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MORPHOLOGICAL AND BIOCHEMICAL PARAMETERS IN Wistar RATS INFLUENCED BY MOLYBDENUM AND ITS OXIDE NANOPARTICLES

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Abstract

Despite widespread use of nanoparticles in industry and medicine, there is very little information about how the newly developed nanomaterials interact with biological objects. Certain properties of the Mo-containing nanoparticles (NPs) suggest their possible toxic effect on warm-blooded animals. In this paper we compared the effect of Mo NPs (at 1 and 25 mg/kg) and its oxide MoO3 NPs (at 1.2 and 29 mg/kg), when administrated parenterally, on metabolic parameters and the exchange of chemical elements in Wistar laboratory rats. There, we assessed the red and white blood cell counts, the hemoglobin level, the activity of catalase (CAT) and superoxide dismutase (SOD) (for oxidative status), the ALT, AST, LDH, GGT, creatine kinase activity, blood creatinine, bilirubin and urea concentrations (for metabolic status) at days 1, 7 and 14. A day after Mo NPs and MoO_3 NPs administration the number of blood leukocyte lowered by 11.3 % ($P < 0.05$) and 58.5 % ($P < 0.01$), respectively. Also, a decrease in monocyte number by 18.9 ($P < 0.05$), 41.9 ($P < 0.01$), 51.7 ($P < 0.05$) and 83.3 % ($P < 0.001$) as depending on NPs chemical composition and doses was characteristic, though on day 14 a significant difference to control (54.5 %, $P < 0.05$) was found only for MoO_3 NPs at a dose of 29 mg/kg. The number of thrombocytes was the highest on day 14 for the maximum dosage of both NPs leading to hindered blood microcirculation. The experiments also showed an increase in serum aminotransferases, GGT and LDH activity. In sum, we observed manifestations of oxidative stress, anemia and capillary-trophic insufficiency in the animals administrated with high doses of molybdenum and Mo oxide NPs. These signs were progressing and the most apparent for molybdenum oxide NPs. Given the comparable doses used, the molybdenum nanoparticles exhibit lower toxicity as compared to its oxide.

Keywords: catalase, superoxide dismutase, glutamyl transferase, lactate dehydrogenase, aminotransferase, nanoparticles of molybdenum, nanoparticles of molybdenum trioxide

Molybdenum nanoforms are widely used in modern technologies [1, 2], e.g. for multifunctional electrocalalysis [3] and production of lubricants [4]. Apart from that, ultradisperse products containing molybdenum and its compounds show unique biological properties, which allows using them for oncotherapy [5], as antibiotics [6, 7] and antifungal agents [8], and for blue-green algae growth promotion [9].

Molybdenum is a well-studied essential microelement. Its participation in enzymatic systems is well-known [10]. Ranges of molybdenum deficiency, sufficiency and toxicity have been described [11]. Background [12], threshold and toxic concentration of molybdenum for invertebrates in soils have been reported [13]. However, the knowledge on consequences of interaction of newly developed molybdenum-based nanomaterials with biological objects is still ex-

tremely poor. At the same time, according to investigative studies, biological effects of nanoforms of molybdenum are much more expressed. Various models have been used to demonstrate this. For example, entry of molybdenum oxide nanoparticles into soil leads to high mortality rate, adaptive changes of antioxidant enzyme activity and inhibition of Cr, Fe, Mg, Mn, Ni, Si, V metabolism in *Eisenia fetida* [14]. The presence of molybdenum nanoparticles in water has a negative impact on cell membrane permeability in *Stylonychia mytilus* and leads to processes, accompanying the damage. Analysis of collected data on biological effects of molybdenum-based nanoparticles suggests that they may have a toxic effect on warm-blooded animals.

Here we for the first time compared the biological effects of molybdenum nanoparticles and molybdenum oxide on rats, the homeothermic mammals.

The objective of this work was the examination of morphological and biochemical blood parameters, morphological and functional characteristics of tissue and chemical element metabolism in model objects due to the effects of molybdenum nanoparticles and molybdenum oxide.

