Evaluation of the proliferative activity of immunocompetent cells in the jejunal and iliac lymph nodes of prepubertal female wild boars diagnosed with mixed mycotoxicosis

Łukasz Zielonka, Ewa Jakimiuk, Kazimierz Obremski, Magdalena Gajęcka, Michał Dąbrowski, Maciej Gajęcki

Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Poland
lukaszz@uwm.edu.pl

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Abstract

The study evaluated the proliferative activity of immunocompetent cells in the jejunal and iliac lymph nodes of prepubertal female wild boars exposed to deoxynivalenol and zearalenone in naturally contaminated feed. The evaluation was performed with the use of the MTT assay and 2 mitogens: lipopolysaccharide (LPS) and concanavalin A. Intensified proliferative processes in T and B lymphocytes were revealed. The mitogenic activity of LPS was more expressed in the lymphocytes of both iliac and jejunal lymph nodes in comparison with the control group. Proliferative activity was higher in iliac lymph nodes than in jejunal lymph nodes. A reverse trend was observed in the percentage of live cells, which was higher in jejunal lymph nodes during the evaluation of lymphocyte proliferation.

Keywords: prepubertal female wild boars, mycotoxins, lymphocytes, jejunum, ileum.

Introduction

Mycotoxins produced by fungi disrupt various physiological processes in animals (8, 13). Fusarium fungi are widespread in feeds. In nature, Fusarium species most commonly produce trace amounts of deoxynivalenol (DON) and zearalenone (ZEN). Long-term exposure to mycotoxin doses below the no observable adverse effect level (NOAEL) causes pathological changes in accordance with the low-dose hypothesis (27) and the principles of hormesis (5). The exposure to endocrine disrupting toxins (EDs) (27), including ZEN, at dose levels below NOAEL may exert stimulating/adaptive effects on prepubertal female wild boars (PFWBs) (17, 9). Mixed mycotoxicoses generally lead to chronic subclinical infections with weakly manifested and non-specific symptoms, which significantly impair diagnosis. It remains unknown whether the above mycotoxins exert stimulating/adaptive or immunosuppressive effects on lymphocyte populations in lymph nodes in different segments of the small intestine.

Chemical compounds, including mycotoxins, disrupt physiological processes in cells, and the resulting changes can be observed with the use of various measurement techniques. The selection of the optimal analytical method is largely determined by the type of compound being analysed and its possible interactions with living organisms and organ tissues. Proliferative activity is evaluated through direct or indirect measurements of changes in cells exposed to a specific compound, such as mycotoxins produced by Fusarium fungi. The MTT assay is the most popular method for evaluating the proliferative activity of immunocompetent cells. Its results are verified with the use of mitogens specific for B and T lymphocytes. The assay is recommended as a reference test by international standard setting organisations (2).

Individual and mixed mycotoxicoses induced by DON and/or ZEN reduce the number of mucus-
secrering cells and decrease glyocalyx production in pigs exposed to trace amounts of these mycotoxins (22). Mycotoxoses caused by higher doses of ZEN alone produce completely different effects by increasing the activity of goblet cells and mucinogen granules (21). Other studies (20) have demonstrated that certain mycotoxins also influence intestinal mucosa and contribute to inflammations and uncontrolled proliferation of cells in the wall of the gastrointestinal tract. The mechanisms responsible for mycotoxins’ influence on mucus secretion have not been described, although some studies have demonstrated that protein synthesis is inhibited by trichothecenes and oestrogenic hormones, which implies that ZEN could be included in that group of compounds (20).

The influence of mycotoxins on the local intestinal immune response has not been explained yet, but research findings imply that DON and, possibly, ZEN induce immunosuppressive effects. DON and ZEN could participate in immunosuppressive processes in intestinal inflammations and hyperadditive (synergistic) interactions (4). Selected mycotoxins exert direct or indirect proinflammatory effects, and thus exacerbate the existing inflammations (19). They can provoke inflammations indirectly by modifying intestinal permeability and facilitating the transport of antigens from the intestinal lumen, or directly by stimulating the secretion of proinflammatory cytokines by the intestinal epithelium (19), or by intensifying lymphocyte proliferation in intestinal lymph nodes in most animal species, including pigs.

