Original article

Zearalenone induces apoptosis and inhibits proliferation in porcine ileal Peyer's patch lymphocytes

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Abstract

Zearalenone (ZEN) is one of the most active natural estrogenic compounds that induces apoptosis. This study has been prompted by the widespread occurrence of ZEN in food and feed and limited knowledge about the effects of exposure to low doses of ZEN on the immune system. The aim of the study was to verify the hypothesis that low doses of ZEN contribute to induction of apoptosis and inhibition of proliferation in lymphocytes of the germinal centers (GC) of ileal Peyer's patches (IPP) in pigs. The experiment was performed on 30 female Polish Large White pigs, aged 2 months, with body weight of 15-18 kg, divided into two groups: control (C, n=15) and experimental (Z, n=15). On days 14, 28 and 42 of exposure to ZEN (100 µg kg^{-1} feed day^{-1}), apoptosis in IPP GC was evaluated histologically in HE-stained specimens, immunohistochemically by active caspase-3 staining and in mononucleosome and oligonucleosome detection-based ELISA. Proliferation was evaluated histologically by mitosis detection in HE-stained specimens, immunohistochemically by PCNA staining and in the MTT tetrazolium salt colorimetric assay detecting mitogenic responses of B cells to LPS. Exposure to low doses of ZEN for several weeks intensified apoptosis and weakened proliferation in IPP lymphocytes. ZEN influences gut-associated lymphoid tissue (GALT) by decreasing the expression of CD21+ on B cells and by increasing the percentage of B$_1$ cell populations.

Key words: zearalenone, lymphocytes, proliferation, apoptosis, germinal center, pigs

Introduction

The global spread of mycotoxins poses a serious health risk for humans and animals. Mycotoxins' adverse effects on the immune system (Murata et al. 2003, Obremski 2013) have been demonstrated by both *in vivo* (Bondy and Pestka 2000) and *in vitro* (Berek et al. 2001) studies, even at doses below the no observable adverse effect level (NOAEL) (Oswald et al. 2005). Zearalenone (ZEN) is one of the most active natural estrogenic compounds (Nikov et al. 2000) produced by various species of the genus *Fusarium* (Bennett and Klich 2003, Obremski et al. 2003a). *Fusarium* colonizes maize, wheat and barley

(Zinedine et al. 2007). ZEN and its metabolites, α-zearalenol and β-zearalenol (α-ZEL and β-ZEL, respectively), disrupt the hormonal balance. The discussed mycotoxin and 17β-estradiol (E2) have some similar effects on the reproductive system (Diekman and Green 1992) because they bind to estrogen receptors (Miksicek 1994). ZEN disrupts the ovulation cycle, induces changes in the reproductive system (Obremski et al. 2003b), affects fertilization, embryo implantation and fetal development. At cellular and molecular levels, ZEN manifests toxic effects by inducing apoptosis (Kim et al. 2003, Abid-Essefi et al. 2004), inhibiting proliferation (Obremski et al. 2003b), fragmenting DNA (Kim et al. 2003), producing micronuclei (Ouanes et al. 2003), promoting chromosome abnormalities (El-Makawy et al. 2001) and the formation of DNA adducts (Pfohl-Leszkowicz et al. 1995).

Ileal Peyer's patches (IPP) play a key role in host mucosal immune protection and food tolerance (Mowat 2003, Artis 2008). T lymphocytes and B lymphocytes are the only cells characterized by unique specificity and immunological memory (Grimaldi et al. 2001). B cells are produced by the bone marrow, and they migrate to secondary lymphatic organs where they respond to foreign antigens (Grimaldi et al. 2001). B cells also proliferate and differentiate in gut-associated lymphoid tissue (GALT), in particular in the germinal centers (GC) of IPP lymphoid follicles (Kalanjati et al. 2011). In IPP GC, B cells differentiate into plasma cells that produce immunoglobulin A and memory B cells (MacLennan 1994). Abnormal proliferation and apoptosis in GC of IPP lymphoid follicles have a negative impact on the immune response. Deregulation of apoptotic signaling often leads to neurodegenerative (Ekshyyan and Aw 2004), neoplastic (Vermeulen et al. 2005) and autoimmune diseases (Mahoney and Rosen 2005).

