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# MANIFESTATIONS OF CHRONIC FEED MYCOTOXICOSIS IN LABORATORY RATS UNDER EXPERIMENTAL CONDITIONS

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## Abstract

Mycotoxicoses are specific human and animal diseases caused by the certain types of microspores fungi that during their life form toxic substances, the mycotoxins. Toxic effects of these diseases are diverse and depend on the dose of toxin, exposure, animal species, age and sex. Lack of data on physiological mechanism of the pathological influence under combined mycotoxicoses significantly reduces the development of new drugs and methods for treatment of animal mycotoxicoses. This paper is our first report on the features of the clinical, biochemical, and pathomorphological signs of associative mycotoxicoses under experimental exposure of laboratory animals to the most common mycotoxins. The aim of the research was studying clinical signs of chronic combined mycotoxicosis and pathoanatomical changes in organs and tissues under experimental mycotoxins in laboratory rats, as well as the characteristics of intoxication symptoms, the effect of mycotoxins on the reproductive function of rats and their progeny, and morphobiochemical blood parameters. Experiments were carried out on 66 white non-linear rats of both sexes with a body weight of 80-120 g (Krasnodar Research Veterinary Institute vivarium, 2018). After 14-day acclimatization (quarantine) the rats matching experimental conditions were divided into two groups, 33 animals each, according to paired analogue criterium. The experimental rats were fed for 21 days with the feed naturally contaminated by mycotoxins. The control rats ate toxin-free feed. The spore counts in the contaminated feed was $5.7\times10^4$ for *Fusarium* sp., $1.2\times10^4$ for *Mucor* sp., and $2.0\times10^4$ for *Penicillum* sp. Mycotoxin concentration in the samples exceeded the maximum permissible level (3.6 mg/kg for zearalenone, 0.2 mg/kg for ochratoxin A, 6.2 mg/kg for fumonisin $B_1$), which was confirmed by bioassay on laboratory animals (mice). During the experimental period, all animals were clinically monitored for general status, feed consumption, behavior, response to external stimuli, motor activity, skin and fur condition, tactile sensitivity, functions of the digestive and urinary organs, corneal and dermal reflexes, and dynamics of weight gain. Hematologic blood tests were also performed. At the end of the study, three rats were euthanized in the experimental group and three rats in control group to identify pathologic and anatomical changes. It was determined that toxic feed leads to a 21 % decrease in body weight compared to the control, and also negatively impacts upon ontogenesis and reproduction causing a higher number of stillborn offspring and lower body weight and vitality of the newborn rats. In the blood of experimental animals the number of erythrocytes decreases by 17.0 % ($p \le 0.05$), hemoglobin by 13.0 % ($p \le 0.05$), total protein by 23.7 % ($p \le 0.01$), glucose by 22.7 %, cholesterol by 28.9 %, and triglycerides by 22.7 % ($p \le 0.05$). Reactive leukocytosis developed as a response of leukopoiesis to intoxication and a possible allergic process. Activity of blood alanine and aspartate aminotransferases was 40.5 and 61.3 % ($p \le 0.01$) higher, respectively, compared to the control rats. We also revealed exudative hemorrhagic inflammation of the mucous membrane of the stomach, thin and thick intestine, swelling and plethora of the lungs, enlarged liver, kidneys and heart. Histological examination of liver tissues showed a decrease in the amount of glycogen in hepatocytes, as well as the areas with granular and fatty dystrophy, vacuolization of hepatocytes, proliferation of the bile ducts, which may indicate severe destructive and necrotic processes. In the kidneys, a granular and fatty dystrophy of the convoluted tubule epithelium, desquamation of epithelial cells and proliferation occur, in the heart there are changes in the transverse striations of fibers, problems with blood circulation in the heart muscle and endocardial thickening. A depletion of lymphoid elements and a decrease in the lymphatic follicles (malpighian bodies) are characteristic

of spleen. Proliferation and mitosis of muscle cells are found in the uterus. Thus, the combined mycotoxicosis deeply violates the homeostasis of laboratory animals and leads to multiple pathological changes in the organs and systems of the body.

