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Original article

Zearalenone-induced changes in the lymphoid tissue and mucosal nerve fibers in the porcine ileum

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Abstract

This is the first study to examine zearalenone-(ZEN) induced changes in the immune system of the ileum and substance P-(SP-) and vasoactive intestinal peptide-(VIP-) immunoreactive nerve fibers in the mucosa, which participate in the regulation of intestinal functions under physiological conditions and during pathological processes. The aim of this study was also to identify potential relationships between selected immune and neural elements in ileal Peyer's patches in pigs that were and were not exposed to ZEN. The experiment was performed on 10 prepubertal gilts divided into two groups: the experimental group (n=5) where ZEN was administered at 0.1 mg kg⁻¹ feed day⁻¹ for 42 days, and the control group (n=5) which was administered a placebo. The tissue levels of cytokines were determined by enzyme-linked immunosorbent assay which revealed elevated concentrations of IL-12/23 40p and IL-1 β in animals exposed to ZEN. Flow cytometry revealed a lower percentage of CD21+ lymphocytes in pigs exposed to ZEN in comparison with control animals. The tissue levels of neuropeptides were evaluated in the dot blot procedure which demonstrated higher concentrations of VIP and SP in experimental pigs. In experimental animals, numerous VIP-like immunoreactive processes were observed, and SP-immunoreactive nerve fibers formed a very dense network. Our results demonstrate for the first time that ZEN can modify the chemical coding of nerve structures in the gastrointestinal system. Those modifications can be attributed to ZEN's impact on estrogen receptors or its pro-inflammatory properties, and they reflect changes that take place in the nervous system at the transcriptional, translational and metabolic level.

Key words: pig, neuropeptides, cytokines, lymphocytes, Peyer's patches, zearalenone

Introduction

Zearalenone (ZEN) is a non-steroidal estrogenic mycotoxin produced by numerous species of *Fusarium*. *Fusarium graminearum* is the most frequently reported species that produces ZEN on a variety of cereal crops. ZEN has at least five metabolic products: zearalanone, α -zearalanol, β -zearalanol, α -zearalenol and β -zearalenol. α -zearalenol is the major ZEN metabolite in ruminant and non-ruminant animals (Fitzpatrick et al. 1989, Obremski et al. 2003a). ZEN is a full agonist of estrogen receptor α (ESR1) and a partial agonist of estrogen receptor β (ESR2) (Zinedine et al. 2007). ZEN and its metabolites are an important group of substances that impair the hormonal balance and, similarly to 17 β -estradiol (E₂), affect reproduction. ZEN causes hyperestrogenism and physiological disruptions in the reproductive system (Obremski et al. 2003b). ZEN also delivers hepatotoxic, hematotoxic and genotoxic effects (Zinedine et al. 2007). The discussed mycotoxin binds to estrogen receptors in target cells of the immune system (Marin et al. 2011), thus interfering with immune functions. ZEN's toxicity is mediated by PTPC-dependent activation of the mitochondrial pathway of apoptosis, which is regulated by Bcl-2 family members (Bouaziz et al. 2009).

Gut-associated lymphoid tissue (GALT) is involved in tolerance to food antigens and commensal bacteria in the intestines. Peyer's patches (PP) are the primary inductive sites for the immune response, and they are the main target of food pathogens such as *Salmonella*, *Shigella* and *Yersinia* (Vazquez-Torres and Fang 2000). Evidences from numerous sources demonstrate the presence of morphological and functional relationships between the immune and nervous systems. The enteric nervous system (ENS) seems to modulate resistance mechanisms involved in the protection of mucous membranes and the entire body. Intestinal neurons innervate intestinal mucosa, including GALT. The innervation of PP includes peptidergic fibers that contain neuropeptides such as vasoactive intestinal peptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP) (Vulchanova et al. 2007). Immune cells express neuropeptide receptors, and their activation can influence antigen presentation, cytokine production and lymphocyte proliferation. Neuropeptides are a potential signaling interface between intestinal nerves and immune cells in PP (Kulkarni-Narla et al. 1999).

