SERUM AMYLOID A CONCENTRATIONS AND LYSOZYME BACTERIOLYTIC ACTIVITY IN THE SERUM OF SHEEP WITH NATURAL ZEARALENOSIS

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Abstract

The aim of the study was to assess bacteriolytic activity of lysozyme and serum amyloid A (SAA) level in sheep affected with zearalenone mycotoxicosis. Bacteriolytic activity of lysozyme, and serum amyloid A (SAA) level are both the elements of innate humoral immunity. Lysozyme bacteriolytic activity in serum was determined by diffusion-plate method with reference to Micrococcus luteus. SAA concentration was determined by the use of commercial ELISA kit (Phase Serum Amyloid A Assay, TP802). The highest lysozyme bacteriolytic activity was observed in sheep with total rectal and intestinal mucosal membrane prolapse. Lysozyme concentration values in animals with partial rectal prolapse were significantly lower. In the control group, lysozyme concentration was low and within the range considered as physiologically normal. The highest levels of SAA, which ranged from 38.5 to 172 μg/mL, were detected in sheep in which the highest lysozyme bacteriolytic activity was noted. From the data obtained, it is undoubtful that changes in SAA level in affected sheep and control sheep are reliable indicators of zearalenone mycotoxicosis progress and termination. The highest levels of SAA were noted in animals, which had the total rectal and intestinal prolapse in course of zearalenone mycotoxicosis, and after returning the rectum to the normal position, mucosal membrane necrosis occurred, which resulted in perforation and peritoneum inflammation. The observed differences in SAA level forming range in particular animals from infected group are conditioned by the character of inflammation process and intensity in organs altered by the disease, which are principal factors inducing changes in its concentration. The level of this protein in serum reflects activation state of immunological system and could be one of the criterions in sheep health assessment.

Key words: sheep, zearalenone, mycotoxicosis, lysozyme, serum amyloid A.

Mycotoxins in the form of secondary metabolites are produced by moulds, which proliferate on plant products or on fodders of plant origin. Zearalenone mycotoxin, which belongs to the group of fusariotoxins, and is produced mostly by Fusarium graminearum, constitutes a serious hazard to human and animal health (4, 9, 18). Mice administered with zearalenone demonstrated significant decrease in percentage of T lymphocytes bearing CD3, CD4, and CD8 antigens, NK cells, and B lymphocytes, and a decrease in proinflammatory cytokine serum levels, as well as of G and M immunoglobulins (2). In our experiments concerning sheep with natural zearalenosis, we noticed a considerable decrease in the percentage of CD4⁺ and CD8⁺ lymphocytes and a significant increase in CD4/CD8 ratio in comparison with healthy animals (8). However, there is no data in the world’s literature regarding the influence of zearalenone mycotoxin on acting of some components of innate humoral immunity and acute phase proteins in sheep with natural zearalenosis.

The aim of the study was to determine if there are significant changes in lysozyme bacteriolytic activity or serum amyloid A (SAA) level during the course of natural zearalenosis. Lysozyme bacteriolytic activity and SAA level are important diagnostic indicators, because they both constitute elements of innate humoral immunity.

Material and Methods

Experimental animals. The study was carried out in a sheep farm with 150 females and 50 males of synthetic meet-prolific line BPC (37.5% Polish lowland sheep, 12.5% Finish or Romanowska sheep, 25% Berrichon du Cher, 25% Charolaise) and SPC (37.5% Polish lowland sheep, 12.5% Finish or Romanowska
sheep, 25% Suffolk, 25% Charolaise) being in a continuous productive cycle. Local Ethics Commission has approved the studies.

