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Research of the Parameters of Acute Toxicity of the Veterinary Drug "Bovistem"

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Abstract

The creation of new effective drugs for the prevention and treatment of animal diseases is one of the most urgent tasks of veterinary pharmacy. A promising direction is the development of agents based on mesenchymal stem cells (MSCs) and a complex of biologically active substances secreted by them, which have a variety of pharmacological effects on the body. The study of the parameters of acute toxicity of potential drugs intended for medical use in veterinary medicine is a mandatory requirement when conducting preclinical studies of new drugs. The aim of our study was to study the parameters of acute toxicity of the new original drug "BoviStem" with a single injection and to identify target organs of potential toxicity of the drug. It was found that a single intramuscular and subcutaneous administration of the drug "BoviStem" to rats and mice did not have a toxic effect on the animal organism, as evidenced by a positive increase in body weight in both males and females. The study of the mass of internal organs and their histological structure (in rats) made it possible to establish sexual sensitivity to the drug and identify target organs, which should be taken into account when prescribing the drug in the clinic. In accordance with the classification of substances according to acute toxicity, the BoviStem preparation, with a single intramuscular and subcutaneous injection to rats and mice, belongs to the VI class of toxicity - "relatively harmless substances" (LD50 > 4.5 ml/kg when administered subcutaneously) and LD50 > 3 ml/kg (intramuscular injection).

Keywords: Polyvinyl Alcohol Foam; Fingerprint; Biomimetic; Bioinspired Design; Medical Device

Abbreviations: LLC: Limited Liability Company; MSC: Mesenchymal Stem Cells: ESIAF: Nuph: Educational and Scientific Institute of Applied Pharmacology of the National University of Pharmacy; RM: Relative Mass; I/M: Intramuscularly; S/C: Subcutaneously.

Introduction

The creation of new effective drugs for the prevention and treatment of animal diseases is one of the most pressing problems of veterinary pharmacy today. A promising

direction is the development of funds based on mesenchymal stem cells (MSC) and a complex of biologically active substances secreted by them, the so-called secretome [1].

Numerous studies have shown that the therapeutic efficacy of MSC is due to their ability to migrate into the damaged area and, through differentiation, compensate for the functional insufficiency of damaged cells [2]. They also have the ability to generate a reparative environment through the paracrine secretion of biologically active macromolecules (growth factors, morphogens, chemokines, cytokines, extracellular vesicles (eg, exosomes) and glycosaminoglycans). These trophic factors or MSC secretions contribute to the immunomodulation of immunocompetent cells (T-lymphocytes, macrophages, mast cells), as well as have antioxidant and antihypoxant effects, stimulate regeneration and affect the processes of apoptosis [1,3]. It should be noted that the secret of MSC is as effective as that of MSC, however, it has a number of advantages over stem cells: during long-term storage, it does not lose its effectiveness, when using it, it is easier to assess the effectiveness of the kinetics of the drug and the possibility of immune rejection, unwanted differentiation and oncogenesis, causes minimal cell transformation [4]. Therefore, the use of drugs that do not contain MSC, but have a similar effect due to the bioactive macromalecules that make up their composition, that is, their secretome, is an effective alternative to stem cells.

All of the above indicates the relevance of studies of the possibility of using pharmacological compositions based on MSC secretome as an effective drug [5-7].

One of these drugs is the original drug "BoviStem" developed by scientists of LLC "NoviStem", which is a protein-peptide complex of bioactive macromolecules. This complex is obtained from a conditioned medium during the cultivation of bovine mesenchymal stem cells (cattle) and has a wide spectrum of action.

One of the mandatory requirements when conducting preclinical studies is to study the parameters of acute toxicity of potential medicinal products intended for medical use in veterinary medicine.

The aim of the study was to investigate the parameters of acute toxicity of the new original drug "BoviStem" with a single injection and to identify target organs of potential toxicity and possible delayed toxicity of the drug.

Materials and Methods

Experimental Animals

White outbred mature rats (males, females) with an initial body weight of 230-250 g and mice (males, females) with an initial body weight of 25.0-27.0 g were used in the study. The average weight of animals in all groups did not differ by more than 10%. Before administration of BoviStem, rats were fasted for 12-14 hours and mice for 4 hours.

