**CONCENTRATIONS OF PROINFLAMMATORY MEDIATORS OF THE ARACHIDONIC ACID CASCADE IN SERUM OF SHEEP WITH NATURAL ZEARALENONE INTOXICATION**

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**Abstract**

The clinical form of natural zearalenone intoxication was observed in sheep that were kept indoors and fed a constant diet of feed concentrates containing high concentrations of zearalenone and its metabolite α-zearalenol. The clinical form of the disease was not noted in the control group, consisting of sheep that were kept on a pasture from spring to late autumn; only in the winter they were fed wheat pellets, in which the zearalenone concentration was determined to be the lowest among all used feed concentrates. During the course of natural zearalenone intoxication, metabolism of arachidonic acid increased, mainly due to enzymes of the cyclooxygenase group, which are responsible for the generation of prostaglandin F\(_2\alpha\) and thromboxane B\(_2\), and an increase in their concentration. Increased production of F\(_2\alpha\) and B\(_2\) was closely correlated with the serum level of SAA, an indicator of the intensity of the inflammatory reaction. This indicates that both compounds participated in the development of inflammatory reactions in the terminal end of the digestive tract that accompanied zearalenone intoxication in sheep. The imbalances noted between the eicosanoid classes investigated in this study were fundamentally responsible for the development of clinical symptoms in sick sheep that showed symptoms of partial or total prolapse of the anus and rectum and prolapse of the large intestine, which were the direct cause of the animals death.

**Key words**: sheep, zearalenone, arachidonic acid, eicosanoids.

The importance of zearalenone (ZEA) and its natural metabolites stems from the presence of a phenolic ring in their chemical structure, which gives these compounds oestrogenic properties and disrupts regulation of the endocrine system. The physiological activity of ZEA and its oestrogenic affinity with 17β-oestradiol explains the competition between ZEA and this oestrogen for specific sites of binding to cytosolic oestrogen receptors present in the reproductive system, brain, or digestive tract (7, 13, 18, 22). A characteristic effect of ZEA on animals is hyperoestrogenism, manifested as vulvar swelling, uterine enlargement, and increased uterine secretions, as well as overgrowth of the mammary gland. Other effects that may occur include infertility, lack or prolongation of the oestrous period, reduced libido, and frequent occurrence of pseudopregnancy (25, 26).

Stimulation of cells in inflammatory reactions and transmission of signal causes rapid changes in cell membrane components. The increased intracellular level of Ca\(^{2+}\) ions in stimulated cells activates membrane-bound phospholipase A\(_2\), which releases polyunsaturated arachidonic acid (AA) – a key compound involved in eicosanoid biosynthesis – from cell membrane phospholipids. Biological derivatives of arachidonic acid play a fundamental role in the development of disease processes by contributing to angiogenesis, inflammation and immunosuppression (5, 10, 23). The available world literature lacks data concerning the effect of ZEA mycotoxin on arachidonic acid metabolism in small ruminants during natural ZEA intoxication.

The aim of this study was to evaluate the biologically active compounds generated by arachidonic acid metabolism in sheep under the influence of ZEA mycotoxin. The study will give a better understanding of the role of eicosanoid biosynthesis pathways in the induction of inflammatory reactions and problems connected with their biological activity during natural ZEA intoxication.
Material and Methods

Experimental animals. The study was carried out on a sheep farm with 150 females and 50 males of the synthetic meat-prolific lines BPC (37.5% Polish Lowland sheep, 12.5% Finnish or Romanov sheep, 25% Berrichon du Cher, 25% Charolaise) and SPC (37.5% Polish Lowland sheep, 12.5% Finnish or Romanov sheep, 25% Suffolk, 25% Charolaise) in an uninterrupted breeding cycle. The study was approved by the Local Ethics Commission. The animals were kept in two breeding systems.

