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Investigation of the long-term toxic effect of nanoparticles of different physical-chemical characteristics

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Abstract. The purpose of this work is to study the effect of metal and oxide nanoparticles on some ecological and functional groups in the soil-plant-animal system to form the stability limits of organisms. Nanoparticles of cobalt, iron, zinc, copper, copper oxide, zinc oxide and titanium dioxide sized 20–80 nm were studied. The concentration range was 0.01–1,000 g of nanoparticles per ton of seeds or soil. Objects suitable for biotesting and environmental monitoring were selected: earthworms (*Lumbricina*), rats (white outbred) and Wistar rats. It was previously found that nanoparticles of the studied metals up to a concentration of 100 g t^{-1} of seeds, unlike oxides, practically do not affect bacterial populations. The use of indicators of biochemical and cytomorphologic reactions of invertebrates seems promising because worms are able to bind pollutants and reduce their penetration into plants. They are also an indicator of soil biotesting for metal contamination. Reactivity and toxic effects of nanoparticles (NPs) in natural conditions depend both on the type of soil and on the size and concentration of nanoparticles. With sizes (NPs) of up to 20 nm (depending on the type of soil and physicochemical characteristics), NPs are much more reactive and reduce the survival of microorganisms. Small nanoparticles (less than 20 nm) are characterized by a large interface. Such nano-objects exhibit high physical-chemical activity and are safe only at very low concentrations. The specifics of the environmental impact of oxide NPs compared to metal NPs was revealed. It was associated with accumulation of oxides in living systems and the peculiarities of changes in the morph physiological, histological and reproductive parameters of organisms and morphological and biochemical parameters of animals. Oxide nanoparticles accumulate in a living organism, exhibit toxic properties, lower the activity of enzymes and hormones and are transferred along trophic chains, which is not typical for metal nanoparticles.

Key words: toxicity, microorganisms, invertebrates, plants, laboratory animals, morphology, cytotoxicity, biochemistry, histology.

INTRODUCTION

In connection with the intensive development of nanotechnology, the flow of artificial nanoparticles entering the environment inevitably increases. This gives rise to the need to study and understand the routes of entry, distribution, accumulation and their effects on living organisms, including the degree of toxicity of nanoparticles, as well as the possibility of their inclusion in biogenic cycles. The environmental effects of nanoparticles of technogenic origin have not been fully studied yet due to the difficulty of isolating them in pure form from natural products for research (Edgington et al., 2010) and conflicting data in scientific studies when assessing their safety. There has been an increase in the number of publications demonstrating the toxic effects of nanoparticles (NPs) with a characteristic size of less than 100 nm (Oberdörster et al., 2005; Alekseeva, 2007; Elsaesser & Howard, 2012). But the properties of nanoparticles depend on many factors: the method of preparation, size, surface area, chemical composition. All these factors, including the effect of small doses, were studied in our earlier works (Churilov et al., 2019) and it was shown that nanoparticles of iron, copper and cobalt with a size of 20–80 nm have biological activity and can be used as growth stimulators of living systems. An analysis of the available scientific data, on the one hand, indicates a high degree of novelty and relevance of the proposed area - the study of the interaction of fine particles of technogenic origin with plant cells to develop technology for their use to stimulate the growth of agricultural plants. And on the other hand it shows the difficulties associated with the probability of bioaccumulation of heavy metals in the form of highly active particles and compounds, which requires special attention to environmental issues and safety of the developed approaches. The ecotoxicological assessment of nanoparticles remains the problem of creating algorithms for their comprehensive investigation, identifying key test objects and test functions, laying the foundations for the development of environmental safety standards. Studies are important to establish tolerance limits and assess the resistance of organisms to this factor of natural and technogenic origin.

The biological activity of metal and oxides nanoparticles indicates that the cause of this phenomenon is the processes associated with the action of a regulatory signal in biological systems (Burlakova et al., 1996; Voronkov et al., 2005; Rajput et al., 2018; Churilov et al., 2019). The biological effect produced by chemical and physicochemical factors is associated with the transfer of information that is universal for any biological objects that operate with a high degree of certainty in the system: an agent (a preparation) - a cell and its structures.

The system of supramolecular structures of the cellular microenvironment satisfies these requirements (Zaytsev et al., 1999). The authors of (Bogatyrenko et al., 1989; Churilov, 2009; Samoylova et al., 2017; Polischuk et al., 2018) showed that the energy of nanoparticles (NPs) of biogenic metals (iron, cobalt and copper) of a certain size (25–60 nm) stimulates the processes of self-organization of biological systems and their adaptation to external conditions. It is assumed that nanocrystalline metals have great potential in mineral nutrition and energy exposure and, thanks to uncompensated bonds, easily form complex compounds with organic substances. As a result, they activate the synthesis of various enzymes that affect carbohydrate and nitrogen metabolism, amino acid synthesis, photosynthesis and cellular respiration (Churilov, 2010; Antoņenko et al., 2016; Argentel-Martínez et al., 2019; Karpenko et al., 2019; Arutyunyan, et al., 2020).

In most cases, maximum activity is observed in a certain dose range, divided by the so-called 'dead zone' or a decrease in the index. For metal nanoparticles, an oscillatory character of the dose dependence is observed. In each case, the significance of the most important biological processes, including participation in gene expression, was identified and proved (Folmanis & Kovalenko, 1999; Churilov et al., 2015; Churilov et al., 2018a). The possibility of penetration and bioaccumulation of nanoparticles (NPs) of copper, zinc and titanium oxides with a size of 20–80 nm in various plant organs was established in (Churilov et al., 2018b; Stepanova et al., 2019). It was shown that bio-accumulation in plants led to transmission of NPs along food chains to animals. When introducing vetch grown after seed treatment with copper, zinc and titanium oxides at a concentration of 100 g t^{-1} into the rabbits' diet, a significant decrease in live weight of animals was observed throughout the experiment in experimental groups by an average of 15–9%.

There is no bioaccumulation for metal nanoparticles (copper, cobalt, zinc), 20–100 nm in size. Additional evidence of the lack of accumulation of metal NPs with a size of 20–100 nm is the effect of the introduction of crops pre-treated with NPs into the diet of animals on the state of animals. Vetch pre-treated with NPs of copper, iron and cobalt affects the growth of live weight of rabbits, increasing it by 12–16% relative to the control, while the main blood indicators were within the physiological norm. Therefore, NPs can be used in feed made of plants pre-treated with NPs of metals at concentrations up to 100 g t^{-1}. Moreover, the increased content of useful nutrients in such plants (Ampleeva, 2006) stimulates the growth and development of rabbits.

The effect of nanoparticles on the biological activity of soils and microorganisms that are sensitive to environmental changes has been studied. Metal nanoparticles have almost no effect on bacterial populations. The response to NPs of copper and zinc oxides is highly dependent on their concentration in the soil. Low oxide concentrations can stimulate bacteria that are thought to use resistance mechanisms to allow them to grow, but at high concentrations, population survival is significantly reduced. NPs in the range of up to 20 nm are much more reactive and dangerous. Oxide nanoparticles are more soluble and interact, which is especially characteristic of ZnO with membrane lipids and thiol groups of the enzyme and proteins, which are important for bacterial respiration, as well as transmembrane and intracellular transport. The generation of ROS (reactive oxygen species) plays a key role in this process, since damage to membranes, DNA and cellular proteins is the result of ROS.

