

DOI 10.2478/pjvs-2014-0007

Original article

Changes in Th1 and Th2 cytokine concentrations in ileal Peyer's patches in gilts exposed to zearalenone

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Abstract

GALT induces tolerance to foreign food antigens and plays an important role in the development of food allergies and the inflammatory bowel disease. The immune function of GALT is significantly influenced by an equilibrium between Th1 and Th2 subpopulations and the cytokines they produce. Th1 cytokines participate in the induction of a cell-mediated immune response, whereas Th2 cytokines induce powerful antibody-mediated responses. Changes in Th1/Th2 cell polarization of an immune response are associated with susceptibility to autoimmune and infectious diseases. This experiment investigated changes in cytokine levels produced by Th1 and Th2 cells in ileal Payer's patches in gilts exposed to ZEN doses below the NOEL (approximately $8 \mu\text{g kg}^{-1}$ BW) for 14, 28 and 42 days. A significant linear increase in IL-4 ($40.32 \pm 1.55 \text{ ng mg}^{-1} - 137.60 \pm 29.96 \text{ ng mg}^{-1}$), and IL-10 ($5.99 \pm 0.15 \text{ ng mg}^{-1} - 16.39 \pm 1.11 \text{ ng mg}^{-1}$) concentrations was observed. An increase in Th1 (IL-2 and IFN- γ) cytokine levels was also noted in the experimental group, but it was not statistically significant. An HPLC analysis of Peyer's patches in group E animals revealed a linear increase in ZEN concentrations ($3.65 \pm 0.91 \text{ ng g}^{-1} - 4.72 \pm 1.85 \text{ ng g}^{-1}$) and an absence of α -ZEL. IL-4 stimulates monocytes and macrophages, it induces the production of proinflammatory cytokines and it may directly and indirectly contribute to the development of inflammatory foci. Higher IL-4 levels could shift polarization toward Th2 cells, stimulate B cells to undergo class switching to produce IgE and contribute to the development of allergies.

Key words: zearalenone, pigs, immunology, Peyer's patches, cytokines

Introduction

Gut-associated lymphoid tissue (GALT) is a host defense mechanism which makes the largest mass of immune cells in the body (Mowat and Viney 1997). It is the central point of the mucosal immune system which contains inductive and effector functional

sites (Brandtzaeg and Pabst 2004). At inductive sites, antigens are sampled from the surface of mucosal tissue, whereas effector sites (lamina propria of the mucous membrane) differentiate lymphocytes and regulate mucosal immune responses (Brandtzaeg and Pabst 2004).

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GALT induces tolerance to foreign food antigens, it constitutes an intestinal immunological barrier and plays an important role in the development of food allergies and the inflammatory bowel disease (IBD). Studies in rodents and epidemiological data pertaining to humans indicate that genetic factors, prenatal and postnatal factors, such as exposure to infections and xenobiotics, play a vital role in programming the immune system (Mouton et al. 1988, Barker 2004).

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin which is synthesized by polyketide synthase genes of several field fungi, including *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium cerealis* and *Fusarium equiseti* (Gajęcki 2002, Bennett and Klich 2003). ZEN is a full agonist of estrogen receptor α (ESR1) and a partial agonist for estrogen receptor β (ESR2) (Zinedine et al. 2007). ZEN and its analogs are an important group of substances that impair the hormonal balance and, similarly to 17β -estradiol, affect reproduction. ZEN causes hyperestrogenism, physiological disruptions in the reproductive system (Dekasto et al. 1995, Obremski et al. 2003b, Zwierzchowski et al. 2005), it impairs fertility, embryo implantation and fetal development. ZEN disrupts the ovulation cycle and decreases the body weight of animals, in particular pigs and rats (Zinedine et al. 2007). The discussed mycotoxin is also able to bind to estrogen receptors in target cells of the immune system (Vlata et al. 2006, Marin et al. 2011).

