Evaluation of biochemical markers in the blood plasma of rats exposed to chronic administration of a mixture of metal nanoparticles

M. Romanko, O. Orobchenko, V. Ushkalov, A. Paliy, A. Palii and H. Chechui*

Abstract

The aim of this study was to determine the effects of a specific mixture of metal nanoparticles (Ag, Cu, Fe and MnO_2) orally administered to rats, on biochemical blood markers as indicators of chronic toxicity. Animals (*Wistar* line rats (n=20)) in experimental groups were given a solution of a mixture of metal salts or a mixture of nanoparticles with food for 90 days. On days 15, 30, 60 and 90 of the experiment, five rats from each group were anaesthetised with CO_2 and decapitated, and blood samples were collected for biochemical studies and spectrophotometric measurement. The toxic effect of the chronic administration of the mixture at a dose of 4.0 mg/kg of body weight in white rats caused partial immunosuppression expressed as hypoproteinaemia, excessive formation of circulating immune complexes and acute phase serum mucoid proteins (P<0.05), and cytolytic damage to hepatocyte membranes. Levels of enzyme activity (AST, GGT, ALT and AP) were significantly elevated (P<0.05). It has been shown that the origin of the toxic effect is due to oxidative stress, which slowed lipoperoxidation along with the elevation of the level of carboxylated proteins, depleting the antioxidant defence resources of the organism, as seen by a decrease in the level of catalase and total antioxidant activity (P<0.05). No such effects were observed at the dose of 0.3 mg/kg as there were no significant negative impacts on the biomarkers. Based on this data, the biochemical markers studied may be suitable for use in pre-clinical *in vivo* toxicological assessment of metal nanoparticle-candidates for pharmaceuticals.

Key words: *NPMe mixture (Ag, Cu, Fe and MnO_2); chronic administration; toxic effect; immunosuppression; oxidative stress; biomarkers*

Maryna ROMANKO, Doctor of Biological Science, Senior Researcher, Oleksandr OROBCHENKO* (Corresponding author, e-mail: toxy-lab@ukr.net), Doctor of Veterinary Science, Senior Researcher, Anatoliy PALIY, Doctor of Veterinary Science, Professor, National Scientific Center Institute of Experimental and Clinical Veterinary Medicine, Kharkiv, Ukraine; Valerii USHKALOV, Doctor of Veterinary Science, Professor, Academician of NAAS of Ukraine, National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine; Andrii PALII, Doctor of Agricultural Sciences, Professor, Helena CHECHUI, Candidate of Biological Science, PhD, Assistant Professor, State Biotechnology University, Kharkiv, Ukraine

Introduction

Colloid solutions of chemically stable essential metal nanoparticles (NPs) have good biocompatibility and thus bear high commercial potential for broad applications in biology and medicine (Singh et al., 2015; Mercier-Bonin et al., 2018; Pereira et al., 2018).

The small dimensions and biological availability of nanomaterials enable them to be in direct contact with infectious agents, toxins, biological systems and individual cell structures (proteins, lipids or nucleic acids) (Bachler et al., 2015; Lee et al., 2016b; Cholewinska et al., 2018). However, concerns have been raised about the impact of NPs on living organisms and the latest regulations require assessing their toxicological properties prior to any biomedical or environmental applications. A relationship between NP size and dose and systemic toxicity to living organisms has been reported in a number of publications (Mate et al., 2016; Zhang et al., 2018), meriting further investigation of the risks associated with the use of NPs at the cellular and systemic levels.

The high affinity of metal-containing NPs (NPMe) with biomolecules in a wide range of a molecular mass, diverse mechanisms of transmembrane transfer into cells, biotransformation and biodegradation result in a great variability of their biological activities, spreading from high toxicity to biocompatibility (Cornejo-Garrido et al., 2011; Pan et al., 2012). A colloidal NPMe micelle has a slightly negative charge formed by its outer shell and developed specific surface area; it exhibits high adsorption capacity and catalytic activity to various metabolites around and inside cell-derived organoids. These mechanisms of receptor-mediated endocytosis and the physicochemical binding of NPMe to the hydrophilic groups of the lipid bilayer and the amino groups of the protein molecules of the cell membrane (Johnston et al., 2010; Lowry et al., 2012), may induce cytotoxic effects.

There is substantial evidence that the cytotoxicity of many nanomaterials is due to structural and functional damages to the integrity of the plasma membrane and inhibition of ATP synthesis and respiration processes (Lara et al., 2011; Canli et al., 2017; Horie et al., 2018), and the oxidation of glutathione and thiol groups of proteins and lipid peroxidation that may lead to oxidative stress (Mao et al., 2018; Canli et al., 2019; Kiyani et al., 2020), genotoxicity and apoptosis (Su et al., 2010; Matsuda, 2011).

The information currently available on the toxicity of NPMe is controversial due to the complex biological activity of the metal components, and the concentration and size effects. Most studies have been limited to assessing their acute toxicity at high (lethal) doses (Garcia et al., 2016; Chinde and Grover, 2017). Our understanding of the biochemical mechanisms underlying sublethal effects caused by chronic uptake of NPMe, including essential ones, is limited.

The number of NPMe synthesized *de novo* is steadily increasing, though there is no consensus among researchers regarding the toxicity risks of these substances or other compounds prepared in a nano-dispersed form. Furthermore, the cytotoxicity and biocompatibility of NPMe are difficult to evaluate and the data available are inconsistent and controversial. There is a need for a more comprehensive toxicological evaluation of NPMe, particularly of their chronic action in sublethal and low doses. Such data will allow for the identification of the most dangerous particles that can affect the biochemical processes and the state and functional activity of cells in various species of organisms.

