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TOXIC EFFECTS OF ULTRA-DISPERSED FORMS OF METALS (Mo AND MoO_3) IN THE EXPERIMENT in vivo

E.V. SHEIDA[1, 2] ✉, E.A. RUSAKOVA[1, 2], O.Yu. SIPAYLOVA[3], E.A. SIZOVA[1, 2], S.V. LEBEDEV[1, 2]

[1]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia, e-mail elena-shejjda@mail.ru (✉ corresponding author), elenka_rs@mail.ru, sizova.178@yandex.ru, Lsv@list.ru;
[2]*Federal Research Centre of Biological Systems and Agrotechnologies RAS*, 29, ul. 9 Yanvarya, Orenburg, 460000;
[3]*Orenburg State Medical University,* 6, ul. Sovetskaya, Orenburg, 460000 Russia, e-mail osipaylova@mail.ru
ORCID:
Sheida E.V. orcid.org/0000-0002-2586-613X
Rusakova E.A. orcid.org/0000-0002-1622-1284
Sipaylova O.Yu. orcid.org/0000-0003-0243-6249
Sizova E.A. orcid.org/0000-0002-5125-5981
Lebedev S.V. orcid.org/0000-0001-9485-7010
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Abstract

Despite the increasing use of nanoparticles (NPs) in industry, there is a serious lack of information regarding their impact on human health and the environment. Thus, nanomaterials based on molybdenum attract attention due to their ultra-high specific surface area and unique optical, electronic, catalytic and mechanical properties. However, having a high penetrating ability, molybdenum can accumulate in excess in organs and tissues of the body, affecting their structural integrity and functional activity. In the present work, the hepatotropic effect of Mo and MoO_3 nanoparticles was first established in experimental rats based on an assessment of the degree of activation of the apoptosis marker, a decrease in the level of motor activity and suppression of the emotional state of animals. A decrease in the body weight of rats and liver weight was recorded as a result of a single intraperitoneal injection of NPs while an increase in brain weight occurred. Our goal was to investigate general effects of Mo and MoO_3 nanoparticles on the growth and development of the internal organs of rats, the peculiarities of their motor and emotional activities, and the hepatotropic effect of the nanoparticles based on the assessment of the Caspase 3 (Cleaved) expression in the cytoplasm and nuclei of liver cells. Biomedical studies were carried out with 30 white Wistar male rats weighing 110-180 g. Mo and MoO_3 NPs were produced by plasma-chemical synthesis. The experimental animals were divided into five groups ($n = 6$ each). For NPs administration, the rats of groups I and II were intraperitoneally once-injected with Mo at 1.0 and 25.0 mg/kg, respectively, and animals of experimental groups III and IV were once-injected with MoO_3 at 1.2 and 29.0 mg/kg, respectively. Animals of the control group were injected with isotonic sodium chloride solution (0.9 % NaCl) in an equivalent volume. The growth of the experimental individuals was monitored daily by individual weighing. At the end of the experiment (day 14), the rats were decapitated under Nembutal anesthesia. Anatomical dissection and weighing of internal organs (liver and brain) were carried out. The absolute and average daily gains were calculated, as well as the weight ratio of the studied organs to the body. To reveal the readiness of liver cells for programmed cell death, expression of caspase 3 (Biocare Medical, LLC, USA) in the cytoplasm and nuclei of hepatocytes was detected immunohistochemically on the stained sections. The open field test was used to assess the emotional, motor activity and behavior of experimental animals. The emotional factor was assessed by the degree of anxiety and fear (the number of fecal boluses), as well as grooming (the number of brushing, washing, and other care elements). A system "Infrared actimeter" with "Panel with holes" (ACT-01, Orchid Scientific & Innovative India Pvt. Ltd., India) was use to assess spontaneous locomotor activity (LMA) of animals. It was shown that Mo and MoO_3 NPs have a toxic effect on the normal functioning of some body systems. In particular, the body weight of rats and the weight of their liver decrease while the weight of the brain increases. It was found out that the maximum decrease in body weight occurs in animals that received Mo at a dose of 25.0 mg/kg and MoO_3 at 1.2 mg/kg. Mo NPs in both low and high doses provoked a significant decrease in liver weight (by 14.3 and 16.1 %) ($p \le 0.05$), and MoO_3 NPs at 1 mg/kg caused a 33.5 % decrease. The injections of Mo NPs at 1.0 mg/kg and 25 mg/kg, and MoO_3 NPs at 1.2 mg/kg led to a significant ($p \le 0.05$) increase in brain weigh (by 10.9, 3.85, and 5.49 %, respectively). This increase is possibly due to edema of the organ, which affects behavioral reactions and motor