The most characteristic structures of GALT are Peyer's patches, ovoid bundles of lymphatic tissue which induce the immune response. The immune function of GALT is significantly influenced by the equilibrium between Th1 and Th2 subpopulations and the cytokines they produce (Swain et al. 1991). Each subpopulation is characterized by unique transcription factors and cytokine patterns. IL-2, IFN- γ and TGF- β (Th1 cytokines) induce the cell-mediated immune response targeted against intracellular pathogens (Chen and Liu 2009). They promote isotype class switching to produce IgG2 (Finkelman et al. 1990, Crawley et al. 2003) and trigger antibody-dependent cellular cytotoxicity. Cytokines such as IL-4, IL-5, IL-10 and IL-13 (Th2 cytokines) induce powerful antibody-mediated responses (Agnello et al. 2003). Antibody isotypes switch to IgG1 and IgE which protect the body against extracellular pathogens, in particular parasites. Th1 and Th2 cytokines are produced by T cells and other immune system cells, and changes in Th1/Th2 cell polarization of an immune response are associated with susceptibility to autoimmune and infectious diseases.

Pigs are characterized by significant variability in

immune responses, such as lymphocyte proliferative responses to *in vitro* mitogens (Jensen and Christensen 1981), but variations in Th1 and Th2 cytokines production in Peyer's patches during prolonged exposure to low doses of ZEA have not been studied to date.

This experiment investigated changes in cytokine levels produced by Th1 and Th2 lymphocytes in young pigs exposed to low doses of ZEN for 14, 28 and 42 days. The experiment verified the hypothesis that xenoestrogens such as ZEN impair cytokine secretion profiles of Th lymphocytes even at doses below the no-observed-effect level (NOEL) (EFSA 2011). Cytokines produced by Th lymphocytes were selected for the study because they have been most extensively researched in pigs, humans and rats. The choice of the above cytokines was also dictated by their varied influence on inflammatory processes. IL-4 and IL-10 inhibit inflammatory responses, whereas IL-2 and IFN- γ demonstrate antagonistic effects.

Materials and Methods

Animals and the Experimental Procedure

The study was performed on 30 female Polish Large White 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 resolution No. 55/2008 of the local Ethics Committee (affiliated with the National Ethics Committee for Animal Experimentation of the Polish Ministry of Science and Higher Education). Gilts were housed in pens with *ad libitum* access to water. They were administered standard feed which was tested for the following mycotoxins: aflatoxin, ochratoxin, ZEN, α -zearalenol (α -ZEL) and deoxynivalenol. The pigs were divided into two groups: a control group (n=15) of healthy animals (group C) and an experimental group (n=15) of healthy pigs administered ZEN (SIGMA-ALDRICH, Cat. No. Z2125, Germany) at $0.1 \text{ mg kg}^{-1} \text{ feed day}^{-1}$ (group E). Analytical samples of ZEN were administered daily *per os* in gelatin capsules before the morning feeding. ZEN samples were diluted in 300 μl of 96% ethyl alcohol (96% ethyl alcohol, SWW2442-90, Avantor Performance Materials Poland S.A., Poland) to produce ZEA doses of $0.1 \text{ mg kg}^{-1} \text{ feed day}^{-1}$. The resulting solutions were added to feed, placed in gelatin capsules and stored at room temperature for 12h to evaporate the solvent.

Biological samples

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. The excised segment of the ileum was opened, and the Peyer's patch was identified. The mucosa was scraped and minced. Peyer's patch samples were stored at -80°C until cytokine analyses. Samples of 1 g of minced tissue were weighed and processed with 2.5 ml of the extraction buffer [PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4), 0.5% sodium citrate (Avantor Performance Materials Poland S.A., Poland), 0.05% Tween 20 (Sigma Aldrich, USA), protease inhibitors (Ref. No. 11 697 498 001, Roche, Germany)] in a homogenizer (Omni-Tips™ Disposable, Omni International, USA). The homogenate was centrifuged at 8600 g for 1 hour in an Eppendorf 5804R centrifuge and supernatant samples were stored at -80°C until analysis.

Determination of cytokine and protein levels in Peyer's patches

Cytokine levels were determined with the use of ELISA kits in accordance with the manufacturer's instructions (IFN- γ – Cat. No. 3130-1A-20, Mabtech, Sweden; IL-2 – Cat. No. CSC1243, Invitrogen, Poland; IL-4 – Cat. No. DY 654 R&D, USA; IL-10 – Cat. No. DY 693B, R&D, USA). ELISA microplates (96-well) were coated with antibodies specific for porcine antibodies in carbonate buffer (16 h, 4°C) and stabilized with 1% BSA (Sigma Aldrich, USA) in PBS (2 hours, 37°C). Extract samples were added and microplates were incubated for 2 hours at 37°C . Biotinylated antibodies specific for porcine cytokines were added and incubated for 2 hours at 37°C . HRP-conjugated streptavidin solution was added and the microplates were incubated for 20 minutes. In each stage microplates were rinsed in PBS with 0.05% Tween 20 (Sigma Aldrich, USA). The specimens were stained with OPD (o-Phenylenediamine, Sigma Aldrich, USA) and hydrogen peroxide (Avantor Performance Materials Poland S.A., Poland). Enzymatic reaction was stopped with 2 M HCl (Avantor Performance Materials Poland S.A., Poland). Absorbance was measured in a spectrophotometer plate reader (TECAN Infinite M200, Switzerland) at $\lambda = 492$ nm. The resulting cytokine levels were compared with protein concentrations in the extract, determined by the modified Bradford method (Bradford