The objective of this study was to determine the effects of a specific NPMe mixture (Ag, Cu, Fe and MnO_2) orally administered to rats, on biochemical blood markers as indicators of chronic toxicity.

Materials and Methods

Preparation of NP dispersions

The particle size and concentration of colloidal dispersions of the tested spherical NPMe are given in Table 1. Colloidal dispersions of NPMe were synthesised at the F.D. Ovcharenko Institute of Biocolloid Chemistry by a chemical reduction of their salts in aqueous media as described in Pertsov et al. (1976). Briefly, NPMe were obtained by a chemical reduction of the corresponding metal salts as follows: $AgNO_3$ with 1% tannin in 0.03 M potassium carbonate; $KMnO_4$ with hydrogen peroxide; and $FeCl_3$ and $CuSO_4$ with sodium borohydride under alkaline conditions.

The NPMe size was calculated from the diffusion rate constant and the hydrodynamic diameter of the particles determined by dynamic light scattering measurements using Zetasizer-3 spectrometer (Malvern Instruments Ltd, UK). All calculations were performed using the manufacturer's software.

In vitro experiments

In the current study, we assessed the effects of NPMe *in vivo*. This work was preceded by experiments *in vitro* (Dybkova et al., 2009; Roman'ko, 2017a), and are briefly described here. In experiments on isolated cells of subcellular fractions of eukaryotic cells of U937 and hamster CHO-K1 cell lines and the apical meristem of *Allium cepa*, we showed that NPAg (size 31.5±0.9 nm), NPCu (70.0±4.0 nm), NPFe (100.0±10.0 nm) and $NPMnO_2$ (50.0±3.0 nm), were not associated with genotoxic, mutagenic or membrane-toxic effects. Under the action of other nanoparticles, such as NPAu (~20 nm), and NPs of cobalt, cobalt hexacyanoferrate and zinc (~100 nm), several negative effects were observed. These effects included: apoptotic DNA comets with a "tail" of isolated eukaryotic cells, slowing of mitotic processes with an increase in the number of aberrant cells of the *Allium cepa* apical meristem, inhibition of membrane Na^+, K^+-ATPase and the release of cytosolic lactate dehydrogenase activity (LDH activity) (Dybkova et al., 2009; Roman'ko, 2017a).

The NPs exhibiting negative effects *in vitro* were excluded from the *in vivo* assessment. The mixture of NPMe that did not show negative impact on cells *in vitro* was selected for further testing *in vivo*.

In vivo experiments

For the *in vivo* experiments, a solution of the NPMe mixture (Ag, Cu, Fe and MnO_2) was used with an initial concentration of 100 µg/mL per metal. In comparison, an aqueous mixture of salts

Table 1. Particle size and concentration of colloidal dispersions of NPMe

NP material (abbreviation and name)	Particle size (nm)	Metal concentration in the initial dispersion (µg/mL)
NPAg (silver)	31.5±0.9	86.4
NPFe (iron)	100.0±10.0	3174.0
NPCu (copper)	70.0±4.0	2680.0
$NPMnO_2$ (manganese dioxide)	50.0±3.0	2785.0

of the corresponding metals ($AgNO_3$, $CuSO_4$, $FeSO_4$ and $MnSO_4$) with an initial concentration of 100 μg/mL per metal was used.

Previously, we studied the effect of a single dose of orally administered NPMe mixture on sexually mature male rats of the *Wistar* line (*n*=144) (Roman'ko, 2017b): a mixture of NPMe at a dose of 0.3 mg/kg body weight produced an adaptogenic effect due to their higher biological availability compared to the mixture of salts. A single treatment with the mixture of NPMe at higher doses of 1.0 to 4.0 mg/kg body weight exhibited a toxic effect, starting from day 3 to day 14 post-treatment. The toxic effect included hyperglycaemia against increased urea production; aspartate aminotransferase (AST activity) and γ-glutamyltranspeptidase (GGT activity) hyperenzymemias; alanine aminotransferase (ALT activity) and alkaline phosphatase (AP activity) hypoenzymemias; albuminemia against the physiological level of total proteins; induction of catalase activity, a decrease in total antioxidant activity (total AOA) and intensity of lipid peroxidation against the increased oxidative modification of proteins, and the formation of toxic immune complexes (P<0.05).

It should be noted that at the end of the observation period, certain clinical and biochemical values of rat blood did not return to normal physiological level, even after only a single exposure to the NPMe mixture.

Experiments to study chronic toxicological effects were performed using four groups of *Wistar* line rats (*n*=20); one control and three experimental groups. Animals in the experimental groups were given a solution of a mixture of metal salts or a mixture of NPMe with food for 90 days. Rats in experimental group I received a mixture of metal salts at a dose of 0.3 mg/kg. Those in groups II and III received a mixture of NPMe at a dose of 0.3 mg/kg and 4.0 mg/kg body weight, respectively. The animals in the control group were given distilled water under similar conditions.

On days 15, 30, 60 and 90 of the experiment, five rats from each group were anaesthetised with CO_2 and decapitated, and blood samples were collected for biochemical studies.

Experimental studies were conducted in specialised laboratories of the National Scientific Centre, Institute of Experimental and Clinical Veterinary Medicine. The research programme was reviewed and approved by the Bioethics Commission of the National Scientific Centre, Institute of Experimental and Clinical Veterinary Medicine in the current order. Animal experiments are in compliance with the current legislation of EU (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, 22 September 2010).

Analytical procedures

The intensity of lipid peroxidation in blood plasma was determined by measuring the concentration of its primary product, diene conjugates (DC) and the end-product, malonic dialdehyde (MDA), using UV spectroscopy. DC and MDA were extracted by a heptane-isopropanol mixture (1:1 v/v) as previously described (Gavrilov and Mishkorudnaya, 1983) and their optical absorbance was measured at wavelengths l=233 nm and l=247 nm, with DC values expressed in mM and MDA in units of specific absorptivity per ml (ΔD/ml).