In the first system, the sheep (50 females and 15 males) were kept indoors throughout the year without seasonal pasture grazing. The animals were fed feed concentrates containing soy, wheat and corn pellets at a proportion of 1:1:2, as well as wheat bran with added vitamins and minerals. Bulky feed consisted of straw and hay (2:1). The animals received water ad libitum. Lambs were with their mothers from birth until they reached slaughter weight. In this group, the symptoms of disease developed regardless of the season and were manifested by prolapse of the anus and rectum and intestinal peristalsis manifesting as continuous straining without defecation. The animals were stooped or lay with their necks stretched out. In the final stage of the disease, intense peristaltic contractions caused rectal and intestinal prolapse, which was the direct cause of numerous deaths. Despite veterinary interventions, i.e. administration of tranquillisers and muscle relaxants, as well as purse-string sutures put in around the prolapsed 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, but from day 14 they also received crushed oat, meadow hay, and dry sugar beet ad libitum. In this group, clinical symptoms of the disease were not observed, but isolated random deaths of lambs due to mechanical injuries were noted.

Tests were performed on 10 sheep from the first breeding system (group I) with clinical symptoms. To ensure uniformity of the material, only sheep in the first stage of the disease (i.e. prolapse of the anus and rectum) were included.

The control group consisted of 10 sheep from the second breeding system (group II) without clinical symptoms. In both groups, ZEA concentration and formation of lysozyme and SAA were determined in blood samples collected from the jugular vein. Furthermore, the presence of ZEA was tested in samples of individual ingredients of the concentrate (soy, wheat and corn pellets, wheat bran and pressed oat) constituting the dietary intake of the group I sheep, and pressed oat constituting the dietary intake of group II sheep.

Determination of zearalenone and α-zearalenol in the concentrate feed. Detection and quantitative determinations were performed using the HPLC method. Determinations involved the injection of 100 µl of the sample on the chromatographic column, maintaining conditions analogous to those for preparation of a standard curve. The obtained results are presented in Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Concentrations of zearalenone and α-zearalenol in concentrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrate type</td>
<td>Zearalenone</td>
</tr>
<tr>
<td>Corn pellets</td>
<td>13.62</td>
</tr>
<tr>
<td>Soy pellets</td>
<td>3.87</td>
</tr>
<tr>
<td>Wheat pellets</td>
<td>3.07</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>14.49</td>
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</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Concentrations of zearalenone and α-zearalenol in the blood serum of the affected sheep (group I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep no.</td>
<td>Zearalenone</td>
</tr>
<tr>
<td>1</td>
<td>6.04</td>
</tr>
<tr>
<td>2</td>
<td>6.52</td>
</tr>
<tr>
<td>3</td>
<td>4.64</td>
</tr>
<tr>
<td>4</td>
<td>8.68</td>
</tr>
<tr>
<td>5</td>
<td>4.44</td>
</tr>
<tr>
<td>6</td>
<td>10.36</td>
</tr>
<tr>
<td>7</td>
<td>9.76</td>
</tr>
<tr>
<td>8</td>
<td>7.24</td>
</tr>
<tr>
<td>9</td>
<td>12.08</td>
</tr>
<tr>
<td>10</td>
<td>15.68</td>
</tr>
</tbody>
</table>
Determination of zearalenone and α-zearalenol in blood serum. The blood samples were immediately placed in pre-chilled heparin tubes and centrifuged at 1,500 x g for 20 min at 4°C. Qualitative and quantitative determinations of ZEA and its metabolites were performed by HPLC analysis. The obtained results are presented in Table 2.

