, may be ignored (dietary tolerance) similarly to the T-regs theory (Silva-Campa et al. 2012) where regulatory T-cells are not activated in response to very low levels of infectious factors; (ii) under prolonged *per os* exposure to ZEN, the absorption of the mycotoxin increases in the host's body (Zielonka et al. 2015) due to its carry-over into the digestive system; (iii) a compensatory effect is also possible (Bryden 2012), whereby the analysed indicators decrease and the original homeostasis is restored (Grenier and Applegate 2013) despite continued exposure to mycotoxins.

Several important observations can be drawn from the results of this study. The highest AI values (Fig. 2A, 2B and 2C) in all groups were found in the lamina propria – a constituent of the mucous membrane, followed by the myometrium and the epithelium. The uterine glands were characterised by the lowest AI values.

Significant differences were observed only in the epithelial cells lining the uterine endometrium between group C and both experimental groups. No significant differences were found between the experimental groups, and insignificantly higher values were determined in group EI in both structures and in all investigated regions, excluding the uterine glands in the body of the uterus (Fig. 2C).

The changes observed are consistent with the results of histological examinations performed by Stopa et al. (2014). They indicate that exposure to ZEN ingested with the administered feed inhibited the activity of uterine glands in the uterine horn proximal to

the ovary in both experimental groups. There was a predominance of coil-shaped uterine glands, whereas spiral-shaped glands were observed only in the fundal layer of shallow uterine glands, which is characteristic of young animals. Extensive gland-free areas were also observed in group EII. No differences between groups were found in the uterine horn proximal to the body of the uterus. Glandular cells in the uterine body were characterised by weak activity levels in all cases.

The changes observed were not consistent with normal stages of physiological development of the uterine wall in bitches shortly after birth. In female dogs, uterine adenogenesis begins at the end of the first week of life. From this point onward, epithelial and stromal cells proliferate less intensely. The development of the uterine glands usually ends at the age of 6 to 8 weeks when oestrogen receptors (ERs) in the uterus are stimulated (Cooke et al. 2012). The results of this study correspond to the age of 16 weeks. The animals were exposed to ZEN in the last 6 weeks when adenogenesis should have been completed. Beagle bitches reach reproductive maturity at the estimated age of 6 months (Concannon 2011), therefore, the evaluated dogs would reach reproductive maturity in other 4-8 weeks. In the analysed period, secretory activity should not be observed in the endometrium (epithelium, uterine glands, lamina propria) (e.g. lower AI values – Fig. 2D and/or higher PI values – Fig. 4F). An analysis of AI values in each group indicates that apoptotic changes were least expressed in group C, but in comparison with other structures, significant differences were noted only in the epithelial cells lining the uterine endometrium as AI values increased in successive uterine regions (UBU and BU) and in groups EI and EII. The AI values of the above structures were lowest in the UO region and highest in the opposite region.

It should also be noted that ZEN is a promiscuous compound which becomes a part of the oestrogen signalling process at the cellular level in pre-pubertal bitches (Greaves et al. 2014). This process takes place with the involvement of: (i) ERs; (ii) enzymes responsible for steroid biosynthesis from cholesterol, steroid transformations and intracrine mechanisms of oestrogen synthesis (Bondesson et al. 2015, Frizzell et al. 2015); and (iii) direct DNA binding, commonly to an oestrogen responsive element (ERE) within the promoter region of the target gene.

AI values were generally higher in both experimental groups than in the control group (a growing trend). The distribution of index values across different quartiles in each group (Fig. 2D) indicates that exposure to ZEN shifted AI values towards the fourth quartile (very high AI>4.5) and suggests that apo-

ptotic processes were intensified to the same extent in both experimental groups. The AI values and the distribution of AI values across quartiles imply that apoptotic processes were induced by exposure to ZEN, regardless of the dose.

Apoptotic processes were intensified in response to a monotonic dose of ZEN administered to pre-pubertal bitches for 42 days (Gajęcka et al. 2013). The metabolism of natural environmental oestrogens, including mycoestrogens, is often perceived as a detoxification process that decreases the concentrations of the parent substance (in this case, ZEN), but also produces new substances with far greater toxicity (e.g. α -ZEL) than the parent compound (Frizzell et al. 2011).