The intensity of processes of oxidative modification of proteins in plasma was determined by the level of formation of carbonyl derivatives with neutral (NC) and alkaline properties (AC), as previously described (Archakov and Mikhosoev, 1998). The detection method is based on the ability of aliphatic amino acid radical residues to form aldehyde

and ketone groups. Upon their reaction with 2.4-dinitrophenylhydrazone, the products are determined using UV-visible spectrophotometry at l=370 nm (NC) and l=430 nm (AC) and expressed in mmol/h per g of protein.

Catalase activity (K. F. 1.11.1.6) in blood plasma was determined using UV-visible spectrophotometry as described in Korolyuk (1988), in an incubation solution of the composition: 0.04 M H_2O_2, 0.01 M KH_2PO_4, 0.1 M Tris-HCl buffer, pH 7.4, and 4.5% ammonium molybdate (VI) at 37±1°C and at l=410 nm; the enzymatic activity was expressed in nmol H_2O_2/s per mg of protein.

The level of total AOA in plasma was determined using UV-visible spectrophotometry as previously described in Klebanov et al. (1988), by the total ability of structural antioxidants to inhibit the accumulation of thiobarbituric acid active products (TBA-active products) induced in a medium of 25 mM $FeSO_4$ in 0.002 M HCl, at l=535 nm; expressed as the percent inhibition of the formation of TBA-active products.

Total proteins, albumin, urea, creatinine, glucose and the level of enzymatic activity [AST (K. F. 2.6.1.1); ALT (K. F. 2.6.1.2); AP (K. F. 3.1.3.1) and GGT (K. F. 2.3.2.2) activity] in plasma were determined using CORMAY (Poland) reagent kits as described in the manufacturer's manual (Vlizlo et al., 2012).

The concentration of circulating immune complexes (CIC) of middle molecular weight was determined by precipitating antigen-antibody PEG-6000 protein complexes as previously described in (Kondrakhin et al., 2004); and seromucoids (Sm) were determined as described by Menshikov et al. (1987); at l=260 and l=280 nm, respectively, and expressed in mg/mL.

Spectrophotometric measurements were performed on the SHIMADZU UV-1800 instrument (Japan).

Statistical analysis

The obtained results were processed by methods of variation statistics using the software package for analysis of variance (ANOVA) StatPlus 5 (6.7.0.3) (AnalystSoft Inc., USA). The probability of the obtained results was evaluated by the Tukey criterion (HSD mean difference) at a probability level of 95.0% (P<0.05).

Results

Clinical observations of rats for 90 days showed that the general condition of animals in both control and experimental (I-III) groups was satisfactory: rats were mobile and adequately responded to external stimuli. No animal deaths were recorded during the observation period. Food and water consumption by rats in groups I-III did not differ from animals in the control group.

On day 15 of the experiment, no pathological abnormalities were found in control rats, in experimental groups I or II, while animals of experimental group III exhibited slight bloating and signs of catarrhal inflammation in the small intestine, and increased size and fatty liver consistency.

After 30 days from the beginning of alimentary intake of the NPMe solution at a dose of 4.0 mg/kg body weight, a change was observed in the colour of the rat liver to clay.

On day 60 of the experiment, rats treated with the solution of a mixture of metal salts at a dose of 0.3 mg/kg body weight (experimental group I) displayed development of catarrhal enteritis of the small intestine. In rats treated with NPMe solution at a dose of 0.3 mg/kg body weight (group II), bloating was recorded in the large and small intestine with signs of enteritis. The most pronounced pathological changes of organs at this time were found in animals injected with NPMe solution in the maximum dose,

particularly bloating of the small and large intestine with signs of enteritis, and an enlarged liver with a fatty, greyish consistency.

On day 90 of alimentary intake of solutions of a mixture of metals in both macro-dispersed (group I) and nano-dispersed forms (groups II and III), pathological changes were observed. The animals of these groups had the same changes: bloating in the small and large intestine with signs of enteritis of varying degrees (significantly expressed in animals of group III), enlarged liver, fatty consistency, with light brown (I and II experiments) or grey (III experiment) colouration, and greyish kidneys. Inflammatory processes were found in the lungs of rats of experimental group III, which received a solution of NPMe at the maximum dose, while these changes were not detected in the other groups.

The detected organic changes were undoubtedly reflected in the biochemical profile of experimental rats in the dynamics of the chronic experiment. Table 2 summarises the protein profile dynamics and the indicators of nonspecific resistance in rat plasma (CIC and Sm) under conditions of a chronic toxicological exposure to NPMe. It was found that the blood plasma of rats in experimental groups I and II did not have statistically significant changes of these indicators during the experiment.