In view of the above, the objective of this study was to evaluate the proliferative activity of immunocompetent cells collected from the jejunal and iliac lymph nodes of PFWBs exposed to DON and ZEN in naturally contaminated feed, and to determine the immunomodulating effects of the analysed mycotoxins on the studied animals.

Material and Methods

Experimental animals. The experiment was conducted on 4 PFWBs, with body weight of 30-35 kg, harvested in boar hunting grounds in the area of Goldap and kept in an enclosure during an adaptation period of 7 d.

The animals were randomly divided into 2 groups: PFWB C (not exposed to mycotoxins – PFWB 1 and PFWB 2) and PFWB E (exposed to mycotoxins – PFWB E1 and PFWB E2). Each group was placed in a separate pen. Feed naturally contaminated with mycotoxins was administered to group PFWB E for 7 d.

PFWBs were kept in cages with ad libitum access to water and were fed diets tested for the presence of the following mycotoxins: aflatoxin, ochratoxin, ZEN, α-zearalenol, β-zearalenol, and DON. Mycotoxin concentrations in the diet were evaluated with the use of common separation techniques involving immunological affinity columns and high performance liquid chromatography (HPLC) (Hewlett Packard, type 1050 and 1100) with fluorescent and/or UV detection techniques. DON and ZEN concentrations in plant material were determined with the use of analytical methods validated for measurement precision and accuracy, linearity, recovery (105.74%, 98.18%), limit of detection (14 μg/kg, 2 μg/kg), and limit of quantification (20 μg/kg, 5 μg/kg).

Cell culture and reagents. Intraoperative samples of jejunal and iliac lymph nodes were placed in neutral PBS (Phosphate Buffered Saline, pH 7.4; Sigma Aldrich) containing 1% AAS (v/v, Antibiotic Antimycotic Solution, Sigma-Aldrich). The fibrous capsule was incised; tissue was sliced with a scalpel and passed through steel wire mesh (100 μm) and nylon mesh (70 μm) in PBS. The resulting cell suspensions were rinsed with PBS and centrifuged (1200 rpm, 5 min). The number of cells was counted with a haemocytometer, and cell viability was determined by staining with 0.4% trypan blue (Sigma Aldrich). Cells with viability of >85% were suspended at the concentration of 3.5 × 10⁴/mL in RPMI 1640 medium (Sigma Aldrich) containing 10% FBS (Foetal Bovine Serum, Sigma Aldrich), 1% AAS, and nonspecific mitogens for T and B lymphocytes – concanavalin A (Con A, Sigma Aldrich) at the concentration of 10 μg/mL, and lipopolysaccharide (LPS, Salmonella enterica serotype Enteritidis, Sigma Aldrich) at the concentration of 10 μg/mL, respectively.

Proliferative activity. Proliferative activity was evaluated in the Laboratory of Alternative Research Methods of the Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland, in 96-well plates (Nunc) filled with 100 μL of the cell suspension per well. The cells were cultured for 48 h in a controlled atmosphere (37°C, 5% CO₂). The proliferative response of immunocompetent cells from the jejunal and iliac lymph nodes of all PFWBs was evaluated with the use of the TOX-1 kit (In Vitro Toxicology Assay Kit MTT Based, Sigma-Aldrich, Germany) according to the manufacturer’s instructions.

In the MTT assay, mitochondrial dehydrogenases, which are active only in live cells, cleave water-soluble yellow tetrazolium salt to form blue formazan crystals that are accumulated inside the cell. After cell lysis (commercial solvent) and substrate dissolution, formazan concentrations were determined in the EIA reader (Multiscan, Labsystems, Finland) at a wavelength of 570/620 nm. The optical density (OD) of staining is proportional to the number of proliferating cells. Each experiment was conducted in 2 replications, and each replication was carried out in 8 series for each treatment. The percentage of viable cells (PVC) was calculated using the formula below:
Viability (%) = 100 x Mean OD of sample exposed to mycotoxins
Mean OD of untreated sample

Statistical Analysis. The results were analysed statistically using Statistica application. The differences between groups of PFWBs were determined by ANOVA. The equality of group variances was tested by the Brown-Forsythe test. When significant differences between groups (P < 0.01 – highly significant differences, 0.01 < P < 0.05 – significant differences, P > 0.05 – no difference) were revealed by ANOVA, Tukey’s test was used to determine the differences between particular groups.

