The effectiveness of a feed additive with galloisite in rat mycotoxicosis

Evgenya Tarasova, Lilia Matrosova, Gleb Kashevarov, Svetlana Tanaseva, Olga Ermolaeva, Anastasia Sofronova, Nailya Mishina, Lenar Valiullin, Rishat Mukhammadiev, and Eduard Semenov

Federal Center for Toxicological, Radiation and Biological Safety, 2 Nauchny Gorodok, Kazan, 420075, Russia

Abstract. The paper presents the results of assessing the effectiveness of a complex feed additive based on halloysite for experimental T-2, afla- and zearalenone toxicosis of white rats in terms of survival, changes in clinical condition, enzyme status of blood serum and spleen ultrastructure. The results presented in the article confirmed the danger of the combined effects of mycotoxins on the body of white rats. The presence of mycotoxins in the feed led to the death of 30.0% of rats with a change in the clinical condition of the surviving animals, a significant increase in the serum levels of aminotransferases, alkaline phosphatase, lactate dehydrogenase, creatine kinase and gamma-glutamyltransferase, as well as changes in the ultrastructure of the spleen. A normalizing effect of a food additive based on halloysite on the studied parameters in experimental combined mycotoxicosis was noted, which was manifested by 100% survival of rats, less pronounced changes in the enzyme status and a protective effect on the ultrastructure of the spleen. This makes it promising for further comprehensive research on productive animal species with the prospect of implementation in animal husbandry as an effective means of preventing combined mycotoxicoses.

1 Introduction

The growth of microscopic fungi and mycotoxin contamination are among the major problems facing agricultural production worldwide [1]. It is difficult to obtain feed completely free of mycotoxins due to their widespread presence in contaminated grain [2, 3]. For the feed industry, mold toxin contamination is a major concern due to a wide range of toxic effects including hepatotoxicity, immunotoxicity, genotoxicity and carcinogenicity [4–6].

A large number of mycotoxins with different physicochemical characteristics have been discovered. Among them, the most significant in terms of prevalence and toxicity are aflatoxin B1, zearalenone and T-2 toxin. Mycotoxin cross-contamination is a real threat as grains can be contaminated by a variety of microscopic fungi that are capable of producing a variety of mycotoxins. In the production of feed, mixing of different types of raw materials is usually used [7, 8].

One of the most toxic and widespread trichothecene mycotoxins is T-2 toxin. It causes a number of acute toxicological effects, including immunotoxicity, vomiting, anorexia, neuroendocrine disorders, growth retardation, etc. [9]. Reprotoxic and endocrine disorders...
caused by zearalenone are well known, including infertility, hormonal dysfunction and hyperplasia of the reproductive tract [10], as well as the hepatotoxic, hepatocarcinogenic and mutagenic properties of aflatoxin B1 [6, 11].

Understanding the underlying mechanisms of toxicity when feed is simultaneously contaminated with aflatoxin B1, zearalenone and T-2 toxins at high doses is necessary to overcome potential threats to farm animals and humans.

Research on the removal of mycotoxins from contaminated feed has recently focused on the degradation, inactivation or removal of mycotoxins using physical, chemical and biological methods.

The optimal strategy for reducing the impact of mycotoxins in livestock farming involves the utilization of specific materials that possess the ability to adsorb mycotoxins, thus restricting their bioavailability within the organism. The list of these substances includes inorganic (bentonites, zeolites, shungite, etc.) and organic adsorbents (for example, plant or yeast β-glucans). Of great interest as a mycotoxin adsorbent is a natural nanomaterial – halloysite, which has not previously been used abroad for combined mycotoxicoses. Halloysite, a two-layer aluminosilicate known for its characteristic hollow tubular shape, boasts nanoscale lumens, cost-effectiveness, and widespread availability. Its exceptional dye adsorption abilities make it a very promising candidate for mycotoxin adsorption [12].

Since a single detoxification method always has its pros and cons, complex-action drugs are of interest, especially in the case of joint contamination. Recognition of the toxicological interactions of mycotoxins, as well as the assessment of the hazards associated with the simultaneous presence of mycotoxins contaminating feed, has created a new serious problem that needs to be studied and new means of reducing the damage caused by mycotoxins, taking into account their combined effects, need to be developed.

Changes in serum levels are an indicator of the degree of liver damage and metabolic pathways. Biochemical parameters are a sensitive indicator of the toxic effects of mycotoxins on target organs and may change before the onset of major symptoms.