Table 2. Protein dynamics and the level of nonspecific resistance indicators in the blood plasma of rats with chronic oral exposure to a mixture of metal salts (experimental group I) and a mixture of NPMe (experimental groups II and III) (M±m; *n*=5)

Animal group	Day	Total proteins (g/L)	Albumins (g/L)	Circulating immune complexes (mg/mL)	Seromucoids (mg/mL)
Control	15	60.70±1.83	36.00±0.20	0.058±0.005	0.069±0.005
	30	60.90±1.73	38.00±1.40	0.068±0.006	0.066±0.008
	60	58.80±3.83	36.40±1.06	0.065±0.003	0.070±0.005
	90	61.60±0.43	39.20±1.40	0.062±0.004	0.067±0.010
I	15	59.30±0.57	35.60±0.82	0.066±0.008	0.061±0.007
	30	61.70±2.17	37.20±1.05	0.073±0.006	0.071±0.003
	60	62.63±3.87	35.00±2.80	0.068±0.010	0.068±0.006
	90	60.80±2.90	36.20±1.40	0.066±0.006	0.068±0.008
II	15	64.30±0.70	38.00±1.25	0.064±0.003	0.070±0.0068
	30	66.80±3.70*	37.70±2.56	0.070±0.007	0.071±0.004
	60	68.33±1.50*	38.00±2.20	0.067±0.008	0.069±0.010
	90	62.53±1.40	36.50±3.40	0.068±0.004	0.066±0.009
III	15	62.00±0.90	36.80±3.50	0.063±0.010	0.105±0.009*
	30	56.90±2.70	37.80±1.00	0.07±0.01*	0.095±0.010*
	60	53.23±1.83*	35.75±3.00	0.09±0.01*	0.120±0.010*
	90	51.90±0.80*	36.2±2.00*	0.10±0.01*	0.125±0.016*

* Indicates significant difference between the control group and the experimental group ($P<0.05$).

On days 60 and 90 of the experiment, the total protein content in experimental group III decreased by 11.2% and 15.7% (P<0.05), respectively. The level of circulating immune complexes of middle molecular weight and seromucoids increased by 36.1% and 62.8% (P<0.05) respectively, compared to the control group.

On the contrary, on day 60, the total protein content in the plasma of rats receiving the NPMe mixture at the dose of 0.3 mg/kg of body weight (experimental group II) increased by 13.0% (P<0.05) relative to the control.

Other indicators of non-specific resistance in all three experimental groups did not differ significantly from the control group.

The chronic effects of exposure to NPMe mixture or metal salts revealed different trends in the intensity of lipid peroxidation processes in the blood of rats (Table 3). In the plasma of rats receiving a mixture of metal salts (group I), on days 60 and 90 of the experiment, the DC content significantly increased by 29.9% and 34.8% (P<0.05) respectively. The MDA content in this experimental group increased by 50.3% and 28.0%

Table 3. The dynamics of lipid peroxidation and oxidative protein modification intensity in the blood plasma of rats with chronic oral exposure to a mixture of metal salts or a mixture of NPMe (M±m; n=5)

Animal group	Day	Products of lipoperoxidation		Products of oxidative protein modification	
		Diene conjugates (μM/L)	Malonic dialdehyde (ΔD/mL)	Formation of carbonyl derivatives with neutral properties (mmoL/h per g of protein)	Formation of carbonyl derivatives with alkaline properties (mmoL/h per g of protein)
Control	15	38.93±2.50	5.52±0.20	549.2±64.2	274.2±24.0
	30	41.51±0.73	4.88±0.16	547.4±44.0	300.5±18.0
	60	39.40±3.20	5.11±0.23	597.9±22.8	302.0±34.3
	90	38.23±2.20	5.40±0.67	539.3±33.36	292.2±26.9
I	15	39.12±1.20	5.46±0.27	568.2±10.2	285.8±21.8
	30	45.81±2.30	4.41±0.22	498.7±62.6	307.1±36.7
	60	51.20±2.60*	6.89±0.12*	544.2±50.2	277.5±26.8
	90	57.40±3.70*	6.91±0.45*	581.4±52.8	311.0±23.4
II	15	37,92±1,50	5.34±0.22	550.1±23.7	277.7±25.0
	30	40.80±0.50	4.80±0.32	573.8±37.4	326.7±18.0
	60	39.50±2.80	5.77±0.20	523.7±41.7	280.7±16.7
	90	28.30±0.80*	5.01±0.28	526.8±26.0	322.6±40.0
III	15	35.60±0.20	5.04±0.32	671.7±26.2*	388.2±25.0*
	30	28.02±0.80*	4.02±0.15*	557.9±33.0	328.4±20.5
	60	26.41±0.53*	4.14±0.12*	587.4±25.6	310.5±32.6
	90	25.80±2.50*	3.11±0.06*	556.2±38.6	302.2±24.8

* Indicates significant difference between the control group and the experimental group (P<0.05).

(P<0.05) respectively compared to control.

In the plasma of animals in group III, no excessive formation of lipid peroxidation products DC and MDA was noticed. Instead, on day 15, an accumulation of NC and AC carbonyl derivatives of proteins was recorded (P<0.05), and starting from day 30 until day 90, the content of both DC and MDA (P<0.05) decreased against the control.

The chronic intake of the NPMe mixture at the dose of 0.3 mg/kg body weight (group II) did not lead to significant changes in the intensity of lipid peroxidation until day 60. However, on day 90, a decrease was observed in the level of DC in the blood plasma of animals in this experimental group against the control (Table 3).

In the plasma of rats in groups I and II receiving a mixture of metal salts or NPMe at the dose of 0.3 mg/kg body weight, no significant changes were observed in the intensity of the oxidative protein modification processes.

In the study of the antioxidant system indices, groups I and II showed an increase in catalase activity (Fig. 1a); this activity was consistently higher (P<0.05) during the study period in group I, which received metal salts compared to the control.

Conversely, in group III, which received a mixture of NPMe at the dose of 4.0 mg/kg, the catalase activity decreased with time (P<0.05) compared to control.

Between the experimental groups, the dynamics of the total AOA in the blood plasma of rats showed different trends (Fig. 1b).

In group I, which received a mixture of metal salts, an increase in total AOA was recorded (P<0.05) on day 60. In group II, which received 0.3 mg/kg of the NPMe mixture, an increase in AOA was recorded on days 15 and 30 (P<0.05) versus the control. In group III, which received 4.0 mg/kg of the NPMe mixture, AOA was gradually reduced, starting from day 30 and this continued until day 90, when the values were lowest (P<0.05).