Technique. Molybdenum and molybdenum oxide nanoparticles (NPs) (Mo NPs and MoO_3 NPs) were obtained by plasma chemical synthesis (OOO Platina, Moscow). Mo NPs products (d = 50 nm, specific surface area 14 m^2/g, Z-potential **–43**±0,52 mV) contained 99,7 % Mo and 0,3 % O_2, and MoO_3 NPs (d = 92 nm, specific surface area 12 $м^2/г$, Z-potential –43±0,21 mV) contained 99,8 % Mo and 0,2 % O_2. The material attestation (determination of particle size, polydispersity, voluminosity, fraction content, surface area) included electronic scanning, transmission and atomic force microscopy using LEX T OLS4100, JSM 7401F и JEM-2000FX (JEOL, Japan). Particle size distribution was examined using a Photocor Compact analyzer (OOO Photocor, Russia). Nanoparticle samples were dispersed in saline solution using UZDN-2T (NPP Akadempribor, Russia) (35 kHz, 300 W, 10 uA, 30 min).

The studies were performed in 75 Wistar white male rats, with the weight of 150-180 g in standard vivarium conditions (experimental biological clinic, Orenburg State University).The diet of animals (State Standard GOST R 50258-92) complied with the requirements of the Good Laboratory Practice in conducting preclinical research in the Russian Federation (State Standard GOST 51000.4-96). The experiments were performed in accordance with the provisions of the Geneva Convention and the principles of Good Laboratory Practice (National Standard of the Russian Federation GOST R 53434-2009), as well as recommendations set out in The Guide for the Care and Use of Laboratory Animals (National Academy Press Washington, DC 1996). After the preliminary period (1 month) the animals were divided into 5 groups (n = 15 per group). Mo NPs were administered intraperitoneally in a single dose of 1 and 25 mg/kg of live weight in groups I and II, respectively, and in groups III and IV MoO_3 NPs were administered at the dose of 1.2 and 29 mg/kg, respectively. Saline solution was injected to control animals.

Biomaterial for the study was obtained after decapitation of rats under Nembutal anesthesia (5 species for each option of the experiment and control in 1, 7, and 14 days after administration of nanopreparations). Blood for examination of morphological parameters was placed in vacuum tubes with anticoagulant, for biochemical studies — into vacuum tubes with a coagulation activator (thrombin). Morphological blood composition and hemoglobin concentration were estimated using an nautomatic hematological analyzer URIT-2900 Vet Plus (URIT Medical Electronic Group Co., Ltd, China). The biochemical blood serum test was performed using an automatic biochemical analyzer CS-T240 (DIRUI Industrial Co., Ltd, China) and commercial veterinary kits (DiaVetTest

by DIAKON-DS, Russia; Randox Laboratories Ltd., Great Britain). The content of chemical elements in the examined samples studied was measured using a mass spectrometer Elan 9000 and an atomic emission spectrometer Optima 2000V (Perkin Elmer, USA). The samples were ashed using microwave decomposition system Multiwave-3000 (Anton Paar, Austria).

For liver microstructure studies, samples were fixed in 10 % neutral formalin and embedded in paraffin mixture HISTOMIX® (OOO BioVitrum, Russia). 5-6 μm thick histological sections were prepared using a semi-automatic microtome (01 MW, Tekhnom, Russia), stained with Mayer's Haematoxylin and Eosin and examined under a light microscope MT 5300L (Meiji Techno Co., Ltd, Japan, ×400).

The data are presented as the arithmetic mean (*M*) with the standard error of the mean (*m*). Statistical analysis was performed using ANOVA (Statistica 10.0 software package, StatSoft Inc., USA) and Microsoft Excel. The validity of differences in the indicators compared was determined by Student's *t*- test. The values were considered statistically significant at $P < 0.05$.

Results. Significant morphological changes of blood were observed as early as 1 day after administration of molybdenum and molybdenum oxide nanoparticles (Table 1). For example, leukocyte counts in groups II and IV have decreased by 11.3 % ($P < 0.05$) and 58.5 % ($P < 0.01$).

