EVALUATION OF SELECTED INDICATORS OF IMMUNE RESPONSE (IL-1β, IL-4, IL-6, SAA, AND Hp) IN PIGS FED DIETS CONTAINING DEOXYNIVALENOL, T-2 TOXIN, AND ZEARALENONE

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Abstract

The objective of this study was to examine the clinical significance of selected indicators of immune response (IL-1β, IL-4, IL-6, SAA, and Hp) in short-term combined intoxication exposure of pigs to low doses of deoxynivalenol, T-2 toxin, and zearalenone present in naturally contaminated feedstuffs during grain growth. Statistically significant differences were not noted after 14 d of feeding diets contaminated with the above mycotoxins. The mycotoxins did not induce inflammatory processes. The subclinical form of combined mycotoxicosis could be due to incorrect immunological response. The identification of the underlying cause would support the development of new methods for the prevention of combined mycotoxicosis.

Key words: pigs, Fusarium toxins, immune response.

Mycotoxins are highly toxic compounds, which destabilize metabolic and homeostatic processes in human and animal cells already in small (µg) doses. In the climate of North-Eastern Europe, special attention needs to be paid to mycotoxins produced by molds of the genus Fusarium sp. including trichothecene, zearalenone, fumonisin, and patulin (1). Various studies demonstrated the immunomodulatory effect of mycotoxins as evidenced by induction and modulation of acute phase responses through cytokines as determinants of cell growth (IL-4), pro-inflammatory (IL-1 and IL-6), and anti-inflammatory properties (IL-4) (1, 8). Acute phase proteins (APP) participating in inflammatory processes may serve as biomarkers of early immunological activation and ligands in immune system cells. Trichothecenes (deoxynivalenol (DON), T-2 toxin) exert pronounced immunotoxic effects on mammals, as well as zearalenone (ZEA), whose effectiveness was attributed to the presence of DON as evidenced by toxicological potentiation between these mycotoxins (5), while immunological activity is low. Trichothecenes decreased noradrenaline (NA) levels resulting in peripheral vasodilation, and hypotension, as well as increased serotonergic (5-HT) levels, resulting in anorectic and emetic responses in the central nervous system (15). Low trichothecenes levels in pigs enhanced intestinal mucosal and bone marrow cell proliferation (2), while higher doses, similar to those used in this study, augmented migration of immunomodulatory cells to the intestinal mucosa and sub- mucosa. Obremski (11) noted the stimulatory effect of mycotoxins on intestinal lymphoid tissue and atrophy of intestinal epithelial cells with a subsequent damage to the duodenal intestinal villi. The histopathological changes in intestinal walls induced by DON, enhanced the absorption of ZEA without biotransformation by enterocytes (4). The phenomenon weakens immune responsiveness, and produces symptoms characteristic of hyperoestrogenism, which may be attributed to potentiation between DON and ZEA (7).

The objective of this study was to examine the effects of DON, ZEA, or T-2 toxin on selected indicators of immune response (non-specific immunity – IL-1β, IL-4, IL-6, serum amyloid A (SAA), and haptoglobin (HP) in pigs. The concentration of mycotoxins was based upon the concentration of no toxicological concern (CoNTC) established by Hardin (6) and added to natural feedstuffs for pigs.

Material and Methods

The experiment was carried out in strict observance of Polish legal regulations, which determine the conditions and the procedure of conducting experiments on animals. The experiment was performed in the course of 14 d on 10 crossbred (Polish Large White x Polish Landrace) pigs. Animals weighing
approximately 45 kg were kept in individual pens, with access ad libitum to water and feed. Pigs were divided into a control group (C) and an experimental group (E) of five animals each. The control group was fed a diet free of mycotoxins (the administered plant material was tested in a lab by HPLC method, and no aflatoxin, ochratoxin, trichothecenes, fumonisins, and zearalenone were detected). The experimental group was administered contaminated feed containing DON, T-2 toxin, and ZEA, at concentrations of 28.9, 11.5, and 33.2 µg/kg of feed, respectively. The feed for both groups comprised winter wheat cv. SUKCES, and the wheat applied in the production of diets for group E was not treated with plant protection chemicals (including fungicides) during the growing period. A similar experimental design was adopted by Tiemann (16).