The previous studies have considered the influence of nanoparticles on the biological activity of soils and microorganisms *Pseudomonas fluorescens*, Trichoderma koningii, *Bacillus cereus* and *E. coli M-17* (test system 'Ekolyum'), which are sensitive to environmental changes. There is some evidence of the effect of metal and metal complex nanoparticles and carbon nanotubes on bacterial populations (Bannikova 2017; Godymchuk, 2012). However, metal nanoparticles with certain physical-chemical characteristics studied in this work practically do not affect bacterial populations. The response of copper oxides and zinc to NPs is highly dependent on their concentration in the soil. Low concentrations of oxides can stimulate bacteria that are thought to use resistance mechanisms to allow them to grow, but at high concentrations, population survival is significantly reduced. When NPs reactivity is in the range of up to ~ 20 they are much more reactive and dangerous. Oxide nanoparticles are more soluble and interact, which is especially characteristic of ZnO with membrane lipids and thiol groups

of the enzyme and proteins, which are important for bacterial respiration, as well as transmembrane and intracellular transport. The generation of ROS plays a key role in this process, since damage to membranes, DNA and cellular proteins is the result of ROS.

Thus, in order to develop a unified approach to assessing the potential dangers of artificial nanoparticles, it is necessary to create an integrated system of phyto-toxicological studies.

The following tasks were set: 1. To reveal the patterns of morph physiological reactions of organisms of such ecological and functional groups as earthworms (*Lumbricina*), white outbred rats and Wistar rats on the effect of colloidal aqueous solutions of nanoparticles. 2. Given the possibility of penetration and bioaccumulation of NPs in tissues of higher plants, to evaluate the resulting biochemical and morph physiological effects. 3. To determine the environmental safety parameters of nanoparticles of different physical-chemical characteristics. The choice of these test objects was determined by the following criteria: a significant role in the functioning of terrestrial ecosystems, the prevalence of ecotoxicological studies in practice and the ease of growing in laboratory conditions.

MATERIALS AND METHODS

Physical-chemical properties of nanoparticles

Nanoparticles were obtained at RTU 'MISiS' by chemical precipitation of metal hydroxides from salt solutions, followed by their low-temperature reduction in a hydrogen stream with subsequent passivation (Dzidziguri et al., 2000; Novakova et al., 2001). Using a scanning electron microscope, the dispersion and morphology of the obtained metal nanoparticles were investigated. Physical-chemical properties of nanoparticles were determined by the following indicators: specific surface area (m^2 g^{-1}), size distribution of nanoparticles and form factor (Table 1). The specific surface area of the nanoparticles under study was measured by low-temperature nitrogen adsorption by BET using the Quantachrome NOVA 1,200e analyzer.

Table 1. Physical-chemical properties of nanoparticles

Parameter	Cu	Co	Zn	CuO	ZnO
Particle Size, nm	34–63	28–46	30–76	28–68	30–80
Specific Surface Area, m^2 g	6.5	52.1	5.7	90.5	37.9
Form Factor (ratio of the maximum size to the minimum one)	< 10	< 10	< 10	< 10	< 10
Solubility in Water wt. %	insoluble	insoluble	insoluble	insoluble	sparingly soluble
Solubility in Biological Fluids	< 1	< 1	insoluble	< 1	> 1
Charge	+	+	+	+	+
Aggregation Resistance	low	low	low	low	low
Hydrophobic Nature	+	+	+	+	+
Ability to Generate Free Radicals	low	low	low	revealed	revealed
Accumulation in Organs and Tissues	not found	not found	not found	revealed	revealed
Acute Toxicity	not hazard (class 4)	low-hazard (class 3)	low-hazard (class 3)	low-hazard (class 3)	low-hazard (class 3)

Toxic properties of nanoparticles

These studies were carried out in accordance with the Methodological Recommendations of the Pharmacological State Committee ('Guidelines for the experimental (preclinical) study of new pharmacological substances', Moscow, 2005, edited by R.U. Khabriev). Advanced development models of nanoparticles of copper, cobalt, iron, zinc, zinc oxide, copper oxide, sized 20–80 nm were used in the studies.

To establish the general toxic properties of the solution, white outbred rats were used. The animals were received from nursery 'Andreevka Branch of the State Center for Scientific Research of Biomedical Technologies of the Russian Academy of Medical Science'. They were grown on purpose and had not previously participated in experiments. The supplier of laboratory animals provides documents proving the last control of their health. The animals, newly arrived at the institute's vivarium, were kept in the quarantine and adaptation room for 14 days. During the quarantine period, their clinical indicators of health status were monitored. Animals were kept in polycarbonate cages, 5 animals each. Sawdust was use as litter. Animals were kept in the vivarium according to sanitary rules and on a standard diet in accordance with the Order of the Ministry of Health of the USSR No. 1045-73 dated April 6, 1973, The rules of laboratory practice and the Order of the Ministry of Health of the USSR No. 1179 dated October 10, 1983. Feeding was carried out in accordance with the Order of the Ministry of Health of the USSR No. 163 dated March 10, 1966 on the ration of laboratory animals, since January 1, 1979 the 'Temporary daily allowance of granular compound feeds for laboratory animals' adopted and approved by the Ministry of Health of the USSR on December 4, 1978 and following the guidelines 'Standardization of the ecological environment of laboratory animals by nutrition factor' (1980), Academy of Medical Science. Compound animal feedstuff extruded for laboratory animals (rats, mice, guinea pigs) GOST R 51849-2011 R.5 was delivered by LLC Labkorm, Moscow. The animals were watered from standard drinking bowls with tap water corresponding to GOST 'Drinking water'

The animals were kept in rooms with natural artificial lighting and controlled microclimate. Daily readings were taken from psychrometric hygrometer VIT-2 (all indications are documented). The temperature and humidity conditions were within normal limits: air temperature 20–22 °C and relative humidity 60–70%.

Preparation for the experiment was carried out in accordance with the instructions 'Toxicity test' GF XI. Before the experiment, food and water were taken from animals. Two hours later, they were weighed and divided into groups. The selection in groups was carried out arbitrarily by the method of 'random numbers', using body weight as a criterion. Individual body mass values did not deviate from the group average by more than 10%. Animals were weighed on a BP-05MS-3 0.5/BR scale (Russia). To conduct experiments on the acute toxicity of nanoparticles, white outbred rats weighing 190–220 g were used. 6 series of experiments were carried out, 48 groups of animals were formed, each of which consisted of 10 outbred rats. Their weight is indicated at the time of the preparation administration.

