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Original article

The effect of low-dose experimental zearalenone intoxication on the immunoexpression of estrogen receptors in the ovaries of pre-pubertal bitches

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Abstract

Zearalenone is an estrogenic mycotoxin that often contaminates plant material used in the production of feeds for companion animals. Small daily doses of ingested zearalenone – a competitive substrate modulating the activity of enzymes participating in estrogen biosynthesis at the pre-receptor level – can induce subclinical symptoms of hyperestrogenism in bitches. The objective of this study was to determine the effects of low zearalenone doses on the presence of estrogen receptors in the ovaries of pre-pubertal Beagle bitches. The bitches were divided into three groups of 10 animals each: experimental group I – 50 µg zearalenone/kg body weight administered once daily *per os*; experimental group II – 75 µg zearalenone/kg body weight administered once daily *per os*; control group – placebo containing no ZEN administered *per os*. The animals were ovariectomized at the end of the experiment, at 112 days of age. Estrogen receptors were detected in ovarian specimens by immunohistochemical methods. The results revealed an absence of estrogen receptors alpha in all groups. In both experimental groups a decrease in the positive response of estrogen receptors beta in specified structures of ovaries was observed. Very low α -zearalenol levels probably attested to the slowing down (hypostimulation) of the biotransformation process. Overall, zearalenone intoxication led to hyperestrogenism during a specific developmental stage of pre-pubertal bitches. As regards hormesis, the threshold dose of zearalenone (adaptive capability) was exceeded in the ovaries of experimental group II animals. The results obtained in both experimental groups suggest that long-term exposure to low-dose zearalenone intoxication decreased the degree of estrogen receptors beta staining in particular structures of ovaries in the experimental bitches, which initiated epigenetic modification mechanisms that inhibited ovarian development.

Keywords: zearalenone, bitches, ovary, estrogen receptors

Introduction

Ovarian dysfunctions frequently occur in bitches (Grundy et al. 2002, Ververidis et al. 2004, Zanghi et al. 2007). It is believed that species-specific hormonal regulation of reproductive processes plays an important role in the etiopathogenesis of ovarian disorders and makes dogs particularly sensitive to estrogens (Grundy et al. 2002). Additionally, the use of hormones for therapeutic and biotechnological (birth control) purposes may pose a health risk (De Bosschere et al. 2002), and it has been suggested that ovarian pathologies in bitches may result from medical errors. Mycotoxins, particularly zearalenone (ZEN), that are ingested with commercial feed may also contribute to ovarian dysfunctions, but this possibility has not been investigated in detail (Gajęcka et al. 2004, Boermans and Leung 2007). Preliminary studies indicate that ZEN concentrations in commercial feeds vary significantly, with some products containing very high levels of this mycotoxin (Zwierzchowski et al. 2004). Overdoses of endogenous or exogenous estrogens (De Bosschere et al. 2002) may lead to various reproductive disorders, including ovarian dysfunction. Long-term exposure to high levels of estrogenic substances during maturation may result in ovarian dysfunction (hypostimulation due to the presence of ZEN in feed) (Gajęcka et al. 2004, Watson et al. 2007).

ZEN and its metabolites are non-steroidal compounds that bind to estrogen receptors (ERs). By competing with estradiol (E_2) for specific ER binding sites, ZEN may have estrogen-like effects in sensitive animals, such as bitches. Estrogens and ERs are vital for ovarian development and function (Knapczyk et al. 2008, Wąsowicz et al. 2011). By forming a complex with the receptor, ZEN initiates conformational changes that cause estrogen-responsive elements (EREs) to bind to DNA, which induces/inhibits the transcription of estrogen-sensitive genes (Mayr et al. 1992, McDonnell et al. 2002). This balance is determined mostly by the type of ERs in the involved tissue, and the relative levels of ligands and receptors (McDonnell et al. 2002, Shang and Brown 2002, Singh et al. 2007). Ligands that bind to estrogen receptors alpha ($ER\alpha$) and estrogen receptors beta ($ER\beta$) domains are similar in structure; therefore, many ligands bind to these ERs with equal affinity; one exception is genistein, which shows higher affinity for $ER\beta$ than $ER\alpha$ (Wąsowicz et al. 2011). Phytoestrogens can activate the transcription of ERs at concentrations of 1-10 nM (Benassayag et al. 2002). The binding affinity of ZEN for ERs in target tissues and cells reaches 1-10% of the values reported for E_2 (Gajęcki et al. 2010). The activity of ZEN and its metabolite zearalenol (ZOL) in target tissues is also determined by the type