1976), and expressed in terms of pg mg^{-1} protein. The measuring range for IL-2, IL-4, IL-10 and IFN- γ was $35 - 570 \text{ pg ml}^{-1}$, $35 - 10000 \text{ pg ml}^{-1}$, $15 - 2000 \text{ pg ml}^{-1}$ and $10 - 4000 \text{ pg ml}^{-1}$, respectively.

Determination of ZEN and α -ZEL concentrations in the liver and Peyer's patches

Samples of 1 g of ileal Peyer's patches and liver tissue were homogenized with 10 ml of MeOH for around 4 minutes. The homogenate with minimal amount of sediment was centrifuged for 10 minutes at 935 g, and the resulting supernatant was diluted in 20 ml deionized water and applied to an immunoaffinity column (ZearalaTest^{WB} Vicam, Naturan, Poland) at the rate of 1-2 drops s^{-1} . Antibody-bound ZEN and α -ZEL were eluted with methanol which was evaporated in a water bath (50°C), and the residues were dissolved in the mobile phase. Quantitative analyses were accomplished by HPLC-fluorescence detection (Hewlett Packard 1100, FLD G1321A) at excitation wavelength of $\lambda_{\text{Ex}} = 218$ nm and emission wavelength of $\lambda_{\text{Em}} = 438$ nm, in a Hypersil ODS column, 5 μm , 4.6mm x 250mm, with a methanol/acetonitrile/water mobile phase (8:46:46; v:v:v) at the flow rate of 1.0 ml min^{-1} (Obremski et al. 2003a).

Statistical Analysis

The results were processed in Excel (Microsoft, USA) and GraphPad Prism 5 (GraphPad Software, USA) applications. Mean values, standard deviation (SD) and standard error of the mean (SEM) were determined for all groups. Population distributions were evaluated by the Kolmogorov-Smirnov test. The results were processed by the unpaired Student's t-test and one-way ANOVA. The results were regarded as statistically significant at $p < 0.05$.

Results

The effect of ZEN on cytokines levels in Peyer's patches

Cytokines secreted by ileal Peyer's patches represent the actual immune response profile of control and experimental group animals. Cytokines produced by Th1 and Th2 cells were evaluated by the ELISA test, and the concentrations of cytokines isolated from Peyer's patches were expressed in of pg mg^{-1} protein according to the Bradford method (Fig. 1).