1. Morphological blood parameters in Wistar rats upon intraperitoneal administration of Mo and MoO_3 NPs at various doses ($M \pm m$, $n = 75$)

Parameter	Control	Mo NPs		MoO_3 NPs	
		group I	group II	group III	group IV
		Day 1			
Leucocytes, $\times 10^9$/l	8.80±0.180	8.85±0.095	7.90±0.080	7.25±0.550	6.55±0.150
Erythrocytes, $\times 10^{12}$/l	8.59±0.120	8.90±0.155	8.65±0.155	9.50±0.380	8.52±0.165
Hemoglobin, g/l	172.5±1.50	189.5±5.50	162.5±4.50	184.0±6.00	133.5±1.50
Platelets, $\times 10^9$/l	170.50±9.500	176.00±12.000	250.00±5.130*	188.50±6.500	184.50±6.500
Lymphocytes, $\times 10^9$/l	4.10±0.010	3.75±0.015	4.35±0.015	2.90±0.030	2.30±0.090
Monocytes, $\times 10^9$/l	2.20±0.090	1.85±0.050*	1.55±0.035*	1.45±0.035**	1.20±0.070**
Granulocytes, $\times 10^9$/l	2.50±0.090	3.25±0.015*	2.00±0.050	2.90±0.090*	2.05±0.055
		Day 7			
Leucocytes, $\times 10^9$/l	7.10±0.120	6.50±0.160	7.85±0.195	6.60±0.110	7.95±0.450
Erythrocytes, $\times 10^{12}$/l	8.12±0.430	7.60±0.360	7.43±0.110	6.62±0.230	7.55±0.050
Hemoglobin, g/l	165.0±10.20	156.0±6.00	143.0±3.00	126.0±5.0	145.0±8.0
Platelets, $\times 10^9$/l	159.5±3.50	162.0±8.00	182.5±2.50*	183.0±8.00	179.5±7.50
Lymphocytes, $\times 10^9$/l	3.80±0.01	3.50±0.07	3.70±0.09	2.90±0.01*	3.40±0.07
Monocytes, $\times 10^9$/l	2.10±0.210	2.35±0.150	2.65±0.300	1.95±0.020	1.40±0.060*
Granulocytes, $\times 10^9$/l	2.50±0.090	1.65±0.035	2.50±0.070	2.75±0.085	3.15±0.015*
		Day 14			
Leucocytes, $\times 10^9$/l	8.15±0.350	8.95±0.150	8.75±0.150	6.26±0.043	7.95±0.250
Erythrocytes, $\times 10^{12}$/l	8.78±0.430	8.62±0.165	6.86±0.105	5.91±0.139	6.76±0.160
Hemoglobin, g/l	166.5±9.50	157.5±5.50	122.5±5.50	108.8±5.25	128.0±8.00
Platelets, $\times 10^9$/l	188.5±7.50	154.5±3.50	352.0±6.00**	149.88±5.13	311.5±9.50**
Lymphocytes, $\times 10^9$/l	4.50±0.012	3.05±0.015	2.20±0.020	2.10±0.099	2.75±0.035
Monocytes, $\times 10^9$/l	2.55±0.015	2.05±0.015	2.10±0.012	2.42±0.038	1.65±0.015*
Granulocytes, $\times 10^9$/l	2.10±0.020	3.85±0.025	4.45±0.035	4.74±0.066	3.55±0.045

Note. Groups I and II — doses of 1 and 25 mg/kg live weight, respectively; groups III and IV — 1.2 and 29 mg/kg.