In view of the widespread occurrence of ZEN in food and feed and limited knowledge about the effects of exposure to low doses of ZEN on the immune system, the aim of this study was to verify the hypothesis that low doses of ZEN (100 μg kg^{-1} feed day^{-1}) influence apoptosis and proliferation of lymphocytes in the germinal centers of ileal Peyer's patches in prepubertal gilts. Apoptosis was evaluated with the use of three methods: histologically in HE-stained specimens, immunohistochemically by active caspase-3 staining and in mononucleosome and oligonucleosome detection-based ELISA performed *in vitro*. Proliferation was also evaluated based on three methods: histologically by mitosis detection in HE-stained specimens, immunohistochemically by staining the proliferating cell nuclear antigen (PCNA) and in the MTT assay detecting

mitogen responses of B cells to LPS *in vitro*. The percentages of CD21+ B cells and CD5+CD8- B1 cells were determined in IPP by flow cytometry on different days of the experiment.

Materials and Methods

Experimental design and animals

The study was performed on 30 Polish Large White female pigs (aged 2 months, body weight 15-18 kg) obtained from a commercial fattening farm in Baldy, Poland. The animals were housed and handled in accordance with the procedures laid down by the local Ethics Commission No. 55/2008 (affiliated with the National Ethics Commission for Animal Experimentation of the Polish Ministry of Science and Higher Education). The pigs were assigned to two groups. Group C (n=15) consisted of healthy animals and served as the control. Group Z (n=15) comprised healthy pigs which were fed the ZEN toxin (Sigma-Aldrich, USA) at 100 μg kg^{-1} feed day^{-1} (EC 2006). The investigated material comprised sections of the ileum sampled from pigs on days 14, 28 and 42 of the experiment. Five randomly selected pigs from each group were euthanized on each of the above experimental days.

Histological examination of apoptosis and proliferation of lymphocytes in GC

Samples of the ileal Peyer's patch were fixed in 10% formalin, neutralized, buffered at pH 7.4, embedded in paraffin blocks and sliced with a microtome. Tissue sections were stained with hematoxylin/eosin (HE), and apoptosis and proliferation were observed in lymphocytes in GC under a light microscope at 400x magnification. Apoptosis and proliferation profiles in IPP GC lymphocytes were evaluated in 3 tissue sections per animal. An arbitrary scale was applied in a semi-quantitative evaluation of apoptosis (cells with morphological features characteristic of apoptotic cells – chromatin condensation, presence of apoptotic bodies) and proliferation (mitosis), where (+) = 0 – 5, (++) = 6 – 10, and (+++) = more than 10 proliferating cells and apoptotic cells in 10 high-power fields of IPP GC. The relative frequencies of apoptosis and mitosis in HE were assessed semi-quantitatively. The evaluation of these structures in the same preparations was performed independently by two investigators.

Immunohistochemical examination of apoptosis and proliferation of lymphocytes in GC