of ERs. ZEN demonstrates higher affinity for $ER\alpha$ than $ER\beta$ in human cells, and it is a powerful agonist of $ER\beta$ (Mueller et al. 2004). According to other authors, zearalenone's *in vitro* binding affinity for $ER\beta$ in human cells varies with its concentration. When ZEN shows higher affinity for $ER\beta$ than $ER\alpha$, its metabolite α -ZOL is more likely to bind to $ER\alpha$ than $ER\beta$, and vice-versa. The degree (%) of binding is always significantly lower than that reported between ERs and E_2 (Kuiper et al. 1998).

The objective of this study was to determine the effect of long-term exposure to low doses of ZEN on the immunoeexpression of ERs in the ovaries of pre-pubertal bitches.

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 10 2006).

Experimental animals

Thirty immature beagle bitches, 70 days of age and with average body weight of 8 kg, were obtained from local breeders (registered at the Polish Kennel Club), and kept under standard conditions with free access to water. Clinically healthy individuals were divided into three experimental groups ($n = 10$ each) and were fed once a day. Experimental group I (EI) received 50 μ g ZEN/kg body weight (Sigma-Aldrich; Germany) (100% NOAEL – no observable adverse effect level – Boermans and Leung 2007); experimental group II (EII) received 75 μ g ZEN/kg body weight (150% NOAEL); and control group (C) received placebo without ZEN. ZEN was administered for 42 days. All bitches were ovariectomized at the end of the experiment, on the approximately 112th day of life.

The animals were kept in common cages with *ad libitum* access to water and were fed standard diets tested for the presence of the following mycotoxins: aflatoxin, ochratoxin, ZEN, 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) (Visconti and Pascale 1998) with fluorescent and/or UV detection techniques. The values obtained were below the sensitivity of the test.

Table 1. The effect of experimental zearalenone intoxication on the immunoexpression of ER β in the ovaries of pre-pubertal bitches.

Experimental groups	EI	EII	C
Oocytes nuclei of primordial follicles	++ (Fig. 1A, 1B)	+ (Fig. 1F)	+++ (Fig. 1I)
Granulosa cells of primordial follicles	–	–	+++ (Fig. 1I)
Oocytes nuclei of primary follicles	++ (Fig. 1B)	–	–
Granulosa cells of primary follicles	+ (Fig. 1B)	+ (Fig. 1F)	+++ (Fig. 1I, 1J, 1K)
Granulosa cells of secondary follicles	++ (Fig. 1B)	+ (Fig. 1E)	+++ (Fig. 1J, 1L)
Interstitial cells	+ (Fig. 1A, 1B)	+ (Fig. 1E, 1H)	++ (Fig. 1I, 1L)

Material sampling and preparation

After 42 days of oral exposure, all bitches were anesthetized. Both ovaries were collected from all the animals. Three sections of each ovary, sampled for immunohistochemical analyses, were fixed in 10% formalin, neutralized, buffered to pH 7.4, and embedded in paraffin blocks. Paraffin blocks were prepared and processed at the Department of Endocrinology and Tissue Culture, Institute of Zoology, Jagiellonian University, Ingardena 6, 30-060 Kraków, Poland.

Immunohistochemistry

Five- μ m paraffin sections were mounted on slides coated with 3'3'-aminopropyl-triethoxysaline (APES, Sigma-Aldrich, Saint Louis, Missouri, USA), deparaffinized in xylene, rehydrated through a series of decreasing ethanol dilutions, and rinsed in water. Sections were then subjected to heat-induced epitope retrieval by microwaving in 0.01 M citric acid buffer (pH 6.0) 3 \times for 4 min at high power (600-W microwave). Endogenous peroxidase activity was blocked by incubation with 0.3% H₂O₂ in TBS (Tris-buffered saline, pH 7.4), and non-specific binding was prevented with the use of 5% normal horse serum (Sigma-Aldrich). ER α was detected using mouse monoclonal anti-human ER α IgG antibody (Dako A/S, Glostrup, Denmark), at a 1:100 dilution. ER β was detected using mouse monoclonal anti-human ER β 1 isoform IgG antibody (Serotec, Kidlington, Oxford, UK), at a 1:20 dilution. The antigens were visualized using biotinylated secondary antibodies horse anti-mouse IgG (1:300, 1.5h at room temperature (RT); Vector Laboratories, Burlingame CA, USA) avidin-biotin-peroxidase complex (1:100, 40 min at RT; StreptABCComplex-HRP, Dako) and 3, 3'-diaminobenzidine (DAB, Sigma-Aldrich) as a chromogen-staining substrate. Additionally, the sections were counterstained with

