Main results obtained in a series of animal experiments for the assessment of the organism's responses to metallic nanoparticles exposure

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Main results obtained in a series of animal experiments for the assessment of the organism's responses to metallic nanoparticles exposure

L I Privalova[1], M P Sutunkova[1], I A Minigalieva[1], V B Gurvich[1], O G Makeyev[2], I V Valamina[2], V Ya Shur[3], E V Shishkina[3], I V Zubarev[3], S N Solovyova[1], S V Klinova[1] and B A Katsnelson[1]

[1] Ekaterinburg Medical Research Centre for Prophylaxis and Health Protection in Industrial Workers, 620014, Ekaterinburg, Russia
[2] Central Research Laboratory of the Ural State Medical University, 620028, Ekaterinburg, Russia
[3] School of Natural Sciences and Mathematics, Ural Federal University, 620000, Ekaterinburg, Russia

privalovali@yahoo.com; privalova@ymrc.ru

Abstract. Nanoparticles (NPs) of Fe_3O_4 were produced by a chemical technique and NPs of Ag, Au, CuO, NiO, Mn_3O_4, PbO, ZnO, TiO_2, SiO_2, and Al_2O_3 – by laser ablation in water. In some experiments we compared particles of a given chemical composition having different diameters, while in others – equidimensional NPs of different metals or metal oxides (Me-NPs). We used two experimental models: a single intra-tracheal instillation of Me-NPs 24 h before the bronchoalveolar lavage procedure (collecting) and the repeated intra-peritoneal injections during 6-7 weeks in non-lethal doses. Besides, we carried out long-term inhalation experiments with NPs of Fe_2O_3, SiO_2, and NiO. We have demonstrated that NPs are much more noxious than their fine micrometric or even submicron counterparts and are usually the more toxic the smaller their dimensions within the nanoscale range. We have found also that toxicity of Me-NPs strongly depends on their chemical nature, solubility, and specific mechanisms of characteristic action of a given metal in any chemical form. Solubility of Me-NPs in biological milieus plays an important role in their toxicokinetics, which can prevail over that of the physiological mechanisms controlling their distribution, retention, and elimination. On the other hand, thanks to the high activity of these mechanisms, the organism is not defenceless against the impact of Me-NPs. The toxicity and even genotoxicity of Me-NPs can be significantly decreased by adequately composed combinations of some bioactive agents in innocuous doses.

1. Introduction

Nanoparticles (NPs) of metals and of their oxides are of special interest for risks assessment and management, because along with engineered NPs, there exists usually a substantial fraction of nanoscale ("ultrafine") particles of the same substances within the particle size distribution of condensation aerosols generated by arc-welding, metallurgical, and some chemical technologies. However, in such industrial aerosols chemically similar micrometre particles, including submicron ones having dimensions >100 nm, are usually present as well. This fact is confirmed by the distribution of particles by size in the submicron range on a filter sampled from the workplace air of a copper smelting and casting facility (Fig. 1) [1,2].

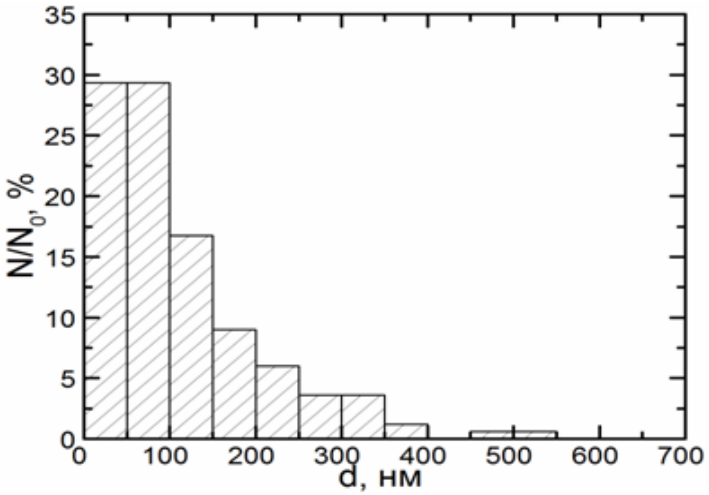

Figure 1. The percentage distribution of particles by size in the submicron range on a filter sampled from the workplace air of a copper smelting and casting facility. N - number of particles of a given diameter; N_0 - total number of particles.

The situation becomes even more complicated, if we take into consideration how rare in such industries the air is polluted by particles of one chemical composition. Indeed, most Me-NP-generating by metallurgical and welding technologies are bound to produce and do produce multi-component mixtures of chemically different particles of similar or dissimilar geometry. For instance, in arc-welding and alloyed steel production, one usually finds different combinations of iron, chromium, nickel, manganese, and silicon oxides in the form of NPs and microparticles (MPs), while in crude copper smelting and refining – those of copper, lead, cadmium, zinc, and arsenic oxides.