*, ** Differences vs. control are statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

The effect of molybdenum-based nanoparticles was characterized by decrease in monocyte counts on day 1 by 18.9 ($P < 0.05$), 41.9 ($P < 0.01$), 51.7 ($P < 0.05$) and 83.3 % ($P < 0.001$) in groups I, II, III and IV, respectively. In 7 days this difference amounted to 55.6 ($P < 0.05$), 27.3 ($P < 0.05$), 7.7 and 50.0 % ($P < 0.05$). At the time of completion of the experiment significant differences were only observed between the control and group IV (54.5 %, $P < 0.05$). The nanoparticle effects with regard to the monocytes observed by us differed dramatically from those reported for polystyrene nanoparticles [16] and Cu and Fe nanoforms [17]. Similar dynamics was observed for lymphocytes, the number

of which in the blood of animals has decreased by 78.6 % ($P < 0.001$) on day 1 and by 63.6 % ($P < 0.001$) on day 14. In other groups significant decrease in the number of lymphocytes was only observed on day 14. Similar dynamics of the number of lymphocytes was previously reported in humans when molybdenum oxide inhalation [18].

On day 7 and 14 upon administration of nanoparticles the rats demonstrated the signs of molybdenum intoxication (decrease in erythrocyte and hemoglobin counts in blood), which were more expressed for MoO_3 NPs due to higher toxicity of the oxide compared to metal [19].

In the groups where the highest doses of molybdenum and molybdenum oxide NPs were administered, the number of platelets has increased by day 14, which led to blood sludging, an increase in viscosity and impaired perfusion through microcirculation vessels. Similar signs have been described earlier for molybdenum-based nanoparticles [20, 21].

2. Biochemical blood parameters in Wistar rats upon intraperitoneal administration of Mo and MoO_3 NPs at various doses ($M \pm m$, $n = 75$)

Parameter	Control	Mo NPs		MoO_3 NPs	
		group I	group II	group III	group IV
		Day 1			
ALT, U/l	59.75±5.890	97,00±7,000	70,65±3,750	80,12±2,720	44,35±1,550*
AST, U/l	140.50±2.200	434,80±16,500*	452,95±14,950*	376,50±16,600	218,00±17,120
LDH, U/l	279.00±12.200	232,00±15,700	272,50±10,150	250,00±13,000	324,50±14,500
GGT, U/l	1.40±0.014	7,00±0,030*	1,00±0,010	15,00±0,120*	3,00±0,150
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2668±104	10983±418	6550±148*	8225±295	5664±231
SOD, %	110.0±5.12	130,0±8,01	128,0±7,81	140,0±2,13*	140,0±3,11*
Creatinine, μmol/l	48.50±1.200	42,45±1,450	49,95±2,850	44,95±0,850	75,60±1,700**
Bilirubin, μmol/l	5.31±0.025	5,13±0,031	5,25±0,018	25,80±0,640**	19,70±0,920*
Urea, mmol/l	5.35±0.051	4,25±0,053	6,30±0,050	4,40±0,070	7,00±0,080**
Creatine kinase, U/l	2948±220	3556±134	5227±107	2693±114	2902±172
		Day 7			
ALT, U/l	67.25±3.450	128,85±17,350	147,60±6,800	111,50±4,800	132,20±3,100
AST, U/l	148.35±7.450	567,60±19,100	664,95±11,950	531,20±25,700	699,25±13,550
LDH, U/l	305.50±9.500	374,50±9,500	309,00±11,000	305,00±14,000	433,00±15,800
GGT, U/l	1.50±0.050	1,50±0,050	5,00±0,025	2,00±0,100	4,50±0,050
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2413±131	3792±147	7486±248	8865±212	22029±931**
SOD, %	136.8±16.20	666,2±6,80**	470,4±14,90	367,0±12,00	396,8±3,00*
Creatinine, μmol/l	47.75±0.650	58,20±1,100	32,15±1,250	44,20±3,500	42,65±3,150
Bilirubin, μmol/l	5.96±0.042	5,86±0,028	5,49±0,067	8,05±0,040	6,90±0,097
Urea, mmol/l	5.70±0.012	4,15±0,050	5,00±0,100	5,80±0,150	5,55±0,170
Creatine kinase, U/l	2726±110	5429±298	4394±115	4278±283	4178±148
		Day 14			
ALT, U/l	65.80±4.900	182,50±8,900	242,25±9,750	142,00±8,300	128,60±7,600
AST, U/l	142.05±11.150	459,70±28,800	629,25±37,850*	147,00±4,280	367,55±15,850*
LDH, U/l	243.00±12.000	236,00±14,000	294,50±8,500	177,88±5,120	291,50±8,500*
GGT, U/l	1.50±0.050	12,50±0,500	8,50±0,120	1,58±0,042	5,50±0,050
Catalase, μmol $H_2O_2 \cdot l^{-1} \cdot min^{-1}$	2532±151	4122±195	6846±285	8764±256*	20021±725*
SOD, %	130.2±8.84	146,5±12,93	108,1±5,82	94,0±5,05	166,3±2,09*
Creatinine, μmol/l	42.60±0.140	48,30±0,240	51,40±0,200*	46,07±0,535	77,75±0,650**
Bilirubin, μmol/l	5.64±0.425	5,25±0,545	12,02±0,615	2,40±0,171	4,58±0,125
Urea, mmol/l	5.60±0.016	5,50±0,030	6,00±0,017	4,21±0,099	10,60±0,080*
Creatine kinase, U/l	2670±68	4090±175	3148±103	3225±118	3971±163