Keywords: mycotoxins, biochemical indicators of blood, laboratory rats, pathological anatomical studies, organs

The productive and physiological health of farm animals largely depends on the composition and quality of the consumed feed rations, which is also determined by the content of mold fungi and mycotoxins [1-4].

Mold fungi use for growth most of the constituent elements of grain, which leads to significant losses of nutrient and biologically active substances. The color, smell, and taste of the grain changes. However, the accumulation of highly toxic metabolites of microsporic fungi, the mycotoxins, is even more dangerous, of which trichothecene mycotoxins (T-2 toxin, deoxynivalenol – DON, zearalenone), aflatoxin, ochratoxin, and sterigmatocystin are the most common [5, 6]. The list of mycotoxins continues to expand; to date, about 350 species of toxin-forming fungi (14 genera) and more than 520 mycotoxins that are dangerous to humans and animals have been identified [7-9].

It has been reliably established that the consumption of feed containing mycotoxins causes a decrease in productivity (and, as a result, a decrease in live weight gain of young animals), overspending of feed per unit of production, and deterioration in product quality [10-12]. Moreover, mycotoxins are detected not only in feed for farm animals and poultry, but can also get into food products that have undergone technological processing, which may lead to the development of a number of human diseases, including oncological ones. This has attracted attention to obtaining biologically complete and harmless livestock products from many transnational communities, i.e. the World Health Organization, Food and Agriculture Organization, United Nations Environment Programme, and International Agency for Research on Cancer [13-15]. With the simultaneous intake of two or more mycotoxins, or their combinations with toxic pollutants (pesticides, dioxins, heavy metals), the mycotoxicological danger increases by many times, which can not only significantly increase the toxicity of the metabolic byproducts of microsporic fungi, but also has a significant negative effect on the health of animals [16, 17].

The action of mycotoxins on the body and the severity of the pathological process depend on many factors, which include doses, the duration of toxins in the body, the animal species, gender and age. However, in all cases, damage to vital organs and body systems occurs [18, 19]. At the same time, the pathological effect of combined mycotoxicoses on physiological systems and organs, as well as the mechanism of such an effect, is still not well understood, which reduces the possibility of developing drugs and methods of treating animals. The increase in export and import of grain between countries and the gradual climate change in the world contributes to a significant increase in the widespread prevalence of feed crops with various mycotoxins, which can lead to uncontrolled contamination of feeds with toxic metabolites of fungi [20-22].

In the case of the combined effect of mycotoxins, in which their combined effect on the organism increases dramatically, it is quite difficult to assess the severity of the pathological development of mycotoxicosis. It depends not only on the association of individual mycotoxins, but also on their concentrations, which raises questions of monitoring the clinical picture of mycotoxicoses in animals at one of the first places in terms of the relevance of research programs on mycotoxicology [23-25].

In this work, for the first time, the clinical, biochemical and pathomorphological manifestations of associative mycotoxicoses were revealed during ex-

perimental exposure laboratory animals to the most common mycotoxins.

The goal was to study the clinical picture of chronic combined mycotoxicosis and pathoanatomical changes in organs and tissues, damaged by mycotoxins, as well as the peculiarities of intoxication symptoms in laboratory rats, the effect of mycotoxins on the reproductive function of animals, offspring obtained from them, as well as morpho-biochemical parameters of the blood.

*Techniques.* An experimental chronic associative mycotoxicosis was simulated on 66 white non-linear rats of both sexes with a body weight of 80-120 g, divided into two groups of 33 animals each (15 females and 18 males) (stationary conditions of the Krasnodar Research Veterinary Institute vivarium, 2018). For the experiment, clinically healthy animals were selected that had a smooth, shiny fur, pale pink color in visible mucous membranes, and a good appetite. The duration of the quarantine (acclimatization period) was 14 days. They were fed at a fixed time with a full standard diet in accordance with established standards. Access to water was not limited.