SP modulates inflammatory processes and lymphocyte proliferation, whereas sympathetic and peptidergic innervation participates in the development and progression of autoimmune diseases, allergies and inflammatory pain. Nanomolar concentrations of

SP activate NF- κ B, an important regulator of cytokine expression (Marriott et al. 2000). SP stimulates immune cells to produce inflammatory cytokines, including IL-1, IL-6, IL-12 and tumor necrosis factor alpha (TNF- α) (Laurenzi et al. 1990, Kincy-Cain and Bost, 1997). SP and its receptor NK-1R are expressed on microglial cells isolated from the brain of human fetuses, stem cells, human mononuclear peripheral blood phagocytes and lymphocytes. The number of SP receptors in blood vessels increases in peripheral inflammatory sites (Mantyh et al. 1988).

VIP is an anti-inflammatory neuropeptide that inhibits the activity of human peripheral blood lymphocytes (Sirianni et al. 1992) and the activity of the spleen, mesenteric lymph nodes and peripheral blood mononuclear cells in mice (Yiangou et al. 1990). VIP is released by nerve endings and immune cells, in particular Th2 lymphocytes. It regulates the expression of co-stimulatory molecules such as B7-2, thus driving the Th1/Th2 balance toward the Th2-mediated response (Delgado and Ganea 2001). VIP exerts anti-inflammatory effects on cells involved in both innate and acquired immunity by inhibiting the production and release of inflammatory cytokines and chemokines (Arciszewski et al. 2008).

ZEN's influence on the enteric nervous system has attracted scientists' interest only recently. The ENS may undergo changes under the influence of various physiological and pathological stimuli such as aging, diet, inflammatory processes and other diseases (Gonkowski et al. 2003, Vasina et al. 2006, Gonkowski and Całka 2012). Lakomy et al. (2009) and Kaleczyc et al. (2010) demonstrated that cytokine expression in Peyer's patches and the correlations between the ENS and the immune system of the digestive tract can change during selected pathological processes.

The aim of the present study was to investigate, for the first time, ZEN-induced changes in the immune system of the ileum and in SP- and VIP-immunoreactive mucosal nerve fibers which participate in the regulation of intestinal functions under physiological conditions and during pathological processes.

This study was also set out to identify potential relationships between immune and neural elements in ileal Peyer's patches in pigs that were and were not exposed to zearalenone.

Materials and Methods

Animals and experimental procedure

The study was performed on 10 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=5) of healthy animals (group C) and an experimental group (n=5) of healthy pigs administered ZEN (Sigma-Aldrich, Germany) at 0.1 mg kg⁻¹ feed day⁻¹ (group Z). Analytical samples of ZEN were administered daily *per os* in gelatin capsules before the morning feeding.

Biological samples

On experimental day 42, all pigs were euthanized and sections of the ileum were sampled for analyses. The excised segment of the ileum was opened, and Peyer's patches were identified. The mucosa was scraped and minced. Peyer's patch samples were stored at -80°C until cytokine analyses.

For the enzyme linked immunosorbent assay (ELISA) and the Dot Blot 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 Na₂HPO₄, 1.5 mM KH₂PO₄), 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 8600 g for 1 hour in an Eppendorf 5804R centrifuge and supernatant samples were stored at -80°C until analysis.

For the immunofluorescence assay, sections of the ileum (approximately 1 cm in length) were immediately fixed by immersion in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min, rinsed several times with phosphate buffer, transferred to 18% sucrose solution and stored at 4°C until sectioning.

Lymphocyte subpopulation study

The excised segment of the ileum was opened, and Peyer's patches were identified. The mucosa was 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. The number of lymphocytes was established in a hemocytometer.