Cleaved Caspase-3 (CCasp3) was investigated in paraffin-embedded IPP by the avidin-biotin peroxidase (ABC) method described by Resendes et al. (2004). Four μm correlative tissue sections were placed on silane-coated slides, they were dewaxed in xylene, rehydrated in graded alcohols and placed in dH_2O. Antigen retrieval was performed by immersing sections in 0.01 M citrate buffer (pH 6.0) in a steam bath at 98°C for 25 min, then rapid cooling the sections for 20 min. After blocking endogenous peroxidase with 3% H_2O_2 in dH_2O (30 min) and washing with 0.1 M Tris-buffered saline (TBS, pH 7.4), non-specific binding was blocked with 2% BSA (Sigma-Aldrich, USA) for 30 min at room temperature. The anti-CCasp3 antibody was used an activation-specific polyclonal antibody (Cell Signalling, USA) that does not recognize the caspase-3 precursor (procaspase-3), but only its active form (CCasp3). The antibody was diluted 1/50 in 2% BSA and incubated overnight at 4°C. After washing in TBS, sections were incubated with biotinylated goat anti-rabbit antibody (1/200 in TBS) (Dako, Denmark) for 60 min at room temperature and subsequently treated with the ABC complex (1/100 in TBS) (Thermo Scientific, USA) for 1 h at room temperature. Finally, sections were incubated in 0.05% diaminobenzidine with 3% H_2O_2 in TBS for 10 min, rinsed in dH_2O, counterstained with hematoxylin, dehydrated and mounted with DPX (Panreac, USA). A porcine liver section with apoptotic hepatocytes was used as positive control. For negative control, the primary antibody was replaced by an irrelevant antibody (isotype-matched control antibody). Apoptosis profiles in IPP GC lymphocytes were evaluated in 3 tissue sections per animal in 10 high-power fields per section. An arbitrary scale was applied in a semi-quantitative evaluation of CCasp3, where (+) = 0 – 5, (++) = 6 – 10, and (+++) = more than 10 CCasp3+ cells in IPP GC. The relative frequencies of CCasp3+ IHC were assessed semi-quantitatively. The evaluation of these structures in the same preparations was performed independently by two investigators.

Proliferating cell nuclear antigen (PCNA) expression was determined in IPP GC lymphocytes. Monoclonal mouse antibodies, designed for paraffin-embedded sections, were used for immunohistochemical staining. PCNA antibodies (Dako, Denmark) of clone PC-10 (Ig G2 κ) were diluted 1:200. Dewaxed and rehydrated sections of IPP were placed in a citrate buffer (pH 6.0) and microwaved twice for 3 min each at 650 W to expose the antigens. 3,3'-dia-

minobenzidine (DAB) was used as the chromogen to obtain a stained reaction product. Negative control was performed with the use of IgG2 κ and IgG1 κ antibodies, and positive control involved DAKO sections. PCNA antigen expression was estimated as a stained nuclei of the lymphocytes to all nuclei of the lymphocytes ratio in high power field. It was defined as cells' proliferation index expressed in percentage. Estimation was performed in 10 high-power fields at high magnification x400.

Apoptosis enzyme-linked immunosorbent assay

Lymphocytes isolated from IPP of group C gilts were cultured *in vitro* in the presence of ZEN. Cultures were established in flat-bottom 48-well plates (6 × 10^6 cells ml^{-1}) on the RPMI 1640 growth medium with L-glutamine (Sigma-Aldrich, USA), enriched with 10% fetal calf serum (Sigma-Aldrich, USA) and 10% penicillin-streptomycin (Sigma-Aldrich, USA). ZEN diluted in ethanol (1μg ml^{-1}) was added (Lioi et al. 2004), and the cultures were incubated without additional stimulation for 7 days at 37°C in a humid atmosphere enriched with 5% CO_2. After lysis, mononucleosomes and oligonucleosomes (apoptotic cell markers) were measured with an enzyme-linked immunosorbent assay kit (Cell Death Detection ELISA) (Roche, Germany) according to the manufacturer's instructions. The number of apoptotic cells in group Z was expressed as a percentage of apoptotic cells in group C (100%).