The animals were kept in the vivarium of the Educational and Scientific Institute of Applied Pharmacology of the National University of Pharmacy (ESIAF NUPh) in accordance with the current rules on devices, equipment and maintenance of vivarium. Animals received standard food in accordance with current regulations [8]. Mice and rats were housed 6 individuals in polycarbonate cages with individual ventilation of the cages. All cages were equipped with food and water facilities. Animals received daily norm of food without restriction, except for the night before weighing and euthanasia. For drinking, we used filtered tap water in standard drinking bottles with free access of animals to water. All manipulations with animals were carried out in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals used for research and other scientific purposes [9].

The Study of the Acute Toxicity

The study of the acute toxicity of the drug "Bovistem" was carried out in accordance with the methodological recommendations [10,11] with intramuscular (i/m) and subcutaneous (s/c) administration. The drug was administered at the maximum dose recommended for intramuscular injection - 25 ml/kg, for subcutaneous injection - 50 ml/kg. During the study, each animal was observed daily in the morning and afternoon. On the day of taking the drug, observation was carried out hourly. The general condition of the animals after administration of the drug was assessed by body weight (the initial weight was measured on days 3, 7 and 14 after administration), by the appearance and main physiological functions of the animals (coat condition, appetite, respiration, salivation, urination and defecation) [10,11]. Survival and clinical signs of a possible toxic effect of the drug were monitored for 14 days.

On the 15th day of the experiment, all animals were killed by inhalation of carbon dioxide (CO^2) followed by macroscopic and morphometric examination of organs. After registration of death, mice and rats were subjected to a complete autopsy, which included examination of the outer surface of the body, all passages, cranial, chest and abdominal cavities with organs and tissues. Lymph nodes, aorta, heart, larynx, trachea, lungs, thymus, esophagus, stomach, small intestine, large intestine, liver with gallbladder (in mice), pancreas, spleen, kidneys, adrenal glands, urinary bladder, ovaries are examined macroscopically uterus, vagina, submandibular salivary gland with lymph nodes, thyroid gland and brain.

After macroscopic examination, the internal organs were removed, weighed, and their relative mass (RM) was determined using the formula:
Relative mass of an organ, % = organ weight (g) / animal weight (g) × 100%.

The Histological Research

The removed internal organs of rats were fixed in a 10% formalin solution, dehydrated in alcohols of increasing strength, and embedded in paraffin. Sections from paraffin blocks 6-7 μm thick were obtained using a sled microtome CM-1, stained with hematoxylin and eosin [12]. The microscope slides were reviewed by a Granum microscope. Microscopic images were taken with a digital video camera Granum DCM 310. The photographs were processed on a Pentium 2.4 GHz computer using the Toup View software. The histological structure of the internal organs: liver, kidneys, cardiac muscle, adrenal glands, spleen, thymus, testicles/ovaries and the site of contact (injection) with the drag BoviStem – muscles and subcutaneous tissue was studied in comparison with the histological structure of similar organs of animals from the intact control group.

Statistical Analysis

Statistical data processing was performed using the STATISTICA for WINDOWS 6.0 software package. Quantitative indicators in the tables are given as the arithmetic mean and its standard error or the minimum and maximum values of the sample. The normal distribution of quantitative data was checked using the Shapiro-Wilk test and the Leuven test. If the data obtained corresponded to a normal distribution, ANOVA was used, and intergroup comparisons of indicators were performed using the Newman-Keuls test. If the obtained data did not correspond to the normal distribution, the general intergroup differences were assessed using the Kruskal-Wallis test. Paired intergroup comparisons of indicators were performed using the Mann-Whitney U-test. The critical value of the significance level was taken to be less than 0.05 ($p < 0.05$) [13].

Results And Discussion

Results of a Study of the Acute Toxicity of the Drug "Bovistem" In Rats

As our observations showed, a single injection of the drug "BoviStem" in doses of 50 ml / kg and 25 ml/kg, s/c and i/m, respectively, did not cause intoxication in animals. The reaction to sound and light stimuli, the processes of urination and defecation were normal. Respiratory disorders were not recorded, convulsions were absent. Observation of the animals for 14 days also did not reveal signs of the toxic effect of the BoviStem drug. The physiological state and behavior of the animals did not differ from the intact control group. No deaths of animals were noted for the entire observation period.

In accordance with the data obtained, the dynamics of body weight gain (Table 1) after administration of the drug "BoviStem" in both males and females did not statistically significantly differ from that in the group of intact animals, which confirms the absence of the toxic effect of the study drug on the body rats.