In the blood plasma of rats given a mixture of metal salts (group I), an increase in enzymatic activity of ALT (Fig. 2a), AST and GGT (Fig. 3a,b) was observed on days 60 and 90, whereas for AP activity (Fig. 2b) an increase was only evident on day 90 (P<0.05).

In the blood plasma of rats receiving the NPMe mixture at a dose of 0.3 mg/kg (group II), only GGT had increased enzymatic activity on day 90 (P<0.05; Fig. 3b). Enzymes ALT, AP and AST showed no significant differences compared to the control group throughout the entire experiment (Fig. 2a, b and Fig. 3a).

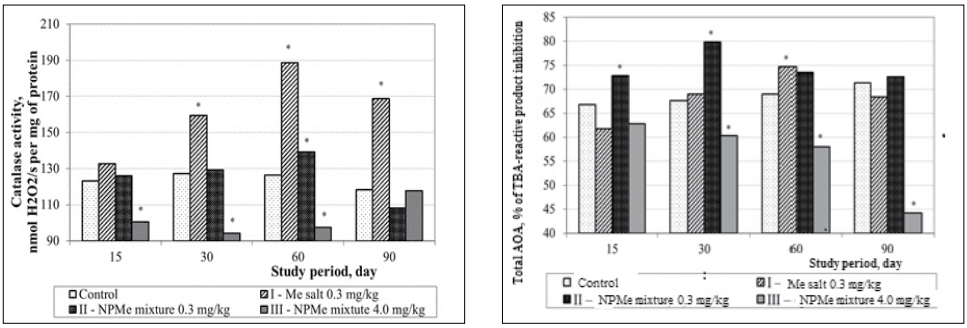
The dynamics of enzymatic activity in the blood plasma of group III indicated an increasing intensity of destructive processes in the liver due to the development of oxidative stress (Fig. 2 and Fig. 3).

The gradual increased activity of ALT and AP in the blood plasma of rats was recorded during the experiment, which reached their peak values on day 60, increasing by 96.8% and 69.1% (P<0.05) against the control, respectively (Fig. 2a, b).

On day 60, the highest levels of AST and GGT activities (Fig. 3a, b) were also determined in the blood plasma at levels 114.8% and 122.3% (P<0.05) higher than in the control group, respectively.

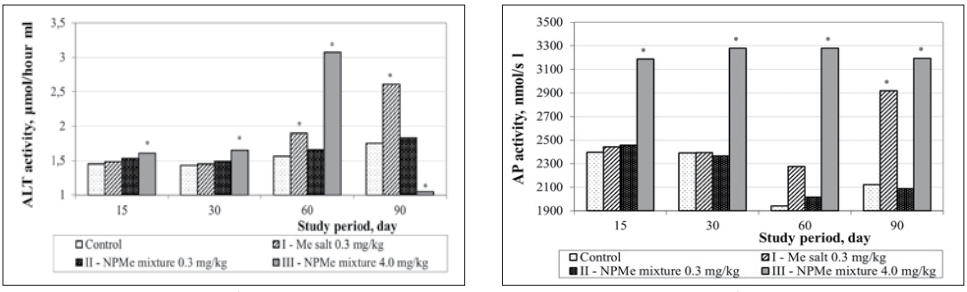
The glucose content in blood plasma of animals in group III increased between days 30 and 60, from 18.4% to 36.1% (P<0.05) against the control. In animals from groups I and II, an increase over the control group was observed only on day 60, by 26.8% and 21.5% (P<0.05), respectively (Table 4).

In group III exposed to the maximum dose of NPMe mixture, urea concentration in plasma was elevated throughout the experiment, reaching the peak value during the period from days 15 to 60 of the experiment. In group



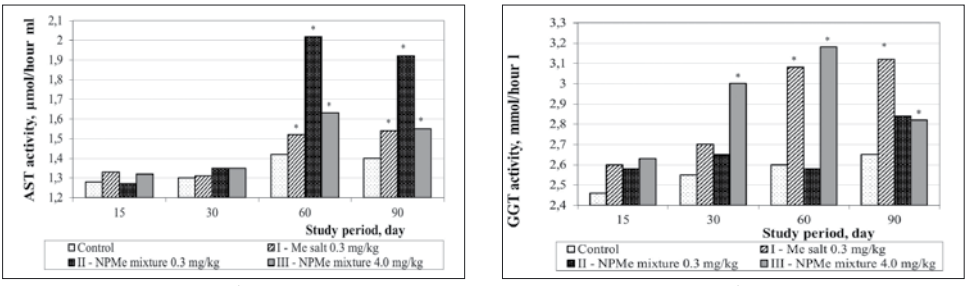
a) b)

Figure 1. (a) Catalase activity and (b) total AOA in blood plasma of rats in the dynamics of chronic effect of the NPMe mixture and the mixture of metal salts (M±m; *n*=5); * – *P*<0.05 against control)



a) b)

Figure 2. Dynamics of ALT activity (a) and AP activity (b) in the blood plasma of rats upon chronic exposure to NPMe mixture and metal salts. (M±m; *n*=5); * – *P*<0.05 against the control)



a) b)

Figure 3. Dynamics of AST activity (a) and GGT activity (b) in the blood plasma of rats upon chronic exposure to NPMe mixture and metal salts (M±m; *n*=5); * – *P*<0.05 – in relation to the control)

I, which received 0.3 mg/g of metal salts, a higher value was observed on day 60 (*P*<0.05). In group II, differences from with control were insignificant throughout the experiment.