The percentages of lymphocyte subpopulations obtained from Peyer's patches were determined with the use of mouse monoclonal antibodies against porcine CD2, CD4, CD8, CD21 (CD2, IgG2a, cat. no. MSA4; CD4, IgG2b, cat. no. 74-12-4; CD8, IgG2a cat no. 76-2-11; CD21, IgG1, cat no. BB6-11C9, all from VRMD, 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. Peyer's patch leukocytes for cytometry were obtained in accordance with the procedure described by Kaleczyc et al. (2010). The samples were analyzed in a flow cytometer (FACScalibur, Becton Dickinson, USA), and the results were processed in the Cell Quest™ 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

IL-12/IL-23 p40 (R&D, USA) and IL-1 β (R&D, USA) levels were determined with the use of ELISA kits in accordance with the manufacturer's instructions. ELISA microplates (96-well) were coated with antibodies specific for porcine 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 h at 37°C. Biotinylated antibodies specific for porcine cytokines were added and incubated for 2 h at 37°C. HRP-conjugated streptavidin solution was added, and the microplates were incubated for 20 min. In each stage, microplates were rinsed in PBS with 0.05% Tween 20 (Sigma-Aldrich, USA). The specimens were stained with o-Phenylenediamine (OPD) (Sigma Aldrich, USA) and hydrogen peroxide (Avantor Performance Materials Poland S.A., Poland). The enzymatic reaction was stopped with 2 M HCl (Avantor Performance Materials Poland S.A., Poland). Absorbance was measured in a spectrophotometer plate reader (TecanInfinite 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 pg mg⁻¹ protein. The values of intra-assay

and inter-assay coefficients of variation (CV%) for IL-12/IL-23 p40 and IL-1 β were determined at 4.58, 5.42, 7.11 and 6.7, respectively.

Dot Blot procedure

To evaluate differential content of neuropeptides VIP and SP in ileal Peyer's patches Dot Blot method was not performed to compare the results to a classic calibration curve, but by antigen titer. Homogenates of Peyer's patches from control and experimental pigs were diluted in PBS to achieve identical protein concentrations in all samples (2 mg ml⁻¹). The protein concentrations in the extract were determined by the modified Bradford method (Bradford 1976). Successive serial dilutions were performed (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280), and 2 μ l of the resulting solution was transferred to the PSQ Immobilon membrane (Merck-Millipore, Germany) activated with methanol (Avantor Performance Materials Poland S.A., Poland). The membrane was blocked with a solution of powdered milk (16 h, 4°C), incubated in a solution of rabbit anti-VIP polyclonal antibodies (Biomol, Germany, cat. No. VA1285, working dilution 1:1000) or rabbit anti-SP polyclonal antibodies (Abcam, USA, cat. No. ab1123, working dilution 1:1000) in 2% BSA solution (16 h, 4°C), and rinsed (3 x 15 min) in PBS with 0.1% Tween 20 (Sigma-Aldrich, USA). The signal was detected using the Vectastain kit (Vector, USA) according to the manufacturer's instructions. The membrane was incubated in DAB solution (Sigma-Aldrich, USA) and dried to visualize the signal. The quantitative analysis of the results proceeded according to the rule: higher concentration VIP or SP, a longer series of dilutions of the sample in the form of spots giving a visible color signal, the higher the titer of the analyzed antigens VIP or SP (determination of the end point of this series). The dilutions were expressed as a decimal fraction and calculated the negative logarithm (similar to the pH scale). The numerical values as the negative logarithm of the dilution [-Log (dilution)] were statistically analyzed and presented graphically.

Immunofluorescence procedure

Ileal sections stored in sucrose solution were frozen and cut in frontal or sagittal planes in the Microm HM 560 cryostat (Carl Zeiss, Germany) to produce slices with a thickness of 10 μ m. The slices