activities in rats, indicating the neurotoxic effect of Mo and MoO_3 NPs. Its severity directly depends on time of exposure and particle dosage. There was a decrease in LMA of rats on days 1 and 7 after Mo administration, and the higher the dosage, the lower the activity. The level of locomotor activity decreased to the lowest level on day 14 after administration of 29.0 mg/kg MoO_3 NPs. The level of emotional activity was lower for all applied dosages of Mo and MoO_3 NPs, and the effect was maximum on days 1 and 7. Evaluation of immunohistochemical expression of activated caspase 3 as a marker of apoptosis in the test with cleaved caspase 3 antibodies revealed an increase in endogenous levels of a larger fragment (p17) of the caspase 3 proenzyme in hepatocytes of male Wistar rats upon administration of Mo and MoO_3 NPs. This confirms the hepatotropic properties of the Mo and MoO_3 NPs. The detected caspase 3 activation depended not only on the dosage and time after injection of NPs, but also on the lesions caused by the NPs. More severe liver lesions occurred when caspase 3 activation was lower compared to control.

Keywords: nanoparticles, rats, caspase 3, apoptosis, internal organs, brain, behavior, locomotor activity, emotionality

Recently, the number of experimental studies to assess the toxic effect of ultrafine particles has lagged far behind the intensive development of nanotechnology. Despite the increasing use of nanoparticles (NPs) in industry, there is a serious lack of information regarding their impact on human health and the environment [1-3]. The study of the biological action and toxic effects of nanosized particles of various origins on the cells and tissues of the body is becoming increasingly important [4-6]. *In vivo* and *in vitro* studies have shown the development of allergic reactions in the offspring of mice intranasally insulated with inhaled titanium dioxide (TiO_2), the adverse effect of NPs on spermatogenesis and histopathological changes in the testes, as well as changes in gene expression in the brain of the offspring of mice after subcutaneous injection of TiO_2 NPs to maternal individuals [4].

NPs of different sizes, such as silver (Ag; 15, 100 nm), molybdenum (MoO_3; 30, 150 nm), aluminum (Al; 30, 103 nm), iron oxide (Fe_3O_4; 30, 47 nm), and titanium dioxide (TiO_2; 40 nm) were also evaluated for potential toxicity by studying the morphological parameters of cells by light microscopy. It was shown that mitochondrial function was significantly reduced in cells exposed to 5-50 μg/ml of Ag NPs. However, Fe_3O_4, Al, MoO_3, and TiO_2 did not have a noticeable effect at lower doses (10-50 μg/ml), while a significant effect was observed at higher doses (100-250 μg/ml). According to microscopic results, cells exposed to NPs at higher doses became abnormal in size, shrinking and becoming irregular in shape. Significant depletion of the glutathione content, a decrease in the mitochondrial membrane potential, and an increase in the amount of reactive oxygen species have been shown, that is, Ag cytotoxicity (15, 100 nm) in liver cells is likely to be mediated by oxidative stress [5]. Also, a number of studies have established the toxic effect of NPs on the nervous system as a whole and on the brain of the offspring when they are transferred from the mother's body to the circulatory system and the fetal body [6, 7].