Nanoparticle bio-testing on representatives of the earthworm family

Objects of study were worms - representatives of the family of earthworms (*Lumbricina*), species: dung worm (*Eisenia fetida*) and White-bellied earthworm (*Octolasion lacteum*). Soil was taken from wheat cultivation sites. The test is considered

reliable according to GOST 33637-2015 when the following criteria are met for controls and experimental samples:

– at the end of the test, the total death during the absorption and elimination phases should not exceed 10% (for earthworms) or 20% (for enchitreides) of the total number of worms introduced into the test;

– the average weight loss for *Eisenia fetida* and *Eisenia andrei*, measured at the end of the absorption and elimination phases, should not exceed 20% compared to the initial wet weight (raw tissue mass).

A series of indicator cytochemical, cytomorphological, reproductive and restoration indicators of worms in contact with soils containing various concentrations of nanoparticles of various compositions and sizes was provided. The effect on the soil (substrate) was analyzed on the following soil options: 1 – a control sample of natural soil from areas where field tests were carried out; 2 – soil containing optimally permissible concentrations of 10 g t^{-1} of soil, 100 g t^{-1} and a maximum of 1,000 g t^{-1} of copper and cobalt NPs up to 20 nm; 3 – soil with copper or cobalt nanoparticles sized 30–80 nm; 4 – soil containing nanoparticles of copper and zinc oxides with a size of 20–80 nm, a concentration of 10 g t^{-1} and 100 g t^{-1}. All components were crushed, moistened and mixed until a homogeneous mass. At the end of the experiment, the worms were selected, washed with distilled water and then weighed. Then the bio-substrates were brought to a constant weight in an oven. Dried samples of soil and worm tissues were used to determine metals.

Determination of antioxidant enzyme activity

After cleansing the digestive tract, the tissues of the worms Eisenia fetida were homogenized with the help of a tissue homogenizer (QIAGEN, Germany). The homogenate was centrifuged for 18 minutes at 9,000 rpm, diluting to 10% solution. The resulting supernatant was diluted with a buffer mixture to 10% homogenate.

The content of lipid peroxidation products, malondialdehyde (MDA), as well as the activity of the key units of the antioxidant defence system (catalase and superoxide dismutase) was determined using a CS-T240 biochemical analyzer (Dirui Industrial Co., Ltd, China) in a homogenate of worm tissues using commercial Randox biochemical kits (USA). For this, hoods were prepared by homogenization in a buffer (Tris 50 mmol L^{-1}, DTT 1.0 mmol L^{-1}, EDTA 1.0 mmol L^{-1}, sucrose 250 mmol L^{-1}, pH 7.5), which was added in a ratio of 1:9. The activity of malonic aldehyde, catalase and superoxide dismutase was determined on a CS-T240 biochemical analyzer using commercial kits from Randox (USA). The activity of antioxidant enzymes was studied in *Eisenia fetida* worms under the action of Zn and zinc oxide nanoparticles sized 20–80 nm at various concentrations on natural soil. To evaluate potential acute and long-term effects, their effects were studied at a concentration of 1, 10, 100 and 1,000 g t^{-1} (g of nanoparticles per ton of soil). The effect of nanoparticles on growth, bioavailability and enzyme activity was determined. The worms were grown in horse manure without any medicine at 22 ± 2 °C. For each repetition, mature worms weighing 400–450 mg were selected, of which 9 groups were formed (n = 10). Group I included worms grown when adding zinc NPs at a concentration of 1 g t^{-1} of soil. Group II was connected with a concentration of 10 g t^{-1} of soil. Group III presupposed a concentration of 100 mg kg^{-1} of soil. Group IV was connected with a concentration of 1,000 g t^{-1} of soil. Group V included worms grown with the addition of zinc oxide nanoparticles at a concentration of 1 g t^{-1} of soil.

Group VI had a concentration of 10 g t^{-1} of soil. Group VII presupposed a concentration of 100 g t^{-1} of soil. Group VIII had 1,000 g t^{-1} of soil and group IX was the control without introducing NPs into the substrate.

Histological studies

Characteristics of the animals used and their conditions

Mature laboratory Wistar rats of both genders and weighing 200 ± 1 g were used in the studies. 96 females were involved in the experiment. All manipulations with laboratory animals were carried out in accordance with the legislation of the Russian Federation and met international principles of good laboratory practice (GOST R 33044-2014), taking into account recommendations for the management and use of laboratory animals (Guide for keeping ... 2001). During the experiment, the animals were kept in cages for rodents with a capacity of up to 13 individuals at a 12-hour light period, temperature of +22 °C and relative humidity of 40–60%. Autoclaved sawdust of deciduous tree species was used as litter. Standard granular feed Compound feed PK-120' specially developed for laboratory rats, mice and hamsters was used.

Five groups of animals, 8 animals each, were formed that received cobalt nanoparticles with a size of 20 nm, cobalt and copper nanoparticles with a size of 30–60 nm and copper and zinc oxides of 20–80 nm in size.

The animals' status observation was carried out within two weeks after the introduction of material with control of lethal action and such parameters of the general state of health as the intensity and nature of motor activity, the state of skin and hair, water and feed. The lethal effect of the introduced NPs was recorded by a number of deaths within the experimental groups of animals throughout the entire controlled period.

Before exposure, animals were kept in quarantine for 10 days. The studied materials were administered once intragastrically at a dose of 0.02, 0.2, 2.0 mg kg^{-1}. The control group was injected with distilled water. At the end of the control period, all animals were euthanized by dislocation of the cervical vertebrae (Mank, 1990). Then, the relative mass of visceral organs was calculated and histological studies were performed.

The liver, kidneys, adrenal glands, brain tissues, reproductive organs (tubes, uterus) were fixed in 10% formalin buffer solution, after which they were processed in a histological processor Tissue-Tek Xpress from SAKURA (Japan) and filled with homogenized paraffin medium for histological filling of tissues 'Meltex' company 'JLS Chemical' (Russia).

Slices with a thickness of 5–7 μm were obtained on a luge microtome 'MS-2' (Russia). For general purposes, connective and muscle tissues were stained with hematoxylin and eosin according to Van Gieson.

RESULTS AND DISCUSSION

Toxicological properties of nanoparticle additives

Nanoparticles were previously mixed with distilled water, kept in a plastic container in an ultrasonic bath for 20 minutes to achieve a uniform suspended state of particles in solution. Immediately after preparation, the suspension was administered to experimental animals once in the stomach using a probe in the form of a provided solution in an amount of 1 ml containing various dosages: 20, 40, 70, 100, 130, 160,

180 mg per animal. This was 1,000, 2,000, 3,500, 5,000, 6,500, 8,000, 9,000 mg kg^{-1} of the body weight and the control without additives.

To calculate the indicators of median lethal dose LD_{16}, LD_{50}, LD_{84}, the Miller-Teitner probit analysis method was used and GOST 12.0.007-76 was used to determine the hazard class.

As a result of studying the toxicological properties of nanoparticle additives, the following results were obtained:

• parameters of acute toxic effects with a single oral administration to white rats;

• parameters of toxic effects with repeated use of additives to white rats (chronic toxicity).