Results

The concentration values of mycotoxins in control feed were below the sensitivity of the method. In experimental feed (complete feed with a high proportion of maize naturally contaminated with mycotoxins), DON concentrations were determined at 1008 μg/kg feed, and ZEN concentrations – at 26 μg/kg feed.

The proliferative activity of immunocompetent cells from the jejunal and iliac lymph nodes of PFWB C and PFWB E (combined exposure to DON and ZEN present in naturally contaminated feed in group E) was evaluated based on OD values determined in the MTT assay. Mean OD values (%) and statistical differences at P ≤ 0.01 are presented in Fig. 1.

Highly significant differences (P ≤ 0.01) in the proliferative activity of immunocompetent cells from jejunal lymph nodes (Fig. 1A) after stimulation with LPS were noted between PFWB E1 vs. PFWB C and PFWB E2 and between PFWB E2 and PFWB C, and after stimulation with Con A – between PFWB E2 vs. PFWB C and PFWB E1. The statistical analysis of the proliferative activity of immunocompetent cells from jejunal lymph nodes, suspended in the analysed mitogens, did not reveal significant differences between the groups (Fig. 1B).

Highly significant differences (P ≤ 0.01) in the proliferative activity of immunocompetent cells from iliac lymph nodes (Fig. 1C) after stimulation with LPS were noted between PFWB C vs. PFWB E1 and PFWB E2 and between PFWB E1 and PFWB E2, and after stimulation with Con A – between PFWB C vs. PFWB E1 and PFWB E2 and between PFWB E1 and PFWB E2. Highly significant differences (P ≤ 0.01) in the activity of immunocompetent cells from iliac lymph nodes stimulated with LPS and Con A were reported in PFWB C, PFWB E1 and PFWB E2 (Fig. 1D).

The percentage of viable cells (PVC) in jejunal lymph nodes (Fig. 1E) stimulated with LPS and Con A was determined at 102.925% and 97.975%, respectively, in PFWB E. The PVC values in iliac lymph nodes (Fig. 1E) stimulated with LPS and Con A reached 96.720% and 87.165%, respectively, in PFWB E.

PVC values were not evenly distributed. The PVC median for both mitogens (LPS and Con A) in jejunal lymph nodes (Fig. 1E) was determined at 102.925% and 97.975%, respectively. For LPS, the lower quartile of PVC values was determined at 101.360%, and the upper quartile – at 104.490% in PFWB E. For Con A, the lower quartile of PVC values was determined at 87.210%, and the upper quartile – at 108.740% in PFWB E. The median for both mitogens in PFWB E reached 102.925% and 97.975%, respectively. The PVC values for the analysed mitogens were classified into 4 activity levels based on median values for the lower and upper quartile: A – very low PVC (PVC < 50%), B – low PVC (50% ≤ PVC < 75%), C – high PVC (75% ≤ PVC < 100%), D – very high PVC (PVC ≥ 100%). Level A (very low activity) was observed only in PFWB C for both mitogens. Level B (low activity) was noted only in PFWB C for LPS. Level C (high activity) was reported in PFWB E for Con A. Level D (very high activity) was noted for both mitogens, where the highest values for LPS were found in PFWB E, and the highest values for Con A – in PFWB C and PFWB E. In PFWB E, the PVC values for both mitogens were characterised by a growing trend in comparison with PFWB C.

In PFWB E, the median of PVC values for LPS and Con A in iliac lymph nodes (Fig. 1E) was determined at 96.72% and 87.165%, respectively. For LPS, the lower and upper quartile of PVC values was calculated at 79.420% and 114.02%, respectively, in PFWB E. For Con A, the lower and upper quartile of PVC values was determined at 71.030% and 103.330%, respectively, in PFWB E. The median for both mitogens in PFWB E reached 96.721% and 87.165%. The PVC values for the analysed mitogens were classified into 4 activity levels based on median values for the lower and upper quartile: A – very low PVC (PVC < 50%), B – low PVC (50% ≤ PVC < 75%), C – high PVC (75% ≤ PVC < 100%), D – very high PVC (PVC ≥ 100%). Level A (very low activity) and level B (low activity) were not observed for either of the analysed mitogens. Level C (high activity) was reported in PFWB E2 for both mitogens. Level D (very high activity) was noted for both mitogens in PFWB E1. The evaluation of the proliferative activity of lymphocytes from iliac lymph nodes revealed a marked increase in the percentage of viable cells.
Fig. 1A. – comparison of the proliferative activity of immunocompetent cells from jejunal lymph nodes in prepubertal female wild boars (PFWBs); B – comparison of the proliferative activity of immunocompetent cells from jejunal lymph nodes suspended in the analysed mitogens; C – comparison of the proliferative activity of immunocompetent cells from iliac lymph nodes in PFWBs; D – comparison of the proliferative activity of immunocompetent cells from iliac lymph nodes suspended in the analysed mitogens; E – percentage of viable cells in jejunal and iliac lymph nodes in PFWBs E in comparison with PFWBs C. LPS – lipopolysaccharide
Discussion