Mayer's hematoxylin. The sections were dehydrated and mounted in DPX. The control sections were incubated with normal horse serum instead of the primary antibody and processed as above. The ovaries with multiparous bitches were used as a positive tissue control for ER α antibodies applied. The rate of immunoreactions was determined in each section. The presence of primordial, primary and secondary ovarian follicles was classified microscopically, depending on the stage of the development. Selected sections were photographed using the Nikon Eclipse E200 microscope and the Coolpix 5400 digital camera (Nikon, Japan) with the corresponding software.

Positive immunohistochemical reactions were graded as weak (+), moderate (++), or strong (+++).

Results

Immunohistochemical analysis

The observed brown staining was not specific to the ovaries; it could have resulted from non-specific tissue staining with DAB (most preparations were non-specifically stained light brown). Assays carried out to detect ER α expression showed that a positive reaction was not observed in any of the groups.

Strong positive response to ER β expression was observed in some structures of the ovarian tissues in pre-pubertal Beagle bitches from C group (except oocytes nuclei of primary follicles – Fig. 1J – where there was no response) (Table 1).

In both experimental groups a decrease in the positive response (moderate or weak) of ER β receptors (hypostimulation) in particular structures of ovaries (except granulosa cells of primordial follicles in both experimental groups – Fig. 1A and Fig. 1F – and oocytes nuclei of primary follicles in group EII – Fig. 1G – where there was no response) was observed (Table 1).