An experiment to determine chronic toxicity and cumulative properties was carried out in 3 series for 60 days using two methods (Lim, 1961; Yu.S. Kagan and V.V. Stankevich, 1968). In the first 5 days, each rat was injected 1/10; 1/20 or 1/50 of the previously established single doses of LD_{50}. Then, every subsequent 5 days, the dose increased 1.5 times from the previous daily administered dose. In the course of the experiment, the phenomena of toxicosis and death of animals were recorded and an autopsy of dead animals was performed.

For nanoparticles with a size of 20–80 nm, toxic characteristics were determined. Parameters of acute toxic effects after a single oral administration to white rats were as follows: LD_{50} (Zn NP) – 379.7 mg kg^{-1}, LD_{50} (ZnO NP) – 292.9 mg kg^{-1}, LD_{50} (Co NP) – 1233.3 mg kg^{-1}, LD_{50} (CuO NP) – 259.2 mg kg^{-1}, respectively, so additives were classified as moderately hazardous substances of hazard class 3. LD_{50} (Cu NP) – 7,000.0 mg kg^{-1}, LD_{50} (Fe NP) – 9,000.0 mg kg^{-1}, respectively, indicated additives belonging to low-hazard substances of hazard class 4 according to GOST 12.1.007-76.

Established chronic lethal doses were as follows: LD_{50} (Zn NP) chron. – 1,284.0 mg kg^{-1} of live weight, LD_{50} (ZnO NP) chron. – 1,183.8 mg kg^{-1}, LD_{50} (Co NP) chron. – 4,180.8 mg kg^{-1}, LD_{50} (CuO NP) chron. – 580 mg kg^{-1}, LD_{50} (Cu NP) chron. – 40,650.9 mg kg^{-1}, LD_{50} (Fe NP) – 56,000 mg kg^{-1}. The results were consistent with previously established hazard classes in experiments on the study of acute oral toxicity.

Biological and environmental safety of nanoparticles by the example of representatives of the earthworm family (*Lumbricina*)

If one compares the chemical method for analysing soil pollution, it shows the concentration of only the substances being determined, and the biological method using worms indicates the presence or absence of toxicity. The main soil load is derived from such pollutants as Cu, Zn, Co, Pb.

Table 2. The metal content in the soil during the experiment

	The content before the experiment, mg kg^{-1}	Content after 8 weeks of experiment, mg kg^{-1}, NP concentration 1,000 g t^{-1}	MPC, mg kg^{-1}
Cu	2.80 ± 0.03	2.9 ± 0.02	3.00
Zn	21.6 ± 0.022	24.0 ± 0.05	23.0
Co	4.60 ± 0.07	4.65 ± 0.04	5.04
Pb	5.40 ± 0.04	5.4 ± 0.03	6.00

The control soil contained these elements below the MPC and the indicators remained in this range for 3 days, 1, 4, and 8 weeks and did not depend on the composition of nanoparticles at the concentration range of 1–100 g t^{-1}. When the concentration of nanoparticles was 1,000 g t^{-1}, the indicators changed remaining in the MPC, excluding the zinc content (Table 2).

After the contact of nanoparticles with the soil, an increase in the weight of the worms (*Octolasion lacteum*) was gradually observed in the first, fourth, and eighth weeks (Fig. 1). The weight of the worms in the control variant was 200 mg, 240 mg, 604 mg, and 805 mg, depending on the time of the experiment. In soils where the content of Cu, Zn, Co nanoparticles was 10 g t^{-1} of soil, an increase in the mass of worms was observed and after 8 weeks it was higher by 28, 29, and 31%, respectively, relative to the control. After the 4th and 8th week the soil, where the content of Cu, Zn, Co nanoparticles was 1,000 g t^{-1} of soil, the mass of worms decreased from 10 to 12% compared with the control. At the same time, after the second week an increase in the mass of worms in soils with the content of ZnO and CuO nanoparticles of 10 g t^{-1} was only by 3 and 5%, respectively. After the 8th week, the mass of worms in this soil decreased by 25% and 45%, and in soils with a content of nanoparticles of 1,000 g t^{-1} the decrease was 46% and 58%, respectively. A similar pattern was observed for *Eisenia fetida*. For copper and zinc nanoparticles with sizes up to 20 nm an increase in the weight of worms was observed by 15% and 35% compared with the control after the 2nd week. After the 6th week the weight of worms decreased in soils where the content of nanoparticles was 10 g t^{-1} by 60% and 65%. The decrease after the 8th week amounted to 75% and in soils with a content of nanoparticles of 1,000 g t^{-1} the worms' death was observed.

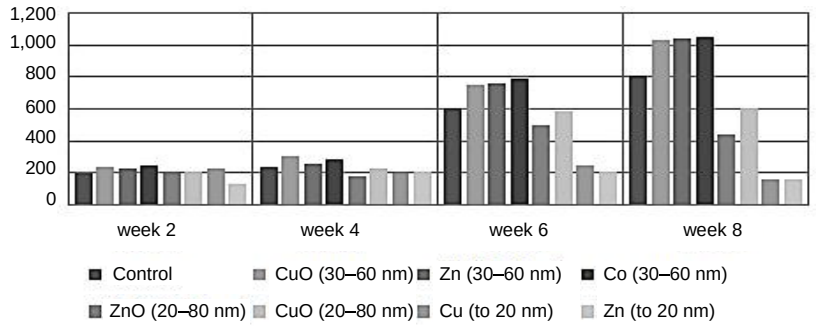

Figure 1. The change in the mass of worms during the experiment at a concentration of nanoparticles in the soil equal to 10 g t^{-1}.

After 5 weeks of the experiment the number of individuals and cocoons of all types of worms in the control soil did not change significantly. The survival rate of individuals was 97% and that of cocoons was 98%. When the content of Cu, Zn, Co, ZnO, CuO nanoparticles was 1–10 g ha^{-1}, the number of individuals, as well as their cocoons, did not change compared to the control. The number of individuals decreased by 15% and that of cocoons was 21% less at a concentration of Cu, Zn or Co nanoparticles of 1,000 g t^{-1} and by 24–29% for ZnO and CuO nanoparticles at a soil concentration of 100 g t^{-1}. The reproductive activity of worms, depending on environmental toxicity, is a reliable indicator of biotesting. It should be noted that the main parameters in the study of the propagation of worms are: the dynamics between the number of individuals and cocoons obtained from them and the number of hatched young individuals from these cocoons. When studying the relationship between the number of cocoons and the appearance of young worms, it was revealed in the third week, that the number of young worms increased on average by 30% compared with the control soil when contact with

metal nanoparticles (1–100 g t^{-1}) and by 5% when contacting oxides. At a higher concentration of oxides in the soil, the number of cocoons decreased with increasing the age of worms, and at the 6th week of ontogenesis they cease to postpone them.

When the concentration of nanoparticles was 100 g t^{-1} of soil the number of large granules in amebocytes increased by 0.6% and small granules decreased by 1.9%. A similar trend remained unchanged for the dung worm. For oxides at a concentration of 1–10 g t^{-1}, the cytochemical parameters in hemolymph cells were as follows: for large granules, an increase of 1.5% was observed and for small granules a decrease of 2–3% was noticed. At a concentration of more than 100 g t^{-1} in the amebocytes of the worms, compared with the norm, the number of large granules increased sharply and small granules practically disappeared. When evaluating the number of large and small glycogen granules in amoebocytes of worms exposed to anthropogenic effects of oxide nanoparticles, it was noted that their change had some negative effect, which was apparently associated with a higher solubility of copper oxides and especially zinc, as well as with an increase in the amount of these metals in the soil.

