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Duodenal morphology and immune responses of broiler chickens fed low doses of deoxynivalenol

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Abstract

Morphometry and flow cytometry for intraepithelial lymphocyte phenotyping were used to determine the changes in duodenal mucosae after administration of low doses of deoxynivalenol in chickens. Moreover, functions of phagocytes and immunocompetent cells in peripheral blood were evaluated by flow cytometry. In total, sixty chickens of Ross hybrid broilers 308 were used in this experiment. Two experimental groups of 20 birds were continually fed for 14 days a diet containing deoxynivalenol at a dose of 1 and 3 mg·kg^{-1}; 20 birds of the control group were fed uncontaminated diet. Morphometry showed only tendency to decrease the height of villi and surface area of duodenal mucosae in chickens fed the diet supplemented with 3 mg·kg^{-1} deoxynivalenol. Phenotyping of intraepithelial lymphocytes showed a decrease of CD45+ ($P < 0.034$) in duodenum of birds fed diets supplemented with deoxynivalenol. Examination of white blood cells showed a decrease of monocytes ($P < 0.020$) in chickens fed 3 mg·kg^{-1} of deoxynivalenol. Both experimental groups revealed higher metabolic burst of peripheral blood heterophils ($P < 0.001$). Phenotyping of immunocompetent cells showed an increase ($P < 0.003$) of CD3+ and a decrease ($P < 0.001$) of MHC II+ cells in peripheral blood of chickens fed with 3 mg·kg^{-1} dose of deoxynivalenol. The experimental feeding of chickens with deoxynivalenol resulted in immunomodulation of immunocompetent cells in duodenum and blood with mild atrophy of intestinal villi, mainly after the feeding of the dose of 3 mg·kg^{-1}. We proved that even low doses of deoxynivalenol can cause changes in haemathological, immunological and morphological profiles already during two weeks, and lead to the activation of compensatory-adaptive mechanisms with unfavourable impact on health and performance of birds.

Intestine, immunity, vomitoxin, morphometry, toxicity, poultry

Deoxynivalenol (DON, vomitoxin) is mycotoxin produced by *Fusarium graminearum* and is included into B type trichothecenes which can cause serious problems of animals and poultry when consumed via contaminated cereal grains. Consumption of lower amounts of fungal toxins may result in impaired immunity and decreased resistance to infection diseases (Oswald et al. 2005).

Mycotoxins may act on all types of immune cells and on different levels of the immune response to produce their adverse effects. Numerous studies conducted on host resistance, antibody responses, and cell mediated immunity have revealed that trichothecenes stimulate or suppress the immune function depending on the dose, exposure frequency, and timing of functional immune assay (Pestka et al. 2004). It is very likely that mycotoxins have their greatest effect on the mucosal lymphoid tissue (particularly gut and bronchial) before they are absorbed and subsequently metabolized (Oswald et al. 2005).

The avian gut associated lymphoid tissue (GALT) comprises a diverse set of cell subsets, distinct from that of systemic tissues but includes representatives of each of the major cell populations found on other sites. Overall the gut is populated with heterophils, macrophages, dendtritic cells, natural killer cell, and B and T lymphocytes, although the proportions of each cell type differ widely according to site and age (Brown et al. 2008). Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) have

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fundamental importance in host prevention after antigenic stimulation in the intestinal tract (Davison et al. 2008).

Enterocytes as part of the integral mucosal immune system are multi-functional epithelial cells that play an important role in the organization and function of the enteric immune system. Studies using the mouse model have shown the importance of enterocyte-expressed chemokines in the recruitment and retention of IEL cell populations (Onai et al. 2002).

Diets contaminated with low DON that induce a negative impact on health and performance could affect small intestinal morphology in broilers by diffusion of mycotoxins. Awad et al. (2006) observed that feeding DON for 21 days to broiler chickens at a concentration of up to 5 mg·kg^{-1} of diet influenced the weight of the small intestine as well as intestinal histology, especially the duodenum, as evidenced by shorter and thinner villi. Changes in the villi may influence also the immunocompetent cells of the intestine.