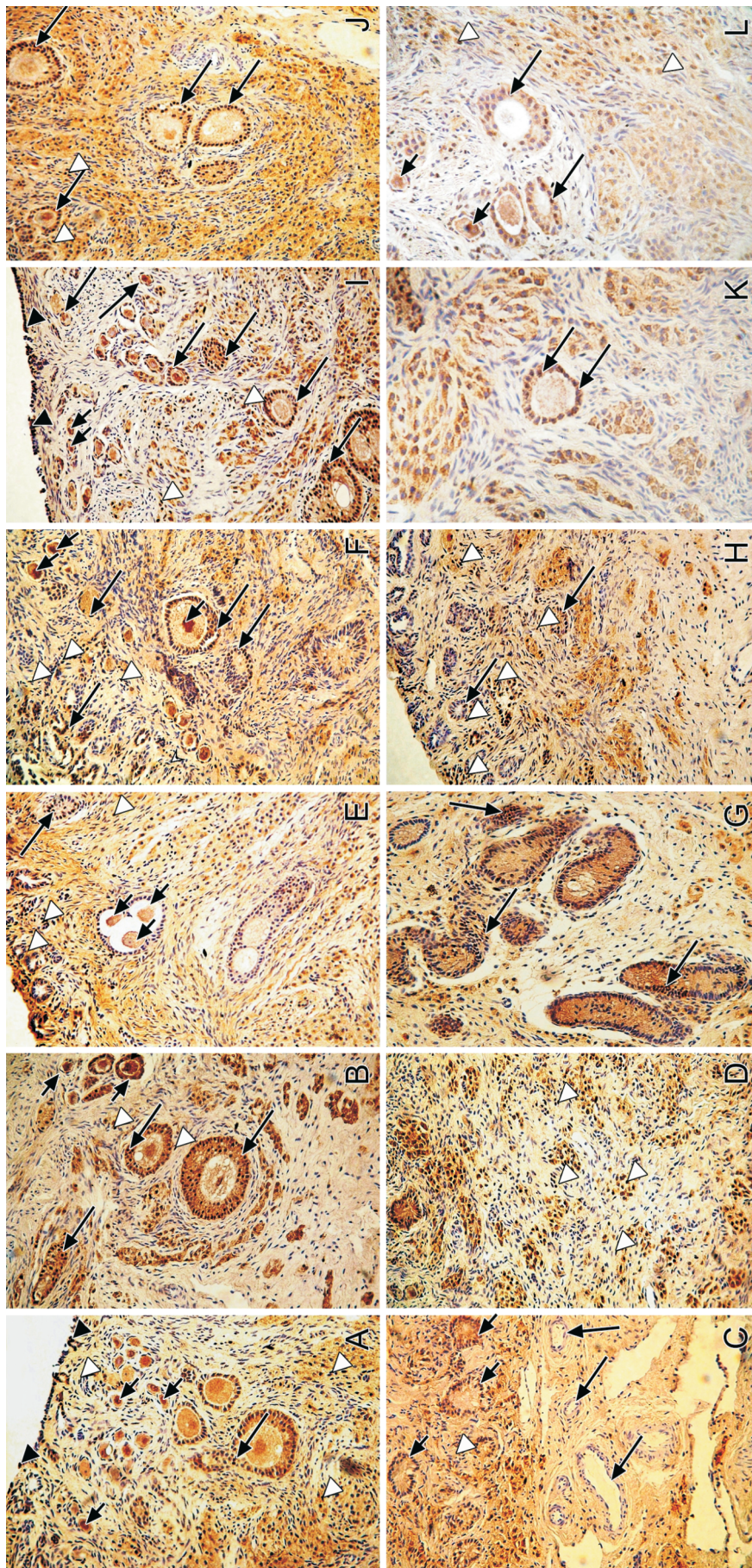


Fig. 1. The presence of ER β in the ovaries of **group EI** animals was determined immunohistochemically. (A) Epithelial ovarian cells (arrowheads), oocyte nuclei (short arrows) of primordial follicles, and granulosa cells of secondary follicles (long arrow). Single interstitial cells are ER β -positive (white arrowhead). (B) Oocyte nuclei of primordial and primary follicles (short arrows), granulosa cells of secondary follicles (white arrowheads). (C) Large blood vessels in the ovarian medulla (long arrows), primary follicles (small arrows), and interstitial cells of the ovarian cortex (white arrowhead). (D) Single interstitial cells of the ovarian medulla (white arrowheads). The presence of ER β in the ovaries of **group EI** animals was determined immunohistochemically. (E) Oocyte nuclei (short arrows) and individual granulosa cells of secondary follicles (long arrows). Single granulosa cells were also ER β -positive (white arrowheads). (F) Oocyte nuclei of primordial follicles and in primary follicles (short arrows). Single granulosa cells of secondary follicles (long arrows), mostly in the ovarian cortex (white arrowheads). (G) ER β -positive cells in follicle-like and canalculus-like structures, which are probably non-functional follicles (long arrows). (H) Absence of ER β -positive cells in the ovarian cortex, except for single interstitial cells (white arrowheads). Granulosa cells of secondary follicles deprived of ER β (long arrows). The presence of ER β in the ovaries of **group C** animals was determined immunohistochemically. (I) Epithelial ovarian cells (arrowheads), oocyte nuclei of primordial follicles (short arrows), granulosa cells of primordial, primary, and secondary follicles (long arrows), and interstitial cells (white arrowheads). (J) Granulosa cells of primary follicles (long arrows) and in secondary follicles (white arrowheads). (K) Granulosa cells of primary follicles (long arrows). (L) Oocyte nuclei (short arrows), granulosa cells of secondary follicles (long arrows), and interstitial cells (white arrowheads). HE. 200 \times magnification (A–J), 400 \times magnification (K, L).

Discussion

Clinical symptoms of zearalenone mycotoxicosis have been described at length in other animal species (Morgavi and Riley 2007, Caloni and Cortinovis 2010, Gajęcka et al. 2011a), but our knowledge of ZEN-induced mycotoxicosis in bitches remains limited (Gajęcka et al. 2004, 2007). The effects of long-term exposure to NOEL doses (low doses) of ZEN on the presence of ERs in the ovaries of pre-pubertal bitches have not been investigated to date, therefore our results cannot be comprehensively benchmarked. The effects of ZEN on the presence of various ERs in the ovaries, as observed in the present study, can be further extrapolated based on basic physiological knowledge of correlations between endogenous steroidal compounds and ERs in bitches.

Trying to explain the decreased degree of ER staining in the ovaries of pre-pubertal bitches subjected to zearalenone mycotoxicosis, we have to account for the fact that ZEN is an endocrine disrupter. Phytoestrogens, including ZEN, exhibit multidirectional biological activity (Wąsowicz 2011). They are capable of modulating the activity of enzymes involved in estrogen biosynthesis (e.g. aromatases, sulfatases, sulfotransferases, 3 β - and 17 β -hydroxysteroid dehydrogenase – HSDs) at the prereceptor level (Wuttke et al. 2002, Tiemann et al. 2003).

