Subpopulation of Lymphocytes CD4+ and CD8+ in Peripheral Blood of Sheep with Zearalenone Mycotoxicosis

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Abstract

In sheep raised in chamber system and fed concentrate fodder containing high dose of zearalenone and its metabolite \((\alpha\)-zearalenol), the clinical course of zearalenonosis was diagnosed. Control animals were kept on grazing-land from the spring to the late autumn, and only in winter time they were fed shredded wheat with the lowest concentration of zearalenone. These sheep showed no clinical symptoms of the mycotoxicosis. Zearalenone and \(\alpha\)-zearalenol cause immunosuppression, which means that lymphocyte T \(CD4^+\) and lymphocyte T \(CD8^+\) percentages decline and \(CD4/CD8\) ratio increases when compared with control animals. Zearalenone mycotoxin suppression effect modulates cellular immunological response, which is essential in suppression of post-vaccination immunity and infectious diseases development despite applying normal specific immunoprophylaxis.

Key words: sheep, zearalenone, mycotoxicosis, lymphocytes, immunosuppression, flow cytometry.

Zearalenone mycotoxin belongs to a remarkable group of mycotoxins, formed as metabolites of filamentous fungi from the genus \(Fusarium\) sp. The phenol ring present in the molecule of zearalenone enables binding to oestrogen receptors of the uterus, vagina, ovary, and oviduct, enhancing cell proliferation and in the end increased synthesis of RNA and proteins in cells of the reproductive tract (3, 12, 15, 22). Zearalenone acts in animals as feminising agent even at low concentrations, but higher concentrations (50-100 ppm) disturb reproduction hindering fertilisation, ovulation, embryos implantation and their normal development, and impairment of newborn animals (14, 23, 24). Previous achievements on the action of zearalenone on the activity of immunological system in polygastric animals are fragmentary and often incoherent (8). Therefore, our studies should clarify the relationship between the presence of zearalenone in blood of sick sheep and formation of subpopulation of lymphocytes CD4+ and CD8+ playing a fundamental role in the development of specific immune response and in immunoregulation.

The aim of the study was the evaluation of the expression of CD4+ and CD8+ on T lymphocytes of peripheral blood of sheep with natural zearalenone mycotoxicosis.

Material and Methods

Experimental animals. The study was carried out in a sheep farm with 150 females and 50 males of synthetic meat-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 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 35 deaths. Despite veterinary interventions, i.e. administration of tranquilisers and muscle relaxants, as well as purgative and muscle relaxants, the condition of the animals did not improve, and mechanical perforation and peritonitis developed.

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 unrestricted 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) and on 10 sheep from the second breeding system (control group II). 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 lymphocyte subpopulations were determined in blood samples collected from the jugular vein. Furthermore, the presence of zearalenone was determined in the samples of individual ingredients of concentrates (soy, wheat bran and corn pellets, wheat bran and pressed oat) constituting the dietary intake of sheep from group II.

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

**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.

**Determination of lymphocyte surface antigens CD4+ and CD8+.** Blood from sheep of both groups was collected from the jugular vein into 2 ml EDTA tubes. Monoclonal antibodies conjugated with fluorochromes (CD8:FITC and CD4 RPE, Serotec Immunological Excellence, England) were used. The antibodies were specific for ovine CD4+ and CD8+ lymphocyte antigens. All tests were done according to the manufacturer’s procedure. For all probes, the flow cytometry method was applied (flow cytometer- Epics XL, Beckman-Coulter, Comesa CH-Werfen Company, USA), and a single step labelling was conducted.

**Statistical analysis.** The obtained results were statistically analysed with Statistica 5.0 programme. The significance of differences between control group and investigated group was verified by t-Student test.

**Results**

Results of mycotoxicological analysis of concentrates used for feeding sheep are presented in Table 1. All fodder samples contained zearalenone mycotoxin, in both forms: the parent compound, i.e. zearalenone and its metabolite - α-zearalenol. The highest levels of zearalenone were found in ground corn meal (14.49 µg/kg) and in wheat bran (13.62 µg/kg), whereas α-zearalenol was detected in ground wheat meal (4.05 µg/kg).

Table 2 illustrates the plasma levels of zearalenone in the affected sheep. Zearalenone, as the parent compound, was present in all plasma samples and its concentration ranged from 4.44 to 15.68 ng/kg. α-zearalenol was detected in six out of ten examined plasma samples; in samples 1 and 2 its levels were higher than those of the parent substance, 13.76 and 7.64 ng/kg, respectively.