For that reason, the aim of the present study was to examine the effects of low doses of DON in naturally contaminated maize on duodenal morphology and immune responses of broilers.

Materials and Methods

Animals, housing and diets

One-day-old sixty chickens of Ross hybrid broilers 308 were randomly divided into 3 groups of 20 birds. The broilers were reared in large pens with wood shavings and had free access to water and feed. The experiment was carried out in accordance with established standards for use of animals. Local ethics and scientific authorities approved the Ro-2518/05-211/c protocol.

Chickens of all groups were fed the commercial diet HYD-02 for 2 weeks, and during the following 2 weeks broilers of DON groups were fed diets contaminated with different doses of DON mycotoxin. Commercial diet of the control group was naturally contaminated with 0.2 mg·kg^{-1} DON. Diets of the second and third groups were experimentally contaminated with 1 mg·kg^{-1} and 3 mg·kg^{-1} DON, respectively. The final mycotoxin contents in the diets for each group of birds are shown in Table 1. Contaminated batches of maize were obtained by their cultivation with *Fusarium graminearum* for four weeks at the Slovak Agriculture University in Nitra (Labuda et al. 2003). To provide stable dietary contents of mycotoxins throughout the whole experimental period, the chickens were fed only one type of diet, HYD-02. The composition of this diet is given in Table 2.

Table 1. Content of mycotoxins in complete diets for control and experimental groups of chickens.

Groups of birds	Mycotoxins (mg·kg^{-1} of complete feed)			
	DON	ZEA	15-ADON	Total aflatoxins
Control group	0.2	0.03	0	0.002
Group fed 1 mg DON	1.0	0.06	0.07	0.002
Group fed 3 mg DON	3.0	0.15	0.24	0.002

DON - deoxynivalenol, ZEA - zearalenone, 15-ADON - 15-acetyldeoxynivalenol

Table 2. Composition of diet HYD-02 fed to broilers during the experiment.

Components	g·kg^{-1}
Wheat ground, 10.5% of CP	260
Soya bean oil	14
Maize ground (8.3% CP)	400
Soya bean extracted ground meal (45% CP, 1.5% fat)	273.5
Fish meal (72% CP)	12.5
Premix HYD-02 (vitamins and minerals)	40

Metabolizable energy (12.75 MJ·kg^{-1} of complete feed), CP - crude protein analysed by Kjelahl method (210.6 g·kg^{-1} of complete feed)

At the age of 4 weeks, 6 randomly chosen chickens from each group were anaesthetized with intraperitoneal injection of xylazine and ketamine (Rometar 2% and Narkamon 5%, Spofa, Czech Republic) at doses of 0.6 and 0.7 ml·kg^{-1} body weight, respectively. After laparotomy, blood was collected into heparinized tubes by intracardial punction and immediately used for counting of leukocytes, and flow cytometry analysis of granulocytes and lymphocytes. Duodenal samples were taken into Hanks solution for flow cytometry, as well as into 10% formaline for histology and morphometry.

Mycotoxin analysis

Mycotoxins in maize were detected using gas chromatography-mass spectrophotometry (GC-MS) method (Raymond et al. 2003). Mycotoxin contents in the basal diet (the part of HYD-02 diet before addition of 40% portion of control or contaminated maize) were analyzed using NOACK kits for enzyme-linked immunosorbent assay with spectrophotometric evaluation.

White blood cell counting

Routine laboratory method using haemocytometer and Hemacolor staining (Merck, Germany) were used for evaluation of total count of leukocytes and their differentiation on blood smears. Absolute numbers (total numbers) of different white blood cell counting (WBC) were counted as follows: total leukocyte count/100 counted cells × relative % of differential cell count.