The results of this study were compared with the findings of experiments performed on other animals, particularly on pigs (11, 14, 15). The results of the study (Fig. 1) indicate that both mitogens had a highly stimulating effect (from ≈ 300% to ≈ 600%) on the proliferative activity of lymphocytes in jejunal lymph nodes of PFWB E (Fig. 1A). The above findings indirectly point to the activation of T and B lymphocytes and, consequently, the immune system.

Similar results were reported in iliac lymph nodes. Mixed mycotoxicosis (DON and ZEN) enhanced the proliferative activity of lymphocytes in iliac lymph nodes (Fig. 1C) after stimulation with Con A (nearly 2- to 2.5-fold) and LPS (from ≈ 400% to ≈ 600%). LPS exerted more stimulating effects (Fig. 1E). The above results point to the relatively strong stimulation of T and B lymphocytes (in particular B lymphocytes), which provoke the immune response to antigens and autoantigens (15, 23). Thus, mixed mycotoxicosis contributed to the stimulation of T and B cell activity in the iliac lymph nodes of PFWB E.

Several factors should be taken into consideration when analysing the above results. One of them is a significant variation in mycotoxin concentrations in experimental feed, which consisted of wheat harvested in a farm-forest ecotone. The daily feed intake of the analysed PFWBs was estimated at 0.8 kg of wheat, therefore, daily exposure to mycotoxins reached approximately 26.880 μg of DON/kg b.w. and 0.693 μg of ZEN/kg b.w. In view of the NOAEL values for pigs, which are set at 12 μg/kg b.w. for DON and 40 μg/kg b.w. for ZEN (4), the concentrations were high for DON, but very low for ZEN. A comparison of mycotoxin concentrations in the administered wheat feed with the results obtained for individual mycotoxics in pigs suggests that lymphocytes should not respond to such low doses of ZEN. Toxodynamic interactions can, however, take place between DON and ZEN, but very little is known about such processes in mixed mycotoxicoses induced by varied toxin concentrations (18, 26, 14). The first study to address the above issue was conducted by Przybylska-Gronowicz et al. (24) who demonstrated that mycotoxin doses below NOAEL induce mild intestinal inflammations in pigs in the first week of exposure.

Another important feature worth consideration is the degree of proliferation of immunocompetent cells obtained from different lymph nodes and the intestinal segment, where the presence of both mycotoxins was signalled. The signal was probably transmitted by dendritic cell projections to lymph nodes (11) where the lymphocyte signalling cascade was initiated. Our results (Fig. 1B and 1D) indicate that a decrease in OD values was accompanied by an increase (%) in the proliferative activity of immunocompetent cells from both types of lymph nodes. In the duodenum, the exposure to mycotoxins intensified the proliferation of T lymphocytes that were stimulated with Con A, and B lymphocytes stimulated with LPS (Fig. 1B) in comparison with PFWB C. Elevated OD values were also reported in the iliac lymph nodes (Fig. 1D), where proliferative activity was higher than in PFWB C. The increase in OD values in the jejunum and ileum was indicative of the proliferation of immunocompetent cells. The observed increase was particularly high in B lymphocytes (LPS), which are responsible for the humoral immune response and the production of antibodies.

The PVC values of lymphocytes stimulated with both mitogens (LPS and Con A) increased in jejunal and iliac lymph nodes collected from animals exposed to DON and ZEN. A greater increase was noted in the lymphocytes of jejunal lymph nodes, compared with iliac lymph nodes (Fig. 1E).