Cytometric analysis of the subpopulations of CD4+ and CD8+ lymphocytes as well as the ratio of CD4+/CD8 lymphocytes are presented in Figs 2, 3, and 4. Fig. 2 demonstrates that the percentage of CD4+ lymphocytes was significantly (P≤0.01) lower in the experimental group (group I) than in controls (group II), both in individual sheep and in relation to its mean values in groups. In group I, the percentage of CD4+ lymphocytes ranged from 18.40 to 28.10% with the mean value of 24.07+/−.9. In group II, the extreme percentages of CD4+ lymphocytes were found to be 26.3% and 40.2% with the mean value of 31.7+/−1.5.

Similar results were obtained for the percentages of CD8+ lymphocytes in the affected sheep compared to controls, both in individual sheep and in relation to mean values in groups (Fig. 3). The ratio of CD4/CD8 lymphocytes in group I was higher than that in controls and ranged from 1.51 to 2.76. In group II, the lowest value of the CD4/CD8 lymphocyte ratio was 1.1 while the highest one - 1.81, and these two values were within normal limits for sheep (Fig. 4).

**Discussion**

The clinical or subclinical course of zearalenonosis in ruminants is highly dependent on the species, breed, and age of animals, as well as feed and season, as the incidence of intoxications in the autumn-winter period is higher. Moreover, our findings show that the system of maintenance and feeding of animals is extremely important, which is indicated by the presence of overt zearalenonosis in the experimental group of sheep (group I). The animals of this group were kept in sheepfolds throughout the year and were fed concentrates with high levels of zearalenone and its metabolite (α-zearalenol) (Table 1). This facilitated the accumulation of zearalenone, reaching the dose, which induced the clinical symptoms of the disease.
Table 1
Concentrations of zearalenone and α-zearalenol in concentrates

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

<table>
<thead>
<tr>
<th>No. of sheep.</th>
<th>Zearalenone</th>
<th>α-zearalenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.04 μg/kg</td>
<td>13.76 μg/kg</td>
</tr>
<tr>
<td>2</td>
<td>6.52 μg/kg</td>
<td>7.64 μg/kg</td>
</tr>
<tr>
<td>3</td>
<td>4.64 μg/kg</td>
<td>0.00 μg/kg</td>
</tr>
<tr>
<td>4</td>
<td>8.68 μg/kg</td>
<td>5.84 μg/kg</td>
</tr>
<tr>
<td>5</td>
<td>4.44 μg/kg</td>
<td>0.00 μg/kg</td>
</tr>
<tr>
<td>6</td>
<td>10.36 μg/kg</td>
<td>5.72 μg/kg</td>
</tr>
<tr>
<td>7</td>
<td>9.76 μg/kg</td>
<td>0.00 μg/kg</td>
</tr>
<tr>
<td>8</td>
<td>7.24 μg/kg</td>
<td>4.16 μg/kg</td>
</tr>
<tr>
<td>9</td>
<td>12.08 μg/kg</td>
<td>10.08 μg/kg</td>
</tr>
<tr>
<td>10</td>
<td>15.68 μg/kg</td>
<td>0.00 μg/kg</td>
</tr>
</tbody>
</table>

Fig. 1. Entire prolapse of the anal and rectal mucous membrane in sheep with zearalenosis.
Fig. 2. Percentage of CD4\(^{+}\) lymphocytes in peripheral blood in sheep with zearalenone mycotoxicosis and in controls (\(\alpha^{+/-}\)SD).

Fig. 3. Percentage of CD8\(^{+}\) lymphocytes in peripheral blood in sheep with zearalenone mycotoxicosis and in controls (\(\alpha^{+/-}\)SD).
No clinical symptoms were observed in the control sheep (group II), which spent the spring-late autumn period on the pasture and were fed concentrates containing ground wheat only during winter, when the lowest levels of zearalenone were found.

The typical symptoms accompanying zearalenonosis in sheep include the 10%-20% decrease in fertility, metritis, and abortions. Our study revealed that zearalenonosis in sheep may also be accompanied by prolapses of the anus and rectal mucosa, increased intestinal peristalsis or complete prolapse of the rectum with intestines in the final stage. Such symptoms were the direct cause of numerous deaths observed in the affected sheep (group I). The pathomechanism of clinical alimentary symptoms in sheep with zearalenone mycotoxicosis is difficult to explain as the literature lacks any reports on the subject. Thanks to the presence of a phenol ring in its structure, zearalenone is capable of binding to the oestrogen receptor competitively to oestradiol (the duodenum and large intestine) (2, 17).