were mounted on gelatinized glass slides and processed by the routine single labeling immunofluorescence method described by Gonkowski et al. (2009). They were air-dried at room temperature for 45 min, rinsed in 0.1 M phosphate-buffered saline (PBS; pH 7.4; 3x10 min) and incubated in a blocking buffer containing: 10% normal goat serum (MP Biomedicals, USA) in 0.1 M PBS, 0.1% donkey serum (Abcam, UK), 1% Triton X-100 (Sigma-Aldrich, USA), 0.05% Thimerosal (Sigma-Aldrich, USA) and 0.01% NaN₃ for 1 h at room temperature to reduce non-specific background staining. The slices were rinsed in PBS (3x10 min) and incubated overnight at room temperature with anti-SP (monoclonal rat anti-serum, Biogenesis LTD, UK, cat. No. 8450-0505, working dilution 1:500) or anti-VIP (polyclonal rabbit antiserum, Biomol, Germany, cat. No. VA1285, working dilution 1:6000) primary antibodies. On the following day, the slices were rinsed (PBS, 3/10 min) and incubated for 2 h with secondary antibodies conjugated with Alexa fluor 488 (donkey anti-rat, cat No. A21208 and donkey anti-rabbit, cat No. A21206, Invitrogen, working dilution 1:1000). Fluorescence samples were viewed under the Olympus BX51 microscope equipped with appropriate fluorescence filter sets. Images were captured with a monochrome camera (Olympus XM 10) and analyzed in Cell F software (Olympus, Japan). Standard controls, including pre-absorption, omission and replacement of primary antibodies by non-immune serum, were performed to test the antibody and the specificity of the method. In pre-absorption tests, ileal samples were incubated with a working solution of primary antibodies that was pre-absorbed for 18 h at 37°C with 20 μ g of SP (Sigma-Aldrich, USA, cat. No. S6883) or VIP (Sigma-Aldrich, USA, cat. No. V6130).

Statistical Analysis

The results of ELISA and Dot Blot procedures (expressed as means \pm SEM) were analyzed by Student's t-test in GraphPad PRISM 5.0 software. Differences were considered significant at $p < 0.05$.

The density of mucosal nerve fibers immunoreactive to SP and VIP was performed by a semi-quantitative evaluation method described by Gonkowski et al. (2013). Nerve profiles were evaluated independently by two investigators in 4 sections per animal (5 fields per section) on an arbitrary scale, where: (-) = absence of fibers; (+) = single fibers; (++) = rare nervous fibers; (+++) = numerous fibers; (++++) = very dense nervous processes.

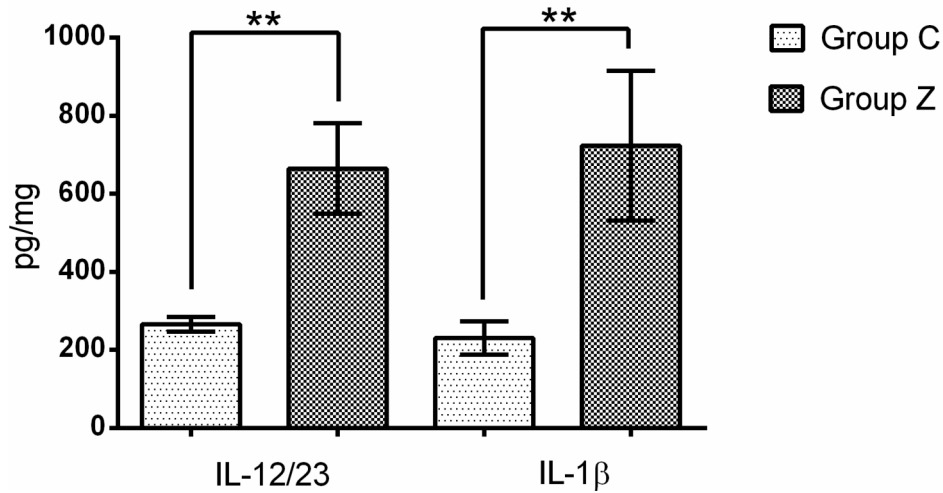


Fig. 1. Tissue concentrations of IL-12/23 40p and IL-1β in ileal Peyer's patches of control and experimental gilts. The results are presented as means ± SEM. Statistical significance was set at $p < 0.05$ (**).

