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Original article

The influence of experimental administration of low zearalenone doses on the expression of Th1 and Th2 cytokines and on selected subpopulations of lymphocytes in intestinal lymph nodes

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Abstract

The aim of this study was to characterize the immune response taking place in ileocecal lymph nodes (ICLN) in control (n=15) and zearalenone (ZEN)-treated (n=15) pigs. The experiment was carried out over 42 days; a dose of 0.1 mg kg^{-1} feed day^{-1} of ZEN was administered to the animals. The dose used in the experiment was at a level where no adverse effects are observed (NOAEL) in the ovaries, uterus and vagina. ICLN samples for analysis were collected on the 14th, 28th and 42nd day of the experiment. The analysis of cytokine concentration in the tissues showed that pigs treated with ZEN had an increased level of cytokines produced by helper Th1 lymphocytes (IL-2, IL-12 and IFN-γ) on the 28th day of the experiment. The level of cytokines produced by helper Th2 lymphocytes (IL-4 and IL-10) was characterized by a statistically non-significant upward trend, as compared with the control group. Flow cytometry showed a linear decrease in the percentage of CD21+ B, CD2+ T and CD4+CD8- T cells and an increase in the percentage of CD8+CD4- and TCRγδ + T cells in pigs treated with ZEN. Both ZEN and α-ZEL (α-zearalenone) concentrations increased over time in the liver, but only ZEN concentration increased in ICLN. The results obtained demonstrate that a NOAEL concentration of ZEN shifts the immune response in pig ICLN towards Th1/Th17, probably with a simultaneous activation of M1 macrophages. Moreover, we observed an increase in humoral cytokine secretion; this can be explained by a negative feedback loop and a phenotypic switch of macrophages from M1 to M2, as well as a switch of immune response from Th1 to Th2 type. ZEN can therefore influence the process of cytokine secretion and the percentage of lymphocytes in ileocecal lymph nodes.

Key words: zearalenone, pig, cytokines, lymphocytes, lymph nodes, low doses

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Introduction

Zearalenone (ZEN), a makrocyclic β-resorcylic acid lactone, is a non-steroidal estrogenic mycotoxin produced as a secondary metabolite by numerous mould species from the genus *Fusarium*, including *F. culmorum*, *F. roseum*, *F. graminearum* and others. These fungi are present on almost all continents and can infest wheat, barley, rice, corn and other plants both before and after harvest, causing food and feed contamination all over the world (Richard 2007, Zinedine et al. 2007). It was demonstrated that 36% of 17 000 feed and feed component samples were contaminated with ZEN in Europe and Asia in 2004-2011. The average ZEN concentration in positive samples was 0.28 mg kg^{-1}, while a maximum of 26.7 mg kg^{-1} was reached (Streit et al. 2013). The above average feed contamination level exceeds the limits determined by EU Commission recommendations, according to which ZEN concentration in pig feed cannot exceed 0.25 mg kg^{-1}, and there is an additional limitation to 0.1 mg kg^{-1} for sows and piglets (European Commission, Commission Recommendation, 2006).

Natural exposure to ZEN in contaminated feed leads to changes in the reproductive system of the animals. This is due to its enormous estrogenic activity, as its hormonal action exceeds most of the naturally occurring non-steroidal estrogens, including isoflavones from soybean and clover (Bennett and Klich 2003). ZEN leads to uterine edema, ovarian cysts and increased maturation of ovarian follicles (Fink-Gremmels and Malekinejad 2007, Obremski et al. 2008). It was reported that relatively low ZEN doses (1.5 to 2 mg kg^{-1} in diet) cause, within three to seven days, swelling and thickening of the vagina and vulva walls, increase in uterus mass, ovarian atrophy and increased proliferation in sexually immature pigs (Obremski et al. 2003a, Kanora and Maes 2009). Long-term exposure to ZEN doses in the range of 1 to 5 mg kg^{-1} leads to hyperestrogenism and increased pig mortality. Doses surpassing 100 mg kg^{-1} cause sow infertility (Zinedine et al. 2007, Kanora and Maes 2009).