Mycotoxins are powerful inhibitors of protein synthesis, which can harm various parts of the immune system. Numerous researches have shown that substances of this class can also modulate immune function [13–15]. These researches demonstrate mycotoxins’ ability to inhibit or facilitate lymphocyte proliferation, cellular and humoral immunity – depending on differences in application conditions. These effects are manifested in the suppression of immunity and in the morphology of lymphoid organs [16]. Many studies have revealed the influence of mycotoxins on the morphology (in particular, the spleen) both at the macro and at the ultrastructural level - as shown in various animal species: calves, rats, mice, poultry [17-22].

Thus, the spleen was chosen for ultrastructural studies because it is a secondary (peripheral) lymphoid organ that is important in the occurrence of an inflammatory reaction and adaptive immunity, responding to mycotoxicosis with obvious morphological changes.

So, the purpose of the study was to examine effectiveness of a complex feed additive in experimental T-2, afla- and zearalenone toxicosis of white rats in terms of survival, changes in clinical condition, enzyme status of blood serum and spleen ultrastructure. The developed feed additive includes substances that have sorption activity (halloysite and β-glucans), as well as those that have hepatoprotective (milk thistle meal), antioxidant (methionine) and immunostimulating effects (β-glucans, milk thistle meal) [23-25].

2 Materials and methods

Groups of white rats weighing between 150 and 170 grams were carefully organized, with 10 animals assigned to each group. To reproduce combined mycotoxicosis, standard samples of aflatoxin B1, T-2 toxin and zearalenone (Sigma-aldrich) were used. Mycotoxins were
added to the feed (T-2 toxin - 5.0 mg/kg, aflatoxin B₁ - 2.5 mg/kg, zearalenone - 2.0 mg/kg of feed) by thorough mixing. Doses of mycotoxins were selected taking into account the reproduction of subacute combined mycotoxicosis with a pronounced clinical picture.

The experimental period lasted for 21 days. The rats were acclimated to laboratory conditions for two weeks.

Rats of the first group (biological control (BC)) received a basal diet free of mycotoxins; the second served as a toxic control ((TC) 3 mycotoxins were added to the feed at once). The rats of the third group were fed the main diet, to which 3 mycotoxins were added at once and an additional complex feed additive based on halloysite was added at the rate of 0.25% of the diet (TC+FA); fourth - the main diet mixed with 0.25% feed additive ((BC+FA) to assess harmlessness).

The basic ration (BR) consisted of complete feed "Chara" for conventional small laboratory rodents (mice, rats, hamsters), previously tested for mycotoxin content by enzyme immunoassay.

During the experiment, the effect of the mycotoxin complex on the survival and clinical condition of animals was studied. Mortality and clinical response were recorded daily.

Blood collection for laboratory tests was carried out on the 21st day of the experiment. Blood sampling was carried out after a 14-15 hour fasting period. Blood samples for biochemical studies were collected on day 21 into Lab-Vac vacuum tubes with a coagulation activator and gel. The study of biochemical parameters was carried out using a microlab 300 biochemical analyzer (Netherlands). Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl aminotransferase (GGT), lactate dehydrogenase (LDH), creatine kinase (CK) and alkaline phosphatase (ALP) were determined.

For ultrastructural studies of the spleen, pieces of tissue up to 1 mm³ in size were fixed in glutaraldehyde and processed using standard electron microscopic techniques (dehydration with ethyl alcohol and acetone) [26].

Semi-thin sections approximately 1.5 µm thick were obtained on an LKB – III 8800 ultramicrotome, stained with a 1% methylene blue solution and viewed in a PZO Biolar light microscope (Poland) in order to select an area for subsequent ultra-thin cutting. Ultra-thin sections with a thickness of 80 nm were obtained on an LKB-III 8800 ultramicrotome and placed on copper grids for electron microscopy with a polymer substrate. The sections were contrasted using the “drop method” in solutions of uranyl acetate (2 hours at 45 °C) and lead citrate (1.5 minutes at 25 °C).

Sections were viewed in the observation field of a JEM 100 CX-II transmission electron microscope at an accelerating voltage of 60-80 kV. Microphotographs were processed using the open source morphometric program FIJI/ImageJ [27].

Statistical treatment of the obtained data has been processed in the MS Excel and Statistica 6.0 software environments. To select a statistical test for intergroup comparison, samples were checked for compliance with normal distribution (Kolmogorov–Smirnov test) and for equality of variances. Based on the results, it was decided to use the nonparametric Mann–Whitney test for unrelated samples with p-value Bonferroni correction. The statistical significance level was set at $\alpha = 0.05$.

### 3 Results and discussion

The presence of mycotoxins in feed, during long-term feeding, led to significant pathological changes in the body of white rats.