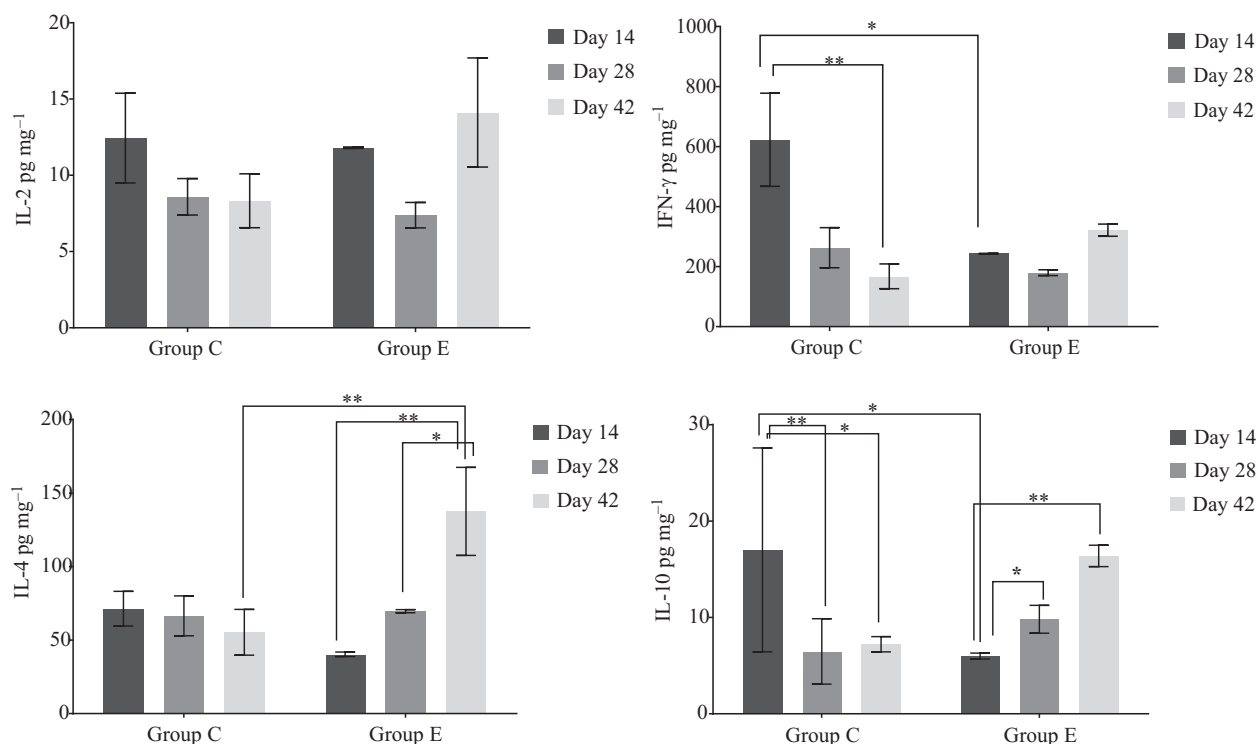


Fig. 1. Comparison of IL-2, IFN- γ , IL-4 and IL-10 concentrations in ileal Peyer's patches between group C (n=5) and group E (n=5) on different days of the experiment. Cytokine concentrations are presented as means value \pm SEM. Differences were regarded as significant at: * $p < 0.05$, ** $p < 0.01$.

Table 1. ZEN and α -ZEL concentrations in porcine livers and Peyer's patches.

Sample	Day of experiment	Concentrations [ng g ⁻¹]*			
		Group C		Group E	
		ZEN	α -ZEL	ZEN	α -ZEL
Liver	14	n.d.	n.d.	10.61 \pm 2.65	26.81 \pm 6.70
	28	n.d.	n.d.	12.22 \pm 1.89	54.20 \pm 33.47
	42	n.d.	n.d.	12.19 \pm 9.12	46.58 \pm 32.70
Peyer's patch	14	n.d.	n.d.	3.65 \pm 0.91	n.d.
	28	n.d.	n.d.	3.94 \pm 1.74	n.d.
	42	n.d.	n.d.	4.72 \pm 1.85	n.d.

* n.d. – not detected; group C (n=5) and group E (n=5) on different days of the experiment; mycotoxin concentrations are presented as means value \pm SD.

During the whole course of the experiment decreased levels of IL-2, IFN- γ , IL-4 and IL-10 were observed in group C. IFN- γ concentrations decreased linearly in group C (622.50 \pm 155.10 pg mg⁻¹ – 167.50 \pm 41.30 pg mg⁻¹) where highly significant differences ($p < 0.01$) were noted between days 14 and 42. A significant increase ($p < 0.05$) in IFN- γ levels was reported in group C relative to group E on day 14. IL-10 concentrations decreased linearly in group C (17.01 \pm 3.74 pg mg⁻¹ – 6.47 \pm 1.39 pg mg⁻¹) where highly significant differences ($p < 0.01$) were noted between days 14 and 28 and significant differences were noted between days 14 and 42 ($p < 0.05$). A significant

increase ($p < 0.05$) in IL-10 levels was reported in group C relative to group E on day 14. Increased levels of Th1 and Th2 cytokines were observed in group E. ZEN contributed to a linear increase in IL-4 concentrations (40.32 \pm 1.55 ng mg⁻¹ – 137.60 \pm 29.96 ng mg⁻¹). Highly significant differences ($p < 0.01$) were noted between experimental days 14 and 42 and significant variations ($p < 0.05$) were observed between days 28 and 42. A highly significant increase ($p < 0.01$) in IL-4 levels was reported in group E relative to group C on day 42. IL-10 concentrations increased linearly in group E (5.99 \pm 0.15 ng mg⁻¹ – 16.39 \pm 1.11 ng mg⁻¹) where significant differences ($p < 0.05$) were

noted between days 14 and 28, and highly significant differences ($p < 0.01$) – between days 14 and 42. A linear increase was also observed in the concentrations of cytokines secreted by Th1 cells (IL-2 and IFN- γ) in the experimental group, but no significant variations were noted.