Determination of eicosanoids. Serum concentrations of thromboxane TXB₂, leukotriene LTB₄, and prostaglandins PGE₂ and PGF₂α were determined using the c-ELISA competition test (R&D Systems, Inc., USA), adapted for use in animal testing (it is intended for testing eicosanoids in human systemic fluids). In order to adapt c-ELISA for use in sheep samples and to obtain repeatable and reliable results for the eicosanoid determinations, the serum was diluted 1:10 for PGE₂, PGF₂α, and LTB₄, and 1:100 for TXB₂. The c-ELISA involved indirect determination of eicosanoid (as an antigen) in the presence of one specific antibody in the liquid phase and another in the solid phase. For this purpose polyclonal rabbit antibody reacted with the eicosanoid under investigation in the presence of the same antigen, with known concentration, labelled with alkaline phosphatase (ALP). As a consequence of the reaction, competition for antibody molecules occurred between the labelled and non-labelled antigen. All of these reagents were in the solution, in the liquid phase, while in the solid phase goat antibodies were present that bound the resultant immune complexes in a herringbone pattern. Free, unbound antigen and conjugate were separated by repeated washing, and p-nitrophenylphosphate (pNPP), a substrate for ALP, was added in the final stage. The intensity of the yellow colour appearing as a result of the colour reaction, which was inversely proportional to the concentration of the factor being tested in the sample, was determined spectrophotometrically at 405 nm wavelength. The results were presented in pg/mL.

Results

The mycotoxicological results for the concentrates administered to groups I and II are presented in Table 1. The data show that all samples of feed with the exception of the pressed oat contained ZEA mycotoxin both in its parent form (ZEA), and its metabolite α-zearalenol. The highest concentrations of ZEA were found in the corn pellets (14.49 µg/kg) and wheat bran (13.62 µg/kg), which constituted the basic dietary intake of the sheep with clinical signs of zearalenosis (group I). The lowest concentration of the parent form of ZEA (3.07 µg/kg) and the highest concentration of its metabolite (4.05 µg/kg) was found in the wheat pellets, which was only the supplemental feed in the control group (group II). However, in the sample of pressed oat neither the parent form nor its metabolite was detected. The serum concentrations of ZEA mycotoxin in affected sheep are shown in Table 2. The parent form of ZEA was found in all of the serum samples, its concentration ranged from 4.44 to 15.68 ng/mL, while α-zearalenol was found in six out of ten samples. In samples 1 and 2, its concentration was higher than that of the parent substance, at 13.76 and 7.64 ng/mL, respectively.

Concentrations of prostaglandin E₂ and F₃α in the serum of sheep with natural ZEA intoxication and in the control group are illustrated in Figs 1 and 2. The data in Fig. 1 show that in the group of affected animals (group I) the serum concentration of prostaglandin E₂ was significantly lower than in the control group (group II), for both individual sheep and in the case of group average. In the sick sheep, the extreme values for prostaglandin E₂ were 1,650 and 6,600 pg/mL, with an average of 4,410 pg/mL for the group, while in the control group it ranged from 3,500 to 9,100 with an average of 6,705 pg/mL. Concentration of prostaglandin F₃α was significantly (P≤0.01) higher in the sick sheep (group I) than in the control group (Fig. 2). In intoxicated sheep, it ranged from 825 do 6,240 pg/mL, with an average of 2,455 pg/mL for the group, while in the control group, the extreme values were 100 and 500 pg/mL with an average of 317 pg/mL.

Serum concentrations of leukotriene B₄ and thromboxane B₂ in sheep with ZEA mycotoxicosis and in the control group are illustrated in Figs 3 and 4. The data in Fig. 3 show that the serum level of leukotriene B₄ was significantly (P≤0.01) lower in the experimental group (group I) than in the control group (group II), for both individual sheep and in the case of the group average. In the sick sheep, the leukotriene B₄ concentration ranged from 370 to 1,940 pg/mL with a group average of 1,155 pg/mL, while in the control group, the extreme values were 1,360 and 4,140 pg/mL and the group average was 2,750 pg/mL. The thromboxane B₂ concentration, on the other hand, was significantly higher in the sick sheep than in the controls (Fig. 4), for both individual sheep and in the case of the group average. In the intoxicated sheep, the extreme values were 12,600 and 72,000 pg/mL with a group average of 35,530 pg/mL, while in the control group the concentration ranged from 2,360 to 7,000 pg/mL with an average of 4,710 pg/mL.