Blood samples for immunological testing were collected on days 1, 7, and 14 of the experiment. The concentrations of selected cytokines (interleukins IL-1β, IL-4, and IL-6) and APP (SAA and HP) were determined with the use of ELISA Quantikine kits (R&D Systems, USA).

The obtained results were validated by the analysis of variance (ANOVA) with the use of STATISTICA 6.0 data analysis software (StatSoft Inc., 2003, www.statsoft.com). Differences between means were regarded as significant at P<0.01. Pearson linear correlation coefficients (r) were additionally determined for selected cytokines and APP to investigate the relationship between the studied indicators. Based on theoretical presumptions, a correlation analysis of the studied immunological indicators needs to be performed for an insight into clinical changes in experimental mycotoxicosis. A correlation indicates the strength of a relationship between two variables. A correlation coefficient is a number indicating the extent to which the analyzed variables are co-dependent, and a measure of correlation between two (or more) variables. The value of a correlation coefficient is <-1; 1> in a closed set. The higher the absolute value the stronger the linear relationship between the variables. rxy = 0 denotes an absence of a linear correlation between variables, rxy = 1 implies a perfect positive linear correlation between variables, while rxy = -1 means a perfect negative linear correlation between variables. The correlation coefficient used was Pearson's r, which determined the strength of linear correlation between random variables. The criterion for significance was set at P<0.05.

Results

As shown in Table 1, no statistically significant differences were determined in the concentrations of selected cytokines and APP at P≤0.01.

IL-6 concentrations remained at a similar level in animals from group C (Fig. 1), while in group E, a rapid initial decrease in IL-6 concentrations was followed by a significant increase on days 7 and 14 of the experiment.

The average Hp concentrations (Fig. 2) were higher in group E throughout the experiment, in particular on the 1st and 7th d of the study.

In most cases, the results of linear correlation analyses (Table 2) are within the rxy < 0.2 range because they are weakly expressed and they indicate an absence of a linear correlation, as shown by the lack of statistically significant differences (Table 1). The reported absolute values (rxy from 0.2 to 0.4) were indicative of a weak correlation between IL-1β and IL-6 (-0.32), IL-4 and SAA (-0.28), IL-4 and IL-6 (0.28). The first two Pearson correlation coefficients rxy are negative (inversely proportional), while the third coefficient is positive (directly proportional).

Discussion

Non-specific immunity is conditioned by a variety of factors and mechanisms, which keep the body in a state of homeostasis. It relies on mechanisms, which are developed early in the phylogensis process and which are found in all multicellular organisms. An innate immunity response is an organism's first line of defence against mycotoxins. Innate immunity mechanisms may be activated directly after exposure to mycotoxins (2), but they are often insufficient to eliminate the pathogen, which enters other parts of the body (intestinal lumen → enterocyte → portal vein → target tissue or cell → liver → enterohepatic circulation → intestinal lumen) (4). They are not as precise as specific immunity mechanisms, and they are not always successful in eliminating foreign antigens. The most important innate immunity responses include the secretion of pro-inflammatory and anti-inflammatory cytokines as well as APP.

Cytokines stimulate fever, they regulate the morphogenesis of cells and tissues, and contribute to pathological processes through their cytotoxic activity. One of such cytokines is IL-1, in particular its structural variant IL-1β, which stimulates the inflammatory response, contributes to the production of other IL-6 cytokines, activates leukocytes and other cells not directly related to the immune system, and participates in APP synthesis. The above dependencies were validated by the obtained results, which indicate that the experimentally induced combined mycotoxicosis lowered the production of IL-1β (by 0.004, 0.003, and 0.002 pg/mL) in comparison with group C, and considerably lowered the share of APP from the group of positive proteins, i.e. serum amyloid A, in comparison with group C (by 0.055, 0.213, and 0.182 µg/mL, i.e. by 19%, 66%, and 58%, respectively on successive experimental days).