Between days 30 and 60 of the experiment, group III showed an increase in creatinine level in blood plasma against the control (*P*<0.05). In group I, an increase was observed on day 90,

Table 4. The dynamics of glucose, urea and creatinine levels in the blood plasma of rats with chronic oral exposure to a mixture of metal salts and a mixture of NPMe (M±m; *n*=5)

Animal group	Day			
	15	30	60	90
Glucose (mmol/L)				
Control	3.37±0.20	3.43±0.18	3.21±0.02	3.49±0.23
I	3.50±0.28	3.60±0.33	4.07±0.19*	3.63±0.22
II	3.46±0.25	3.53±0.28	3.90±0.16*	3.38±0.36
III	3.73±0.26	4.06±0.20*	4.37±0.24*	3.72±0.16
Urea (mmol/L)				
Control	5.93±0.46	6.07±0.21	6.04±0.18	6.99±0.25
I	6.63±0.51	7.00±0.10*	7.32±0.27*	6.71±0.35
II	6.03±0.42	6.57±0.80	6.47±0.15	7.45±0.38
III	10.05±0.50*	10.67±0.74*	7.72±0.35*	7.84±0.35*
Creatinine (μmol/L)				
Control	159.1±5.7	149.80±8.22	152.3±10.2	160.2±10.5
I	160.0±10.1	153.4±10.8	162.0±18.0	188.4±11.1*
II	159.8±6.9	138.6±13.45	142.6±8.1	174.8±9.8
III	167.40±8.55	168.3±7.8*	183.8±13.3*	178.5±10.0

* Indicates significant difference between the control group and the experimental group ($P<0.05$).

and in group II, the difference with the control was insignificant throughout the experiment.

Therefore, the maximum severity of metabolic changes in animals during chronic exposure to the NPMe mixture at 4.0 mg/kg/day was observed on day 30, and in animals exposed to metal salts on day 60 of the experiment.

In group II, chronic administration of NPMe at the dose of 0.3 mg/kg/day showed good adaptability of animals as evidenced by the indicators of innate immune resistance and antioxidant activity, as they did not differ significantly from the control group that received no metals in any form. The optimal duration of chronic administration was up to 30 days. Since the overall body resistance, immune status and metabolism require the presence of essential metals, a mixture of NPMe at the dose of 0.3 mg/kg of body weight by its biotic influence could be used as a prodrug and/or a component of feed additives for animals and birds.

Discussion

Summarizing the above results of clinical and pathological studies, alimentary intake of a mixture of metals in macro- and nano-dispersed forms resulted in pronounced, chronic, dose-dependent intoxication. A toxic effect of NPMe solution is the development of inflammation of the digestive tract of rats, liver and lungs, which is consistent with the data (Kiyani et al., 2020).

Oxidative stress plays a leading role in the toxicity of nano-sized materials. Its consequences include the development of cytolytic syndrome and genotoxicity, which reflect their ability to penetrate through the pores of the cell and

nuclear membrane. Thus, nanoparticles of various chemical origins can act as prooxidants through their membrane-toxic action (Pan et al., 2009; Shim et al., 2017; Pereira et al., 2018), and, conversely, as antioxidants blocking the cytolytic syndrome (Stoliar and Falfushynska, 2012; Sutherland et al., 2012).

Our results show that the cytotoxic action of metals in different dispersion states is dose-dependent. The maximum toxicity of the metal salt mixture administered at the dose of 0.3 mg/kg and NPMe mixture at the dose of 4.0 mg/kg of body weight of rats per day was determined on day 30 of the experiment. Chronic administration of these mixtures resulted in changes in the activities of most enzymes studied and levels of metabolites, as markers of hepatotoxicity, cardiotoxicity and nephrotoxicity. Such changes indicated the toxic effects of NPMe mixture (from day 30) and metals salts (noticeable from day 60).

Both the chronic exposure to the mixture of metal salts, which intensified lipid peroxidation, and exposure to the mixture of NPMe administered at the dose of 4.0 mg/kg/daily, which suppressed lipid peroxidation but increase protein oxidation, demonstrate their pro-oxidant effects. Both these observations are characteristic of the development of peroxidation processes, which lead to the disruption of the antioxidant system as seen in the changes in catalase and AO activities discussed below.

Catalase plays a major role in the adaptation of cells to increased intensity of catabolic and destructive processes (Marques et al., 2015). The gradual decrease in the plasma catalase activity and the gradual depletion of structural antioxidant resources as seen in the decrease in total AOA ($P<0.05$) under the action of NPMe mixture at a dose of 4.0 mg/kg/daily that was observed from day 15 of treatment, had a compensatory nature. This effect is a sign of the development of destructive processes associated with the denaturation of antioxidant enzymes by toxic products of the oxidative modification of proteins and other metabolites.

An increase in the level of enzymatic activity starting from day 30 of the experiment in the experimental group receiving a mixture of metal salts, is likely a sign of the adaptive response of cells to chronic accumulation stress in the rat organism.

On the contrary, the slowed accumulation of toxic products of lipid peroxidation and carbonyl products of oxidative protein modification following chronic administration of NPMe mixture at 0.3 mg/kg of body weight, was likely due to the action of antioxidant resources, both the enzymatic (on day 60) and non-enzymatic parts of the antioxidant system (days 15 and 30; $P<0.05$).

The results obtained demonstrate the regulatory function of the endogenous antioxidant system, which is realised via activation of glutathione-dependent enzymes in Phase II of detoxification, and structural antioxidants present in the animal organism triggered by a cytotoxic action of a low-dose NPMe mixture. These results and conclusions are in agreement with the views reported in literature (Connolly et al., 2015).

A similar response of antioxidant enzymes, in particular, an increase in catalase and glutathione (GSH)-dependent enzyme activity, was reported when nanoparticles of copper, aluminium and titanium oxides were orally administered to rats even at the lowest dose of 0.5 mg/kg of body weight (Canli et al., 2019).