Phagocytosis and oxidative burst assay

The functions of polymorphonuclear cells were measured by flow cytometry in whole heparinized blood (heparin 10-20 U·ml^{-1} in PBS, Zentiva, Czech Republic). A commercial Phagotest and Bursttest kits (ORPEGEN®Pharma, Germany) were used for examination of phagocytosis and metabolic activity by the manual instructions.

Flow cytometry

Duodenal intraepithelial lymphocytes were isolated by modification some methods and published in detail by Levkut et al. (2009). Mononuclear cells from blood and intestine were separated over Histopaque-1077 gradient sedimentation (Sigma, Germany). Indirect and direct immunofluorescence methods of single staining cells were used. Labelled and unlabelled primary mouse anti-chicken monoclonal antibodies (Serotec, GB, and Southern Biotechnology Associates, Inc., Birmingham, USA) were used (Table 3). Polyclonal goat anti-mouse FITC-conjugated immunoglobulins F(ab')$_2$ fragment (Dako, Denmark) at a working dilution 1:50 with phosphate-buffered saline and 0.1% natrium azide (PBS+NaN_3) was used for staining lymphocytes in indirect immunofluorescence.

For each cell suspension (1.10^6 lymphocytes in PBS), cell population acquisition and analysis was carried out based on 10,000 cells using FACScan flow cytometer and Cell Quest programme (Becton Dickinson, Germany). For each marker, the relative percentage of fluorescent positive cells was recorded and absolute subpopulation's lymphocyte counts in peripheral blood were computed as follows: absolute lymphocyte counts/100 × relative % subpopulation's lymphocytes.

Table 3. Primary mouse anti-chicken monoclonal antibodies used in the experiment.

Specificity	Clone	Isotype	Dilution
CD3-UNLB	CT-3	mouse IgG1	1:50
CD4-FITC	CT-4	mouse IgG1κ	1:25
CD8α-PE	3-298	mouse IgG_{2b}κ	1:25
IgM-UNLB	M-1	mouse IgG_{2b}κ	1:10
CD45-UNLB	LT40	mouse IgMκ	1:25
MHC Class II-FITC	2G11	mouse IgG1κ	1:25

UNLB – unlabelled, FITC – fluorescein isothiocyanate, PE – phycoerythrin

Histology and morphometry of duodenum

Routine histological method with haematoxylin-eosin staining was used. Photos of histological sections were taken by Nikon LABOPHOT-2 with camera adapter (DS Camera Control Unit DS – U2) with × 4 magnification. The height and surface area of villi were measured by NIS-Elements programme. The height of villi was measured from the base to the apex.

Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) with *post hoc* Tukey multiple comparison test. Differences between the mean values for the groups of chickens were considered significant when $P < 0.05$.

Results

Determination of peripheral blood heterophils demonstrated their increased ($P < 0.002$) frequency in birds fed with the 3 mg·kg^{-1} DON dose than in chickens fed with the 1 mg·kg^{-1} DON dose. On the contrary, values of monocytes were lower ($P < 0.02$) in chickens fed with the 3 mg·kg^{-1} DON dose than in control. Metabolic burst of heterophils was higher ($P < 0.001$) in birds fed with 1 mg·kg^{-1} and 3 mg·kg^{-1} DON doses compared to control (Table 4).

Density of lymphocyte subpopulations in peripheral blood revealed higher frequency of CD3+ cells in birds fed with 3 mg·kg^{-1} DON dose than in 1 mg·kg^{-1} DON dose ($P < 0.003$) and in control ($P < 0.003$). Numbers of CD4+ and CD8+ cells were higher ($P < 0.003$, $P < 0.016$, respectively) in birds fed with 3 mg·kg^{-1} DON dose than in chickens fed with 1 mg·kg^{-1} DON dose. However, density of MHC II+ cells was lower ($P < 0.001$) in birds fed with 3 mg·kg^{-1} DON dose than in control (Table 5).