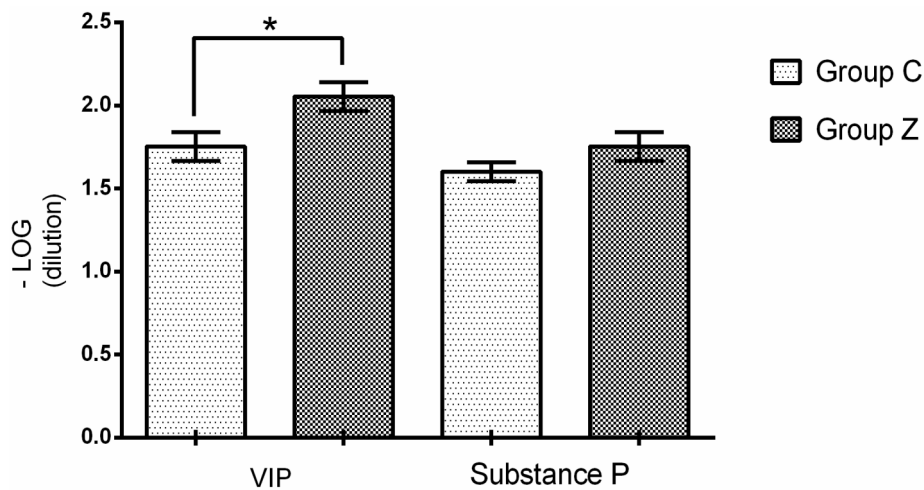


Fig. 2. Tissue concentrations of neuropeptides in ileal Peyer's patches of control and experimental gilts. The results are presented as means ± SEM. Statistical significance was set at $p < 0.05$ (*).

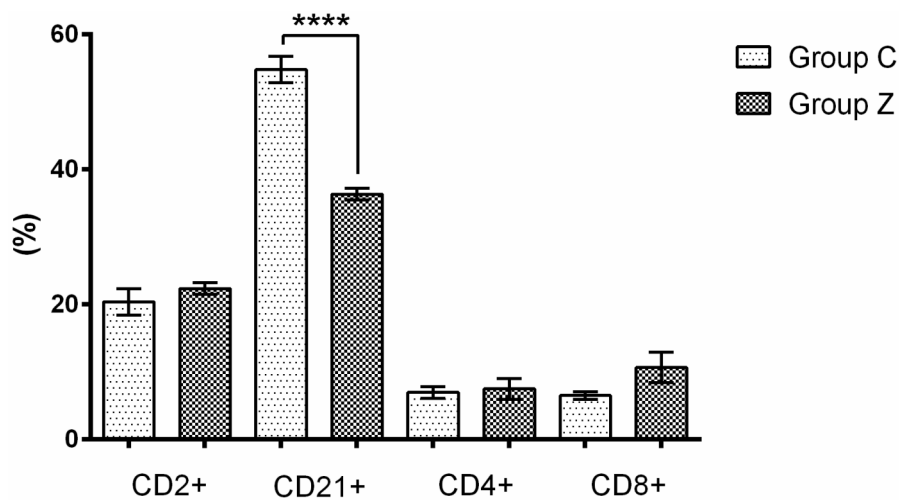


Fig. 3. Lymphocyte subsets in ileal Peyer's patches of control and experimental gilts. The results are presented as means ± SEM. Statistical significance was set at $p < 0.0001$ (****).

Results

ZEN effects on the percentage of lymphocytes

The results of the study indicate that the ZEN can modify the percentages of T and B lymphocyte subpopulations in Peyer's patches. The effects of chronic exposure to low ZEN doses on the percentages of lymphocyte subpopulations isolated from porcine ileal Peyer's patches were determined by immunophenotyping and cytometric analysis. A significant decrease ($p < 0.0001$) in the percentage of CD21+ cells was observed in ileal Peyer's patches of experimental animals (36.31 ± 0.86) in comparison with control (54.76 ± 1.96) (Fig. 3). The percentages of CD2+, CD4+ and CD8+ lymphocytes were higher in experimental (22.33 ± 0.88 , 7.46 ± 1.55 , 10.64 ± 2.22 , respectively) than in control pigs (20.34 ± 1.93 , 6.92 ± 0.85 , 6.48 ± 0.57 , respectively), but the noted differences were not statistically significant (Fig. 3).