Discussion

ZEA binds with type α- and β-oestrogen receptors, initiating a mixed agonist-antagonist interactions, which induces the syndrome known as hyperoestrogenism in animals. In ruminants, ZEA is metabolised to α-zearalenol mainly by rumen protozoa and enterocytes, and by the liver in further stages of its metabolism. α-zearalenol has 3-4-fold higher affinity for oestrogen receptors and is more hormonally active than the parent substance (4, 14, 17, 20, 24).
Fig. 1. Serum concentration of prostaglandin E\(_2\) (PGE\(_2\)) in sheep with ZEA mycotoxicosis and in the control group (\(\alpha \pm SD\)).

* P≤0.05 in comparison of mean values of experimental group with controls.

Fig. 2. Serum concentration of prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) in sheep with ZEA mycotoxicosis and in the control group (\(\alpha \pm SD\)).

** P≤0.01 in comparison of mean values of experimental group with controls.
Fig. 3. Serum concentration of leukotriene B₄ (LTB₄) in sheep with ZEA mycotoxicosis and in the controls (±SD).

** P≤0.01 in comparison of mean values of experimental group with controls.

Fig. 4. Serum concentration of thromboxane B₂ (TXB₂) in sheep with ZEA mycotoxicosis and in the controls (±SD).

** P≤0.01 in comparison of mean values of experimental group with controls.
In the present study, high concentrations of α-zearalenol (4.16-13.76 ng/mL) were noted in six out of ten sheep from group I (Table 2), which could have been the reason for the more rapid progression of the disease. An excess of oestrogen-like substances was a factor initiating inflammatory states of varying intensity in the digestive tract and disturbances in local blood circulation. Moreover, changes noted in the mucous membrane of the terminal end of the digestive tract during the course of natural ZEA intoxication in sheep may indirectly indicate stimulation of oestrogen receptors and their effect on the development of clinical symptoms (2, 4, 6, 9, 13, 14, 24). ZEA, due to the presence of a phenolic ring in its chemical structure, can also bind to cellular oestrogen receptors in the duodenum and large intestine, and after forming a complex with the receptor it initiates conformational changes in it, which lead to the binding of ERE (oestrogen-responsive elements) to DNA. This induces or inhibits transcription of oestrogen-sensitive genes. Stimulation of type β-oestrogen receptors contributes to changes in the (negative) metabolic profile of cells sensitive to the mycotoxin, causing their degeneration or substantial atrophy (1, 4, 14).

Eicosanoids affect cellular functions, because of the presence of specific receptors for them. They exhibit a broad spectrum of biological activity, playing a key role in the regulation of inflammatory processes, including contraction and relaxation of blood vessels, platelet aggregation and chemotraction of neutrophils, as well as in fibroblast biology and collagen production. Without doubt, the most important process modulated by these compounds is the inflammatory reaction, which causes many clinical symptoms accompanying various diseases (8, 10, 19, 21, 23). No studies were found in the available literature concerning metabolism of arachidonic acid during the course of natural ZEA mycotoxicosis in sheep, so it is difficult to compare the results of the present study with those of other researches. The data obtained indicate that increased metabolism of arachidonic acid in sheep with natural ZEA mycotoxicosis is mainly mediated by enzymes of the cyclooxygenase group, which catalyse its conversion to prostanoids, including prostaglandin, prostacyclin, and thromboxane. The observed significant increase in PGF$_2\alpha$ and TXB$_2$ in the serum of sick sheep, compared with the control group, constitutes a good evidence of this effect (Figs 3, 5). It appears that in the sheep with ZEA mycotoxicosis with intensification of clinical symptoms involving the digestive tract, the main source of cellular mediators were neutrophils infiltrating the focus of inflammation, which upon stimulation exhibit expression of enzymes necessary for their synthesis. In the focus of inflammation, PGF$_2\alpha$ can be produced by epithelial and endothelial cells during transcellular metabolism (11, 23), while leukocytes can utilise arachidonic acid released from epithelial cells as a substrate. Transcellular biosynthesis between epithelial or endothelial cells and leukocytes might have been the cause of overproduction of these mediators and led to excessive intensification of the inflammatory reaction in the terminal end of the digestive tract in the sick animals.