Determination of the activity of antioxidant enzymes in *Eisenia fetida* worms

To confirm the different toxicities of zinc and zinc oxide, the activity of the antioxidant enzymes of *Eisenia fetida* worms was studied. The tissues of the worms (after cleaning the digestive tract) were mixed with a homogenizer (QIAGEN, Germany).

The homogenate was centrifuged for 15 minutes at 10,000 rpm, diluting to 10% solution.

The activity of malonic aldehyde catalase and superoxide dismutase was determined on a CS-T240 biochemical analyzer using commercial kits from Randox (USA). The activity of antioxidant enzymes in *Eisenia fetida* worms was studied under the action of Zn nanoparticles and zinc oxide with a size of 20–80 nm, in natural soil. To evaluate the potential acute and long-term consequences of their impact, the effect of nanoparticles on growth, bioavailability and enzyme activity was determined at a nanoparticle concentration of 1, 10, 100 and 1,000 g t^{-1} of soil.

On the 30th day of exposure to zinc oxide nanoparticles, the development of oxidative stress was manifested in a sharp increase in the level of enzymes.

When a dose of Zn NP of 100 mg kg^{-1} the maximum level of malondialdehyde (MDA), the main indicator of lipid peroxidation, was higher than the control by 18%. At low and medium concentrations (1–10 g t^{-1}), the activity of MDA was in the range of 8–12% relative to the control values. The activity of superoxide dismutase (SOD) and catalase enzymes for zinc oxide nanoparticles increased, but deviations from the control were less than 30% and reached 70–120%. Evaluation of the antioxidant status of antioxidant enzymes, superoxide dismutase and catalase showed that under the influence of zinc NPs there was a slight increase in SOD at concentrations of 10 and 100 g t^{-1}, followed by a decrease of 32% at a dose of 1,000 g t^{-1}, respectively. An increase in load leads to inhibition of enzymatic reactions. There is a decrease in catalase activity by 10% relative to the control. Against the background of an increase in the level of antioxidant protection, the worm's body is able to withstand the amount of zinc NPs much higher than 100 g t^{-1} and, consequently, for metal NPs of 35–60 nm in size the stability limit of adaptation mechanisms is a concentration significantly exceeding 100 g t^{-1} and for oxides it exceeds 10 g t^{-1}.

The bio-accumulation of nanoparticles in the structure of worms depends on the level of nanoparticles in the soil substrate and on the ability of metal ions to return to the soil substrate. The level of zinc bio-accumulation in the presence of zinc and zinc oxide is 1.3 and 18.02, respectively, at a dose of 100 g t^{-1}, whereas it is 2.3 at 10 g t^{-1}. The degree of absorption and the rate of accumulation of metals in the body of the worms increased depending on the level of NP dose in the soil substrate. In particular, the degree of absorption in the control corresponded to a value of 0.48 mg % at 100 g t. With an increase in the concentration of zinc and zinc oxide, respectively, to 1,000 g t^{-1}, the rate of accumulation and degree of absorption of zinc was 2.3% and 29.8%, respectively, more than the control. The highest bio-accumulation value was found at a dose of oxide nanoparticles of 1,000 g t^{-1}, where the absorption and accumulation rates increased linearly with increasing the load on the soil substrate. It can be argued that the nanoparticles of oxides accumulate in structures of living systems, complicating their development and reducing survival.

Cytotoxicity of nanoparticles of copper, cobalt and oxides of copper and zinc for rats

With the introduction of suspensions of NPs of copper, cobalt and their oxides in a dose of 0.02 mg kg^{-1} of live weight to rats, blood hematological and biochemical parameters were studied for a month.

The analysis results showed that cobalt (Co NP) and copper (Cu NP) nanoparticles with particle sizes of up to 20 nm and 35–65 nm, respectively, did not significantly affect the blood hematological parameters of experimental animals compared with the control group. A slight change in the myelogram was observed: for example, leukocytes increased by 38% in the case of Cu NPs and by 39.3% in the case of Co NPs compared to the control, remaining within the normal range, which can be explained by stress during blood sampling. The percentage of components has not changed.

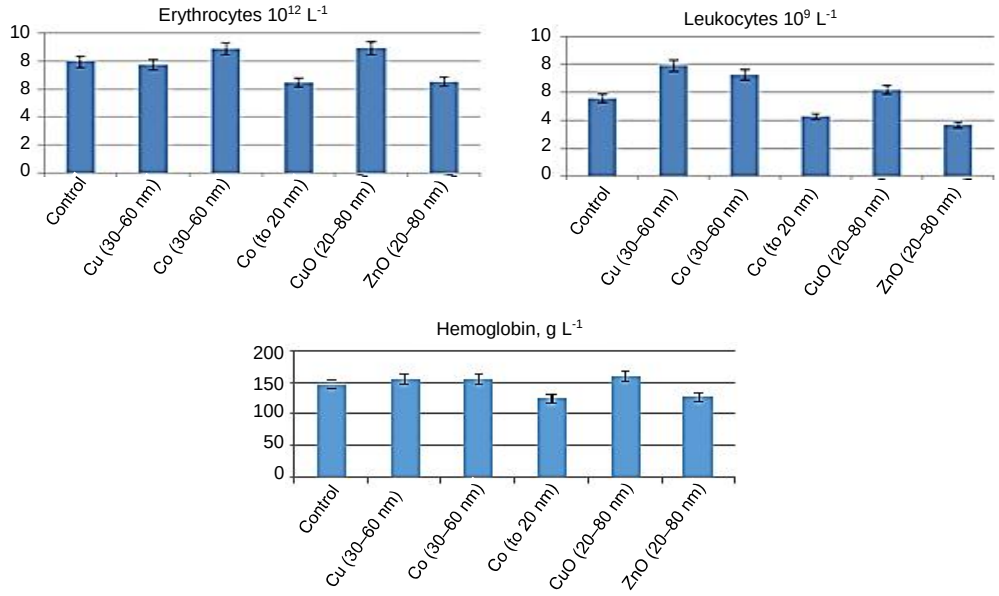

Figure 2. Hematological blood parameters of experimental animals.

When ZnO NPs (particle size 20–80 nm) were introduced into the diet of rats, the number of erythrocytes, leukocytes and hemoglobin decreased by 17.7% and 18.5%, 34.1% and 20.5%, 13.6% and 15%, respectively, compared with the control (Fig. 2). The total volume of erythrocytes, their distribution in blood and lymphocytes increased by 13.9%, 59.7% and 18.5%, respectively. ESR increased relative to the control by 5.5–8 times, which indicated the toxicity of ZnO and Co NPs (to 20 nm).