Terms of observation	Animal groups		
	Intact control group	Drug "BoviStem"	
		50 ml/кg (s/c)	25 ml/кg (i/m)
males			
original mass	239±10	233±5	233±6
3 days	239±10	233±3	231±6
7 days	240±10	234±4	226±5
14 days	248±10	237±4	227±5
females			
original mass	257±13	267±8	261±10
3 days	258±12	261±7	259±10
7 days	258±12	277±7	268±11
14 days	261±11	278±7	273±11

Note: n – number of animals in the each group.
Table 1: The results of the effect of the drug "BoviStem" on the dynamics of body weight (g) of rats (males, females) with a single (s/c, i/m) of administration, n= 6, (M ± m)

On dissection of rats, the internal organs of both males and females were located anatomically correctly. Macroscopic examination of the internal organs did not reveal any signs of pathological processes: in size, color and consistency they did not differ from the organs of intact animals. Natural passages were clear, visible mucous membranes without inflammation. The gastric mucosa had a characteristic fold relief, without hemorrhage, edema, and erosive lesions. The mucous membrane of various parts of the intestine without visible signs of irritation.

Indicators of the relative mass of the internal organs of rats are given in table 2. It was found that a single s/c and i/m administration of the drug "BoviStem" in doses of 50 ml/kg and 25 ml/kg, respectively, does not lead to statistically significant changes in the relative mass of internal organs rats (males and females) in comparison with similar indicators of animals from the intact control group, with the exception of a statistically significant increase in the mass of the lungs and testes in males and a decrease in the mass of the thymus in females with the i/m administration of the drug "Bovistem" (Table 2).

Indicators	Animal groups		
	Intact control	Drug "BoviStem»	
		50 ml/кg (s/c)	25 ml/кg (i/m)
males			
Liver	3,00 (2,77÷3,50)	2,82 (2,69÷3,02)	2,73 (1,98÷3,12)
Kidneys	0,59 (0,55÷0,62)	0,62 (0,58÷0,66)	0,60 (0,55÷0,65)
Heart	0,31 (0,27÷0,34)	0,34 (0,32÷0,36)	0,32 (0,26÷0,37)
Lungs	0,57 (0,50÷0,67)	0,65 (0,48÷0,78)	0,72 (0,63÷0,89) *
Spleen	0,30 (0,26÷0,35)	0,31 (0,27÷0,37)	0,29 (0,18÷0,44)
Adrenal glands	0,022 (0,017÷0,029)	0,023 (0,016÷0,032)	0,020 (0,017÷0,022)
Thymus	0,090 (0,032÷0,130)	0,092 (0,008÷0,129)	0,086 (0,037÷0,117)
Testicles	1,24 (1,15÷1,35)	1,26 (1,15÷1,33)	1,46 (1,20÷1,63)*
females			
Liver	2,65 (2,47÷2,95)	2,98 (2,29÷3,44)	2,92 (2,58÷3,28)
Kidneys	0,58 (0,48÷0,74)	0,51 (0,36÷0,60)	0,53 (0,36÷0,65)
Heart	0,39 (0,27÷0,70)	0,31 (0,24÷0,36)	0,33 (0,28÷0,38)
Lungs	0,86 (0,64÷1,11)	0,81 (0,66÷1,20)	0,85 (0,56÷1,50)
Spleen	0,33 (0,25÷0,46)	0,37 (0,26÷0,59)	0,35 (0,28÷0,39)
Adrenal glands	0,029 (0,020÷0,036)	0,025 (0,018÷0,035)	0,025 (0,013÷0,030)
Thymus	0,130 (0,070÷0,181)	0,108 (0,069÷0,133)	0,066(0,041÷0,089)*

Note: 1. * – - the differences are statistically significant when compared with the intact control group (Mann-Whitney test);
2. n – number of animals in the group.

Table 2: Influence of the drug "BoviStem" on the relative mass (%) of internal organs of male and female rats, n=6 ($\tilde{O}(X_{\min} \div X_{\max})$

Comparison of the obtained data with the literature data [12] allows us to conclude that the deviations in the mass of the lungs and testes in males are within the physiological norms. However, in females, the weight of the thymus was statistically significantly reduced by 2 times with the intramuscular injection of BoviStem at a dose of 25 ml/kg. Analyzing the data obtained in this study, it can be assumed that the high bioavailability with the i/m administration of the drug, which is a complex of biologically active macromolecules (various growth factors, etc.), leads to excessive stimulation of the thymus in response to administration. This leads to active formation, differentiation of T-lymphocytes, their migration into the blood and, as a consequence, a decrease in the mass of the thymus, which in female rats reaches a statistically significant level. The results obtained allow us to speak about the presence of sex differences and the greater sensitivity of females to the action of this drug, as less emotionally stable and more sensitive to stressful influences (such as the introduction of the drug in large doses) compared to males.