The fact that lipoperoxidation and oxidative modification of proteins in the group of rats chronically administered 0.3 mg/kg of NPMe mixture remained within the physiological level could be explained by the adaptive induction of structural endogenous antioxidants,

such as ascorbate, SH-groups, GSH, ceruloplasmin, cytochrome, metallothioneins, etc.), known due to the high content of sulfhydryl groups (Stoliar and Falfushynska, 2012; Sutherland et al., 2012). Therefore, it can be assumed that the binding of metal ions released from the colloidal NP micelle during the biotransformation process, to the functional groups of metallothioneins, decreases their putative toxicity. In contrast, unbound ions can participate in other interactions and can be accumulated by cells that determine their biocompatibility, in this case, antioxidant properties. Both indirectly and directly, the cytolytic syndrome is actively blocked through the normalisation of free radical oxidation; *i.e.*, NPMe in this lower dose of 0.3 mg/kg can act as an antioxidant to 'trap' radicals (Stoliar and Falfushynska, 2012).

Since the liver is the main organ of the synthesis of serum proteins, any toxic effect disrupting its functioning should affect the total protein level. The type of protein changes in the experimental group III (4.0 mg/kg NPMe mixture) showed the domination of catabolic over anabolic processes in the animals.

The reduction of total protein level against the albumin increase on days 60 and 90 ($P<0.05$) in group III determines the impact of the NPMe mixture on the liver and kidney function of animals. This observation is in agreement with the toxicokinetics (biodistribution and accumulation) determined for NPs of metallic copper and silver and oxides of copper, aluminium, titanium and tungsten in the tissues of liver, spleen and kidneys of rats (Lee et al., 2016; Chinde and Grover, 2017), and isolated rat kidney mitochondria (Ansari and Kurian, 2018) and correlates with the dynamics of immune responses and biochemical processes in the respective organs and tissues of animals and fish (Al-Akel et al., 2010; Thummabancha et al., 2016).

Circulating immune complexes, an important component of the mechanism for maintaining the immune homeostasis, are constantly present in the blood. Their elimination occurs by cells of the reticuloendothelial system (RES) (Levinsky, 1981). It should be noted that an increase in CIC, as a physiological product of the 'antigen-antibody' reaction found in group III (4.0 mg/kg NPMe mixture), is likely to be caused either by antibody production due to the forced dysfunction of the mechanisms of non-specific defence, or disruption of the elimination path of CIC from circulation by the RES. This fact, along with an increase in the formation of acute-phase proteins, seromucoids ($P<0.05$), as a result of NPMe mixture exposure, may reflect the phases of immunotoxic reactions development in the rats. Immunosuppression and the induction of fibrotic changes in the rat liver have been observed following ingestion of copper nanoparticles for 28 days (Lee et al., 2016b). These effects have been shown to be due to the inhibition of lymphocyte proliferation and inhibition of B- or T-lymphocyte-mediated immune reactions.

The gradual increased activity of ALT and AP enzymes ($P<0.05$) in the blood plasma during the experiment in group III indicates a hepatotoxic effect of the NPMe mixture administered at the dose of 4 mg/kg, with the most prominent effect recorded on day 60.

The magnitude of the induction of these enzymes in the blood is considered proportional to the degree of destruction of hepatocytes and the activity of the pathological process (Valko et al., 2007). The increase in the levels of liver enzymes (AST, ALT and AP activity) in the blood serum of rats was demonstrated starting on day 14 of administering titanium dioxide nanoparticles in the dose range of 0.5-50.0 mg/kg of body weight, whereas for nanoparticles of copper and

aluminium oxides, only AP level was elevated (Canli and Canli, 2017).

The AST and ALT increase activity caused by NPMe mixture in the present study could be a consequence of parenchymal cell injuries and an additional toxic load on the myocardium and liver. Mitochondria are targets for the damaging effects of toxic peroxidation products caused by disrupting their membranes and increasing their permeability by either forming pores or initiating the opening of temporary permeability pores (Begriche et al., 2011; Shan et al., 2015), which in turn could trigger apoptotic processes in cells. This is confirmed by the induction of reactive oxygen species along with depletion of the endogenous antioxidant system at the exposure of isolated liver mitochondria of rats to titanium and silver nanoparticles and the synergistic effect of their composition (Pereira et al., 2018; Zhang et al., 2019).

The gradual increase in the AP and GGT activity under the influence of NPMe mixture at 4 mg/kg can be considered a common sign of the development of cytotoxic processes and the pathological state (Whitfield, 2001; Babij et al., 2010; Joshi et al., 2014).

Stress in higher vertebrates initiates the release of glucocorticoids and catecholamines, which cause an increase in gluconeogenesis (Polakof et al., 2012). The observed increased glucose levels in rats of all experimental groups on day 60 may perhaps indicate an increase in energy consumption aimed at intensifying the elimination of metals. At the end of the experiment (day 90), glucose levels in the blood plasma of experimental rats were not significantly different from the control group.

Furthermore, the supply of the NPMe mixture at the highest dose (group III) and the mixture of metal salts (group I) led to an increase of plasma urea content starting from day 15 and day 30, and a significant increase of creatinine ($P<0.05$) from day 30 and day 90. This indicates an increase of proteolytic processes and the intensity of glomerular filtration in the kidneys of experimental rats.

The dynamics of glucose, urea, and creatinine level in rats in groups I and III, especially on days 30 and 60 illustrates the adaptive mobilisation of energy and purine metabolism resources and the prevalence of catabolic processes over anabolic ones due to long-term metal exposure (Lee et al., 2016a).