There is evidence that estrogens can have an influence on effector cells of the immune system; this is related to the presence of estrogen receptors (ER) in immune cells. Two forms of ER were identified in humans: ERα and ERβ. Estrogen can have various effects depending on the receptor to which it binds, and the final effect of the hormone is strictly related to the subtype of receptor present in a given cell type. For example, increased expression of ERβ versus ERα in some inflammations can be responsible for the pro-inflammatory effect of estrogen (Straub 2007, Cutolo and Straub 2009). The lymphatic tissue of the intestine is a part of the immune system, especially important for creating a tolerance to environmental antigens in the organism. Selective treatment of factors delivered in food by the organism is a prerequisite for proper human and animal existence, so that the homeostasis is not disturbed, and the chances of survival are not decreased. In this respect, a very important role is attributed to the gut-associated lymphoid tissue (GALT). The processes of tolerance to food antigens and formation of immunological memory to trophoallergens that reach the lymphatic structures via lymph and blood are taking place in GALT (Macpherson and Smith 2006). Mycotoxins use exactly the same pathways to reach the lymph nodes. Although many mycotoxins are able to influence the function of the immune system and of the gut (Bouhet and Oswald 2005), no ultimate assessment of the potential relation between exposure to mycotoxins and chronic inflammatory disorders in animals and in humans (ulcerative colitis, Coeliac disease or Crohn's disease) was established (Maresca and Fantini 2010).

In contrast to estrogens, studies of the immunoregulatory function of ZEN are rare. Although ileocecal lymph nodes (ICLN), unlike small intestine absorptive cells, are not structurally associated with the intestinal epithelium, they constitute an integral part of GALT. Immunological tolerance to food antigens is also established in ICLN (Faria and Weiner 2005). Due to the lack of direct contact with the intestinal lumen, one could think that the reaction to ZEN in ICLN may lead to ambiguous effects in cytokine production or in the variation of the percentage of given cells involved in the immune response, as was observed in small intestine Peyer's patches (Obremski 2014).

Due to a lack of sufficient information on the influence of ZEN on GALT, we have conducted a study, which is the first attempt to demonstrate the influence of ZEN on processes taking place in GALT. The aim of this study was to characterize the immune response taking place in ileocecal lymph nodes in control and ZEN treated sexually immature female pigs, keeping the administered mycotoxin at a level where no adverse effects are observed (NOAEL) in the ovaries, uterus and vagina.

Materials and Methods

Animals and the experimental procedure

All experimental procedures involving animals were conducted according to Polish legal regulations concerning experiments on animals (following deci-

sion No. 55/2008 issued by the Local Ethics Committee for Experiments on Animals).

The experiment was conducted at the Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland. 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 gilts were divided into groups and penned with *ad libitum* access to water. The animals were randomly assigned to an experimental group (Group E) and control group (Group C) with 15 gilts in each. Each day, the animals in the experimental group were administered ZEN *per os* 0.1 mg kg^{-1} of feed. The amounts of toxin application were dependent on the amount of feed consumed and updated weekly. Analytical samples of ZEN were administered *per os* daily in gelatine capsules before the morning and the afternoon feeding. A placebo was administered to the control group. The experiment was conducted over 42 days. Gilts were slaughtered on a two week basis. Every two weeks, 5 gilts from the experimental and control group were slaughtered (10 gilts each time).

Determination of lymphocyte subpopulation

For cytometric analysis, one half of a cut lymph node was placed in a cold Petri dish with the cut surface directed upwards. Lymph node stroma was finely chopped with a scalpel, and the tissue was subsequently placed in 1.5 ml of ice-cold PBS (PBS, pH 7.4, 0.1 M). The disaggregated tissue was mixed for 1 minute in PBS and left for sedimentation (2 minutes). The supernatant was collected, and the extraction was repeated with another 1.5 ml of ice-cold PBS. The combined supernatants were filtered through a polyester filter placed in 2 ml single-use syringes, and the lymphocyte concentration was determined using a haemocytometer.