The results of this study contradict the popular belief that all concentrations of DON deliver immunosuppressive effects. Our findings indicate that mycotoxin doses below NOAEL (hormesis) have a stimulating and beneficial influence because they prevent inflammation (12). Our previous study (13) demonstrated that the mRNA expression of type-1 and type-2 nitric oxide synthase (NOS1 and NOS2) in the digestive tract is decreased for two reasons. Firstly, fungal mycotoxins have bactericidal properties and they reduce the number of microbiological pathogens – proinflammatory agents which stimulate the production of NO (7). Secondly, mycotoxins inhibit the mRNA expression of both NOS genes, which could have therapeutic implications (16). Feed contaminated with ZEN and DON doses below NOAEL could inhibit inflammatory processes in the digestive tract. Similar observations were made by Lun et al. (18), who evaluated the influence of anti-inflammatory substances in vivo. In their study, the analysed compounds decreased the production of NO, a signal molecule, in mouse peritoneal macrophages, and induced the proliferation of splenocytes stimulated with Con A and LPS. Our findings and the results of other studies suggest that at given concentrations of DON and ZEN, mixed mycotoxicoses exert anti-inflammatory effects in intestinal mucosa and stimulate lymphocytes in iliac and jejunal lymph nodes.

Mycotoxin doses which exceed NOAEL only in moderation (e.g. by 124%) can exert a highly significant, stimulating effect (5) on the immune system despite the fact that DON is rapidly absorbed in proximal segments of the small intestine (1). DON is more hydrophobic than ZEN, which facilitates its transport to the top part of enterocytes and its deacetylation. As a result, DON is accumulated in enterocytes in the first hours of mycotoxin exposure, mainly in the duodenum and ileum (29).

It should be noted that even low concentrations of ZEN can trigger synergistic interactions in the immune system. Similar results were reported by Alassane-
Kpembi et al. (1) in a study on pigs. Synergistic interactions can occur at different stages in the same toxicity pathway, or when the presence of one mycotoxin increases absorption or decreases the metabolic degradation of another mycotoxin (27, 28, 6).

Unpublished results of histological analysis of porcine tissues indicate that both mycotoxins stimulated mucus secretion in the small intestine, particularly in the first week of the experiment, and lymphocyte infiltration of intestinal lamina propria (24). The above findings are consistent with the results of our previous study, in which mycotoxin concentrations in the porcine digestive tract were determined by the period of exposure to mycotoxins (29). The highest mycotoxin concentrations were reported in the duodenum and distal sections of the small intestine in the first week of the six-week exposure period. At the observed concentrations of DON, smaller doses of the mycotoxin reach distal segments of the small intestine, and in accordance with the principle of hormesis, they can stimulate proliferative processes without exerting adverse effects on the body (5).

The immune system of boars is probably more tolerant to low mycotoxin doses, or such doses do not provoke a response to toxic substances ingested in small amounts with feed, in accordance with the Tregs hypothesis (25); this is because wild boars are omnivorous scavengers which do not eat a monodiet. Therefore, the species can tolerate widely varying levels of undesirable substances, including mycotoxins, at least in initial stages of exposure, as at the dose levels applied in our study, the exposure period of 7 d was very short not only for PFWBs. The results of other studies (1) on mixed mycotoxicoises induced by Fusarium species suggest that low doses of two or more mycotoxins in feed and natural diet exert more toxic effects than predicted for individual mycotoxins. This observation has significant biological implications in view of the mycotoxin doses ingested by PFWBs.

Wheat is an abundant source of digestible energy for biotransformation processes, and it does not provoke pathological changes in the gastrointestinal tract, such as mucosal inflammation or allergy (12, 20).

The results of this study indicate that mixed mycotoxiosis induced by DON and ZEN in naturally contaminated feed enhances proliferative processes in immunocompetent cells of jejunal and ileal lymph nodes in PFWBs. The mitogenic activity of LPS was more expressed in both jejunal and ileal lymph nodes. Higher OD values were observed in ileal than in jejunal lymph nodes. A reverse trend was reported in analysis of PVC values, which were higher in jejunal lymph nodes during the evaluation of lymphocyte proliferation. The differences observed in different segments of the small bowel could be attributed to the fact that mycotoxins are toxic metabolites of fungi, LPS is a bacterial mitogen, and Con A is a plant mitogen.

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References