Microscopic Examination

Liver: In intact rats, both male and female, the structural organization of the hepatic parenchyma is typical for this animal species. The pattern of the lobules is blurred, their boundaries were determined by the zones of triads (portal tracts). The stromal connective tissue in the zones of the triads is poorly represented. In these zones, as in the parenchyma of the lobules, no cellular infiltration was observed. The radial orientation of the hepatic plates (beams) is not disturbed. Hepatocytes had a characteristic shape and size, rather clear cell boundaries. The centrally located cell nuclei varied moderately in size and contained 1-2 nucleoli. The pool of binucleated cells was visually within the normal range. Sinusoidal hemocapillaries, vessels of portal tracts and central veins of normal blood filling. The state of the Kupffer cells without features (Figure 1A).

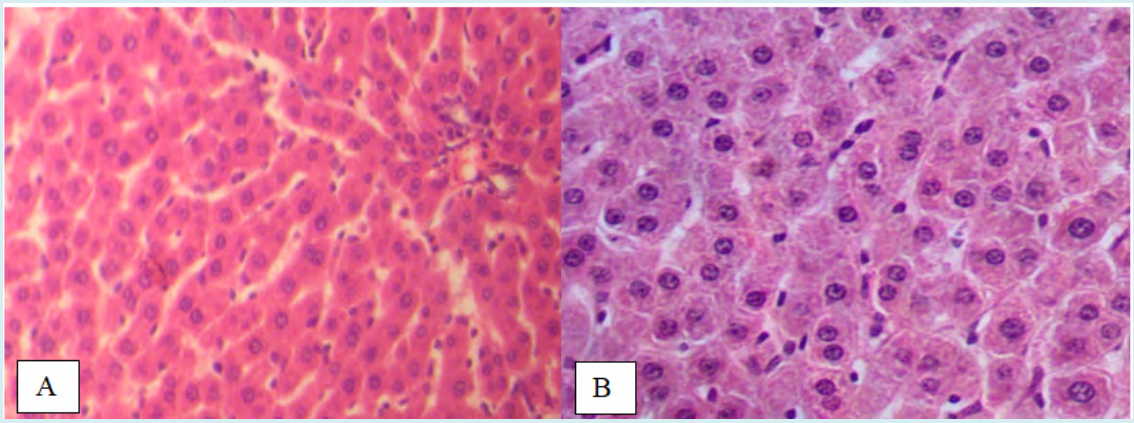

Figure 1: Rat liver fragment: A – intact rat. The normal pattern of the hepatic corpuscles, the state of the sinusoidal capillaries, the triad zones are unchanged. Staining with hematoxylin and eosin x 250. B - after subcutaneous administration of "BoviStem". Moderate proliferation and activation of Kupffer cells. Hematoxylin and eosin stainin x 400.

On the 14th day after a single subcutaneous injection of the drug "BoviStem" in all males and in 3 females of 6 rats in the parenchyma of the hepatic lobules, round-cell infiltration of a greater / lesser part of the portal tracts of different intensity was revealed. In one of the males, along with infiltration, there was a pronounced expansion and plethora of sinusoidal hemocapillaries, an uneven content of lymphoid cells in them. In almost all animals, anisonucleosis, more pronounced than in intact animals, is noticeable - fluctuations in the size of hepatocyte nuclei (Figure 2) and moderate proliferation of Kupffer cells (Figure 1B), which indicates the activation of adaptation processes in response to an excessive dose of BoviStem ".