ZEN effects on IL-12 and IL-1 β concentrations

Cytokines secreted by ileal Peyer's patches represent the actual immune response profile of control and experimental group animals. Cytokines produced were evaluated by the ELISA test, and the concentrations of cytokines isolated from Peyer's patches were expressed in pg mg^{-1} protein according to the Bradford method (Fig. 1). A significant increase ($p < 0.01$) in IL-12 and IL-1 β levels was noted in pigs administered ZEN (723.10 ± 191.5 pg mg^{-1} , 664.80 ± 115.90 pg mg^{-1} , respectively) for 42 days in comparison with control (265.80 ± 19.07 pg mg^{-1} , 230.60 ± 43.02 pg mg^{-1} , respectively). IL-12 and IL-1 β concentrations in the experimental group were more than three-fold higher than in the control group.

ZEN effects on SP and VP concentrations

The differential content of neuropeptides VIP and/or SP was observed in the ileum under physiological conditions and after the administration of ZEN (Fig. 2). A significant increase ($p < 0.05$) in VIP and not statistically significant increase in SP (1.75 ± 0.09 , 2.05 ± 0.09 and 1.53 ± 0.07 , 1.75 ± 0.09 , respectively) concentrations (-Log dilution) were observed in pigs administered ZEN for 7 weeks in comparison with control group animals.

ZEN effects on the distribution of nerve fibers immunostained for VIP and SP

Mucosal nerve fibers immunoreactive to VIP and/or SP were observed in the ileum under physiological conditions and after the administration of ZEN. In control animals, VIP-like immunoreactive nerve processes were rare (++) (Fig. 4A), whereas fibers immunoreactive to SP were more numerous (+++) (Fig. 4B). In control group animals, VIP- and/or SP-positive nerves were delicate and thin. The administration of ZEN induced pronounced changes in the density of mucosal fibers immunoreactive to both VIP and SP. In experimental animals, both VIP and/or SP-like immunoreactive processes were abundant and formed a very dense network (++++) (Fig. 4C, Fig 4D, respectively). Moreover, the appearance of SP-positive fibers also changed under exposure to ZEN. Those fibers formed large nerve bundles, they were thicker and more visible than under physiological conditions.

Discussion

The interactions between the immune system and the nervous systems are characterized by highly dynamic changes. The immune system remains under powerful psychoneural influences (Weihe et al. 1991), whereas in humans, the intestinal immune system can affect the development of various disorders that are expressed by the nervous system, including depression (higher IL-1 β levels in the blood), schizophrenia and autism, and are characterized by elevated levels of both pro-inflammatory and anti-inflammatory cytokines.

Neuropeptides and cytokines participate in neuro-immunoregulation by interacting with specific receptors on immune and neuroendocrine cells. T and B cells possess receptors for various neurotransmitters (Levite et al. 1998) and are capable of responding to those substances. Neurotransmitters induce proliferative and/or antiproliferative responses in mucosal lymphocytes and intestinal epithelial cells, thus influencing cytokine and immunoglobulin synthesis.

This is the first ever study to demonstrate significant differences in the tissue concentrations of SP, VIP, IL-12 and IL-1 β in ileal Peyer's patches of healthy pigs and pigs exposed to ZEN. The tissue levels of both neuropeptides were higher than in animals administered ZEN than in control pigs. The distribution pattern of VIP- and SP-immunoreactive nerve fibers in the ileal mucosa of healthy pigs and pigs exposed to ZEN point to the stimulating role of ZEN. The above implies that SP and VIP could play