The percentages of lymphocyte subpopulations obtained from the ileocecal lymph node were determined with the use of mouse monoclonal antibodies against porcine CD2, CD4, CD5, CD8, gamma-deltaTCR, CD21 (CD2, IgG2a, cat. no. MSA4; CD4, IgG2b, cat. no. 74-12-4; CD5, IgG1 cat. no. PG114A; CD8, IgG2a cat. no. 76-2-11; TCR1-N7, IgG1 cat. no. PT79A; CD21, IgG1, cat. no. BB6-11C9, all from Veterinary Medical Research and Diagnostic (VMRD), Pullman, Washington, USA) and secondary (Biotinylated rat anti-mouse, IgG2b, cat. no. 550333; streptavidin-PE, cat. no. 554061; FITC rat anti-mouse IgG2a, cat. no. 553390; PE rat anti-mouse IgG1, cat. no. 550083, all from DB Pharmingen, USA) antibodies. The ileocecal lymph node leukocytes for cytometry were stained in accordance with the procedure described by Kaleczyc et al. (2010). The samples were analysed in a flow cytometer (FACScalibur, Becton Dickinson, USA), and the results were analysed 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.

Determination of cytokine and protein levels

For the ELISA (enzyme linked immunosorbent assay) procedure, 1 g samples 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) and protease inhibitors (Roche, Germany)] in a homogenizer (Omni International, USA). The homogenate was centrifuged at 8 600 g for 1 hour in an Eppendorf 5804R centrifuged, and supernatant samples were stored at -80°C until analysis.

Cytokine levels were determined with the use of ELISA kits in accordance with the manufacturer's instructions (IL-2 – cat. no. CSC1243, Invitrogen, USA; IL-4 – cat. no. DY 654 R&D, USA; IL-10 – cat. no. DY 693B, R&D, USA; IL-12/IL-23 p40 – cat. no. P1240 R&D, USA; IFN-γ – cat. no. 3130-1A-20, Mabtech Sweden). ELISA microplates (96-well) were coated with antibodies specific for porcine antibodies in a carbonate buffer (16 h, 4°C) and stabilized with 1% BSA (Sigma Aldrich, USA) in PBS (2 h, 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. An HRP-conjugated streptavidin solution was added, and the microplates were incubated for 20 minutes. In each stage, the 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 λ=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, IL-12 and IFN-γ was 35 – 570 pg ml^{-1}, 35 – 10 000 pg ml^{-1}, 15 – 2 000 pg ml^{-1}, 47 – 3 000 pg ml^{-1} and 10 – 4 000 pg ml^{-1}, respectively. The values of the intra- and inter-assay coefficients of variation (CV%) for IL-2, IL-4, IL-10, IL-12, IFN-γ were 4.14, 4.66, 4.48, 5.02, 2.84, 2.19, 4.58, 5.42, 3.86 and 4.19, respectively.

Determination of ZEN and α-ZEL

Samples of 1 g of the ileocecal lymph (ICLN) node and liver tissue were homogenized with 10 ml of MeOH for approx. 4 minutes. The homogenate, with a 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 a 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 the HPLC method (Obremski et al. 2003b).

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 the 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

ZEN effects on the percentage of lymphocytes

The evaluation of the influence of a low dose of ZEN on the percentage of lymphocyte populations in ileocecal lymph nodes was conducted using immunophenotyping and flow cytometry analysis. Figure 1 shows the percentage values of CD2+ lymphocyte, CD4+CD8-helper T-lymphocyte, CD8+CD4-cytotoxic T cell, TCRγδ+ T cell and CD21+ B cell subpopulation in ileocecal lymph nodes of pigs that were intoxicated *per os* with a daily dose of ZEN equal to 0.1 mg kg^{-1} feed over 14, 28 and 42 days. No statistically significant differences were found after the selected experimental periods among CD2+, CD4+CD8-, CD8+CD4- and T TCRγδ+ cells of group C and E. The percentage of CD2+ and CD4+CD8- cells obtained from intoxicated animals showed a linear decrease; this was in contrast to the linear increase of the percentage of subpopulations of CD8+CD4- and TCRγδ+ cells. During the entire experimental period, CD21+ B lymphocytes in the placebo-receiving group showed a linear increase; this was in contrast to a decrease in the percentage of these lymphocytes in animals treated with ZEN. As compared with group C, group E had a clearly lower CD21+ B cell percentage, with statistically lower values observed on the 28th ($p<0.05$) and 42nd ($p<0.0001$) day of the experiment.