Interactions between cytokines should also be considered. Cytokines should be regarded not only as proteins that have a local effect, but also as a group of molecules that have a profound effect on the functioning of organisms (10). The most widely investigated cytokine to date is interleukin 1 (IL-1). It is a collective name referring to cytokines that are vital for the inflammatory process and are characterised by a broad spectrum of activity. The interleukin is secreted in response to, among others, mould antigens.
Table 1
Concentrations of selected cytokines and APP

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>IL-1β pg/mL</th>
<th>IL-4 pg/mL</th>
<th>IL-6 pg/mL</th>
<th>Hp mg/mL</th>
<th>SAA µg/mL</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>C</td>
<td>0.059</td>
<td>0.104</td>
<td>69.57</td>
<td>0.58</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.007</td>
<td></td>
<td></td>
<td>0.114</td>
<td>0.289</td>
</tr>
<tr>
<td>1</td>
<td>E</td>
<td>0.055</td>
<td>0.100</td>
<td>72.40</td>
<td>0.18</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.005</td>
<td></td>
<td></td>
<td>0.182</td>
<td>0.234</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>0.063</td>
<td>0.103</td>
<td>66.78</td>
<td>0.43</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.005</td>
<td></td>
<td></td>
<td>0.549</td>
<td>0.279</td>
</tr>
<tr>
<td>7</td>
<td>E</td>
<td>0.060</td>
<td>0.122</td>
<td>56.24</td>
<td>0.73</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.010</td>
<td></td>
<td></td>
<td>0.678</td>
<td>0.162</td>
</tr>
<tr>
<td>14</td>
<td>C</td>
<td>0.058</td>
<td>0.101</td>
<td>57.79</td>
<td>0.26</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.006</td>
<td></td>
<td></td>
<td>0.537</td>
<td>0.106</td>
</tr>
<tr>
<td>14</td>
<td>E</td>
<td>0.056</td>
<td>0.114</td>
<td>61.61</td>
<td>0.54</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.006</td>
<td></td>
<td></td>
<td>0.553</td>
<td>0.112</td>
</tr>
</tbody>
</table>

Table 2
Values of Pearson linear correlation coefficient ($r_{xy}$) between cytokines and APP in pigs at a significance level of $P \leq 0.05$

<table>
<thead>
<tr>
<th>Specification</th>
<th>x</th>
<th>IL-1β</th>
<th>IL-4</th>
<th>IL-6</th>
<th>SAA</th>
<th>Hp</th>
</tr>
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<tr>
<td>$y_{IL-1β}$</td>
<td>0.08</td>
<td>-0.32</td>
<td></td>
<td></td>
<td>0.15</td>
<td>-0.15</td>
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<tr>
<td>$y_{IL-4}$</td>
<td>0.08</td>
<td>0.28</td>
<td></td>
<td></td>
<td>-0.28</td>
<td>0.14</td>
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<tr>
<td>$y_{IL-6}$</td>
<td>-0.32</td>
<td>0.28</td>
<td></td>
<td></td>
<td>0.07</td>
<td>-0.10</td>
</tr>
<tr>
<td>$y_{SAA}$</td>
<td>0.15</td>
<td>-0.28</td>
<td></td>
<td></td>
<td>0.07</td>
<td>-0.14</td>
</tr>
<tr>
<td>$y_{Hp}$</td>
<td>-0.15</td>
<td>0.14</td>
<td></td>
<td></td>
<td>-0.10</td>
<td>-0.14</td>
</tr>
</tbody>
</table>

Fig. 1. Graphic presentation of IL-6 cytokine concentrations on different days of the experiment.

Fig. 2. Graphic presentation of Hp acute phase proteins on different days of the experiment.
IL-1 is also capable of inducing the secretion of other pro-inflammatory cytokines such as IL-6 (9), which has not been confirmed by statistical results presented in Table 2. The reported findings suggest that the experimentally induced, low-dose combination mycotoxicosis caused a weak correlation (-0.32) between IL-1β and IL-6 in terms of absolute values. A negative Pearson coefficient $r_{xy}$ indicates that the observed correlation (interaction) is inversely proportional, i.e. it does not fulfill innate immunity requirements in a state of intoxication, which is undesirable from the clinical point of view.

IL-4 is a cytokine produced by Th2 lymphocytes, mastocytes, and basophils. It shows a broad spectrum of activity, affects different immune cell populations, has an antagonistic effect (in most cases) on IFN-γ, and directly and indirectly contributes to the development of the inflammatory focus. IL-6 is one of the most important cytokines with a broad spectrum of activity. It is secreted mainly by monocytes and macrophages under the influence of IL-1 and other pro-inflammatory cytokines. It strongly stimulates inflammatory processes, but also inhibits TNF production. The key properties of IL-6 are its pyrogenic activity and the ability to stimulate the production of APP.