Proliferative response of Peyer's patch lymphocytes

Mitogenic response of Peyer's patch lymphocytes B was determined in the MTT colorimetric assay (Mosmann 1983). On days 14, 28 and 42, cells from group C and group Z were suspended in the RPMI 1640 growth medium containing mitogens – lipopolysaccharide (LPS) from *Salmonella enterica* (Sigma-Aldrich, USA) at a concentration of 10 μg ml^{-1} as the B cell mitogen, and 100 μl of the suspension was added to each well of microtiter plates. The mixture was cultured for 72 h. After incubation, 25 μl of the solution containing 7 mg ml^{-1} of MTT (3-[4, 5 dimethylthiazoly-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) in PBS was added, and the plate was incubated for 4 h. The supernatant was removed, and 100 μl of DMSO was added to each well. Optical density was measured at 570 nm wavelength. All samples were tested in triplicate. The results of the proliferation assay were expressed

Table 1. Relative frequency of apoptosis and proliferation in the germinal centers of ileal Peyer's patches in pigs orally administered ZEN.

Parameters	Group	Experimental day		
		14	28	42
Apoptosis	C	++	+++	++
	Z	+++	+++	+++
Mitosis	C	++	++	++
	Z	+	+	+

The number of proliferating cells and apoptotic cells was evaluated in a high-power fields of view in 10 germinal centers of lymphoid follicles in ileal Peyer's patches (magnification 400x) where: (+) = 0-5, (++) = 6-10, (+++) = more than 10 proliferating cells and apoptotic cells.

in terms of the stimulation index (SI), which was calculated by dividing the mean O.D. of stimulated cultures by the O.D. of non-stimulated (control) cultures.

B-cell subpopulations

The excised segment of the ileum was opened, and Peyer's patch was identified. The mucosa and submucosa were scraped, minced and placed in 1.5 ml of ice-cold phosphate buffered saline (PBS, pH 7.4, 0.1 M). Minced tissues were shaken for 1 min in PBS, the sediment was allowed to settle for 2 min, and the suspensions were removed. The "extraction" was repeated with 1.5 ml of ice-cold PBS. Pooled suspensions were filtered through polyester wool in 2 ml disposable syringes. Lymphocyte counts were established in a hemocytometer. The percentages of subpopulations obtained from Peyer's patches were determined with the use of mouse monoclonal antibodies against porcine CD5, CD8, CD21 (VMRD, USA) and secondary antibodies (BD Pharmingen, USA). Payer's patch CD21+ B cells and CD5+CD8-B_1 cells for cytometry were obtained in accordance with the procedure described by Kaleczyc et al. (2010). The samples were analyzed in the FAC-Scalibur flow cytometer (Becton Dickinson, USA), and the results were processed in the Cell QuestTM program (Becton Dickinson, USA). Lymphocytes were gated based on forward/side scatter cytograms, and lymphocyte subpopulations were identified based on the fluorescence intensity of dot-plot quadrant statistics.

Statistical Analysis

IHC, ELISA and flow cytometry data (expressed as the mean ± SEM) were analyzed by the unpaired Student's t-test and two-way ANOVA using the GraphPadPrism 5.0 software package. Differences were regarded as significant at $p < 0.05$.

Results

Histological examination of apoptosis and proliferation

Proliferative changes followed a different pattern because the relative frequency of mitosis in the experimental pigs was lower than that found in the control animals throughout the experiment (Table 1).

On days 14 and 42, the frequency of apoptosis in the germinal centers of IPP lymphoid follicles was higher in gilts receiving ZEN than in control animals (Table 1).

Immunohistochemical examination of apoptosis and proliferation

Cleaved caspase-3 form was more active in IPP GC in group Z than in group C on all experimental dates. On day 14, the difference between groups Z and C was statistically significant ($p < 0.05$). In group Z, the highest levels of active caspase-3 were reported on day 28, and they were significantly higher than on day 42 ($p < 0.01$). In group C, the highest levels of active caspase-3 were also observed on day 28, and they were significantly higher than on days 14 and 42 ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 1).