Evaluation of intraepithelial lymphocytes showed lower ($P < 0.034$) density of CD45+

Table 4. Number of peripheral white blood cells ($G{\cdot}l^{-1} = 10^9{\cdot}l^{-1}$) and functions of phagocytes in broilers fed diets contaminated with deoxynivalenol.

White blood cells	Control group	1 mg DON group	3 mg DON group	*P* values
Leukocytes	4.68 ± 0.62	4.30 ± 0.42	5.13 ± 0.94	0.545
Lymphocytes	3.54 ± 0.45	3.52 ± 0.53	3.58 ± 0.72	0.985
Heterophiles	1.06 ± 0.33	0.73 ± 0.27[b]	1.51 ± 0.35[a]	0.002
Monocytes	0.09 ± 0.03[a]	0.06 ± 0.03	0.05 ± 0.01[b]	0.020
Phagocytic activity (%)	35.00 ± 7.50	33.00 ± 21.10	32.00 ± 14.00	0.965
Metabolic burst	50.00 ± 9.10[a]	74.70 ± 6.40[b]	66.00 ± 8.90[b]	< 0.001

DON - deoxynivalenol. Data are presented as means ± SD (n = 6), different letters within the same row mark significant differences (*P* < 0.05).

Table 5. Actual count of immunocompetent cells ($G{\cdot}l^{-1} = 10^9{\cdot}l^{-1}$) in the peripheral blood of broilers fed diets contaminated with deoxynivalenol.

Subpopulation of lymphocytes	Control group	Group fed 1 mg DON	Group fed 3 mg DON	*P* values
CD3	0.95 ± 0.13[b]	0.76 ± 0.14[b]	1.88 ± 0.61[a]	0.003
CD4	0.83 ± 0.18	0.59 ± 0.20[b]	1.22 ± 0.36[a]	0.003
CD8	0.40 ± 0.11	0.27 ± 0.12[b]	0.56 ± 0.21[a]	0.016
CD45	1.89 ± 0.39	1.38 ± 0.53	2.15 ± 0.87	0.129
MHC II	0.30 ± 0.09[a]	0.21 ± 0.06	0.15 ± 0.09[b]	< 0.001
IgM	0.21 ± 0.07	0.16 ± 0.05	0.12 ± 0.07	0.120

DON - deoxynivalenol. Data are presented as, means ± SD (n = 6), different letters within the same row mark significant differences (*P* < 0.05).

Table 6. Relative percentage of duodenal intraepithelial lymphocytes of broilers fed diets contaminated with deoxynivalenol.

Subpopulation of lymphocytes	Control group	Group fed 1 mg DON	Group fed 3 mg DON	*P* values
CD3	40.66 ± 9.16	34.77 ± 5.64	34.65 ± 7.43	0.349
CD4	4.32 ± 3.00	5.01 ± 0.70	2.31 ± 0.39	0.063
CD8	12.15 ± 6.40	18.66 ± 3.55	17.54 ± 2.07	0.093
CD45	74.83 ± 10.72[a]	59.72 ± 7.89[b]	59.25 ± 9.50[b]	0.034
IgM	0.41 ± 0.39[b]	2.13 ± 0.02[a]	0.62 ± 0.48[b]	< 0.001

DON - deoxynivalenol. Data are presented as means ± SD (n = 6), different letters within the same row mark significant differences (*P* < 0.05).

cells in both experimental groups fed with DON than in control chickens, but with tendency in increase of CD8+ cells (Table 6).

During the experiment, chickens did not reveal clinical signs. Similarly, no gross and histological lesions were found in the intestine of birds fed the diet naturally contaminated with deoxynivalenol. Duodenal morphology demonstrated only tendency to decrease the height of villi and surface area of villi in birds fed with 3 $mg{\cdot}kg^{-1}$ DON dose compared to 1 $mg{\cdot}kg^{-1}$ and control (Table 7).

Table 7. Morphometry of duodenal villi in broilers fed diets contaminated with deoxynivalenol.