The first clinical signs (depression of general condition, weakness, lethargy, revival only when feeding, decreased motor activity, impaired coordination of movements, disheveled hair, loss of shine of the coat, refusal to feed, increased water consumption) in the toxic
control group of white rats were observed on the fourth day introduction of mycotoxins, on the eighth day six individuals developed indigestion (diarrhea). Diarrhea was observed in all toxic control rats starting from the eighth day of the experiment.

When a feed additive was introduced into toxic food, clinical changes manifested themselves in some rats as lethargy.

An external examination of the dead rats revealed cyanosis of the mucous membranes of the oral cavity with areas of necrosis and contamination of the fur with fecal matter. There were no deaths of rats when the developed feed additive was introduced into either "clean" or experimentally contaminated food.

It was shown that in the toxic control group, the death rate of white rats was 30.0%. During the experiment, three rats died, one each on the fifth, seventh and sixteenth days.

The feed additive used in the experiment had a positive effect on the general condition of the rats, while safety in the third and fourth groups, respectively, was 100.0%.

A clinical examination alone is not enough to objectively show the severity of the disease, while biochemical analysis makes it possible to assess the functioning of internal organs, identify hidden disorders, as well as the degree of development of a particular pathological process, predict the course of the disease, and justify the therapy used at the organo-enzyme level.

With inflammatory lesions of the liver, a fair amount of enzymes of intracellular localization are released from its tissue. The study of ALT, AST, ALP, LDH, CK and GGT is of diagnostic importance.

The accumulation of lipid peroxidation products, superoxide anion radicals, glycosylated proteins and carbohydrates leads to the formation of oxidative stress and can cause cytolysis of hepatocytes, the main markers of which are liver enzymes - ALT, AST, ALP, LDH, GGT, CK.

High concentrations of these enzymes are a biomarker of liver damage and indicate impaired membrane integrity, bile duct obstruction, or kidney problems [28-30]. It has been shown that mycotoxins lead to liver dysfunction, with a significant increase in the level of liver enzymes in the blood serum [31-35].

Table 1 shows the enzymatic status of white rats with mixed mycotoxicosis and the use of the developed feed additive.

Table 1. Enzyme status of blood serum of white rats with mixed mycotoxicosis and the use of a complex feed additive based on halloysite.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>1 group BC</th>
<th>2 group TC</th>
<th>3 group TC+FA</th>
<th>4 group BC+FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, U/l</td>
<td>52.06±2.08</td>
<td>221.66±8.77***</td>
<td>69.00±2.11*</td>
<td>54.88±1.98</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>63.93±2.49</td>
<td>126.83±3.16***</td>
<td>77.80±3.49*</td>
<td>66.51±2.35</td>
</tr>
<tr>
<td>Ritis coefficient</td>
<td>1.24±0.07</td>
<td>0.58±0.03***</td>
<td>1.14±0.05</td>
<td>1.21±0.04</td>
</tr>
<tr>
<td>ALP, U/l</td>
<td>179.80±2.04</td>
<td>235.11±6.67***</td>
<td>202.29±6.47*</td>
<td>173.10±2.71</td>
</tr>
<tr>
<td>LDH, U/l</td>
<td>410.70±4.35</td>
<td>574.21±6.68***</td>
<td>458.70±5.31*</td>
<td>400.30±4.72</td>
</tr>
<tr>
<td>CK, U/l</td>
<td>153.40±5.25</td>
<td>202.76±4.03***</td>
<td>174.29±5.44*</td>
<td>145.20±4.15</td>
</tr>
<tr>
<td>GGT, U/l</td>
<td>1.60±0.06</td>
<td>1.98±0.09***</td>
<td>1.73±0.11</td>
<td>1.55±0.09</td>
</tr>
</tbody>
</table>

*p<0.05 differences were revealed in comparison with biological control; **p<0.01, differences were revealed in comparison with biological control; ***p<0.001 differences were revealed in comparison with biological control

A significant increase in the levels of AST (1.98 times), ALT (4.26 times) and ALP (30.76%) was observed in the second group, which is associated with the destruction of the structural integrity of hepatocytes.

Elevated AST levels are a response to the degree of hepatocyte damage, and increased ALT levels reflect the intensity of liver damage [36].
The use of a halloysite-based feed additive as a prophylactic agent reduced high levels of AST, ALT and ALP compared to the group receiving only mycotoxins, indicating the effectiveness of the developed feed additive in suppressing mycotoxin-induced damage to hepatocytes. Thus, the level of AST, ALT and ALP in the third group of rats was lower than that of the biological control by 1.22, 1.33 times and 12.51 % respectively.