On day 42, the percentages of PCNA+ cells in groups C and Z were significantly lower than on days 14 and 28 ($p < 0.0001$ and $p < 0.01$, respectively). The percentage of PCNA+ cells was significantly lower in group Z than in group C on each day of the experiment ($p < 0.0001$) (Fig. 2).
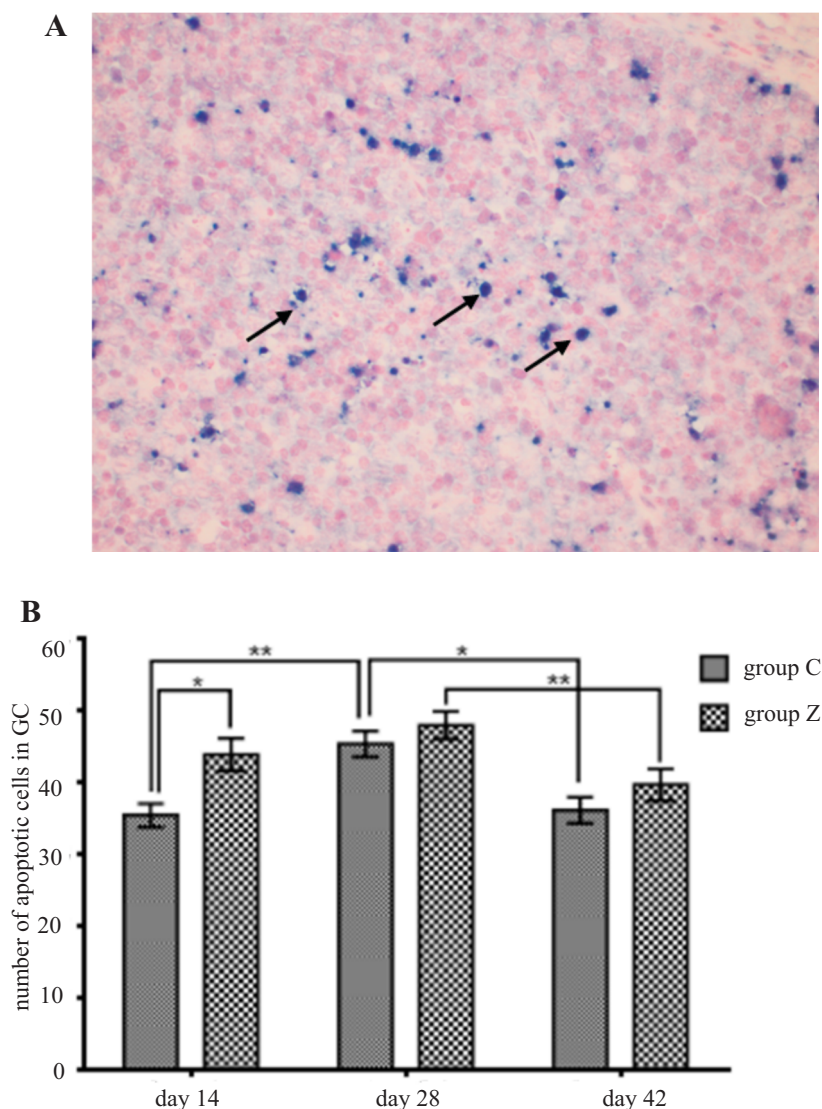
Fig. 1. ZEN induces apoptosis in the germinal center (GC) of lymphoid follicles in ileal Peyer's patches (IPP). A: a representative immunohistochemical macroscopic image of active caspase-3 in IPP GC of group Z pigs on day 28 of ZEN administration (arrows). IHC staining, x100. B: quantitative representation of the number of apoptotic cells in 10 high-power fields (x400). The values are expressed as means ± SEM. Differences were regarded as statistically significant at $p<0.05$ (*) and $p<0.01$ (**).

Apoptosis enzyme-linked immunosorbent assay

The percentages of released mononucleosomes and oligonucleosomes, the building blocks of chromatin that are made of histones (H1, H2A, H2B, H3, H4) wrapped around DNA, were significantly higher in *in vitro* lymphocyte cultures in group Z than in group C ($p<0.05$) (Fig. 3).