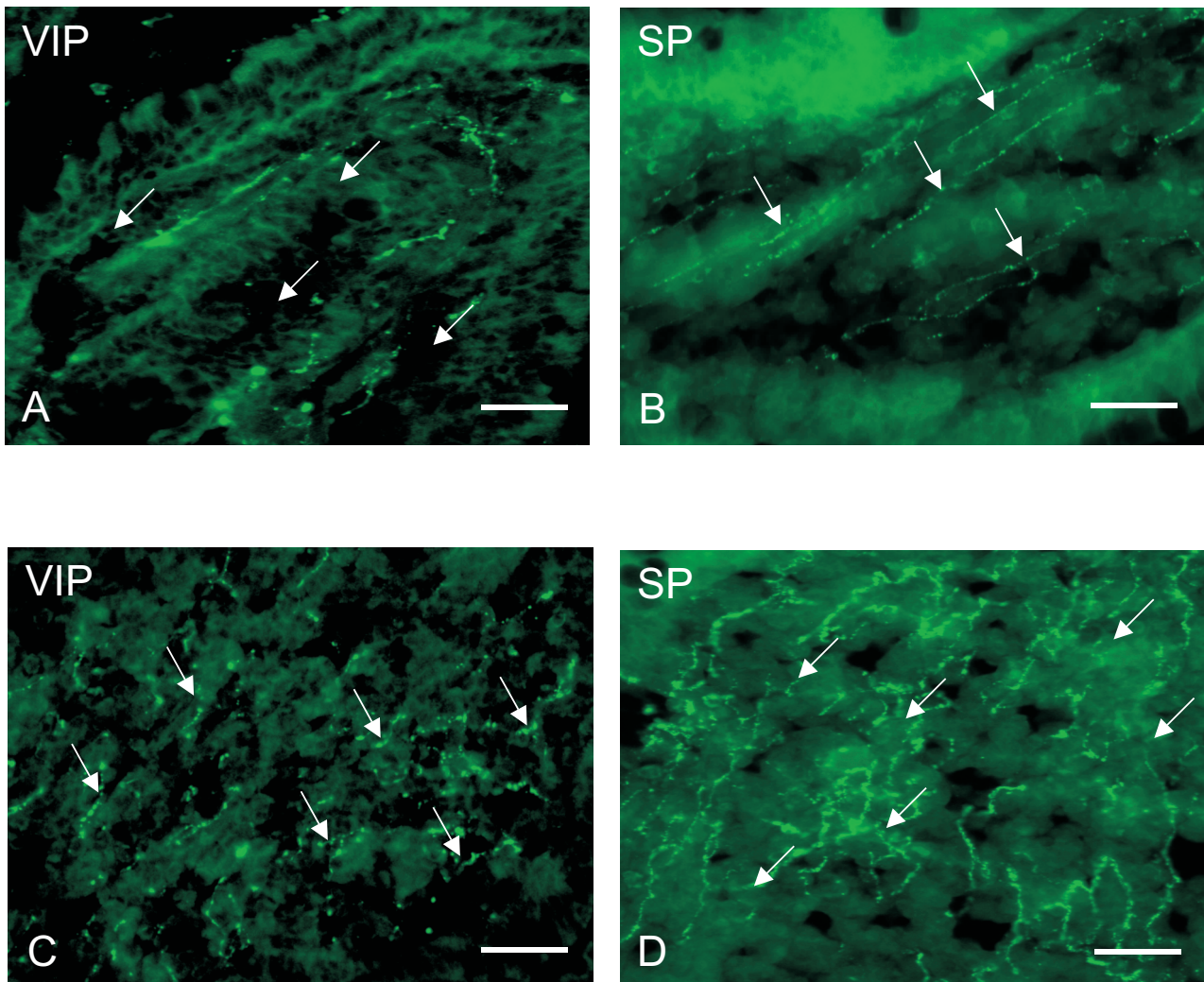


Fig. 4. Distribution pattern of nerve fibers (arrows) immunostained for VIP and SP in the mucosal layer of the porcine ileum under physiological conditions (A, B, respectively) and after exposure to ZEN (C, D, respectively). Scale bar = 40 μ m.

an important role in local neuroendocrine systems during ZEN-induced inflammations of porcine ileal mucosa.