ZEN effects on cytokines concentrations

Cytokine secretion in ileocecal lymph nodes represents the actual profile of the immune response of animals treated with ZEN or with the placebo (Fig. 2). The ELISA method was used to evaluate the secretion of pro- and anti-inflammatory cytokines by lymph node cells. Measured cytokine concentrations were calculated per mg^{-1} of protein (pg mg^{-1}), which was determined using the Bradford method. An increased secretion of cytokines by Th1 cells was observed in group E. The concentrations of IL-2, IL-12 and IFN-γ on the 28th day of experiment (11.31±1.85 ng mg^{-1}, 577.80±33.59 ng mg^{-1}, 270.80±56.92 ng mg^{-1}, respectively) were statistically significantly higher ($p<0.01$) than the concentrations recorded in group C (3.31±0.52 ng mg^{-1}, 294.50±18.74 ng mg^{-1}, 76.28±16.36 ng mg^{-1}, respectively). The concentration of IFN-γ in pigs treated with ZEN on the 14th and 28th day of the experiment was statistically significantly different ($p<0.05$). The level of IL-2 on the 28th day in the placebo receiving group showed a statistically significant decrease as compared with the 14th and 42nd day of the experiment ($p<0.05$, $p<0.01$, respectively). The level of cytokines produced by Th2 lymphocytes (IL-4 and IL-10) in group E tended to increase, but the difference was not statistically significant. On the 28th day of the experiment, the concentration of IL-10 in the group of intoxicated animals was statistically higher as compared with that found in the placebo receiving group ($p<0.05$, 15.97±1.02 ng mg^{-1}, 5.23±0.66 ng mg^{-1}, respectively). Moreover, IL-10 concentration in group C on the 28th day of ZEN administration was statistically lower than that measured on the 42nd day ($p<0.05$).