ZEN and α -ZEL concentrations in the liver and Peyer's patches

The daily dose of ZEN administered to experimental group animals was 100 μg per kg feed⁻¹. ZEN and α -ZEL concentrations in the liver and Peyer's patches were evaluated by HPLC on different days of the experiment (Table 1). The presence of ZEN and α -ZEL was not observed in liver or Peyer's patch samples of group C animals. In the liver tissue samples of group E pigs, ZEN and α -ZEL concentrations were determined at 10.61 ng g⁻¹ – 12.22 ng g⁻¹ and 26.81 ng g⁻¹ – 54.20 ng g⁻¹, respectively (Table 1). A chromatographic analysis of Peyer's patch samples from group E animals revealed a linear increase in ZEN concentrations (3.65 ± 0.91 ng g⁻¹ – 4.72 ± 1.85 ng g⁻¹) and an absence of α -ZEL (Table 1).

Discussion

The experiment aiming to determine the effect of low ZEN doses on cytokine secretion profiles of Th lymphocytes in the GALT system was prompted by the results of previous research. Our study of 30 gilts is probably the first systematic attempt to document the cytokine microenvironment (Th1 and Th2 subpopulations) of ileal Peyer's patches in pigs exposed to ZEN. Zearalenone mycotoxicosis was induced by administering ZEN to gilts in a daily dose of 100 μg kg feed⁻¹, which is the maximum approved level for ZEN toxins in pig feeds (European Commission, Commission Recommendation 2006). The results of chromatographic analyses of ZEN and α -ZEL levels in porcine livers and Peyer's patches indicate that even very low ZEN concentrations in animal feed lead to the accumulation of the discussed toxin in the body tissues and potential disruptions of homeostasis.

ZEN is a xenoestrogen and a non-steroidal agonist for estrogen receptors which affects cell proliferation (Coffey 2001, Tiemann et al. 2008). In pigs and probably in humans, ZEN is quickly absorbed after oral administration and it is metabolized by intestinal cells. ZEN undergoes hydroxylation in the intestines and it is reduced to α -zearalenol (α -ZEL), β -zearalenol (β -ZEL), zearalanone (ZAN), α -zearalanol (α -ZAL) and β -zearalanol (β -ZAL).

ZEN is transformed to α -ZEL mainly in the liver. α -ZEL is the predominant ZEN metabolite in pigs (Malekinejad et al. 2006) with the highest estrogen receptor-binding affinity (Biehl et al. 1993).

Previous research indicates that prolonged exposure to estrogens may influence T and B cell homeostasis and lead to autoimmunization (Chighizola and Meroni 2012) because estrogens significantly influence the innate and adaptive immune systems (Straub 2007). Estrogens regulate the expression of cytokines, molecular messengers which activate and deactivate various cells (Sattar Ansar 2000). Estrogens inhibit the production of Th1 cytokines such as IL-12, TNF- α and IFN- γ , and they stimulate the secretion of anti-inflammatory Th2 cytokines, including IL-10, IL-4 and TGF- β (Salem 2004). Estradiol (E₂) was also found to stimulate the Th1 response (Maret et al. 2003, Delpy et al. 2005).

GALT is the main system which protects the gastrointestinal system against external pathogens. Peyer's patches are major inductive sites of immune responses which promote tolerance to food antigens. In this *in vivo* experiment we evaluated the effect of *per os* administration of ZEN on cytokine secretion profiles of Th lymphocytes, an important aspect of the immune response induced by ileal Peyer's patches in pigs. The concentrations of ZEN and α -ZEL were determined in samples of liver and intestinal lymphoid tissue. Th1 and Th2 cytokine levels were compared with protein concentrations determined in Peyer's patches by the Bradford method.