At the site of the inflammatory reaction, different groups of eicosanoids have an inhibitory or stimulatory effect on each other, and their diverse activity enables a temporary increase in the concentration of one or more active eicosanoids, as well as increased expression of their specific receptors (7, 10, 20). This was confirmed by the results of the present study, i.e. by the fact that the serum concentration of prostaglandin E$_2$ declined significantly (P≤0.01) in the affected sheep, while F$_2\alpha$ concentration increased in comparison with the controls, in the case of both individual sheep and group average (Figs 2, 3). Similarly, in the experimental group a significant (P≤0.01) decrease in leukotriene B$_4$ concentration was observed and an increase in that of thromboxane B$_2$ (Figs 4, 5). Because eicosanoids regulate the inflammatory reaction, collagen production and blood vessel functions, we assumed that imbalances between different classes of eicosanoids are fundamentally responsible for the development of clinical symptoms of ZEA intoxication in sheep, as well as for the course and outcome of the disease.

The results of the study indicate that PGF$_2\alpha$ concentration in the serum of the group I sheep was significantly lower, and that of PGF$_2\alpha$ higher than in control sheep. The obtained data indicate that one of the mediators of the inflammatory reaction during the course of ZEA mycotoxicosis in sheep is prostaglandin PGF$_2\alpha$. The sites of its excretion, and the effector cells of the reaction, are mainly macrophages and endothelial cells. The initially local inflammatory reaction mobilises defence mechanisms and exhibits activity that is beneficial to the system. However, its uncontrolled development leads to the acceleration of self-destructive processes. In the sheep with natural ZEA intoxication, there was also an increase in the production of thromboxane TXB$_2$, correlated with the serum level of SAA, which is an indicator of the intensity of the inflammatory process in the terminal end of the digestive tract in sick animals (15). Thromboxane caused platelet aggregation and vasoconstriction, which resulted in local ischemia and hypoxia in cells and tissues, leading to activation of immune inflammatory processes and necrosis. Moreover, acting on the lamina vascularis mucosae and excretory functions of the digestive tract it caused partial or complete prolapse of the anus and rectum, and later prolapse of the large intestine, which was the direct cause of death of the sheep. In contrast, concentration of leukotriene B$_4$ in the serum of sick sheep was significantly lower than in the controls and was negatively correlated with SAA level, which indicates that cyclooxygenases, generating and increasing the concentration of prostaglandin F$_2\alpha$ and thromboxane B$_2$, play the most important role in the development of inflammatory processes. Leukotriene B$_4$ does not appear to play a major role in the development of inflammatory reactions in ZEA intoxication because its concentration in the serum of the affected sheep was much lower than in the control group, and thus not sufficient to induce a strong biological effect in the form
of increased permeability of blood vessels and leukocyte chemotaxis (3, 12, 16).

To sum up, the role of eicosanoids in the development of inflammatory reactions in sheep during the course of natural ZEA intoxication appears to be irrefutable, while the mechanism of their activation and the source of their production are difficult to determine. Most likely, the production of these mediators is a process activated during many co-existing mechanisms. Research on the course of the inflammatory reaction and the role of molecular mediators will contribute to a better understanding of the pathogenesis of natural ZEA mycotoxicosis in sheep. As there is no causal treatment, research should seek possibilities for effective inhibition or weakening of the inflammatory process, including substances that block excessive eicosanoid metabolism or medications blocking specific receptors.

References