Molybdenum (Mo) is one of the most important chemical elements in a living organism. It is a part of xanthine oxidase, aldehyde oxidase, and sulfite oxidase [8, 9], participates in protein metabolism, sulfur exchange [10], as well as transport and excretion of iron [11]. Nanomaterials based on molybdenum have recently attracted attention due to their ultra-high specific surface area and unique optical, electronic, catalytic, and mechanical properties [12-14]. In a living organism, molybdenum NPs have a high penetrating ability and can accumulate in excess amounts, acting as antagonists of other vital elements, in particular copper, the deficiency of which affects the functional activity of the hematogenous system [15, 16]. At the same time, nanoscale Mo can provide protection against the effects of increased concentrations of heavy metals, such as Cd and Hg. It was suggested

that Na_2MoO_4 was capable of removing the acute toxicity of $CdCl_2$ in rats, and the protective mechanism of this metal was partially associated with increased induction of the synthesis of Cd-metallothionein in the liver [17]. The accumulation of Cd in sheep tissues decreased as the amount of Mo and sulfur in the diet increased [18, 19].

This work for the first time established the hepatotropic effect of Mo and MoO_3 NPs in experimental rats, which was expressed in an increase in the endogenous level of caspase 3, the apoptosis marker. A decrease in motor activity and suppression of the emotional state of animals was also noted, as well as a decrease in their bodyweight and liver weight and an increase in brain weight after a single intraperitoneal injection of Mo and MoO_3 NPs.

The goal of the research was to study the overall effect of Mo and MoO_3 NPs on the growth and development of the internal organs of rats, on the motor and emotional activity of animals, as well as to establish the hepatotropic effect of NPs based on the assessment of the expression of the marker of activated caspase 3 as an indicator of the development of apoptosis in liver cells.

Methods. A total of 90 male Wistar rats with body weight of 110-180 g were used in the biomedical studies according to the methodological recommendations (Assessment of the safety of nanomaterials; approved by order of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare dated October 12, 2007 No. 280; https://www.rags.ru/stroyka/text/52003/#i396117), as well as guidelines [20]. Prior to the experiment, the animals were kept in the laboratory of biological tests and examinations of the Federal Research Center of Biological Systems and Agricultural Technologies RAS and fed a standard diet for laboratory animals (GOST R 50258-92) in accordance with the requirements of laboratory practice during preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST 51000.4-96). The experiments were carried out within the framework of the requirements for the humane treatment of animals [21], with the confirmation of the ethics committee (Minutes No. 3).

The sources of trace elements were Mo and MoO_3 NPs obtained by plasma-chemical synthesis. Particle sizes were estimated based on measurements of the specific surface area using a Sorbi®-M device (META LLC, Russia). The microstructure of the powders was analyzed using a Philips CM-30 transmission electron microscope (Philips, Japan). To determine the phase composition, a Rigaku D/MAX-2200VL/PC diffractometer (Rigaku Corporation, Japan) was used, Cu Kα radiation. When obtaining lyosols, aqueous suspensions of Mo and MoO_3 NPs were treated with ultrasound on the dispersant UZDN-2T (NPP Akadempribor, Russia) at 35 kHz, 300/450 W, 10 μA for 30 min. The resulting lyosols of NPs were used for injections.

Experimental rats were divided into five groups (n = 18 each) and kept under the same conditions on a standard balanced diet for laboratory animals. The control and experimental groups were formed from individuals of the same age. The spread over the initial mass did not exceed 10%. Rats of experimental groups I and II were once intraperitoneally injected with Mo NPs at a dose of 1.0 and 25.0 mg/kg; animals of experimental groups III and IV were injected with MoO_3 NPs at a dose of 1.2 and 29.0 mg/kg. Animals of the control group were injected with isotonic sodium chloride solution (0.9% NaCl) in an equivalent volume during the experiment. The selected concentrations of ultrafine particles were within the maximum tolerated doses for the metal under study. On days 1, 7, and 14 of the experiment, the rats were decapitated under Nembutal anesthesia. After that,

anatomical cutting was performed to take liver samples for morphological analysis. The changes in the mass of internal organs (liver and brain) were taken into account on day 14 of the experiment.

The growth of experimental individuals was monitored daily by individual weighing in the morning before feeding (error ±2 g). The data obtained were used to take into account changes in the absolute body weight (BW) and calculate the ratio of the mass of the studied organs to BW.