Note. Groups I and II — doses of 1 and 25 mg/kg, respectively; groups III and IV — 1.2 and 29 mg/kg live weight. ALT, AST, LDH, GGT, SOD — alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, γ-glutamyl transferase, superoxide dismutase.

*, ** Differences vs. control are statistically significant at $P < 0.05$ and $P < 0.01$, respectively.

Thus, the signs of anemia, leukopenia, sludge, local inflammatory reactions reflect the development of capillary-trophic insufficiency in case of MoO_3 NPs application. The doses of molybdenum and molybdenum oxide nanoparticles used are comparable, so we can conclude that the former are less toxic.

An increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in animals was indicative of cytolysis, which became

more severe in the course of time (Table 2). For example, with regard to ALT and total bilirubin, the difference as compared to control amounted to 18.2-62.0 % on day 1, 119.0 % on day 7, and 272.0 % on day 14. The difference was even more significant for AST, i.e. 222.0-352,0 %; with regard to bilirubin, significant (4.8-fold) increase was observed for MoO_3 NPs as early as on day 1. Significant ($P < 0.05$) decrease in ALT activity was observed for maximum dose of MoO_3 NPs (29 mg/kg) on day 1. This can be indicative of glomerular filtration impairment, which is confirmed by high values for creatinine (14.3-fold difference with the control, $P < 0.01$) and urea (30.8 % higher, $P < 0.01$).

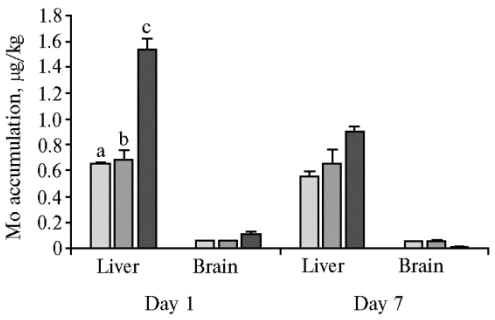
We did not detect an increase in serum γ-glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activity. The values close to upper limit of normal were detected on day 1 for minimum doses of Mo NPs and MoO_3 NPs, as well as on day 7 and 14 at the minimum doses of both nanoparticles. Such dynamics of GGT and LDH activity may be indicative of membrane destruction in a small part of cell population and weak microsomal oxidation induction due to nanoparticles of transition metals [22]. At the same time, with increase in duration of impact up to 14 days at the minimum dose of Mo NPs GGT activity increased 5-fold, which may be considered as a sign of oxidative stress due to effect of Mo NPs. This phenomenon was previously reported for mouse fibroblasts (line L929) when generation of active oxygen forms with subsequent decrease in glutathione content and catalase activity [23]. LDH activity only increased at high doses of the agent (mostly MoO_3 NPs), which may be considered as moderately toxic, as compared to other transition metals [24].