The animals were kept in two breeding systems. The first group of the sheep (50 females and 15 males) was kept in the stabling system under a roof throughout the year without seasonal pasture grazing. The animals were fed the concentrate feed containing soy, wheat, and corn pellets in 1:1:2 proportion, as well as wheat bran with added vitamins and minerals. Bulky feed consisted of straw and hay in proportion 2:1. The animals received water *ad libitum*. Lambs were with their mothers from the birth until they reached slaughter weight. In this group, zearalenone mycotoxicosis developed regardless of the season and manifested itself in the prolapse of the anus and rectal mucosa. The prolapsed mucosa was in light to dark red colour and was covered with grey fibrinous exudate. The affected sheep showed increased intestinal peristalsis manifesting itself in continuous tensing without defecation. The animals were stooping or lying with their necks stretched. In the final stage of the disease, intense peristaltic contractions caused the rectal and intestinal prolapse, which was the direct cause of numerous deaths. Despite veterinary interventions, *i.e.* administration of tranquilizers and muscle relaxants, as well as purse-string sutures put in around the reponated rectum and anus, necrosis of the mucosa set in, leading to its perforation and peritonitis.

In the second system, the sheep (100 females and 35 males), were kept on a pasture from spring until late autumn and additionally fed wheat pellets and hay. The animals had permanent access to water. Lambs stayed with their mothers for 70 d, however, from day 14, they additionally received crushed oat, meadow hay, and dry sugar beet *ad libitum*. In this group, the clinical symptoms of the disease were not observed; however, isolated deaths of some lambs caused by mechanical injuries were noted.

The examinations were performed on 10 sheep from the first breeding system (group I) with clinical symptoms and on 10 sheep from the second breeding system (control group II) without clinical symptoms. To provide uniformity of the material, only sheep in the first stage of the disease (*i.e.* prolapse of the anus and rectum) were included (Fig. 1).

In both groups, the concentration of zearalenone and formation of lysozyme and SAA were determined in blood samples collected from the jugular vein. Furthermore, the presence of zearalenone was examined in the samples of individual ingredients of concentrates (soy, wheat bran and corn pellets, wheat bran and pressed oat) constituting the dietary intake of group I sheep and pressed oat constituting the dietary intake of group II sheep.

**Determination of zearalenone and α-zearalenol in concentrates.** The detection and quantitative determinations were performed using the HPLC method. Analyses involved the injection of 100 μl of the sample on the chromatographic column maintaining the conditions similar to the ones for preparation of standard curve. The obtained results are presented in Table 1.

**Determination of zearalenone and α-zearalenol in blood plasma.** The blood samples were immediately placed in the pre-chilled tubes containing heparin and centrifuged at 1,500 x g for 20 min at 4°C. Qualitative and quantitative determinations of zearalenone and its metabolites were performed by HPLC analysis. The obtained results are presented in Table 2.

![Fig. 1. Entire prolapse of anal and rectal mucous membrane in sheep with zearalenosis.](image)

<table>
<thead>
<tr>
<th>Concentrate type</th>
<th>Zearalenone</th>
<th>α-zearalenol</th>
<th>μg/kg</th>
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<tr>
<td>Corn pellets</td>
<td>13.62</td>
<td>3.44</td>
<td></td>
</tr>
<tr>
<td>Soy pellets</td>
<td>3.87</td>
<td>2.87</td>
<td></td>
</tr>
<tr>
<td>Wheat pellets</td>
<td>3.07</td>
<td>4.05</td>
<td></td>
</tr>
<tr>
<td>Wheat barn</td>
<td>14.49</td>
<td>3.46</td>
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</tr>
</tbody>
</table>
Table 2
Concentrations of zearalenone and α-zearalenol in blood plasma of the affected sheep (group I)

<table>
<thead>
<tr>
<th>Sheep no.</th>
<th>Zearalenone</th>
<th>α-zearalenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/kg</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.04</td>
<td>13.76</td>
</tr>
<tr>
<td>2</td>
<td>6.52</td>
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<td>5.72</td>
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<td>7</td>
<td>9.76</td>
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<tr>
<td>8</td>
<td>7.24</td>
<td>4.16</td>
</tr>
<tr>
<td>9</td>
<td>12.08</td>
<td>10.08</td>
</tr>
<tr>
<td>10</td>
<td>15.68</td>
<td>0.00</td>
</tr>
</tbody>
</table>

** - P≤0.01, comparison of arithmetic means of lysozyme concentrations between group I and group II of sheep.

Fig. 2. Comparative results of bacteriolytic activity of lysozyme in serum of sheep with natural zearalenosis and sheep without signs of zearalenosis.
Determination of lysozyme bacteriolytic activity (LZM). Lysozyme bacteriolytic activity in serum was determined by diffusion-plate method with reference to Micrococcus luteus (Serva) according to the method described by Hankiewicz (6). Lysozyme concentration in all investigated serum samples was calculated using variance analysis $y=\exp(a+bx)$, applying STATGRAF programme with $R=93.51\%$. The obtained results were expressed in mg/L.