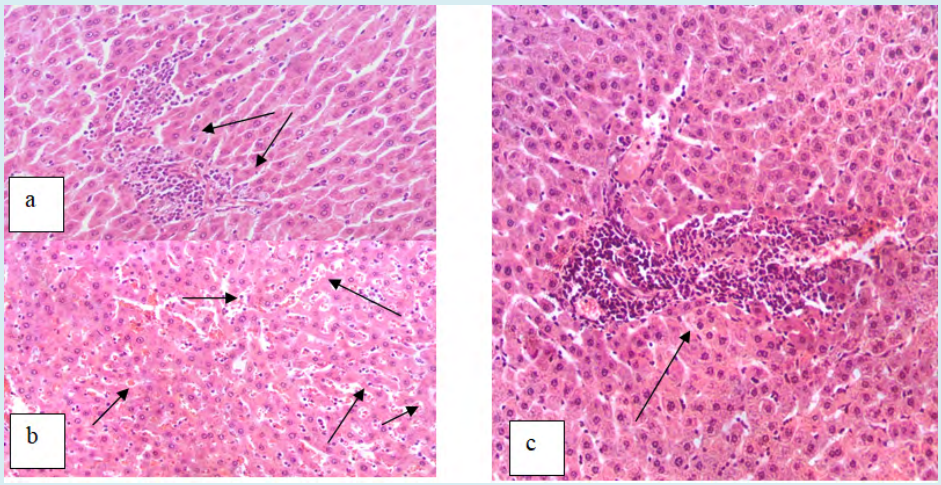

Figure 2: Fragments of the liver of male (a-b) and female (c) rats after subcutaneous injection of the drug "BoviStem". Round-cell infiltration of the portal tracts (a, c), marked plethora of sinusoidal capillaries (b). Hematoxylin and eosin staining. X 200.

In all animals, incidental involution of the thymus was revealed - inversion of the medula and cortex of the organ was observed, which is a manifestation of adaptive processes in response to stressful exposure - the introduction of the study drug (Figures 3,4). However, these changes are reversible. In the medulla and cortical layers of the thymus, areas of lymphocyte death were visualized, which was light-optically manifested as a "picture of the starry sky." It should be noted that the established accidental involution of the thymus had hemodynamic consequences (a decrease in the relative weight of the thymus) only in females after i/m administration of BoviStem, which indicates the effect of gender and route of administration on the sensitivity of animals to the studied drug.

According to the results of histological examination, it can be concluded that on the 15th day after taking the drug "BoviStem" in toxic doses, changes in the structure of the heart muscle, lung, kidneys, adrenal glands, gonads, spleen are noticeable at the light-optical level were not detected in comparison with the intact control. Periportal focal clusters of round cells, similar to lymphocytes, found in rat liver after subcutaneous administration of "BoviStem", are apparently associated with resorption or excretion of metabolic products and are a reflection of immunological reactions in response to drug administration.

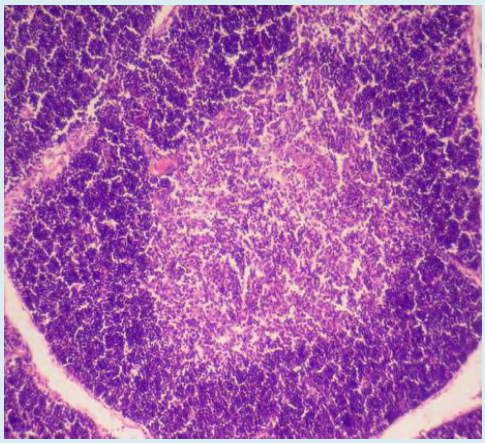

Figure 3: Thymus of an intact rat. A clear division of the glandular tissue of the lobule into bark and medulla. Hematoxylin and eosin staining. x200.