The introduction of CuO NPs (particle size 20–80 nm) contributed to an increase in all parameters (Fig. 2). So, erythrocyte distribution in blood increased by 17.7%, hemoglobin by 4.8% and segmented neutrophils by 18.75%. All NPs, except cobalt sized 20 nm and zinc oxide (20–80 nm) did not have any negative effect on morphological parameters of blood.

Nanoparticles of copper and cobalt with a size of 35–60 nm changed the biochemical parameters of blood approximately to the same extent within the physiological norm (Figs 3–8). NPs of copper and cobalt caused an increase in creatinine by 18.5% and 18.05% and glucose by 25.2% and 26.0%, respectively. The decrease in urea was 11.0% and 11.8%, the total bilirubin was 23.0% and 22%, respectively. The increase was insignificant and indicated the absence of toxic effects of NPs on hepatocytes.

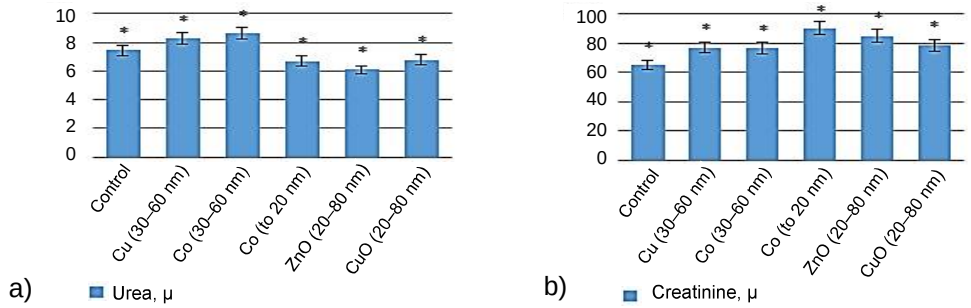

Figure 3. The effect of nanoparticles on the content of urea (a) and creatinine (b) in rats' blood (hereinafter, in section *, options are indicated in which a significant difference with control values is noted at $p < 0.05$).

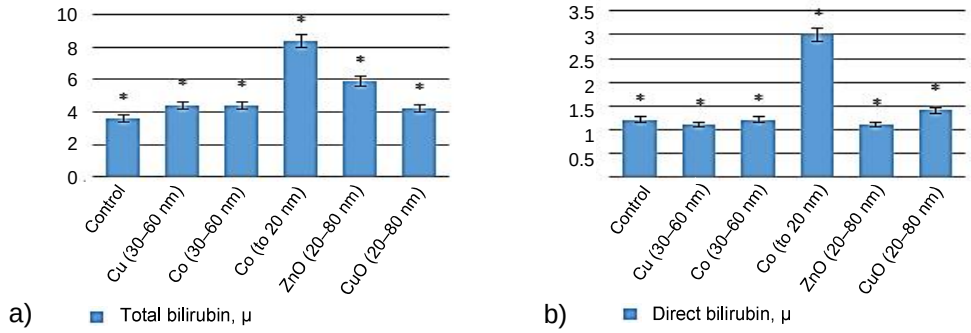

Figure 4. The effect of nanoparticles on the content of total bilirubin (a) and direct bilirubin (b) in rats' blood.

To determine the effect of nanoparticle sizes, rats were injected with Co NP, 18–20 nm in size (Figs 3–8). For this group there was an increase in comparison with the control values: creatinine by 38.46%, total bilirubin by 133.3%, direct bilirubin by 60.0%, glucose by 17.5%, albumin by 39.3% and cholesterol by 31.3%. AST increased by 15%. ALT decreased by 47.5% and de Ritis coefficient decreased by 2.3 times. At the same time, total protein and urea decreased by 5.8% and 9.8%, respectively. All these data indicate the toxic effect of Co NPs (to 20 nm) on internal organs (liver, kidneys). Such nanoparticles are extremely reactive and dangerous for living systems.

For CuO NPs the values of the parameters were as follows: urea decreased by 8.7%, AST increased by 14% and ALT became 55% lower, de Ritis coefficient decreased by 20%, relative to the control. Total bilirubin increased by 16.7%, alkaline phosphatase by 38.7%, total protein by 7.42% and globulins by 6% (Figs 3–8).

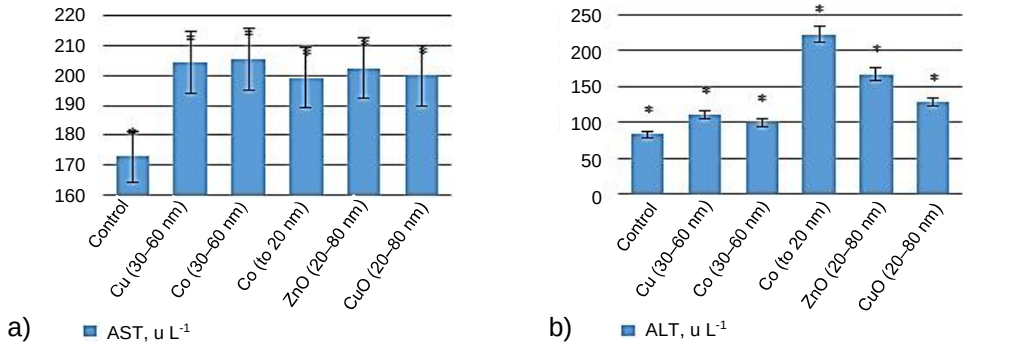

Figure 5. The effect of nanoparticles on the content of AST (aspartate aminotransferase) (a) and ALT (alanine aminotransferase) (b) in rats' blood.

When rats got ZnO NPs, urea decreased by 18%, total protein by 12.8% and the globulin fraction by 18.4%. The content of alkaline phosphatase increased by 4 times, AST by 17%, ALT by 2 times and de Ritis coefficient decreased by 1.7 times, which indicated the toxic effect of NPs on bone tissue, liver and kidney (Figs 3–8).

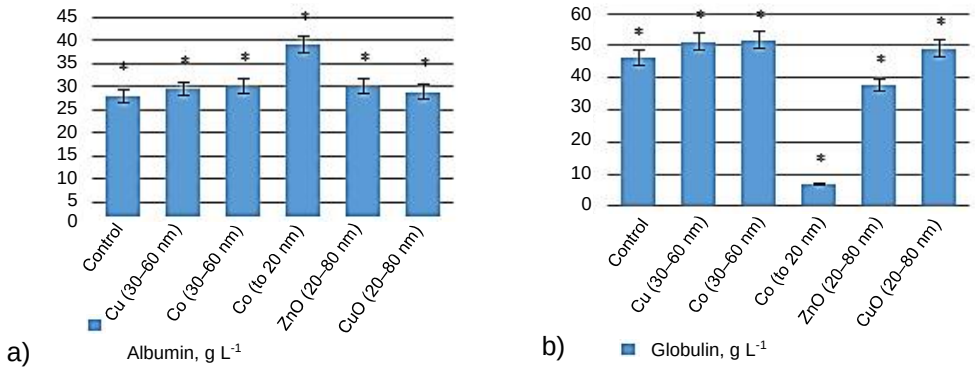

Figure 6. The effect of nanoparticles on the content of albumin (a) and globulin (b) protein fractions in rats' blood.