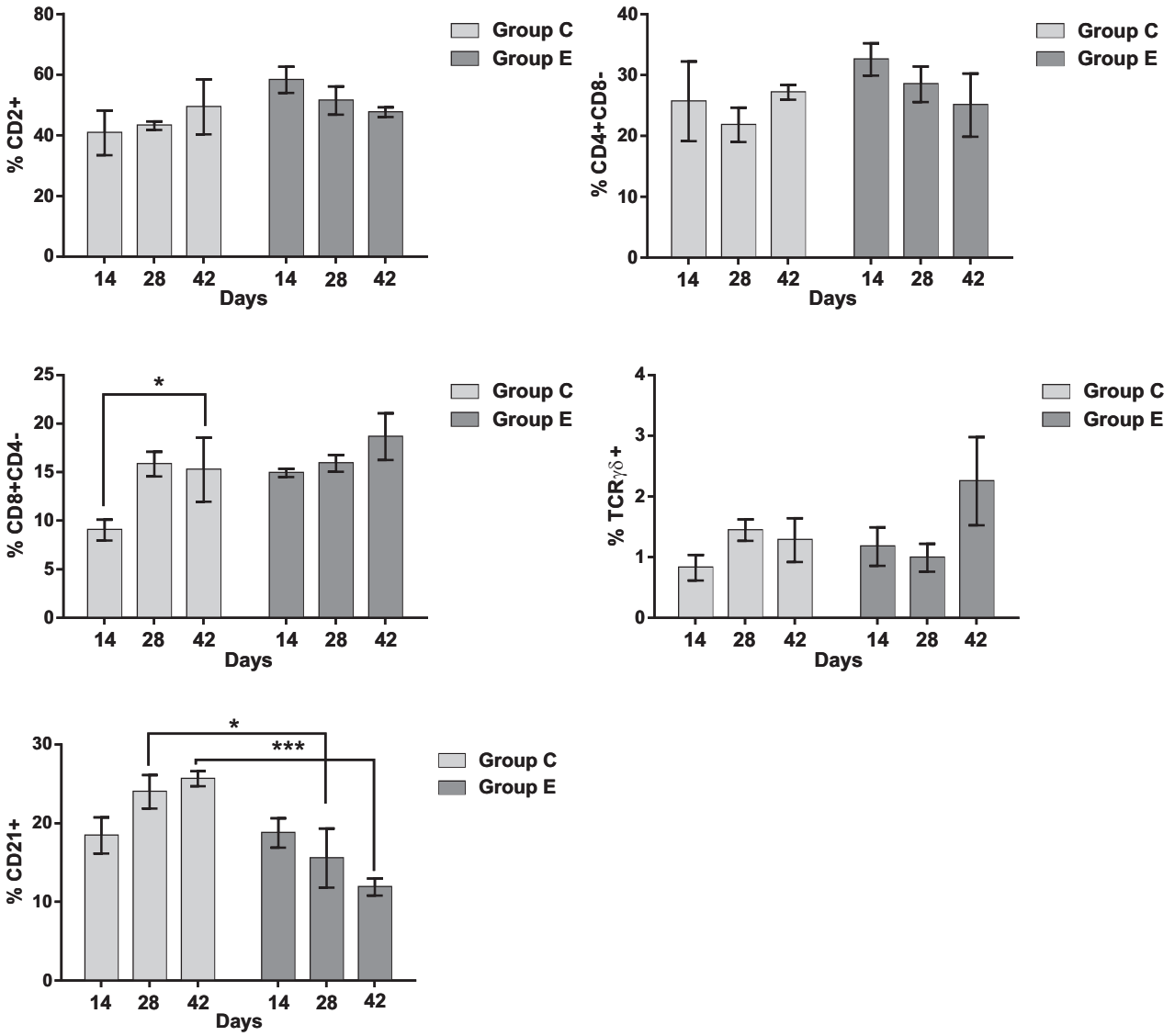

Fig. 1. Cytometric analysis of CD2+, CD4+CD8-, CD8+CD4-, CD5-TCR+ (TCRγδ+) and CD21+ cells isolated from the ileocecal lymph nodes of the control and zearalenone-administered gilts. The graphs present mean values ± SEM (n=5 each). Differences were regarded as significant at: *p<0.05, **p<0.01, *** p<0.001.

ZEN and α-ZEL concentrations

Evaluation of the levels of ZEN and its metabolite α-ZEL in the tissues after treating the pigs with a daily dose of 0.1 mg kg^{-1} feed was conducted using HPLC and is presented in Table 1. No ZEN and α-ZEL were detected in samples taken from the liver and ileocecal lymph nodes of the control group in the case of all tested durations of ZEN exposure. In the livers of animals exposed to ZEN, the concentrations of the parent substance and its metabolite were in the range of 10.61±2.65 ng g^{-1} – 12.22±1.89 ng g^{-1} and 26.81±6.70 ng g^{-1} – 54.20±33.47 ng g^{-1}, respectively; no statistically significant differences were found. Chromatographic analysis of samples taken from ileocecal lymph nodes of group E demonstrated the presence of ZEN ranging from 7.41±1.85 ng g^{-1} to 9.13±5.93 ng g^{-1} and the absence of α-ZEL. In the case of both liver and ileocecal lymph node samples, the highest level of ZEN and α-ZEL was measured on the 28th day of the experiment.