ZEN is a mycoestrogen which, in addition to its widely established hyperestrogenic activity, is capable of modulating the immune response towards the anti-inflammatory state (Obremski 2014). The above is manifested by increased secretion of IL-2 and IFN- γ (Marin et al. 2013) and an intensified cytotoxic response characterized by a drop in the population of B cells and an increase in the population of cytotoxic CD8+ T cells. In the present experiment, significant differences in lymphocyte subpopulations were observed only in CD21+ B cells. The above lymphocytes produce antibodies for fighting infections, and the observed significant drop in their subpopulation is surprising. The concentrations of IL-1 β and IL-12/23 40p in lymphoid tissue (Peyer's patches) were higher in pigs exposed to ZEN than in control animals. IL-1 β

and IL-12/23 40p are secreted during activation by antigens of monocytes and macrophages belonging to phenotype M1 IL-10lo, IL-12hi and IL-23hi (Mantovani et al. 2007). Identical cytokine secretion patterns are observed in Crohn's disease where macrophages in intestinal lymphoid tissue increase their secretion of IL-12/23 40p (Strober et al. 2010). For this reason, the response of Th1/Th17 lymphocytes should dominate over the Th2 response and the pro-inflammatory response. A similar relationship is observed in SP whose concentrations were higher in Peyer's patches of animals exposed to ZEN. SP is secreted by nerve endings, including in intestinal epithelium and Peyer's patches. In the current study, the production of IL-1 β and IL-12 was increased, which confirms the pro-inflammatory effects of SP (Laurenzi et al. 1990). According to the literature, VIP delivers effects opposite to SP. Through VIP-R1 and VIP-R2 receptors on lymphocytes (Delgado et al. 1996), VIP contributes to

increased levels of anti-inflammatory cytokines (IL-10, IL-4) associated with Th2 and regulatory lymphocytes. It also decreases the concentrations of IL-6, IL-12 and IFN- γ . In our study, however, cytokine levels did not decrease in response to higher levels of VIP expression. The increase in VIP levels could be attributed to negative feedback which downregulates pro-inflammatory and cytotoxic responses. A strong pro-inflammatory/cytotoxic response is confirmed not only by an increase in IL-12/23 40p concentrations, but also by a decrease in the subpopulation of CD21+ B lymphocytes. IL-2 and IL-4 are required for the proliferation and survival of CD21+ cells. High concentrations of IL-12 lead to the secretion of IFN- γ with strong anti-proliferative effects that significantly contribute to the differentiation of B cells. Higher IFN- γ levels and activation of cytotoxic lymphocytes provide indirect evidence for the cytotoxic activity of IL-12. The populations of Th1 and Th17 lymphocytes probably increased in the CD4+ T cell pool. The above observation is based on the characteristic activity of IL-12 and IL-23 which share a common p40 subunit. According to Eisenberg et al. (1990), IL-1 β stimulates differentiation of human virgin CD4+ Th cells, including Th17 cells (Hus et al. 2010). A considerable increase in the subpopulation of CD8+ T lymphocytes was observed in experimental animals relatively to control. CD8+ T lymphocytes stimulated by IFN- γ can „kill” B cells by interacting with FAS-FASL (CD95-CD178).

The results of this study and literature data indicate that pro-inflammatory and cytotoxic responses are intensified when the intestinal immune system is exposed to low concentrations of ZEN, probably due to the activity of M1 macrophages that produce IL-12, IL-23 and IL-1 β (Van Ginderachter et al. 2006). The above generates an immune response associated with Th1/Th17 lymphocytes which synthesize IFN- γ and stimulate cytotoxic CD8+ T cells that can significantly reduce the percentage of CD21+ B cells. The described responses are also stimulated by the enteric nervous system by increasing SP concentrations in intestinal epithelium and lymphoid tissue. The described mechanisms also explain the increase in the levels of VIP which can stimulate IL-10 synthesis (possible protective effect) to inhibit IFN- γ and downregulate the immune response. Our results demonstrate for the first time that ZEN can modify the chemical coding of nerve structures in the digestive tract. Those modifications can be attributed to ZEN's influence on estrogen receptors (Zinedine et al. 2007) or its pro-inflammatory properties (Marin et al. 2013), and they can reflect changes that take place in the nervous system at the transcriptional, translational and metabolic level. ZEN's impact on nerve structures

in the digestive tract remains unknown and requires further investigations.

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