Indicator	Control group	Group fed 1 mg DON	Group fed 3 mg DON	*P* values
Height of villi (mm)	1.651 ± 0.188	1.661 ± 0.067	1.379 ± 0.116	0.069
Surface area of villi (mm^2)	198.85 ± 29.98	191.91 ± 25.56	156.09 ± 20.07	0.169

DON - deoynivalenol. Data are presented as means ± SD (n = 6).

Discussion

The gastrointestinal tract is the first barrier against ingested chemicals, feed contaminants, and natural toxins such as deoxynivalenol. Alterations of the gastrointestinal tract including duodenitis, jejunitis, intestinal bleeding and necrosis have been associated with the exposure to DON (D'Mello et al. 1999). Dänicke et al. (2002) reported that dietary concentration of DON exceeding 5 mg·kg^{-1} is needed to cause a detrimental effect and dietary treatments with 17.6 mg·kg^{-1} *Fusarium* contaminated maize caused decreased weight of the small intestine. Awad et al. (2006) reported that in broilers fed wheat artificially contaminated with DON (10 mg·kg^{-1}) small intestinal morphology was altered, especially in the duodenum and jejunum. On the other hand, Canady et al. (2002) suggested that artificially contaminated diets are less toxic than naturally contaminated diets and Awad et al. (2008) supposed higher toxicological risk of naturally contaminated grains because of synergic interactions between multiple mycotoxins. In our study, only mild alterations indicative of atrophy of intestinal villi were found after the addition of 3 mg·kg^{-1} DON to diet for 2 weeks of the experiment.

Administration of 3 mg·kg^{-1} DON dose to diet in our experiment resulted in decreased numbers of monocytes in the peripheral blood of broilers.

On the other hand, there was a tendency to increase the number of circulating heterophils. Heterophils play an integral role in inflammation, arriving first at damaged tissues. Certain alterations in intestine morphology could activate heterophils which is consistent with increased metabolic burst in heterophils in our experiment. If we calculate that DON is a potential inflammatory irritant mainly in the gastrointestinal tract, increased number of damaged cells in the target organ could therefore promote increased production of heterophils in the bone marrow after application of 3 mg·kg^{-1} DON in diet.

To receive more information about the sensitivity of immunocompetent cells included into natural and acquired immunity to DON feeding we chose to follow selected lymphocyte subpopulations and macrophages. Flow cytometry examination of decreased MHC II bearing cells density in peripheral blood in our study suggests that certain populations of haematopoietic cells are in lower frequency in animals fed 3 mg·kg^{-1} DON. The trend to decrease the number of MHC II+ cells was demonstrated in peripheral white blood cells, however, due to large variations in the data from animals fed the 1 mg·kg^{-1} DON dose, this was not significant. Decreased frequency of IgM+ lymphocytes in peripheral blood could be explained by their moving and higher differentiation into plasma cells expressing IgA in the intestine. It was suggested that the DON related up-regulation of pro-inflammatory cytokines produced by T-lymphocytes and macrophages was essential for the differentiation of B-cells to IgA secreting plasma cells (Pestka 2003).

It is known that DON is absorbed through the intestinal epithelium by simple diffusion, inhibits intestinal cell proliferation (Sergent et al. 2006) and alters immune cells in the gut (Levkut et al. 2011). This data indicate that tendency to elevate CD8+ cells could be

associated with restoration of gut immunity. Decrease of cells with common leukocytic antigen (CD45) suggests to be confined with shifting of CD3+ and CD4+ cells in the intestine of animals fed the 3 mg·kg^{-1} DON dose in our study.

In conclusion, the results demonstrated that the most sensitive immunocompetent cells at administration 3 mg·kg^{-1} DON in feed were monocytes. Mycotoxin-induced changes also involved metabolic burst of heterophils and activation of T lymphocytes. It is suggested that mild morphological alterations in duodenum and mild activation of immune cells are produced in broiler chickens fed low DON diets.

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