During 21 days, the experimental group of rats received feed, naturally contaminated with mycotoxins, the control group received high-quality feed. In both groups, water was given ad libitum. From the date of replanting males into groups when pregnancy was detected, the effect of toxic feed on embryonic development and generative function of animals was determined.

In the process of mycological, toxico-biological and enzyme-linked immunosorbent assay of samples taken in accordance with the regulations for the selection and transportation of feed for sanitary-hygienic and chemical-toxicological studies (as per GOST 13586.3-83), the content of fungi spores was determined. During the experimental period, all animals underwent clinical control according to the following criteria: general condition, feed intake, behavior, reaction to external stimuli, nature of motor activity, condition of the skin and fur, tactile sensitivity, functions of the digestive and urinary organs, corneal and dermal reflexes, and dynamics of bodyweight gain.

Blood for research was taken from five rats from each group at the end of the experimental period directly from the heart under ether anesthesia. Hematological blood tests were performed on an automatic hematological analyzer for in vitro diagnostics Mythic18 (C2 DIAGNOSTICS S.A., Switzerland/France), biochemical tests were performed on an automatic biochemical analyzer Vitalab Flexor Junior (Vital Scientific N.V., Netherlands) using kits of the company ELITech Clinical Systems (France).

At the end of the study, three rats were killed in the experimental and control groups using ether anesthesia (following the principles of bioethics) to identify pathoanatomical changes. The effect of mycotoxins on the macro- and microstructure of the internal organs of white rats was evaluated by post-mortem examination of the animals with complete removal of the internal organs. The material was fixed in 10% neutral formalin; the diagnosis was conducted by methods generally accepted in pathomorphology [26]. The samples were stained with hematoxylin and eosin. For microphotography, an MS-300 microscope (Micros, Austria) and a digital 10-megapixel camera Digital IXUS 970 IS (Canon, Inc., Japan) were used; magnification ×150 (ocular ×15, lens ×10); ×300 (ocular ×15, × lens 20) and ×600 (ocular ×15, lens ×40).

The results were processed using the software package Statistica 6.0 (StatSoft, Inc., USA). The data were presented as mean ($M$) and standard error of the mean (±SEM). The significance of differences between the series was determined using Student's $t$-test.

*Results.* The contamination with fungal spores of the feed, which was given to the animals of the experimental group, exceeded the maximum permis-

sible level (MPL) (5.7×104 per 1 g feed), that is, the feed was found to be toxic. The number of spores was $2.5\times10^4$ for *Fusarium* sp., $1.2\times10^4$ for *Mucor* sp., and $2.0\times10^4$ for *Penicillum* sp. The mycotoxins in the sample (zearalenone — 3.6 mg/kg, ochratoxin A — 0.2 mg/kg, fumonisin $B_1$ — 6.2 mg/kg) also exceeded the MPLs, which was confirmed by bioassay in laboratory animals (mice).

The first signs of intoxication with mycotoxins in rats from the experimental group were already recorded on day 5 to day 7 of the experiment. This was manifested by excessive timidity with a simultaneous increase in excitability against together with a decrease in spontaneous motor activity and the development of adynamia. The fur was ruffled, not shiny, with areas of loss and contamination of wool and alopecia. There was an increase in thirst with a decrease in appetite, which led to a noticeable growth lag by the end of the first stage of the experiment (Table 1).

**1. Live weight of non-linear white rats fed with feed contaminated with spores and metabolites of microscopic fungi** ($M$±SEM, $n$ = 33)

| Group | Body weight, g | | Average daily gain, g | To the control, % |
|---|---|---|---|---|
| | initial | final | | |
| Control | 106.1±2.38 | 131.7±3.12 | 1.22±0.03 | 100 |
| Test | 103.8±2.33 | 124.2±2.94 | 0.97±0.04* | 79.5 |

N o t e. For a description of the groups, see the Techniques section.П р и м е ч а н и е. Описание групп см. в разделе «Методика».