Pieces of liver were fixed in 10% neutral formalin and embedded in HistoMix paraffin mixture (BioVitrum LLC, Russia). To reveal the readiness of liver cells for programmed cell death, the expression of the caspase 3 enzyme in the cytoplasm and nuclei of hepatocytes was immunohistochemically detected during the staining of the sections in accordance with the standard procedure recommended by the manufacturer of the kit (Biocare Medical, LLC., USA; antibodies to caspase 3). Immunopositive cells were counted per 1000 cells and expressed in % (light optical microscope MT 5300L, Meiji Techno Co., Ltd., Japan).

Behavioral tests were performed on days 1, 7, and 14 in the morning before feeding the animals. The open-field test was used to assess the emotional, motor activity, and behavior of experimental animals. The emotional factor was assessed by the degree of anxiety and fear (the number of fecal boluses), as well as grooming (the number of combing, washing, and other care elements).

Spontaneous locomotor activity (LMA) of the animals was assessed using an Infrared Actimeter system complete with a Perforated Panel system (ACT-01, Orchid Scientific & Innovative India Pvt. Ltd., India). Movement and curiosity were recorded using an infrared sensor system as the animals moved freely over a 16-hole panel. The rats crossed the holes or immersed in them, while the intersections and immersions were recorded by sensors by the refraction of rays in the X and Y planes.

The Statistica 10.0 software package (StatSoft, Inc., USA) was used for statistical data processing. Results are presented as arithmetic means (*M*) and their standard errors (±SEM). The significance of the differences between the compared indicators was determined by Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Results. The Mo NPs used in the work contained no less than 99.7% Mo and 0.30% O_2, their size was 50.0±0.56 nm, and the specific surface area was 14.0 m^2/g. For MoO_3 NPs, the indices were as follows: 99.8% MoO_3 and 0.20% O_2, 92.0±0.54 nm, 12.0 m^2/g.

The introduction of Mo NPs at concentrations of 1.0 and 25.0 mg/kg (experimental groups I and II) led to a decrease in BW on day 14 of the study by 2.14% and 7.04%, respectively, compared to the control ($p \leq 0.05$). A similar trend occurred when exposed to MoO_3 NPs at doses of 1.2 and 29.0 mg/kg (groups III and IV), that is, BW decreased by 6.41 and 1.51% ($p \leq 0.05$) compared to the control (see Fig. 1, A). Weight of liver (WL) under the influence of Mo in experimental groups I and II significantly decreased by 14.3 and 16.1% ($p \leq 0.05$), under the influence of MoO_3 (group III), this decrease was maximum (by 33.5%) ($p \leq 0.05$), while in group IV, there was an increase in WL by 21.6% ($p \leq 0.05$) relative to the control group (see Fig. 1, B). The WL/BW ratio was maximum at a concentration of MoO_3 NPs of 29.0 mg/kg, which quantitatively was 23.4% more than in the control. A 25.0 mg/kg concentration of Mo NPs caused an insignificant decrease in WL/BW (by 9.80%). The maximum decrease in this indicator (by 29.0%) was established at 1.2 mg/kg MoO_3 NPs (see Fig. 1, C).

The concentration of Mo NPs 1.0 mg/kg led to the maximum (by 10.9% ($p \le 0.05$) increase in the weight of the brain (WB) of rats. In experimental groups II and IV, there was a uniform increase in WB by 3.85 and 5.49% ($p \le 0.05$), in contrast to group III where the WB decreased by 1.10% compared to the control (see Fig. 1, D). The ratio WL/WB was maximum at a concentration of Mo NPs of 1.0 mg/kg, which was 13.4% ($p \le 0.05$) more than in the control. The trend towards an increase in the WL/WB ratio persisted in groups II (by 11.7%), III (by 5.67%), and IV (by 7.11%) ($p \le 0.05$) (see Fig. 1, E).