When comparing the effect of NPs of cobalt and copper, sized 35–60 nm, on morphological and biochemical parameters of blood, one can say they have a positive effect on the animal's organism, stimulating metabolic processes. Cobalt NPs, having a particle size of up to 20 nm, have a toxic effect, so they are dangerous for living systems. The effect of copper oxide NPs is not toxic, but it inhibits the metabolic processes of both plants and animals. Zinc oxide has a toxic effect and this is possibly due to higher solubility of zinc oxide in biological fluids.

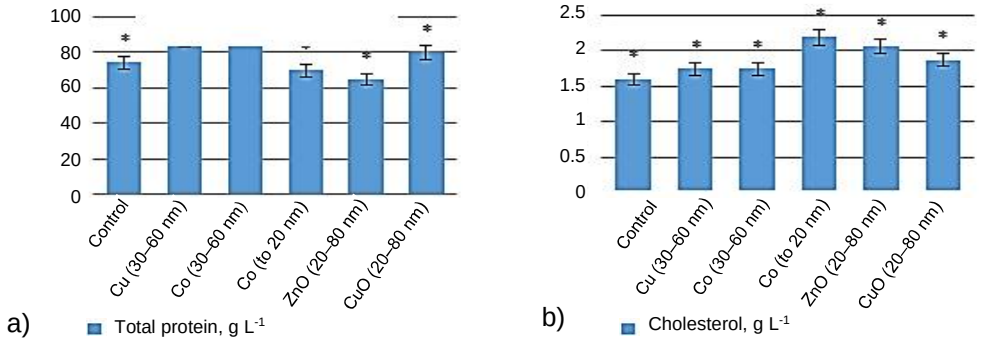

Figure 7. The effect of nanoparticles on total protein (a) and cholesterol (b) in rats' blood.

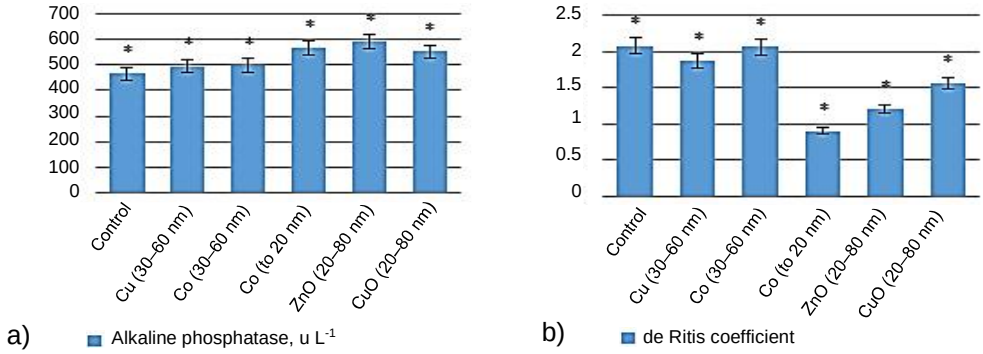

Figure 8. The effect of nanoparticles on the activity of alkaline phosphatase (a) in rats' blood and on the value of de Ritis coefficient (b).

Determination of the level of malondialdehyde and the activity of superoxide dismutase showed a different ratio for all studied groups. The MDA content in the group getting metal NP corresponds to the control group and is less than in the groups treated with oxides NP and especially cobalt, up to 20 nm in size. A decrease in the content of MDA indicates a decrease in the level of lipid peroxidation processes.

A change in the level of malondialdehyde, one of the final products of the lipid peroxidation process, allows to judge the intensity of processes that inevitably increase in pathogenic conditions. A decrease in the MDA level for 35–60 nm nanoparticles of copper and cobalt indicates a decrease in free-radical oxidative processes, i.e. a decrease in the level of oxidative stress, relative to the effect of cobalt oxide NPs and cobalt NPs, 20 nm in size.

Table 3. The content of malondialdehyde and the activity of superoxide dismutase in plasma and hemolysates of rat erythrocytes with the introduction of NP in a dose of 0.02 mg kg^{-1}

Indicator	Malondialdehyde, mol ml Hb		Superoxide dismutase, c.u. g Hb	
	Hemolysates	Plasma	Hemolysates	Plasma
With natural pathomorphism	17.8 ± 1.5	2.10 ± 0.32	1.45 ± 0.2	13.04 ± 0.73
NP of copper, size 35–60 nm	17.0 ± 2.6	2.04 ± 0.01	1.42 ± 0.3	12.32 ± 0.54
NP of cobalt, size 35–60 nm	17.1 ± 1.8*	2.0 ± 0.021	1.40 ± 0.41	12.42 ± 0.65
NP of copper oxide, size 20–80 nm	18.0 ± 2.6	2.09 ± 0.01	1.42 ± 0.3*	13.09 ± 0.65
NP of zinc oxide, size 20–80 nm	19.1 ± 2.6	2.36 ± 0.01	1.35 ± 0.3	13.45 ± 0.65
NP of cobalt, size 20 nm	19.5 ± 2.2	2.8 ± 0.5	1.33 ± 01	14.06 ± 1.01

*-$P \leq 0.05$.

The decrease in SOD activity against the background of an increase in the MDA level (Table 3) may indicate a decrease in the protective mechanisms of the cell, which is typical for the action of zinc oxide nanoparticles, 20–80 nm in size and cobalt NPs, sized 20 nm, at a concentration of 0.2–2.0 mg kg^{-1}. In this experiment, under the influence of copper and cobalt nanoparticles with a size of 35–60 nm, SOD decreases proportionally with a decrease in MDA, which suggests a decrease in the number of superoxide anions on the cell surface, i.e. a decrease in the intensity of peroxide oxidation processes.

Histological studies

For histological studies, 8 animals were selected. Samples were taken from 1.5 to 2.0 cm in size. A 10.0–12.0% formalin solution was used to fix the material. Samples were filled in paraffin blocks (7–8 days). Sections with a thickness of 5–7 μm were obtained on a luge microtome 'MS-2' (Russia) and stained with hematoxylin and eosin.

Some moderate expansion of the vessels of the venous channel with their pronounced plethora was noted in the liver of female rats treated with Cu NPs sized 35–60 nm at a dosage of 2.0 mg kg^{-1}. Some blood vessels of rats treated with 2.0 mg kg^{-1} CuO NPs were empty. Dystrophic processes developed in hepatocytes with the appearance of grains and vacuoles in the cytoplasm of cells. A moderate polymorphism of hepatocyte nuclei was observed, both in size and in the degree of chromatin staining.

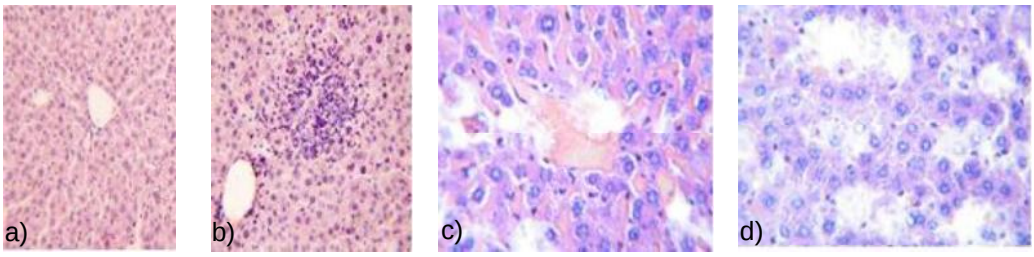

Figure 9. Histological studies of rats' liver: a – control, mp ×100; b – pathological changes in the experimental group, ZnO, mp ×400; c – expansion of the central veins and centrolobular sinusoids; d – focal necrosis of hepatocytes, ZnO, mp ×640.