An insignificant increase in IL-4 and IL-6 levels was noted in group E in comparison with control on the 7th and 14th d of the experiment (by 0.019 and 0.004 on day 7 and by 0.013 and 0.003 on day 14, respectively). In view of the values of Pearson correlation coefficient $r_{xy}$, the above points to a weak correlation of 0.28. As it is a positive value, it is indicative of a directly proportional correlation (interaction) between the two interleukins (anti-inflammatory and pro-inflammatory) in this experiment, suggesting an atypical innate immunity response (3) to a subclinical form of combined mycotoxicosis (14).

The above is supported by the concentrations of selected APP (Hp and SAA) in group E. These blood serum proteins are released by the liver as part of the body's immune response to the infection. They stimulate phagocytosis and facilitate the elimination of pathogens. They modify other immunity proteins, protecting them from the destructive effect of plasma and cell enzymes that reach the bloodstream from the damaged organs. Their concentrations are also higher in chronic inflammations and malignant tumours (13).

An increase in the levels of APP is part of the body's innate immune response, which aims at quick elimination of damaging and toxic factors, such as mycotoxins, and restoration of homeostasis. The APP is stimulated by mononuclear phagocytes, which release cytokines such as IL-1 and IL-6 during infection. Under their influence, hepatocytes synthesise and secrete APP. They are a group of serum proteins with various physical and chemical properties and a broad spectrum of biological functions (13). The following APP are of significance in the clinical diagnostics of breeding animals: haptoglobin (Hp), serum amyloid A (SAA), CRP, fibrinogen, α1-acid glycoprotein, α1-antitrypsin, ceruloplasmin, and seromucoid. The above APP prevent the inflammation from spreading, they limit the scope of tissue damage (SAA), and affect the proliferation of B-lymphocytes (Hp).

In view of the type of the experiment and pig species involved in this study, the authors decided to determine the levels of only the first two of the above APP. Hp levels in breeding animals are popularly determined with the use of quantitative methods. Haptoglobins are practically not found in healthy animals, and the rapid increase in Hp levels, that accompanies an inflammation, makes Hp a highly useful diagnostic tool in the even-toed ungulates. Hp concentrations usually increase proportionally to the intensity of clinical symptoms and the severity of the disease (13). SAA is becoming an increasingly popular clinical diagnostic tool for monitoring the patient's health condition. Its concentrations do not exceed 5 µg/mL in healthy animals. SAA levels usually increase after 4-5 h and peak concentrations are observed 24-36 h after activation by the inflammatory stimulus.

SAA concentrations noted in this study were within normal limits (9, 13) and they were consistent with the results reported in the control group. The above authors have suggested that Hp levels usually increase (as observed in group E on the 7th d of the experiment) in response to a pathological stimulus, indicating that the disease has a subclinical form, i.e. it does not produce pathognomonic clinical symptoms. SAA is an apolipoprotein and a pro-inflammatory mediator. In this experiment, SAA levels decreased in comparison with group C, suggesting that pathogens (mycotoxins) did not cause an inflammation in the studied gilts. An analysis of Pearson correlation coefficients did not reveal such interactions between APP or with cytokines. The reported values are within the boundaries of weakly expressed values, and the absence of a linear correlation points to the lack of statistical differences. Yet the presence of a negative absolute value within the weak correlation interval (-0.28) between IL-4 and SAA suggests an inversely proportional correlation between two mediators that are generally considered to be anti-inflammatory mediators. Upon contact with a pathogen, the levels of IL-4 increase, stimulating the growth of SAA concentrations (12). A reverse situation was observed in this experiment, suggesting that a dysfunction of the innate immune system takes place in the presence of the analysed mycotoxins.

The results of this experiment show that combined mycotoxicosis (DON, ZEA and T-2 toxin), induced by very low doses of the analysed mycotoxins, produced no immune response or a very weak immune response from selected cytokines (IL-1β, IL-4, and IL-6) and APP (SAA and Hp). Changes in correlations ("non-physiological" and undesirable) between cytokines (IL-4 and IL-4), and between cytokines and APP (IL-4 and SAA) were observed. The findings of this study lead to the conclusion that subclinical forms of combined mycotoxicosis (DON, ZEA, and T-2 toxin) could be due to an incorrect immunological response. The identification of the underlying cause would support the
development of new methods for the prevention of combined mycotoxicosis (12).

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References