Discussion

Estrogens influence the function of many non-reproductive tissues, especially of the immune system. The influence of estradiol (E2) depends on the concentration, length of administration, type of target cell and the type of receptor present on/in the cell. The

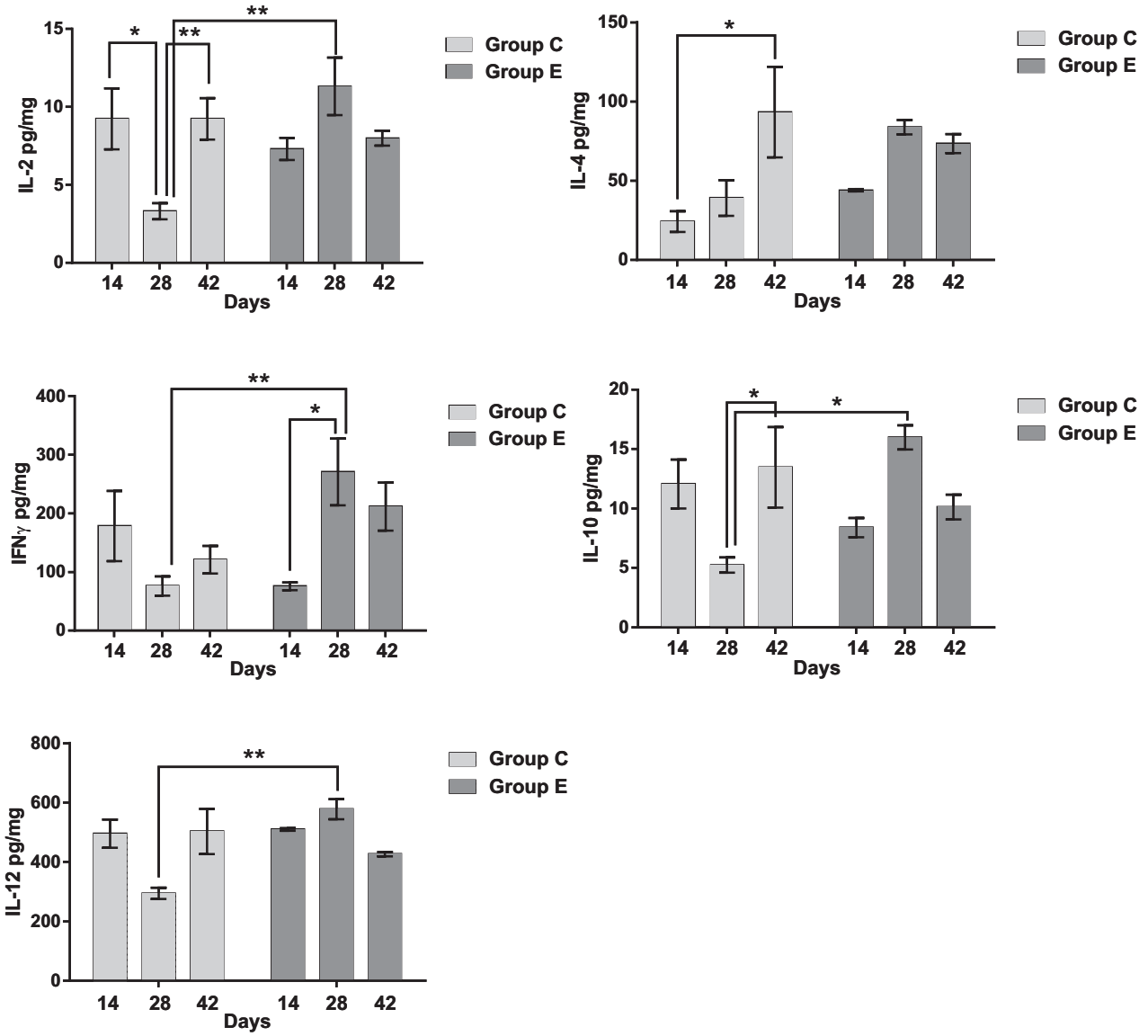

Fig. 2. Comparison of IL-2, IFN-γ, IL-12, IL-4 and IL-10 concentrations in the ileocecal lymph nodes between control (C; n=5) and experimental (E; n=5) group on different days of the experiment. Cytokine concentrations are presented as means value ± SEM. Differences were regarded as significant at: * $p<0.05$, ** $p<0.01$.

Table 1. ZEN and α-ZEL concentrations in the porcine livers and in the ileocecal lymph nodes.