Thus, solving some general problems in relation to Me-NPs that nanotoxicology faced from the very beginning of its evolution as a special branch of the toxicological science was not only theoretically challenging, but also very urgent for the everyday practice of occupational health risks assessment and management. Starting from the very first nanotoxicological work in 2008-2009 [3,4] up to now, our team has been conducting *in vivo* animal experiments involving Me-NPs and has thus accumulated a certain wealth of relevant knowledge. This paper summarizes mostly our experience [3-5].

2. Materials and methods

Some suspensions of NPs were produced by laser ablation of metal targets (99.99% purity, 1-mm thick) placed on the bottom of a glass vessel with 5-30 mL of deionized water. NPs size distribution was obtained by a direct measurement using scanning electron microscopy. Suspension stability was characterized by zeta potential measured in a Zetasizer Nano ZS analyser (Malvern, UK) and it was high enough to increase the concentration by partial water evaporation at 50°C [2].

The experiments were carried out on outbred white male and female rats from our breeding colony 3-4 month old with an initial body weight of 150-220 g. Both the exposed and the control groups contained at least 12 animals. The rats were housed in conventional conditions (dry bulb temperature 20-22ºC, relative humidity 50-60%), breathed unfiltered air, and were fed with standard balanced food.

The experiments were planned and implemented in accordance with the «International guiding principles for biomedical research involving animals» developed by the Council for International Organizations of Medical Sciences (1985) and were approved by the Bio-Ethics Committee of the Yekaterinburg Medical Research Centre for Prophylaxis and Health Protection in Industrial Workers.

In animal experiments we estimated toxicity of different metallic particles in nanometre and micrometre ranges using three experimental models:

- a single intra-tracheal instillation of Me-NPs 24 h before collecting the bronchoalveolar lavage to obtain a liquid sample for cytological and biochemical assessment [6];
- repeated intra-peritoneal injections during 6-7 weeks in non-lethal doses to assess the thus induced subchronic intoxication by a lot of functional, biochemical, and morphological indices;
- long-term inhalation experiments with NPs of Fe_2O_3, SiO_2, and NiO [6,7].

All comparative assessments were based on parallel experiments.

An intratracheal instillation of 1 ml of Me-NP or Me-MP water suspension (or of sterile deionized water from the same batch, without any particles) served as an experimental model for the response of the lower airways to particle deposition. To this point, cell population and some biochemical characteristics of bronchoalveolar lavage fluid (BALF) obtained 24 h after the instillation were studied.

The subchronic toxicity of the Me-NPs and some Me-MPs was investigated by means of repeated intraperitoneal (i.p.) injections of the same suspensions in sub-lethal doses 5 times a week during 5-7 weeks. NP inhalation by laboratory rodents is not an ideal model of real human exposures as it is often deemed to be [8], but the intraperitoneal animal model circumvents these interspecies differences. Dosing by injection is much more accurate, reliable, and reproducible compared to the more "natural" experimental methods, such as inhalation ones.

The rats were killed by decapitation and their blood was collected by exsanguination. The liver, spleen, kidneys, and brain were weighed. The assessed blood biochemical indices included the total serum protein, albumin, globulin, triglycerides, cholesterol, high and low density lipoproteins, bilirubin, ceruloplasmin, reduced glutathione (GSH), malondialdehyde (MDA), alkaline phosphatase, alanine and aspartate transaminases ALT, AST), catalase, gamma-glutamyltransferase, creatinine, and, in some experiments, thyrotropic hormone of hypophysis, thyroxin, triiodothyronine, follicle-stimulating and luteinizing hormones, progesterone, dehydroepiandrosterone, estradiol, and neuron-specific enolase.

The level of genomic DNA fragmentation as an index for the metals' in vivo genotoxicity was assessed using the Random Amplification of Polymorphic DNA (RAPD) test. The method is based on the fact that, unlike a fragmented DNA, which forms the so-called "comet tail" during agarose gel electrophoresis, a non-fragmented DNA has a very low degree of migration and virtually stays at the same place ("comet head"). The degree of migration is directly related to the extent of DNA fragmentation. To characterize the extent of damage to DNA tagged with tritium, we used "coefficient of fragmentation", i.e., the ratio of total radioactivity of all "tail" fractions to the radioactivity of the "head" [4]. To study structural and ultrastructural used changes in rats' viscera, we used optical and transmission electron microscopy.