Gajęcka et al. (2013) demonstrated that the exposure to low ZEN doses intensified the production of steroid hormones, in particular progesterone (P_4). By contrast, E_2 concentrations increased relative to group C. The above changed the sequence of the physiological increase in steroid hormone concentrations (contrary to the hypothesis proposed by Van Cruchten et al. 2004) during maturation, which is referred to as „hormonal non-equivalence” (Vandenberg et al. 2012, Stopa et al. 2014). The changes observed can lead to false behavioural symptoms (false oestrus), sexual maturation (specific morphotic changes in the uterus) or histological changes in oestrogen-dependent tissues (e.g. intensified secretory activity of uterine glands) (Taylor et al. 2010, Waters 2014), thus contributing to incorrect clinical diagnoses of the reproductive tract in pre-pubertal bitches.

The above observations were validated by PI values (Fig. 4) and changes in their distribution (%) across quartiles (Fig. 4F). An increase (including a significant increase) in PI values was noted in the lamina propria in all investigated regions in the experimental groups relative to group C (Fig. 4A, 4B and 4C). A similar increase was observed in the uterine glands, but only in the UBU region (Fig. 4B). PI values in the epithelium decreased in all groups in regions UO to BU (Fig. 4D-4E). In the remaining structures, PI values increased in all the groups, and the greatest increase was observed in the lamina propria where the differences between regions UO and BU were statistically significant.

The findings of Taylor et al. (2010), who investigated the location of various ERs in reproductive systems of different animal species with a different health status, should be taken into account because ZEN (similarly to 17β -oestradiol – E_2) has greater affinity for $ER\alpha$ than for $ER\beta$ (Warner and Gustafsson 2015). The location of ERs in the uterus suggests that the changes observed could be induced by ZEN and α -ZEL via $ER\alpha$ (Gajęcka 2012). It should also be

noted that an increase in concentrations of ZEN and other exogenous oestrogens in feed decreases the number of $ER\alpha$ in uterine tissues (Schlafer and Gifford 2008), which may result from the non-genomic action of oestrogens (Bishop and Stormshak 2008). ZEN is one of such oestrogens and an exogenous ligand that provokes an increase in E_2 concentrations (Gajęcka and Przybylska-Gornowicz 2012), which leads to considerable vasodilation (Freeman et al. 2013c). The above observations contradict Barton’s hypothesis (Barton 2012) postulating that vasodilation should be accompanied by cross-talk between ERs. The above could result from neoangiogenesis and/or vasodilatation (Freeman et al. 2013c). Those mechanisms lower blood pressure because total blood volume increases when the volume of the circulatory system is stable. The flow of blood through uterine tissues is slowed down, which provides ZEN and α -ZEL with easier access to oestrogen-dependent uterine cells that contain ER proteins. The decrease in E_2 concentrations in group C (Gajęcka et al. 2013) probably resulted from the presence of $ER\alpha$ in the uterus. Monomeric ERs influence constitutive gene expression without the involvement of oestrogen (Liu et al. 2008). Therefore, uterine cells can develop despite very low concentrations of E_2 . ZEN decreases the number of those ERs due to functional redundancy caused by excessive levels of oestrogen-like substances of various origin. The drop in E_2 concentrations and the increase in the levels of P_4 provoked by the presence of ZEN and its metabolites in feed (Gajęcka et al. 2013) is the most probable cause of the increase in AI values and the decrease in PI values in the uterine mucosa, as demonstrated *in vitro* by Galabova-Kovacs et al. (2004) and *in vivo* by Cooke et al. (2012). The latter authors revealed that the administration of P_4 to bitches during the early neonatal period can lead to infertility due to histological changes in the uterus that resemble sterilization.

The mechanisms observed, which result from the activity of the lamina propria and uterine glands, could be attributed to hormesis, a biological phenomenon whereby toxin doses below NOAEL (threshold) values have a stimulating/adaptive effect on the exposed organism, but produce an inhibitory effect when administered at doses above NOAEL values (Heberer et al. 2007, Dobrzyński and Fornalski 2011). PI values in the lamina propria and uterine glands were higher in group EI (lower ZEN dose) than EII, which is consistent with the principle of hormesis (Calabrese 2005). In both experimental groups, a greater shift of PI values to quartile IV was observed in comparison with that found in group C (Fig. 4F).

The results of this study do not provide a clear answer as to whether the determined changes are reversible or whether they can be classified as etiologic factors of the spontaneous endometritis-pyometra complex (Ververidis et al. 2004). For this reason, we will refrain from predicting the possible consequences of the observed mechanisms.

The following conclusions can be formulated based on the results of immunohistochemical analyses of uterine tissues of pre-pubertal bitches that were orally administered ZEN doses at and above NOAEL over a period of 42 days:

- apoptotic processes are enhanced only in the epithelial cells lining the uterine endometrium;
- lower ZEN doses (e.g. in group EI) provoke greater proliferative effects than higher doses, e.g. in the lamina propria and uterine glands.

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