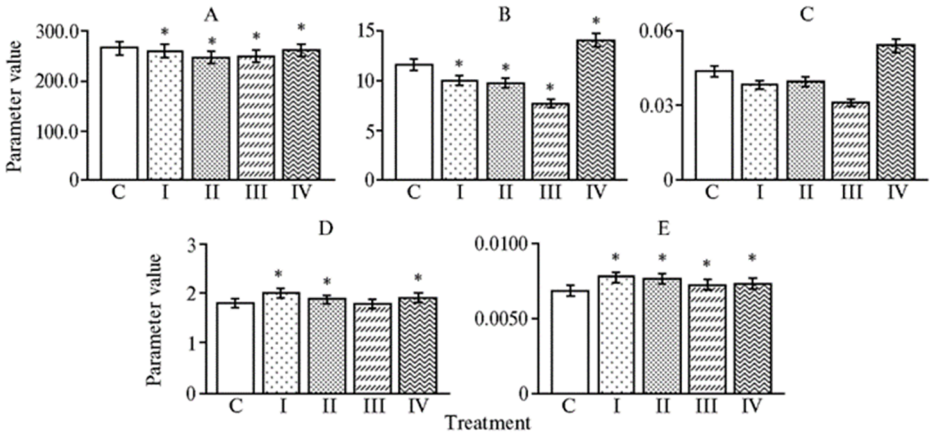

Fig. 1. Body weight, g (A)**, weight of liver, mg** (B)**, weight of liver to body weight ratio, mg/g** (C)**, weight of the brain, mg** (G)**, and weight of the brain to body weight ratio, mg/g** (E) **in male Wistar rats on day 14 after intraperitoneal injection of various forms of molybdenum NPs:** C — control, I — Mo, 1.0 mg/kg; II — Mo, 25.0 mg/kg; III — MoO_3, 1.2 mg/kg; IV — MoO_3, 29.0 mg/kg (n = 6, M±SEM).

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

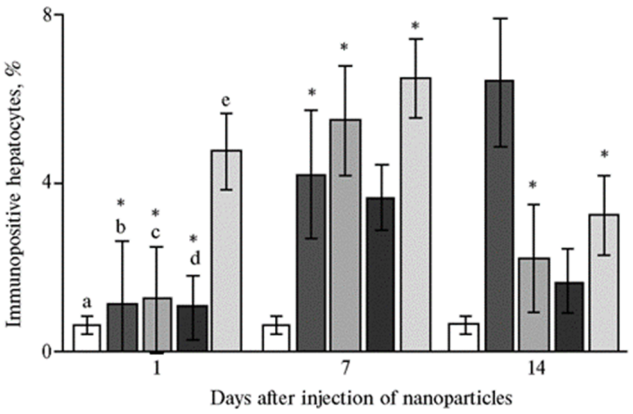

Fig. 2. Expression of the antigen of activated caspase 3 in liver cells of **male Wistar rats depending on time period from the intraperitoneal injection of various forms of molybdenum NPs:** a — control; b — Mo, 1.0 mg/kg; c — Mo, 25.0 mg/kg; d — MoO_3, 1.2 mg/kg; e — MoO_3, 29.0 mg/kg (n = 6, M±SEM).

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

Caspase 3 is one of the enzymes involved in apoptosis [22], which, in turn, acts as a fundamental and general biological mechanism responsible for maintaining the constancy of the cell number, cell formation and culling of defective cells. Expression of caspase 3 serves as a marker of apoptosis activation [23], which can be detected immunohistochemically using specific antibodies. In our experiments, an immunohistochemical study of caspase 3 activation revealed a dependence of the apoptosis marker expression on the administered dose of Mo and MoO_3 NPs, as well as on the time elapsed after injection (Fig. 2).

The counting of immuno-positive hepatocytes showed that intraperitoneal injection of Mo NPs at a dose of 1.0 and 25.0 mg/kg induced apoptotic changes in cells, especially on day 7 after injection and at an increased dose ($p \le 0.05$)

(Fig. 3, A). On day 14, the effect was opposite, i.e., the expression of the pro-apoptotic protein was the highest in the animals that received a smaller amount of NPs, despite the better morphofunctional state of the liver during this period (see Fig. 3, B) [24]. Apparently, this was influenced by the formation of a larger number of irreversibly damaged areas of the organ in rats, injected with a higher dose of Mo NPs (25.0 mg/kg).