Proliferative response of Peyer's patch lymphocytes

The proliferative response of porcine IPP B cells to the mitogenic activity of LPS (10 µg mL^{-1}) was lower in group Z than in group C on all days of the experiment, but the differences observed were not significant (Table 2).

B cell subpopulations

In group C, the percentage of CD21+ B cells continued to increase throughout the experiment. No significant differences in the percentage of CD21+ B cell populations were observed between the experimental and control groups but in group Z, the percentage of CD21+ B cells was lower than that found in group C. On day 28, the percentage of CD5+CD8-(B$_1$) cells was significantly higher ($p<0.05$) in the experimental than in the control animals (Table 3).
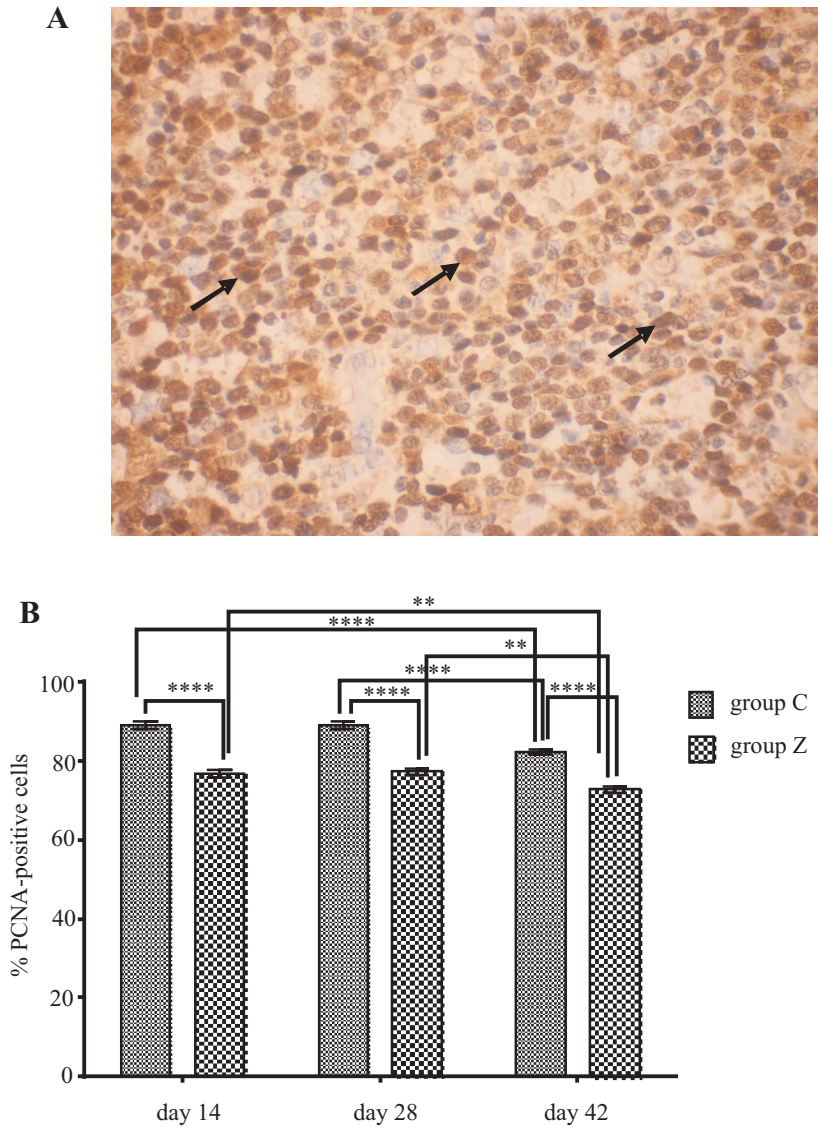
A