Determination of SAA level. SAA concentration in serum was determined with the use of commercial ELISA kit (Phase Serum Amyloid A Assay TP802, Tridelta Development Limited, Ireland). The test was carried out according to the procedure attached to the kit and the results were read off by the use of ELISA LAP-SYSTEM at 450 nm with reference at 630 nm. SAA concentration was expressed in μg/mL.

**Results**

The mycotoxicologic results for concentrates fed to groups I and II of sheep are presented in Table 1. The data show that all samples of feed, with the exception of pressed oat, contained zearalenone mycotoxin both in its parent form, *i.e.* zearalenone, and its metabolite - α-zearalenol. The highest concentrations of zearalenone were found in corn pellets (14.49 μg/kg) and in wheat bran (13.62 μg/kg), which constitute the basic dietary intake of sheep with clinical signs of zearalenosis (group I). The lowest concentration of the parent form of zearalenone (3.07 μg/kg) and the highest concentration of its metabolite α-zearalenol (4.05 μg/kg) was found in wheat pellets, which was only additional feed in control group (group II). However, in the pressed oat sample, the presence of both: the parent form (zearalenone) and zearalenone metabolite (α-zearalenol) was not detected.

The concentrations of zearalenone mycotoxin in blood plasma of the affected sheep are shown in Table 2. Zearalenone in its parent form was found in all plasma samples, and its concentration ranged from 4.44 to 15.68 ng/kg; α-zearalenol was found in six out of ten plasma samples, and its concentration was higher in samples 1 and 2 compared to the parent substance - 13.76 and 7.64 ng/kg, respectively.

Lysozyme bacteriolytic activity in serum of sheep with zearalenosis and in control group is presented in Fig. 2. According to the presented data the serum lysozyme concentration in each of the sheep in the group I was conditioned by character and intensity of clinical symptoms, which were noticed during zearalenone mycotoxicosis course (Fig. 2). The highest lysozyme bacteriolytic activity was observed in sheep numbered 1, 2, 5, and 8, which had the total rectal and intestinal mucosal membrane prolapse during the disease course. In case of other animals from group I, which suffered from partial rectal and intestinal mucosal membrane prolapse, lysozyme concentrations were significantly lower. However, in the control group, lysozyme concentrations in all sheep were low and

*P≤0.05; comparison of arithmetic means of SAA level between group I and group II of sheep.

**Fig. 3.** Comparative results of SAA level in serum of sheep with natural zearalenosis and control sheep (α±SD).
within the range considered as physiologically normal (Fig. 2).

The data presented in Fig. 3 show that serum SAA level in individual sheep from group I was conditioned by the intensity of clinical symptoms, which occurred during the course of zearalenone mycotoxicosis. The highest levels of SAA, which ranged from 38.5 to 172 μg/mL, were detected in sheep numbered 1, 2, 5, and 8 in which the highest lysozyme bacteriolytic activity was noted (Fig. 2). In these sheep, the total rectal and intestinal prolapse occurred during the disease, and after returning rectum to the normal position mucosal membrane necrosis, and in consequence perforations and peritonitis were found. In the rest of sheep from group I, in which only partial rectal and intestinal prolapse occurred, SAA concentration values were significantly lower and ranged from 2.5 to 23.5 μg/mL. In the control group, SAA values ranged from 0.2 to 0.5 μg/mL, which are considered to be physiological (Fig. 3).

Discussion

Breeding environment of animals greatly influences not only their behaviour but also the efficiency of the immune system. This influence may enhance or inhibit defence reactions, and what plays here an important role is feeding with fodder in which heterogenous substances with immunostymulating and immunosuppressive activity are present. Mycotoxins, secondary metabolites of molds, which mostly exist in plant material or in fodders of plant origin, are the classical example of this. Metabolites of Fusarium species – cereal facultative pathogens, which infect ears of wheat, triticale, rye, barley, and oats and in result cause the cumulation of mycotoxin in grain even before harvest, are the particular group of mycotoxins.