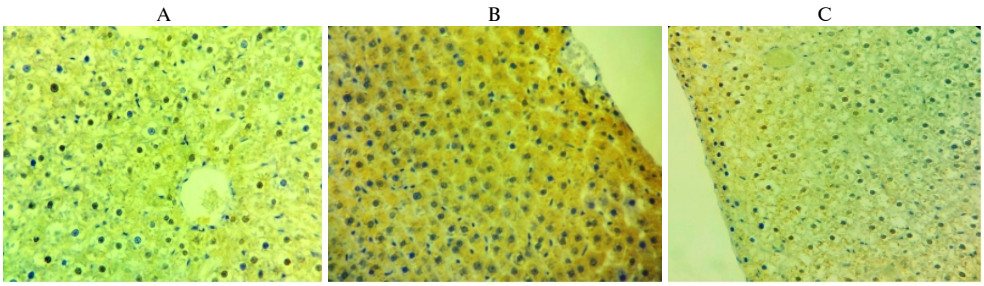

Fig. 3. Micrographs of hepatocytes in male Wistar rats: A — day 1 after injection of Mo (1.0 mg/kg), expression of activated caspase 3 in the nuclei and cytoplasm of hepatocytes (yellow-brown staining) along the periphery of the lobules under the capsule; B — day 7 after injection of Mo (25.0 mg/kg), expression of activated caspase 3 in the nuclei and cytoplasm of hepatocytes of the periportal zone; C — day 14 after injection of MoO_3 (29 mg/kg), weak expression of activated caspase 3 in the nuclei and cytoplasm of hepatocytes along the periphery of the lobules under the capsule (magnification ×400, light optical microscope MT 5300L, Meiji Techno Co., Ltd, Japan)..

An increase in the dose of MoO_3 NPs in group IV caused an increase in the expression of activated caspase 3 to a greater extent than in group III and control groups at all periods of the study, reaching a maximum value 7 days after administration. It can be noted that with an increase in necrobiotic processes in the liver tissue on day 14 in group IV, the degree of activation of the pro-apoptotic marker decreased and weakly stained immunopositive hepatocytes were mainly located along the periphery of the lobules under the capsule (see Fig. 3, C).

The changes in the motor activity of the experimental animals during the study in the "infrared actimeter" system corresponded to the data obtained in the "open field" test. LMA on days 1 and 7 of the experiment in the experimental groups decreased, that is, the higher the dosage of the introduced Mo, the lower the LMA was. On day 14, LMA in experimental groups I and II, on the contrary, was higher relative to the control. With the introduction of MoO_3 NPs, LMA in the experimental groups decreased in comparison with the control, and to a greater extent in the groups with the highest dosage and duration of exposure (on day 14).

The degree of anxiety and fear on day 1 of the study was the highest in animals of the control group and the lowest in experimental group I (by 86%) ($p \leq 0.05$). The grooming frequency in group II was 89% ($p \leq 0.05$) lower than in the control. On day 7 of the experiment, the indicator characterizing the emotional state decreased by 32% in group I and by 48% in group II compared to the control. The total indices of grooming were significantly lower in groups I and II than in the control (by 83 and 92%, respectively; $p \leq 0.05$). By the end of the experiment, the indicators in the groups receiving ultradispersed molybdenum particles were 22% lower compared to the control.

Upon injection of MoO_3 NPs, the degree of emotional activity (grooming, defecation) in the experimental groups of the animals on day 1 of the experiment decreased in groups III and IV by 52 and 69%, respectively ($p \leq 0.05$), compared to the control. On day 7, emotional activity was also suppressed, and, as on day 1 after application of NPs of MoO_3, a greater decrease was noted in animals that were injected with MoO_3 at a lower dosage. On day 14 after the injection of NPs,

emotional activity in both groups III and IV continued to decrease compared to the control (by 33 and 22%, respectively, $p \leq 0.05$).