B

Fig. 2. ZEN inhibits proliferation in the germinal center (GC) of lymphoid follicles in ileal Peyer's patches (IPP). A: a representative immunohistochemical macroscopic image of PCNA+ cells in IPP GC of group Z pigs on day 28 of ZEN administration (arrows). IHC staining, x400. B: changes in proliferation expressed as percentage of PCNA+ cells calculated in 10 high-power fields (x400). The values are expressed as means ± SEM. Differences were regarded as statistically significant at $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).
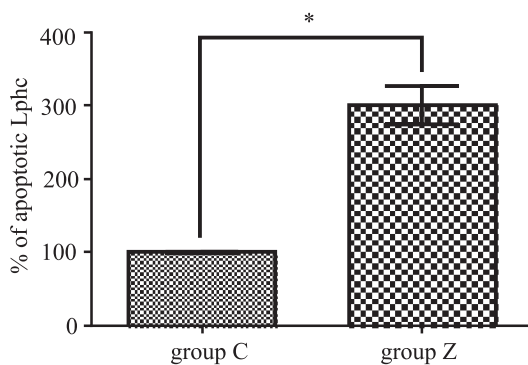

Fig. 3. ZEN induces apoptosis in an *in vitro* culture of lymphocytes (Lphc) from porcine ileal Peyer's patches (6×10^6 cells ml^{-1}). The values are expressed as means ± SEM. Differences were regarded as statistically significant at $p<0.05$ (*).

Discussion

The objective of this study was to verify the hypothesis that low ZEN doses induce apoptosis and inhibit proliferation in the porcine IPP, where both processes are intensified under normal conditions (Kalanjati et al. 2011). Similarly to mesenteric lymph nodes, IPP are part of GALT and are situated along the route of intestinal xenobiotic absorption. IPP play a key role in host protection and food tolerance (Mowat 2003, Artis 2008). IPP GC are the main source of memory B cells and plasma cells that produce high-affinity antibodies for protection against microbial invasions (MacLennan 1994). GALT is weakly developed in newborn piglets, and an abnormal response to feed antigens and/or bacterial

Table 2. Proliferative response of B cells in ileal Peyer's patches (SI) of pigs orally administered ZEN.

Mitogen	Group	Experimental day					
		14		28		42	
		mean	SEM	mean	SEM	mean	SEM
LPS	C	1.09	0.06	1.17	0.27	1.24	0.16
(10 µg/mL)	Z	0.99	0.08	1.12	0.08	1.11	0.08

The results are expressed as means ± SEM (standard error of the mean).

Table 3. Analysis of B cell subpopulations (%) in ileal Payer's patches in pigs orally administered ZEN.

Marker type	Group	Experimental day					
		14		28		42	
		mean	SEM	mean	SEM	mean	SEM
CD21+	C	28.81	14.46	40.50	9.13	54.76	4.80
B cells	Z	28.28	11.72	23.99	13.25	36.31	2.12
CD5+CD8-	C	9.70	2.99	10.47	4.18	8.40	3.89
B$_1$ cells	Z	11.32	3.03	15.23*	2.23	12.22	3.53

The results are expressed as means ± SEM (standard error of the mean)
The p-values (*$p<0.05$) indicate significant differences between the control and experimental groups.

antigens after weaning can lead to severe allergic and inflammatory reactions (Bailey and Haverson, 2006).