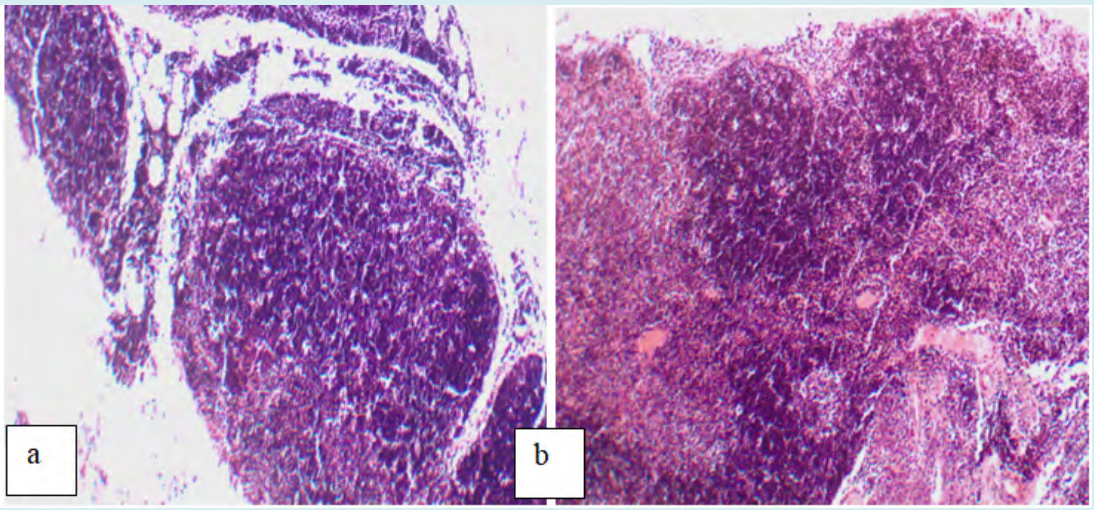

Figure 4: Rat thymus after intramuscular injection of Bovistem. A decrease in the size of lobules, a picture of the "starry sky", migration of lymphocytes (a), a decrease in the density of lymphocytes in the cortex, inversion of layers (b). Staining with hematoxylin and eosin. x100.

Accidental involution of the thymus gland, found in all animals, is a manifestation of the adaptive syndrome in response to the stress effect of the investigated drug "BoviStem" [14,15]. According to the classification of the stages of random involution of the thymus gland, the severity of the observed signs is reversible [16]. The histologically established random involution of the thymus gland had hemodynamic consequences (a decrease in the relative mass of the thymus gland) only in females after i/m administration of the drug, which indicates the effect of gender and the route of administration on the sensitivity of animals to the drug "BoviStem".

Results of the Study of Acute Toxicity of the Drug "Bovistem" In Mice

In accordance with the data obtained, a single subcutaneous injection at a dose of 50 ml/kg and an

intramuscular injection at a dose of 25 ml/kg of drug "BoviStem" did not lead to the death of the animals. No signs of intoxication were observed against the background of the drug. During the entire observation period, the animals were tidy, active, and normally responded to sound and light stimuli. Food and water intake was normal. The processes of urination and defecation were normal, breathing disorders and seizures were not observed. The physiological state and behavior of the animals did not differ from the mice from the intact control group.

The dynamics of the body weight of male and female mice (Table 3) at all periods of observation did not statistically significantly differ from both the initial data and the weight of the intact control, which indicates the absence of the toxic effect of the drug "BoviStem" on the body of animals in case of overdose.

Terms of research	Animal groups		
	Intact control	Drug "BoviStem»	
		50 ml/кg (s/c)	25 ml/кg (i/m)
males			
original mass	26,50±1,73	26,17±1,80	26,50±1,71
3 days	27,33±1,74	27,17±1,87	21,17±1,68
7 days	27,00±1,88	27,33±1,74	27,67±1,61
14 days	27,50±1,89	27,83±1,56	27,83±1,72
females			
original mass	22,50±0,72	22,67±0,88	25,00±0,73
3 days	22,67±0,92	22,83±0,79	25,17±0,60
7 days	22,83±0,87	23,17±0,91	24,83±1,08
14 days	23,00±0,93	23,50±0,85	25,67±1,05

Note: n – number of animals in the group.

Table 3: The results of the effect of the drug "BoviStem" on the dynamics of body weight (g) of male and female mice with a single (s/c, i/m routes of administration, n=6 (M±m).

At external examination of mice, which were injected with the drug "BoviStem" s/c at a dose of 50 ml/kg and i/m at a dose of 25 ml/kg, the coat was shiny, neat in appearance. No discharge from natural orifices was observed. Visible mucous membranes are pale pink, shiny, smooth. Macroscopic examination at autopsy showed that the internal organs of the mice corresponded to the physiological norm and did not differ in color, consistency and location from those in intact animals.

Conclusion

A single intramuscular and subcutaneous injection of the drug "BoviStem" to rats and mice did not have a toxic effect on the animal organism, as evidenced by a positive increase in body weight in both males and females. The study of the mass of internal organs and their histological structure (in rats) made it possible to establish sexual sensitivity to the drug and the target organ, which should be taken into account when prescribing the drug in the clinic. In accordance with the classification of substances for acute toxicity, the drug "BoviStem" with a single intramuscular and subcutaneous administration to rats and mice belongs to the VI class of toxicity – "relatively harmless substances" (LD50> 4.5 ml/kg with subcutaneous administration and LD50> 3 ml/kg – for intramuscular injection).

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