* Differences with control are statistically significant at $p \le 0.05$.

The average daily weight gain in rats of the experimental group was lower if compared to the control. The excess of this indicator in control analogs was 21% ($p \le 0.05$). In absolute units, the average body weight of the control animals exceeded that of rats from the experimental group by 5.2 g.

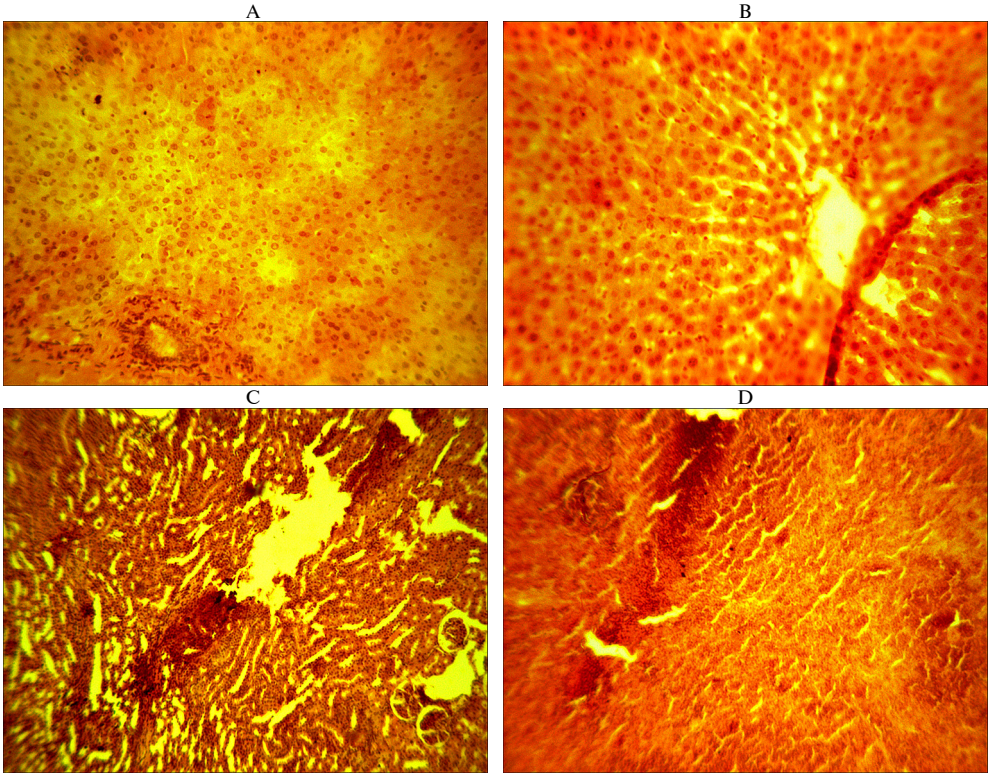
Long-term consumption of toxic feed had a negative effect on gynogenesis and animal development. By the end of the studies, 6 pregnant females were detected in the experimental group, and 9 ones in the control group. Although in rats receiving feed affected by mycotoxins, the pregnancy ended in 23-24 days with natural birth and did not differ in duration from pregnancy in control females, the number of newborn rats in such animals was insignificant (1-2 rats per a female). At the same time, out of the total number of young rats that were born to females of the experimental group (10 animals), two were stillborn, three more were not viable and died during the first 2 days. However, deformities and anomalies in their development were not noted. The average weight of young rats in the experimental group was 2.9±0.2 g. In the control group, 29 rats were born with an average weight of 3.5±0.3 g, of which two were non-viable.