The development of oxidative stress was also confirmed by the dynamics of catalase (CT) and superoxide dismutase (SOD) activity. The peak values for CT were recorded at the high dose of MoO_3 NPs (29 mg/kg) on day 7 and day 14, with 9.0-fold ($P < 0.01$) and 7.9-fold ($P < 0.05$) difference, respectively, compared to control. Minimum doses on day 1 caused a rapid increase in catalase activity (4.1-fold compared to control); on day 7 the values decreased to 1.5-fold difference, and by day 14 increased again, but not up to the initial level. Presumably, catalase activation takes place in response to increase in lipid peroxidation and accumulation of hydrogen peroxide and other oxidative stress products, as catalase metabolizes them and prevents their accumulation in cells. However, according to some researchers, Mo NPs are not toxic and can act as antioxidants, e.g. by showing protective effect in contact with peroxide compounds (H_2O_2) and ZnO-NPS, which has been demonstrated for cell lines of human mammary gland adenocarcinoma (MCF-7) and fibrosarcoma (HT-1080). Mo NPs have been reported to significantly increase glutathione content in MCF-7 line (1.6-fold) and HT-1080 line (1.3-fold), which could be compared to the effect of antioxidant drug N-acetylcysteine (NAC) [25].

Both minimum microstructural changes (granular degeneration, hepatocyte hypertrophy and hyperchromia of their nucleus) in case of low doses of Mo NPs and MoO_3 NPs, and significant pathological changes (large areas of hepatosis and necrosis) in case of high doses of MoO_3 NPs were observed in liver.

Antagonistic interactions of molybdenum with other microelements could contribute to effect of molybdenum nanoparticles in animals [26]. Analysis of liver composition, muscle tissues and brain of animals for 25 chemical elements has revealed significant changes related to three of them, i.e. Mo, Fe and Ca. Thus, in liver in groups I and II a decrease in Fe content by 31.1 ($P < 0.01$) and 38.9 % ($P < 0,001$) was observed on day 1, and by 24.0 ($P < 0.01$) and 76.1 % ($P < 0.001$) on day 7, respectively. The difference for brain tissues amounted to 48.3 ($P < 0.001$) and 90.1 ($P < 0.001$), and 21.1 ($P < 0.01$) and

41.5 % ($P < 0{,}001$), respectively. Application of MoO_3 NPs was accompanied by similar changes. With regard to Ca, significant increase in accumulation in liver was only observed on day 1, by 17.1 % ($P < 0.05$) in group I and by 26.3 % ($P < 0.01$) in group II. Mo level analysis has demonstrated the same dynamics (Fig.). Peak values were observed on day 1 and were directly dependent on Mo administration dose with clinical difference (136.9 %) for liver. During the next 7 days the amount of Mo in liver decreased in animals of group II by 41.55 %, of group I — by 4.41 %, as compared to the value on day 1.



Mo accumulation in organs of Wistar rats upon single administration of Mo nanoparticles at various doses: a — control, b — 1 mg/kg, c — 25 mg/kg.

Accumulation in brain at a dose of 1 mg/kg was comparable with that of intact animals. Increase of the dose up to 25 mg/kg led to an increase in Mo content by 83.3 % on day 1 and its decrease on day 7 up to the values below control ones.

The obtained results are generally expectable. It is known that molybdenum is one of the essential microelements and its deficiency is accompanied by development of a number of pathologies in humans and animals [27]. However, reports exist on toxic effect of molybdenum in the body, a strong connection between excessive Mo content and development of asthma [28], alveolar and bronchial adenomas and carcinomas [29], etc.

So, administration of Mo nanoparticles in rats is accompanied by capillary-trophic insufficiency, signs of oxidative stress, with more clear manifestation in case of molybdenum oxide nanoparticles. Considering the comparable doses of the agents, we may suggest that Mo nanoparticles are less toxic than nanoforms of its oxide.

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