B cells are sensitive to cell death at different stages of peripheral differentiation and during the immune response, and their apoptosis can be initiated by mitochondria and death receptors. GALT is particularly sensitive to ZEN which is widely spread in the environment and can be ingested with feed. In line with legal regulations, a ZEN dose of 100 µg per kg^{-1} feed (EC 2006) should not cause reproductive disorders, but research indicates that the immune system is sensitive to even such small doses of the mycotoxin. In pigs receiving 100 µg ZEN kg^{-1} feed per day^{-1}, free ZEN was identified in IPP in the amount of 4 ng g^{-1} (Obremski 2014). Due to the absence of relevant literature data, the influence of the above ZEN levels on the immune system is difficult to determine. The majority of studies into ZEN addressed the effects of high ZEN doses on apoptosis and proliferation in the immune system and in other tissues, whereas the consequences of natural exposure to low doses of ZEN have not been investigated in detail. According to Lioi et al. (2004), ZEN is cytotoxic for bovine lymphocytes and induces their apoptosis. In the cited study, ZEN did not induce apoptosis in lymphocytes exposed to a ZEN dose of 1.2 µg ml^{-1} for 24 hours. In a study by Vlata et al. (2006), higher doses of ZEN intensified apoptosis. Minervini et al. (2006) observed that low doses of ZEN stimulated proliferation of ovarian follicular granulosa cells in horses, whereas high doses down-regulated proliferation indicators in GC, which was attributed to an increase in α-ZEL levels. In our

study, chromatographic analyses did not reveal the direct presence of α-ZEL in IPP, but the immune system could be influenced by α-ZEL, as demonstrated by the presence of α-ZEL in the porcine liver (Obremski 2014).

Apoptosis can be induced by a wide variety of stimuli and conditions, and the sensitivity to apoptotic processes varies among different types of cells. Estrogens can both inhibit and stimulate apoptosis, subject to cell type and baseline conditions (Lewis-Wambi and Jordan 2009). ZEN exerted different effects on the size of selected B cell populations. The percentage of mature CD21+ B cells decreased, whereas an increase was observed in the percentage of B$_1$ cells which participate in autoimmune processes. A study of BALB/c mice revealed that elevated estradiol (E$_1$) levels activate autoreactive B cells that would normally undergo apoptosis (Grimaldi et al. 2001). Ayed-Boussema et al. (2008) reported on the proapoptotic effects of ZEN, which were manifested by an increase in active caspase-3 levels and enhanced release of mononucleosomes and oligonucleosomes. The cited authors demonstrated that ZEN induces apoptosis and inhibits proliferation in human HepG2 hepatocytes. A reduced number of cells undergoing mitosis and a significant drop in PCNA, the proliferation marker which is synthesized in the late G1 phase and in the S phase of the cell cycle, indicate that proliferation in IPP CG was reduced after several weeks of exposure to ZEN (Grossman et al. 1991). LPS, a component of the bacterial cell wall, should stimulate blastic transformation of B cells, during which

quiescent cells move from phase G0 to G1. B cells increase their volume and are transformed to lymphoblasts with organelle-rich cytoplasm and higher RNA content. The above processes are not observed under exposure to ZEN because, as demonstrated by this experiment, the mitogenic response of B cells is reduced by 10-12%.

Surface glycoprotein CD21 is required to generate a strong humoral response to T-dependent antigens (Makar et al. 2001), and it contributes to the survival of B cells in primordial lymphoid follicles and germinal centers (Carroll 2000). By lowering the expression of the CD21 complex, ZEN can significantly decrease the viability of memory B cells. Decreased expression of complement receptor type 2 (CR2, CD21) on B cells and follicular dendritic cells and low activity of the complement system in newborns can lead to abnormal activation of B cells. Decreased expression of CD21+ B cells under the influence of ZEN can distort the immune response to polysaccharides (Griffioen et al. 1993), disturb innate immune functions and disrupt B cell responses (Dempsey et al. 1996), which was manifested by a drop in the proliferative index in the MTT assay.

In this study, the administration of low doses of ZEN to prepubertal gilts for several weeks intensified apoptosis and decreased proliferation in ileal Peyer's patches. ZEN influences GALT by decreasing CD21+ expression on B cells, which could distort the immune response to polysaccharides, disturb innate immune functions, disrupt B cell responses and contribute to the development of autoimmune diseases due to an increase in the percentage of B_1 cell subpopulations.

Acknowledgements

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