Detoxication of Fusarium mycotoxins, including zearalenone (ZEA), occurs mainly in the liver and, to lesser degree, in the gastrointestinal tract; in ruminants most likely in the terminal part of the masseter with the bacterial flora (20). These changes precede absorption. Almost 90% of ZEA is reduced to α-ZOL and β-ZOL. According to Dänicke et al. (4), ZEA and its metabolites are detected in ruminant bile in the amounts reaching 68% β-ZOL, 24% α-ZOL, and 8% ZEA. Recently, a number of reports concerning biotransformation of mycotoxins in various animal species by intestinal microorganisms were published. The majority of them describe changes in deoxynivalenol and Fusarium mycotoxin levels in pigs (5, 7, 10). As far as ZEA is concerned, it was suggested that intestinal microorganisms hydrolyse the parent compound only to α-ZOL (11). Our results confirm that biotransformation processes occur earlier in the plant material (Table 1), which is demonstrated by the presence of α-zearalenol, whose hormonal activity is several times higher than that of the parent substance, i.e. zearalenone. On the other hand, results of mycotoxicological analysis, especially samples 1 and 2, which contained higher amounts of α-zearalenol than the parent compound, demonstrate that intoxication was long lasting or biotransformation, or rather biostimulation of zearalenone (i.e. enzymatic conversion of zearalenone to α-zearalenol), was quicker than the process of detoxication (21).

Beside their toxic action, Fusarium mycotoxins show adverse immunosuppressive effects impairing cellular and humoral mechanisms of non-specific and specific immunity (19). A few studies concerning the effects of Fusarium mycotoxins on the immune system indicate imbalance in the production of cytokines by helper lymphocytes Th1 and Th2 (1). In pigs receiving the feed with fumonisin B₁, the level of IL-4 decreased whereas the production and release of IFN-γ by T lymphocytes increased (6). In piglets, fumonisin B₁ inhibited the specific humoral immunity, which was manifested in decreased serum levels of specific antibodies after vaccination, and reduced expression of mRNA for IL-10 (19). Moreover, in vitro studies demonstrated that fumonisin B₁ had proinflammatory properties visible in increased production of nitrous oxide by macrophages in rats and increased TNF-α synthesis by LPS – stimulated macrophages in mice (1).

Our results demonstrate explicitly that in sheep, zearalenone and its metabolite (α-zearalenol) induced a significant decrease in the percentage of lymphocytes TCD4⁺ and TCD8⁺ as well as an increase in their ratio compared to the control group. There is lack of reports in the literature concerning immune phenomena during natural zearalenone mycotoxicosis in sheep and therefore it is difficult to confront our findings. Similar results concerning mice receiving zearalenone were published by Ben Salah-Abbes et al. (1), who demonstrated that the percentage of CD3⁺, CD4⁺, and CD8⁺ T lymphocytes as well as percentage of B and NK cells in the peripheral blood decreased significantly. Moreover, levels of proinflammatory cytokines and G and serum M immunoglobulins decreased. Most likely,
immunosuppression accompanying the zearalenone mycotoxin-induced intoxication cannot be explained by a single mechanism.

In the immune response to infection or vaccine antigen, the helping/inducing CD4+ and suppressive/cytotoxic CD8+ lymphocytes act as regulatory cells; their proportion determines the profile of the induced immune response (9, 16). In healthy animals, their suitable number and appropriate CD4+/CD8+ ratio are maintained. This fact was confirmed by our results in control sheep (group II) where the CD4+/CD8+ proportions were within normal limits (13). However, in the affected sheep (group I), the percentage of CD4+ and CD8+ lymphocytes was found to be significantly reduced and their ratio markedly increased when compared to group II. Mycotoxins could both stimulate and suppress immunological system, leading often to hypersensitivity (8). Thus, it may be assumed that the suppressive action of zearalenone observed in our study modulates the cellular specific immune response. Immunosuppression of cellular mechanisms of anti-infectious immunity plays a significant role in overcoming the post-vaccination immunity in sheep, which may result in increased susceptibility of animals to infections and decreased effectiveness of vaccines used for specific immunoprophylaxis (18, 19).

References