It was established that in rats exposed to the NPMe mixture at 0.3 mg/kg, the values of the studied markers remained within the levels of the control group. According to the indicators of innate immune resistance and endogenous antioxidant system in the rat organism, it is possible to assert that the NPMe mixture is biocompatible at this dose with an optimal period of administration of no more than 30 days. At the same dose, metal salts show significant toxicity as does the NPMe mixture at the dose of 4 mg/kg of body weight.

The overall organism resistance, its metabolism and innate immune status are generally largely dependent on the presence of certain metals, in particular those metals with known pharmacological effects (e.g., Cu, Mn, Ag and Fe) (Al-Akel et al., 2010; Arami et al., 2015; Thummabancha et al., 2016). Therefore, the proposed mixture of NPMe mixture at a dose ensuring its biocompatibility could be used as a prototype for drugs and component of biologically active substances. This is seen in the use of this substance in poultry, with proven safety for laying hens at a dose of 0.3 mg/kg body weight and a positive effect on productivity, reproductive capacity and quality of the resulting young (Orobchenko et al., 2019; 2020). Furthermore, it is necessary to carry out a complex of veterinary and

sanitary measures at poultry enterprises (Paliy et al., 2018; 2021).

Thus, the results obtained demonstrate the dependence of NPMe mixture toxic effects on the dose and duration of administration. The studied biochemical markers may be used as the basis for an algorithm for pre-clinical testing of pharmaceuticals based on metal nanoparticles in toxicological experiments *in vivo*.

Conclusions

The chronic administration of the NPMe mixture (Ag, Cu, Fe and MnO_2) at the dose of 4.0 mg/kg of body weight to white rats over the course of 90 days resulted in the development of inflammation of the digestive tract of rats, liver and lungs, in partial immunosuppression evidenced by hypoproteinemia, excessive formation of circulating immune complexes and proteins of acute phase (seromucoids), and cytolytic damage to hepatocyte membranes expressed by AST, GGT, ALT and AP hyperenzymemias. These changes are caused by oxidative stress that slows lipid peroxidation, in conjunction with an accumulation of carbonyl products of oxidative protein modification and depletion of the antioxidant defence system. These biochemical and immunological signs are informative biomarkers of the transition from the physiological state to a pathological one. Increased levels of glucose, urea and creatinine (P<0.05) in the blood plasma of rats depends on the dose and time of administration metals in both ionic (salt) and NP mixtures and indicates the adaptive mobilisation of carbohydrate and protein metabolism in rats. The chronic administration of metal containing nanoparticles at a dose of 0.3 mg/kg of body weight did not cause a negative impact on the innate immunity resistance and endogenous antioxidant defence system in rats, proving their better biocompatibility in comparison with the chronic intake of the solution of the mixture of their salts at the same dose of metals.

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Procjena biokemijskih markera u krvnoj plazmi štakora izloženih kroničnoj primjeni mješavine nanočestica metala

Maryna ROMANKO, Doctor of Biological Science, Senior Researcher, Oleksandr OROBCHENKO* (Corresponding author, e-mail: toxy-lab@ukr.net), Doctor of Veterinary Science, Senior Researcher, Anatoliy PALIY, Doctor of Veterinary Science, Professor, National Scientific Center Institute of Experimental and Clinical Veterinary Medicine, Kharkiv, Ukraine; Valerii USHKALOV, Doctor of Veterinary Science, Professor, Academician of NAAS of Ukraine, National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine; Andrii PALII, Doctor of Agricultural Sciences, Professor, Helena CHECHUI, Candidate of Biological Science, PhD, Assistant Professor, State Biotechnology University, Kharkiv, Ukraine

Cilj je naše studije bio utvrditi učinke mješavine nanočestica određenih metala (Ag, Cu, Fe i MnO_2) koje su oralno davane štakorima na biokemijske markere krvi kao indikatore kronične toksičnosti. Životinjama (štakorima soja *Wistar* (*n*=20)) je u eksperimentalnim skupinama hranom tijekom 90 dana davana je otopina mješavine metalnih soli ili mješavine nanočestica. Na 15., 60. i 90. dan eksperimenta, pet štakora je iz svake skupine pomoću CO_2 anestezirano i dekapitirano te su prikupljeni uzorci krvi za biokemijske studije. Spektrofotometrijska mjerenja su provedena uporabom SHIMADZU UV-1800 instrumenta. Toksični je učinak kronične primjene NPMe mješavine pri dozi od 4,0 mg/kg tjelesne mase u bijelih štakora izazvao djelomičnu imunosupresiju izraženu kao hipoproteinemija, pretjerano formiranje cirkulirajućih imunokompleksa i mukoidne proteine u serumu (P<0,05) akutne faze te citolitičko oštećenje membrana hepatocita. Razine enzima (AST, GGT, ALT i AP aktivnost) su značajno povećane (P<0,05). Dokazano je da je uzrok toksičnog učinka oksidativni stres, koji je usporio lipoperoksidaciju uz povećanje razina karboksiliranih proteina, iscrpljujući antioksidativnu obranu resursa organizma, očitovanu smanjenjem razine katalaze i ukupne antioksidativne aktivnosti (P<0,05). Takvi učinci pri dozi od 0,3 mg/kg nisu zamijećeni, jer nije bilo značajnih negativnih učinaka na biomarkere. Na temelju naših podataka zaključujemo da je proučavane biokemijske markere moguće izabrati kao osnovu za predkliničku toksikološku procjenu obećavajućih metalnih nanočestica - kandidata za *in vivo* lijekove.

Ključne riječi: *NPMe mješavina (Ag, Cu, Fe i MnO_2), kronična primjena, toksični učinak, imunosupresija, oksidativni stres, biomarkeri*