The introduction of the studied feed additive with the main diet did not lead to changes in liver biomarkers, which indicates its safety.

Increased activity of these enzymes is a reliable marker of liver damage. But considering that AST, unlike ALT, is found in almost all organs in addition to the liver, the de Ritis coefficient has the greatest clinical significance. In the experiment, there was a tendency towards a decrease in the de Ritis coefficient by the 21st day of the experiment, which may be the result of pronounced destructive processes in hepatocytes. In the second and third groups, a decrease in the de Ritis coefficient was recorded by 53.23% (p <0.05) and by 8.06%, respectively.

Liver damage is indicated by the positive dynamics of LDH activity, which has a pronounced increase towards the end of the experiment. LDH activity in the second and third groups of white rats was higher than in the biological control by 39.81% (p<0.001) and 11.69% (p<0.05), respectively.

Creatine kinase is an enzyme found in various types of tissue in the body, especially muscle and the heart. If creatine kinase is present in the blood at elevated concentrations, this indicates damage to the cells that contain it. In the second and third groups, creatinase activity was higher than in animals from the biological control group by 32.18% (p<0.001) and 13.62% (p<0.005).

Gamma-glutamyltransferase is a microsomal enzyme involved in the metabolism of amino acids. It is found in many parenchymal organs; maximum GGT activity is observed in the kidneys, liver, and pancreas. Even minor deviations in the GGT indicator are almost 100% likely to indicate pathology. In white rats of the second and third groups, GGT activity was lower by 23.75% (p <0.01) and 8.13%, respectively.

**Fig. 1.** Ultrathin sections of the white pulp of the spleen of rats from different groups: 1 – 1 group (biological control (BC)): normal nucleus and mitochondria with an electron-dense matrix (indicated by a white arrow); 2–4 – toxic control group (TC): mitochondria with partially and completely lost cristae, electron-transparent matrix (2, 3); clearing of the cytoplasm is observed (3), expansion of the perinuclear space (3) and marginalization of chromatin in the nucleus (4) (pathological changes are indicated by black arrows); 5 – preventable group (TC+FA): cytoplasm of medium electron density, mitochondria of normal structure (indicated by a white arrow) and with a cleared matrix (indicated by a black arrow); 6 – group for assessing the safety of feed additives (BC+FA).
Also in the toxic control group, morphological changes in the white pulp of the spleen were observed at the ultrastructural level. Cellular structures show a picture generally consistent with that described by other authors in conditions of mycotoxicosis [18-20]. Despite the absence of nuclei with disrupted membranes, we observed mitochondria that had undergone cristae lysis, marginalization of nuclear chromatin, and expansion of the perinuclear space (Fig. 1).

Taking into account not only the cytoplasmic, but also the mitochondrial (AST) localization of enzymes, it can be assumed that the negative consequences of the metabolic transformations of mycotoxins are profound changes in hepatocytes, which can lead to the progression of liver failure. Our results on changes in enzyme status are consistent with data published by other authors [36-41].

Ultrastructural changes that characterized the spleen of animals in the TC group were less pronounced in the TC+FA (prevented group) and occurred sporadically.

Less pronounced changes in the enzyme status and ultrastructural organization of the white pulp of the spleen when adding a complex feed additive to a toxic diet indicate its protective effect against the cytotoxic activity of mycotoxins.

4 Conclusion

Despite many years of research and intervention at various levels, mold toxins still pose major problems in livestock production. As poor agricultural practices remain, the use of various methods to detoxify feeds containing mold toxins has become increasingly important to reduce health risks to humans and animals. Among the mycotoxins identified, the most common are T-2 toxin, aflatoxin B1 and zearalenone. Long-term intake of mycotoxins was accompanied by characteristic clinical signs of mycotoxicosis, death of animals, an increase in the level of serum enzymes and morphological changes in the white pulp of the spleen. The use of a developed food additive based on halloysite mitigated the negative effects of T-2 toxin, zearalenone, and aflatoxin B1. This was expressed in the biochemical parameters of blood serum and the ultrastructural organization of the spleen of white rats. The use of a feed additive with halloysite in experimental combined mycotoxicosis of white rats reduced high levels of liver enzymes in the blood serum. Less pronounced morphological changes in the white pulp were recorded. Also, the introduction of the feed additive itself did not have a negative impact on the animals. The effectiveness of the prophylactic agent is achieved by the versatile nature of the action of the incoming components. The data obtained confirm the effectiveness and prospects of its further comprehensive study as a means of preventing combined mycotoxicosis.

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