In the present experiment, the animals were exposed to ZEN intoxication over a period of 42 days. It should be noted that when the levels of phase I detoxification enzymes (normally cytochrome P450 are involved in phase I detoxification and antiport activity at enterocyte level – Boermans and Leung 2007, Gajęcka et al. 2009) are low relative to the quantity of ingested ZEN, the detoxification balance is disrupted between phase I (hypostimulation) and phase II. The above imbalance supports the assimilation of α -ZOL (1-2 ng/ μ l; unpublished data) and β -ZOL (0 ng/ μ l; unpublished data) metabolites; similar observations have been made by other toxicologists (Plewka 2011). These metabolites can significantly alter activities of enzymes that participate in steroidogenesis and hormonal regulation at the prereceptor level, depending on the dose of substrates (ZEN and its metabolites, where the latter produce an inversely proportional effect). The results of our recent study (Gajęcka et al. 2011b) show that NOAEL doses (group I) do not induce significant increases in mRNA levels of 3 β -HSD and CYPsc genes relative to group C, implying that the body is capable of effectively fighting minimal ZEN intoxications (adaptation mechanism). In animals administered higher ZEN doses (150% NOAEL values), we observed a significant, seven-fold increase ($P = 0.016$) in the mRNA levels of 3 β -HSD, which is crucial for maintaining steroidogenesis. This change

may result from the accumulation of the ZEN substrate (Gajęcka et al. 2009) and could also be attributed to the fact that progesterone levels are generally very low in pre-pubertal bitches, which does not support the biotransformation of ZEN into α -ZOL (Tiemann et al. 2003). The increase in the levels of 3 β -HSD was accompanied by a two-fold increase in CYPsc mRNA levels, at the threshold of significance ($P = 0.076$).

These results suggest that an absence of ER α and the exclusive presence of ER β in the ovaries of group C animals attenuate E2 concentrations. Monomeric ERs mediate constitutive gene expression without hormone involvement. This is particularly true of ER β which can directly bind to selectively sensitive sites in EREs in DNA (Liu et al. 2008), significantly contributing to bodily development (Słomczyńska 2002). These observations imply that ovarian cells can develop at very low physiological concentrations of E₂. The presence of ZEN decreased the share of those receptors, as shown in Fig. 1.

ERs can also affect mechanisms of epigenetic modification (Taylor et al. 2010), leading to changes in activities of genes in the cell nucleus, which “regress” the cell to an earlier developmental phase. The expression of ERs is inhibited by reversible methylation and acetylation of histones at the promoter (Leader et al. 2006). Da Silva Faria et al. (2008) reported morphological changes (“regression”) in the ovaries, increased E₂ serum concentrations, and decreased mRNA expression of ovarian receptors ER α , ER β 1, and ER β 2 in the female offspring of rats suffering from protein-energy malnutrition.

Ligands binding ER α and ER β domains are similar in structure, this is why many ligands bind to those receptors with equal affinity. There are numerous exceptions, however, including genistein, a phytoestrogen whose affinity for ER β is five-fold higher than that for ER α . Genistein is an example of a selective ER modulator (SERM), and this group is also inclusive of ZEN (Gajęcka et al. 2009). SERMs are commonly found in plant material and the natural environment. By binding to the receptor, ZEN changes its conformation (spatial rearrangement of molecules) the ligand’s specificity; it modifies the co activator and activates the receptor as an agonist or an antagonist. The agonistic/antagonistic balance is determined mostly by the sub-type of the ERs, the tissue type, and the relative levels of ligands or receptors (McDonnell et al. 2002). Therefore, it is possible that ZEN, by binding to ER β , changed the receptor’s conformation and activated it as an antagonist, thus causing an inconsiderable drop in the degree of ER β staining in the ovaries of group EI animals, and a considerable decrease in the degree of ER β staining in group EII animals, in comparison with group C (Fig. 1I – 1L).

The discussed mechanism could be an example of epigenetic and/or hypostimulating activity.

Conclusions

Immunohistochemical analyses revealed an absence of a positive response to the presence of estrogen receptors ER α , but a positive response to ER β expression was observed in the ovarian tissues of pre-pubertal Beagle bitches (control group).

The results obtained in both experimental groups suggest that long-term exposure to low-dose ZEN intoxication (100% and 150% NOAEL values) decreased the positive response of ER β receptors (hypostimulation) in the ovaries of experimental bitches, which initiated epigenetic modification mechanisms that inhibited (rather than regressed) ovarian development.

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