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±2.65	26.81±6.70
	28	n.d.	n.d.	12.22±1.89	54.20±33.47
	42	n.d.	n.d.	12.19±9.12	46.58±32.70
Ileocecal lymph nodes	14	n.d.	n.d.	7.41±1.85	n.d.
	28	n.d.	n.d.	9.13±5.93	n.d.
	42	n.d.	n.d.	5.82±2.51	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 ± SD.

mechanism of E2 function mainly occurs through intracellular estrogen receptors that are expressed in all cells of the immune system (Pernis 2007, Cunningham and Gilkeson 2011). Intracellular expression of ERα isoforms was shown in T and NK cells, as well as in macrophages, while ERβ was present in B lymphocytes and monocytes (Lang 2004). ERα46 seems to be the most common isoform in lymphocytes, though without any significant differences between subpopulations. ERβ isoform is also common; it shows, however, a lower expression level (Pierdominici et al. 2010). As shown by Kuiper et al. (1998), ZEN acts as a full agonist of ERα, and a mixed agonist-antagonist of ERβ. α- and β-zearalenone (α- and β-ZEL) are reduced ZEN metabolites formed during the process of biotransformation in the gastrointestinal tract and in the liver; they show a much higher affinity to ER than ZEN.

Cases of inflammatory bowel diseases (IBD) occurring with increased incidence include principally two disease entities: Crohn's disease and ulcerative colitis (UC). In both cases, the aetiology of the disorder is not fully understood. Nevertheless, an increasing number of data suggests the important role of immunological factors (Maresca and Fantini 2010), including the imbalance between pro- and anti-inflammatory factors and the increased activity of Th1 lymphocytes. The character of these disorders is important due to its effect not only on Th1 lymphocytes, but also Th17, regulatory and suppressor T cells. Looking from the perspective of immune disorders that are characteristic for IBD, a similarity to the effect of the zearalenone mycotoxin in pigs can be observed. The results obtained in the experiment show that ZEN influence on the ileocecal lymph nodes is linked to an inflammatory response, related to an increased pro-inflammatory response connected with Th1 lymphocytes.

We have conducted a study in which animals were administered ZEN doses of 0.1 mg kg^{-1} feed day^{-1} over 42 days; such a dose in feed is considered as acceptable in EU countries and does not lead to observed adverse effects in the reproductive system (NOAEL, 10 μg kg^{-1} m.c. day^{-1}) (EFSA 2011). Reduced metabolites, α- and β-zearalenone (α- and β-ZEL), are formed in the gastrointestinal tract and in the liver as a result of ZEN biotransformation; they show a much higher affinity for ER than the parent compound.

Analysis of selected pro- and anti-inflammatory cytokines (IL-2, IL-12, IFN-γ, IL-4 and IL-10) demonstrated that the course of their concentration changes in the placebo group and showed significant differences as compared with animals treated with ZEN. A similar profile of IL-2, IL-10, IL-12 and IFN-γ secretion in the group of control animals reflects naturally occurring changes in the immune response of animals that have permanent contact with a wide range of food antigens (Obremski 2014). Increased IL-4 production in the group of control sows can be attributed to its secretion, among others, by follicular lymphocytes (T_{FH}) (Seder et al. 1992); their role is to stimulate B lymphocytes of germinal centres in lymph nodes to produce antibodies (Deenick et al. 2011). The remaining cytokines were secreted according to the cascade: antigen presenting cells (APC) – effector cells. It can be presumed that antigen presenting cells, e.g. macrophages, stimulated Th1 lymphocytes to produce IFN-γ via IL-12 and IL-2 secretion (Coffelt et al. 2009), but also IL-10, which can be secreted by regulatory lymphocytes Treg (Pot et al. 2011). A similar profile of IL-2 and IL-10 concentration changes can result from the fact that IL-2 is necessary for T lymphocyte survival, including FOXP3-expressing regulatory T lymphocytes survival (McMurchy et al. 2011). The secretion of IFN-γ by Th1 lymphocytes upon IL-12 produced by APC cells (classical APC activation by, for example, M1 macrophages) is a similar phenomenon (Murray and Wynn 2011).