**2. Morphological and biochemical blood parameters in non-linear white rats fed with feed contaminated with spores and metabolites of microscopic fungi** ($M$±SEM, $n$ = 5)

| Indicator | Group | |
|---|---|---|
| | test | control |
| Erythrocytes, $\times10^{12}$/l | 5.4±0.23*** | 6.3±0.31 |
| Leukocytes, $\times10^9$/l | 9.8±0.57** | 8.07±0.29 |
| Hemoglobin, g/l | 113±3.74*** | 127.3±4.25 |
| Total protein, g/l | 64.6±4.33** | 79.9±2.94 |
| Aspartate aminotransferase, units/l | 150.7±6.41* | 93.4±3.06 |
| Alanine aminotransferase, units/l | 97.8±3.47* | 69.6±6.13 |
| Alkaline phosphatase, units/l | 621.5±13.07 | 547.3±21.84 |
| Glucose, mmol/l | 6.6±0.42 | 8.1±0.63 |
| Urea, mmol/l | 8.4±0.31 | 7.5±0.47 |
| Creatinine, μmol/l | 28.3±1.15 | 25.3±1.1 |
| Cholesterol, mmol/l | 1.76±0.05* | 2.27±0.14 |
| Triglycerides, mmol/l | 0.44±0.03*** | 0.54±0.03 |

*, ** and *** Differences with control are statistically significant at $p \le 0.001$; $p \le 0.01$ and $p \le 0.05$.

The intoxication was assessed by the morpho-biochemical profile of the blood of experimental animals (Table 2). In rats from the experimental group, a decrease in hematological parameters was found. By day 21, there was a decrease in the number of erythrocytes by 17.0% ($p \leq 0.05$), in hemoglobin by 13.0% ($p \leq 0.05$) and an increase in leukocytes by 21.4% ($p \leq 0.05$) compared to the control. Therefore, it may indicate the inhibition of erythro- and hematopoiesis as a result of long intoxication. The presence of reactive leukocytosis in the blood, which occurs as a response of leukopoiesis to intoxication and a possible allergic process, confirms this assumption.




**Granular dystrophy** (A) **and areas of fatty degeneration of the liver** (B)**, proliferation in the kidneys** (C) **and spleen** (D) **in non-linear white rats with the consumption of feed contaminated with spores and metabolites of microscopic fungi.** Light microscopy (MS-300, Micros, Austria), staining with hematoxylin and eosin, magnification ×150 (A), ×300 (B, D) and ×600 (C).

At the end of the study, during the external examination in rats of the experimental group, we noted cyanosis of visible mucous membranes, rumpledness and dullness of the fur. A visual examination of the internal organs of rats from the experimental and control groups did not reveal any abnormalities in their location and structure. However, pathological changes were revealed in rats of the experimental group as hemorrhagic inflammation of the gastric mucosa, thin and thick intestines, swelling and pulmonary congestion. The liver was dark red, flabby, enlarged, in some places there were grayish areas of necrosis, the gall bladder was full. The heart was enlarged, with foci of micronecrosis, the heart muscle was flabby. The kidneys were enlarged, pink-gray, the vulva of the females was swollen, the testes of the males were enlarged.

During histological examination (Fig.), the most characteristic changes were found in liver tissues: the amount of glycogen in hepatocytes decreased, there were areas with granular and fatty degeneration in the cytoplasm, hepatocyte vacuolization and proliferation in the bile ducts were visible. In the hepatic

lobules, the radial arrangement of the blocks was disturbed due to the rounding of hepatocytes. All this indicated the presence of severe destructive and necrotic processes in the liver. Granular and fatty degeneration of the convoluted tubule epithelium, desquamation of epithelial cells, and proliferation were observed in the kidneys, in the heart there were changes in the transverse striation of the fibers, impaired blood circulation in the heart muscle, and thickening of the endocardium. In the spleen, depletion of lymphoid elements and a decrease in the Malpighian layer occurred. Proliferation and mitosis of muscle cells were noted in the uterus.