Mycotoxins produced by molds from Fusarium sp. genus display carcinogenic, mutagenic, teratogenic, oestrogenic, and immunosuppressive activities in various extent, the consequence of which are low breeding results, as well as increased animal susceptibility to infections and lower efficacy of vaccines applied in specific immunoprophylaxis (3, 9, 16, 18).

The results of numerous experiments concerning the influence of fusariotoxins on the immune system indicated direct immunotoxic effect, balanced disturbance between cytokine production by helper lymphocytes Th1 and Th2, and considerable decrease in lymphocyte T percentage, serum level of α, β1, β2, and γ-globulins, complement C3 factor, as well as M and A immunoglobulins (2, 5, 10, 11, 13, 15). It was demonstrated that in sheep with natural zearalenosis, a marked increase in lysozyme bacteriolytic activity occurred. The interpretation of the obtained results is difficult. It is worth mentioning that in previous investigations it was found that within the same group of sheep under influence of zearalenone and α-zearalenol, the decrease in percentage of lymphocyte CD4 and CD8 occurred, and CD4/CD8 ratio increased when compared to control animals (8).

Thus it can be assumed that cell-mediated response suppression, which takes place in the first stage of the disease, is compensated by an increase in serum level of lysozyme, which is a component of innate humoral immunity. The increase in lysozyme bacteriolytic activity in sheep with mycotoxicosis gives evidence of efficiency of phagocytic cells, which are the main centre of lysozyme production and which are not negatively affected by zearalenone mycotoxin. Lysozyme is one of the most important mechanisms of non-specific humoral immunity, as it takes part in oxygen independent killing of the range of bacteria by phagocytic cells.

The acute phase reaction, the aim of which is to restrict inflammation, to eliminate damaging factor and to reconstruct affected tissues and organs, is significant for the reliable assessment of the process and termination of the disease. The acute phase response is a component of non-specific immunity, and it involves the earliest changes which occur in the organism under influence of damaging factors such as: injury, surgical interventions, necrotic and tumour processes, and others. One of the effects of acute phase response is a change of level of certain acute phase proteins (1, 7, 12).

Hence our investigations were concentrated on the usefulness of SAA determination in monitoring of zearalenone mycotoxicosis progress in sheep. There is no information in the literature referring to the usefulness of SAA determination in the monitoring of natural zearalenosis course in sheep, which hinders the interpretation of the obtained results. From data obtained from our research, it is undoubted that changes in SAA level in affected and control sheep are reliable indicators of zearalenone mycotoxicosis progress and termination of the disease. SAA is the most important acute phase protein in sheep, and the initiation of its production in liver cells as a result of inflammatory reaction is mostly influenced by inflammatory cytokines, while the scale and duration time of this production is influenced by a number of factors acting at the same time and present even before the reaction started (7, 14, 17).

In the group of affected sheep (group I), formation of SAA level was influenced by the character and intensity symptoms in individual animals. The highest levels of SAA were noted in animals, which had the total rectal and intestinal prolapse in course of zearalenone mycotoxicosis, and after returning the rectum to the normal position, mucosal membrane necrosis occurred, which resulted in perforation and peritoneum inflammation. Taking into consideration that these animals were kept in the same zoohygienical conditions and fed in the same manner, it could be assumed that the observed differences in the forming range of SAA level in particular animals from infected group are conditioned by the character of inflammation process and intensity in organs altered by the disease, which are principal factors inducing changes in its concentration.

The formation of SAA level is also the reflection of activation degree of the immune system in
unsettled internal homeostasis in sheep with natural zearalenosis. On the basis of the obtained results, it could be affirmed that formation of the serum SAA level is a reliable marker of unsettled internal balance assessment in sheep with natural zearalenosis. The level of this protein reflects activation state of the immunological system and could be one of the criterion in assessment of sheep health.

References