In this experiment, cytokine concentrations in experimental group animals increased with duration of exposure to ZEN. The above was clearly demonstrated by cytokine levels determined on various days of the experiment (Fig. 1). A gradual drop in the concentrations of pro-inflammatory (IFN- γ , IL-2) and anti-inflammatory cytokines (IL-4, IL-10) was observed in the control group, where IL-4 levels decreased at the slowest rate (Fig. 1). The above can be attributed to the immunosuppressive function of Peyer's patches where food antigens come into contact with the intestinal system. Decrease of cytokine secretion in the GALT occurs due to the very nature of the intestinal immune system. When applying food antigens the natural consequence of the immune response is the formation of immunological memory and food tolerance. The development of food tolerance involved three main mechanisms: clonal anergy, clonal deletion and active suppression. They can occur separately or in a single stage and a crucial factor in determining the type of leader is the mechanism of antigen dose and frequency of administration (Strobel and Mowat 1998). The anergy is characterized by a lack of lymphocytes proliferation in response to

antigen and the lack of cytokine production, in addition to the production of small amounts of IL-10 (Sundstedt et al. 2003). Additionally, anergic lymphocytes absorb the environmental IL-2 producing an excitatory signal, thereby delaying the proliferation of other cells present in the environment. During pigs weaning period, in which were the animals in our experiment, fluctuations in the concentrations of Th1 and Th2 cytokines are also observed. Vázquez et al. in study on mice showed that the increase in the production of both pro-inflammatory cytokines and inflammatory Peyer's patches cells took place until 14 days after weaning and then was followed by the gradual drop (Vázquez et al. 2000). A similar situation occurred in our experiment in the control group, where the initial high production of cytokines by Peyer's patches lymphocytes may indicate the start of the mechanisms involved in the maturation and differentiation of intestinal immune system in weaning piglets. In the group of pigs fed ZEN, the concentrations of pro-inflammatory and anti-inflammatory cytokines increased with the duration of toxin exposure and the differences in cytokine levels on different days of the experiment were statistically significant, in particular those noted between days 28 and 42 (Fig. 1). In comparison with control, IL-2, IL-4, IL-10 and IFN- γ levels in experimental animals increased gradually with prolonged toxin exposure and ZEN accumulation in GALT, reaching the highest values on day 42 (Table 1).

Endogenous estrogens – estrone, E₂ and estriol – deliver similar effects. Estrogenic hormones exert a considerable influence on the immune system and, subject to concentration, they can produce different effects. For example, low concentrations of E₂ in the periovulatory period stimulate the secretion of IL-4, IL-10 and IFN- γ , whereas no such effect is observed with regard to TNF- α which is released by Th1 cells during pregnancy when E₂ levels are high. The above inhibits the secretion and activity of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, MCP-1 and it stimulates the pro-inflammatory response by enhancing the production of IL-4, IL-10, TGF- β , TIMP and osteoprotegerin (Fass et al. 1999). Estrogens may thus exert an inhibiting effect on Th1 cell populations and components of the cellular immune response. During pregnancy estrogens do not inhibit B cell populations and E₂ contributes to an increase in the levels of Th-2-dependent transcription factor GATA-3 and CD4+CD25+ FoxP3 regulatory T cells (Polanczyk et al. 2004). Endogenous estrogens and ZEN produce similar effects in the immune system. In the discussed experiment, pigs were exposed to ZEN doses below the NOEL (approximately 8 $\mu\text{g kg}^{-1}$ BW), but the effect of such low toxin concentrations on cytokine se-

cretion was similar to that produced by periovulatory levels of E₂. It should also be noted that IL-2 levels increased gradually with the time of exposure to ZEN. ZEN inhibits Th1 cells whose proliferation is mediated by IL-2. IL-2 also promotes the proliferation of CD4+CD25+ FoxP3+ Treg cells. Regulatory T cells secrete high amounts of IL-10 and TGF- β which inhibit the pro-inflammatory response. IL-4 is produced mainly by Th2 cells, but also by mastocytes and basophils. It has a broad spectrum of activity and, in most cases, it has an antagonistic effect on IFN- γ . In this experiment, an increase in IL-4 levels could be responsible for the stimulation of B cells and antibody class switching to produce IgE, which is an important consideration in the pathomechanism of allergies. IL-4 also directs T cells to produce Th2 cytokines. IL-4 stimulates monocytes and macrophages, it induces the production of proinflammatory cytokines and it may directly and indirectly contribute to the development of inflammatory foci.

The results of this study suggest that ZEN and its metabolites disrupt the immune response by influencing the secretion of cytokines from Th1 and Th2 cells in GALT. Our findings could have clinical applications by contributing to the mitigation of the risk posed by low ZEN concentrations in feeds and foodstuffs.

Acknowledgements

The study was conducted as a part of research project No. N N308 237936 financed by the National Science Centre in Poland.

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