The situation is different in ileocecal lymph nodes from ZEN intoxicated pigs, as there is a change in the similarity of the dynamics of secreted cytokine concentration levels. In the case of IL-4, there was a dynamics of changes analogical to IFN-γ. In contrast to the control group, IFN-γ in the experimental group showed a slightly different secretion profile than IL-12. This result can indicate that one cell population produced both IL-4 and IFN-γ or that one cytokine caused the secretion of the other in two different populations. However, it is known that IL-4 together with IFN-γ can be secreted by NKT lymphocytes (Morris et al. 2006).

ZEN, a compound exhibiting estrogenic properties, has an influence on the cells of the immune system, such as, among others: dendritic cells, macrophages, T lymphocytes and B lymphocytes (Obremski 2014). In the course of the experiment, the percentage of the following cells was monitored: CD2+ T lymphocytes, CD4+ helper T (Th) cells, CD8+ cytotoxic T (Tc) cells, CD21+ B lymphocytes and CD5-/TCR+ Tγδ cells. The decreasing trend of CD2+ T cell percentage in the group treated with ZEN as compared with a similar trend in the placebo group is strictly related to IL-12 and IFN-γ cytokine secretion; their concentration in the lymph nodes of intoxicated animals was elevated. The examined pro-inflammatory and cytotoxic cytokines probably promoted the response of Th1 cells and M1 macrophages (Martinez and Gordon 2014); this could have contributed to a decreased percentage of other populations. The decrease in CD21+ B cell percentage observed in the

experiment within the group treated with ZEN can result from the activation of NKT cells; NKT cells can also decrease the percentage of Th cells. Moreover, it should be noted that the group of ZEN-treated animals showed an increasing percentage of CD8+ T cells (Tc); Tc cells have well-known cytotoxic properties. This is in agreement with the observations of Hueza et al. (2014); the authors observed an increase in the number of CD3+CD8+ Tc cells and a decrease in the number of CD3+CD4+ helper T (Th) cells and of IgM+CD45R+ B lymphocytes in the thymus of rats administered ZEN. Tc cells stimulated by IFN-γ during the experiment could have induced the apoptosis of various populations of immune cells (Obremski and Poniatowska-Broniek 2015). Similar properties characterize Tγδ lymphocytes, whose level increased especially on the 42nd day of intoxication. The dynamics of changes in this population were smaller in the lymph nodes of the control pigs; this observation additionally draws the attention to the particular impact of ZEN on the immune system.

The changes in ICLN after ZEN intoxication were reminiscent of IBD symptoms developing in GALT and, to a certain extent, of the polarization of immune response occurring in the course of Crohn's disease (Fuss 2008). In this case, we would probably observe a stimulation of cytokine secretion by macrophages with a M1 phenotype; in normal conditions, M1 macrophages develop from monocytes stimulated by LPS derived from natural intestinal microflora (Murray and Wynn 2011). M1 macrophages secrete IL-12, which modulates the immune response towards a cellular response and stimulates the differentiation of Th0 lymphocytes to Th1 and the production of IFN-γ. In Crohn's disease, this process is excessive, and autoimmune reactions are observed. In previous studies conducted on pigs treated with ZEN, the authors observed a higher IL-12 concentration in the ileal absorptive cells as compared with the control group (unpublished data).

The results show that ZEN at NOAEL concentration shifts the immune response in pig ileocecal lymph nodes towards Th1/Th17, probably with a simultaneous activation of M1 macrophages. The secretion of cytokines was, however, not as enhanced as it was previously observed in the ileum (Obremski 2014). At the same time, increased humoral cytokine secretion was observed; this can be explained by a negative feedback loop and a switch in macrophage phenotype from M1 to M2 (Sica et al. 2010, Martinez and Gordon 2014), as well as a switch from Th1 to Th2 response. This was especially clear upon comparison with the control group, which, in contrast to intoxicated animals, was characterized by an increased IL-4 and IL-10 secretion. The significant impact of ZEN is thus emphasized.

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