Laboratory rats are the most common type of experimental animals for developing models of the consequences of acute and chronic intoxication. Currently, more than 100 separate outbred stocks and inbred lines of laboratory rats have been bred. Wistar rats, Bio Breeding Sprague-Dawley, C57BL, CFI, C3H are most often used in toxicological studies. In our studies of the toxic effect of ultrafine particles of molybdenum and its oxide, male Wistar rats were a bio-model. Previously, it was shown that a single intraperitoneal injection of Mo and MoO_3 NPs led to morphological changes in liver tissue in experimental animals of this line, the severity of which (from adaptive to necrobiotic) and reversibility depended on the dose and time elapsed after injection. An increase in the NP dose was accompanied by significant pathological changes, namely, the appearance of extensive areas of large vacuole hepatosis or foci of necrosis, or both, and the MoO_3 NPs exerted the most damaging effect on the liver tissue [24].

It can be assumed that the basis of pathomorphological changes in the liver against the background of the intake of Mo and MoO_3 NPs is their direct destructive effect on the vascular system of the animal organism and, as a consequence, the development of tissue hypoxia and necrobiotic changes. This assumption is based on the results of studies by Sherkhova et al. [25] who established the effect of an excess of Mo administered *per os* in the form of a salt ($Na_2MoO_4 \cdot 2H_2O$) on the vascular system of rats, including the liver. Despite the fact that in our studies, Mo was used in the nanoform, and not in the composition of the salt, the results obtained can be compared, since the possibility of a partial transition of NPs in the internal environment of the body to the ionic form is not excluded [26, 27].

In turn, destructive changes in cells can lead to the activation of the system of mononuclear phagocytes, which actively capture and accumulate nanosized particles [24], to the development of inflammation, and also, possibly, to the induction of apoptosis in the liver tissue, namely, to the activation of the proapoptotic protein caspase 3 [28] that we revealed in the liver of rats of the experimental groups.

Possessing a high penetrating power, NPs affect the organs and systems of the body, including the nervous one. When observing rats that were injected with NPs, the authors found signs of intoxication of the nervous system, in particular, inhibition of motor activity and an increase in brain mass. The results obtained indicate the absence of addiction in animals receiving NPs of molybdenum and its oxide. Previously, we have shown the toxic effect of ultrafine particles of iron, titanium, and titanium dioxide on the manifestation of cognitive functions in animals and the morphological structure of the brain, which confirms the data of other researchers on an increase in the absolute mass of the brain and changes in the emotional state of animals under the influence of NPs of trace elements metals [29-31].

Thus, the performed immunohistochemical analysis revealed an increase in the expression of the apoptosis marker, the enzyme caspase 3, in hepatocytes of male Wistar rats upon administration of Mo and MoO_3 NPs (NPs). The detected activation depended not only on the dose and time after injection but also on the degree of destructive changes in the organ during NPs administration. More severe liver damage was accompanied by a weaker activation of the proapoptotic enzyme compared to the control. A possible reason for this was the development of extensive necrobiotic processes in organ tissue without the initiation of apoptotic cell death. Mo and MoO_3 NPs had a toxic effect on the functioning of some

body systems. The maximum decrease in BW occurred in animals treated with Mo at a dose of 25.0 mg/kg and MoO_3 at a dose of 1.2 mg/kg. Both low and high doses of Mo NPs led to a significant decrease in WL (by 14.3 and 16.1%), and 1.2 mg/kg MoO_3 caused a 33.5% decrease. Mo at doses of 1.0 mg/kg, 25 mg/kg and MoO_3 at a dose of 1.2 mg/kg caused a significant increase in WB (by 10.9; 3.85 and 5.49%, respectively, $p \leq 0.05$). In turn, an increase in brain mass (possibly due to organ edema) led to changes in the behavioral reactions and LMA of rats, which indicated the neurotoxic effect of Mo and MoO_3 NPs, the severity of which directly depended on the time after administration and dosage of the particles. There was a decrease in LMA in rats on days 1 and 7 after Mo administration, and the activity was more strongly suppressed with increasing dosage (the higher the dosage, the lower the activity). The smallest values of indicators characterizing LMA were obtained on day 14 after the introduction of MoO_3 NPs at a dose of 29.0 mg/kg. It was shown that the emotional activity of rats decreased after the introduction of Mo and MoO_3 NPs in all studied dosages, with the greatest effect from the effect of NPs recorded on days 1 and 7 of the experiment.

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