Pathological changes were found in kidney tissues of animals in groups receiving Co NPs sized 20 nm and CuO and ZnO NPs sized 20–80 nm (Fig. 9). These included

congestive plethora of central veins (b) and expansion of the central veins and centrolobular sinusoids (c). One can see multiple focal necrosis of hepatocytes (d). However, in the case of cobalt with a size of 35–60 nm, the liver was characterized by plethora of central and portal veins, moderately expressed focal necrosis of hepatocytes.

In all experimental rats treated with nanoparticles of zinc, copper and cobalt oxides sized 20 nm the brain layer and a glomerular zone of the adrenal glands were with protein dystrophy, which was possibly associated with a decrease in the content of mineral corticoids in them. After NPs of copper and cobalt, sized 35–60 nm, the rats had moderate protein glomerular dystrophy. The reproductive function (tubes, uterus) in all groups, including the control, did not have gross violations.

Noticeable changes were detected in kidney tissues of animals in groups receiving cobalt NPs sized 20 nm and copper and zinc oxides of 2.0 mg kg^{-1} (Fig. 10). For the kidneys of the experimental groups (Fig. 10: 2, 3, 4), plethora of the capillary network and veins of the cortical substance was observed. In all series of experiments, the histo-structural organization of the proximal tubules is disrupted. This include multiple foci with turbid swelling of the nephocyte cytoplasm with fine granularity, a flattened form of nephrocytes, pycnosis of the nuclei and a large, filled lumen. In addition, in all series of experiments, one of the types of disturbance is observed. This is granular degeneration of cellular protein metabolism due to the decay of lipoprotein complexes, which form the basis of the membrane structures of the cell, primarily the mitochondria and the endoplasmic reticulum. The pronounced plethora of capillaries of the renal corpuscles and capillaries of the peritubular network found in all series can be the cause of granular dystrophy (Chen et al., 2007; Serrano et al., 2015).

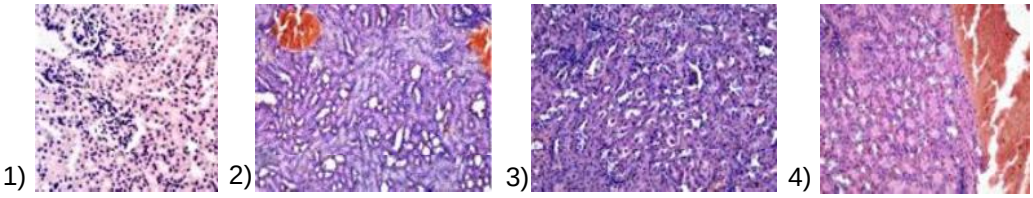

Figure 10. Histological studies of rats' kidneys in the control (1) mp ×400 and experimental groups at a nanoparticle concentration of 20 mg kg^{-1}: copper oxide (2); zinc oxide (3); cobalt (4). Stained with hematoxylin and eosin mp 10×16×160.

The observed effects were dose dependent 0.02, 0.2, 2.0 mg kg^{-1}. At the maximum dosage of cobalt NPs, 20 nm in size, pathological changes were manifested on the macroscopic level. The rats treated with NPs of metals and copper oxide in the same dosage did not have such violations.

The effect of nanoparticles on living systems depends on many factors. Important factors of their biological activity are the chemical interaction of nanoparticles with a liquid medium and a change in the pH of the medium due to the high reduction ability (Aleksanyan et al., 2005). These factors increase the permeability of membranes for nanoparticles, contributing in certain conditions to their bio-accumulation, which depends on the information (those properties) that particles of different sizes, composition and physicochemical properties possess (Auffan et al., 2009; Nel et al., 2009). When studying the dependence of the effect of nanoparticles on the oxidative

modification of proteins it was established that there was a decrease in the possibility of renewal of thymic tissue proteins due to low activity of cellular protease systems in groups of rats receiving nanoparticles of copper and zinc oxides at doses of 0.001 and 0.1 mg kg^{-1} (Abalenikhina, 2012), which directly depended on the activity of the biosynthetic mechanism of the cell. Histological studies in rats receiving 2.0 mg kg^{-1} of copper oxide and cobalt NPs sized 20 nm and zinc oxide NPs sized 20–80 nm showed that there was a change in nuclear cytoplasmic ratios in hepatocytes. There was moderate polymorphism of the hepatocyte nuclei, 'perforated nuclei' and dystrophy. In the cells of the kidneys and brain, protein dystrophy and pycnosis of the nuclei were noted, which mainly indicated some violation of the permeability of the nuclear membrane and indicated the decay of nucleoproteins and the release of nucleic acids. In addition, there was one type of violation in all the experiments, i.e. granular degeneration of cellular protein metabolism. All this indicated the influence of nanoparticles to varying degrees on the metabolism of protein synthesis and, in particular, on DNA.

CONCLUSIONS

To create environmentally friendly norms of nanoparticles in contact with biological objects, it is first necessary to determine the effects of nanoparticles on microorganisms, invertebrates (worms), plants and animals, studying their effect in the soil - plant - animal system. In this case, it is necessary to take into account the chemical composition and physical parameters of low frequencies, biocompatibility and bioaccumulation in the presence of the effect of 'low doses'. Nanoparticles are exposed to all components of a living organism, including bio-macromolecules, hormones, enzymes, especially cell membranes. Influence on the membrane structure leads to a change in the functional state of the cell, which changes the mechanism of action of nanoparticles depending on their concentration, structure and size.

Nanoparticles of copper, zinc and cobalt oxides belong to the III moderate toxicity class, but they accumulate in living systems, prolonging the time of interaction with them, which affects the survival of microorganisms, a decrease in the enzymatic activity of invertebrates, plants and animals (mice, rats, rabbits) biological, biochemical parameters of blood, the destruction of the protein structure and a decrease in the ability to update tissue proteins, which is confirmed by histological studies of internal organs.

Oxide nanoparticles accumulate in a living organism, exhibit toxic properties, lower the activity of enzymes and hormones and are transferred along trophic chains, which is not typical for metal nanoparticles.

Small nanoparticles (less than 20 nm) are characterized by a large interface. Such nano-objects exhibit high physical-chemical activity and are safe only at very low concentrations. Representatives of the family of earthworms, mice, rats are exposed to their negative effect, caused not by the toxicity of the particles themselves, but by a destructive effect on all metabolic processes and this leads to the accumulation of radicals (oxidation products) and the destruction of cell membrane proteins. Therefore, talking about favorable concentrations is impossible, they are dangerous.

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