In the study, we found high contamination of the feed used in the experiment with spores of fungi producing the main types of mycotoxins — zearalenone, ochratoxin A, fumonisin $B_1$, which are classified as potent and highly toxic compounds. Such a combination leads to an increase in the synergistic effect of mycotoxins on the organism, as a result of which the clinical signs of toxicosis were noted in rats already during week 1 of the experiment. Further consumption of toxic feed increased the negative effect of mycotoxins and led to serious physiological changes and pathological processes in the reproductive organs. In our opinion, such manifestations are associated with zearalenone which is 1.8 times higher than the maximum permissible level. It was established that this mycotoxin can cause infertility, abortion and cyst formation in animals. The negative effect of the association of zearalenone, ochratoxin A, and fumonisin $B_1$ on the conception in rats and the embryonic development of the offspring, which was found experimentally, is consistent with the established properties of T-2 toxin and DON to induce apoptosis in animals' embryos, including poultry [27].

The presence of high doses of ochratoxin A (4 times higher than the maximum permissible level. MPL) in the feed leads to inhibition of hemo- and leukopoiesis, synthesis of protein and a number of enzymes, damage to the liver and kidneys, a decrease in live weight and growth retardation, and a combination of several mycotoxins enhances their joint pathological effect on the organism [28]. It was reported that experimental mycotoxicosis in laboratory mice, caused by compound feed contaminated with ochratoxin and T-2 toxin, was manifested by the following clinical signs: hyperemia of the visible mucous membranes, disturbance of the nervous system, impaired feed intake, decreased live weight, gastrointestinal tract damage, a change in the biochemical parameters of blood, i.e. a decrease in the content of amylase and cholesterol while increasing the amount of urea and creatinine [29].

A number of researchers note that when consuming feed contaminated with mycotoxins in vivo, the toxic effect is more pronounced than when an equivalent amount of pure mycotoxin was received in the experiment. In respect of T-2 toxin and aflatoxin, trichothecenes and fusaric acid, zearalenone and deoxynivalenol, there are data confirming the effects of their synergism [30-34].

When discussing the problem of chronic mycotoxicoses, it should be emphasized that the increased lipid peroxidation, observed during mycotoxicoses, leads to damage to the membranes of hepatocytes, inhibition of various liver functions, and ultimately to the development of hepatopathies [35-38]. Our research indicates that under combined natural contamination of feed with zearalenone, ochratoxin A and fumonisin $B_1$, pathological processes in the liver developed in accordance with fatty degeneration type.

Thus, in non-linear white rats, experimental combined mycotoxicosis was characterized by multiple toxic manifestations, i.e. excessive timidity, rumpledness and dullness of the fur, alopecia, increased thirst and decreased appe-

tite, lag in growth and development, negative impact on fertility of females and fetal development (a small number of newborn rats, high level of stillbirth and mortality, low body weight at birth). Morpho-biochemical blood parameters of rats with associative mycotoxicosis were lower in erythrocytes and hemoglobin levels (by 17.0 and 13.0% at $p \leq 0.05$ compared to animals from the control group) together with the development of reactive leukocytosis, hypoproteinemia and hypoglycemia, lipid metabolism disorders show a simultaneous increase in activity of liver transaminases (by 40.5% for alanine aminotransferase and by 61.3% for aspartate aminotransferase at $p \leq 0.01$). Histological examination of the liver of test animals revealed areas of granular and fatty degeneration, vacuolization and a decrease in the amount of glycogen in hepatocytes, as well as proliferation in the bile ducts. Granular and fatty dystrophy of the convoluted tubule epithelium, desquamation of epithelial cells, as well as proliferation occur in the kidneys, in the heart there are changes in the transverse striation of the fibers, impaired blood circulation in the heart muscle and the thickening of the endocardium. In the spleen, there is a depletion of lymphoid elements and a decrease in lymphatic follicles (Malpighian bodies), in the uterus the proliferation and karyokinesis of muscle cells occurs. The results of the research indicate deep violations of homeostasis in laboratory animals, as well as multiple pathological changes in organs and systems of the body caused by combined mycotoxicosis.

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