3. Results and discussion

Figures 2 and 3 illustrate the dependences based on values of the neutrophil leukocytes to alveolar macrophages count ratio in broncho-alveolar lavage fluid, which is a very informative index for comparative cytotoxicity and pulmonary toxicity of particles deposited in the lower airways. We present the examples of the responses to iron oxide (magnetite) particles of different diameters and to gold and silver particles of different or of virtually equal dimensions [9].

Of a special significance is the affinity of metallic NPs to mitochondria with more or less prominent damage to these organelles; it was reported by many authors with the so-called oxidative stress resulting in generation of free radicals – one of the key primary mechanisms of the DNA damage [10]. Thus, NPs of silver and copper oxide are more likely to damage mitochondria than Au NPs. Figure 4 demonstrates that Ag NPs are also more genotoxic as compared with Au NPs [10].

If metallic NPs of a given composition are always the more cytotoxic the smaller their dimensions, the dependence of their organ-systemic chronic resorbtive toxicity on the diameter within nanoscale range is not so unique due to its intricate (complicated) and often contra-directional (antagonistic) influences of this parameter on toxicodynamics and toxicokinetics.

Unlike the effects on liver and other organs of the reticuloendothelial system (RES), the toxic action of metallic NPs on kidneys is probably less associated with their bio-persistency than with a quick dissolution of NPs in biological milieus.

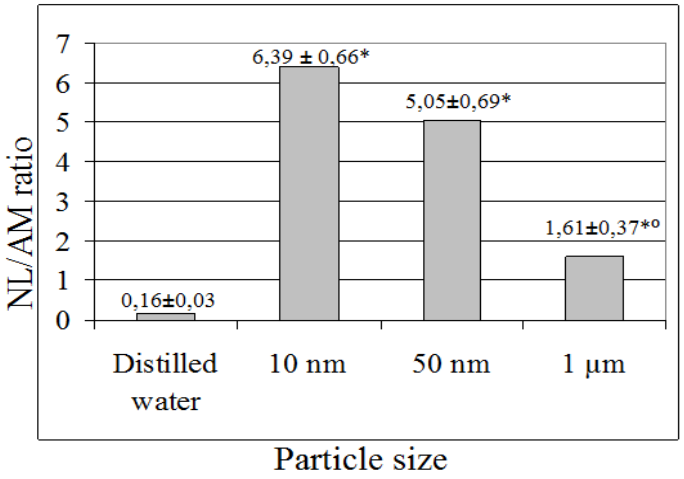

Figure 2. The neutrophil leukocytes to alveolar macrophages count ratio (NL/AM) in the BALF or rats 24 hr after i.t. instillation of Fe_3O_4 particles depending on their mean diameter. * statistically significant ($P < 0,05$) difference from controls; ° - the same from 10 nm.

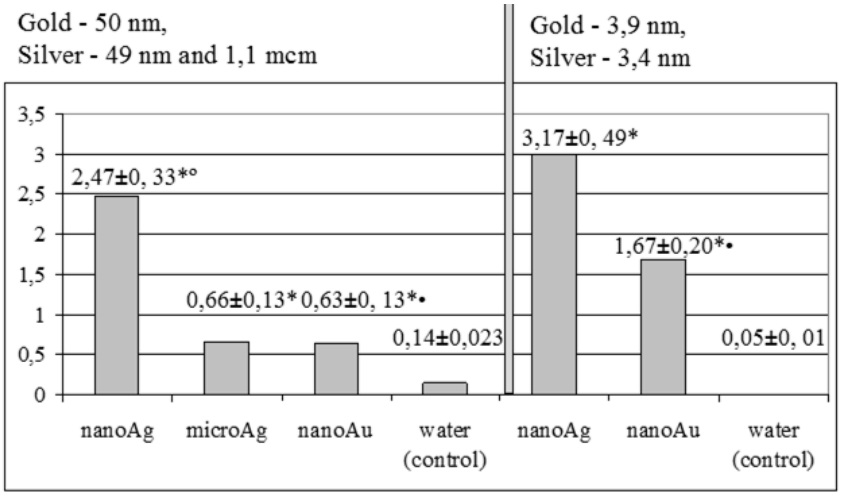

Figure 3. Index for comparative cytotoxicity of silver and gold particles. * statistically significant ($P < 0,05$) difference from controls, ° - the same from nanoAg, ▪ - the same from microAg.

Table 1. Morphometric indices for the state of rat's brain after repeat intraperitoneal injections of NiO or Mn_3O_4 NPs (X±s.e.) Note: * statistically significant difference from the control group ($p < 0.05$ by Student's t-test).

Index	Control	Mn_3O_4-NP	NiO-NP
Caudate nucleus			
Cells without nucleoli (%%)	30,50±2,77	69,90±1,79*+	47,36±2,45*
Hippocampus (CA 1)			
Cells without nucleoli (%%)	30,50±2,30	70,45±2,31*	35,8±2,21

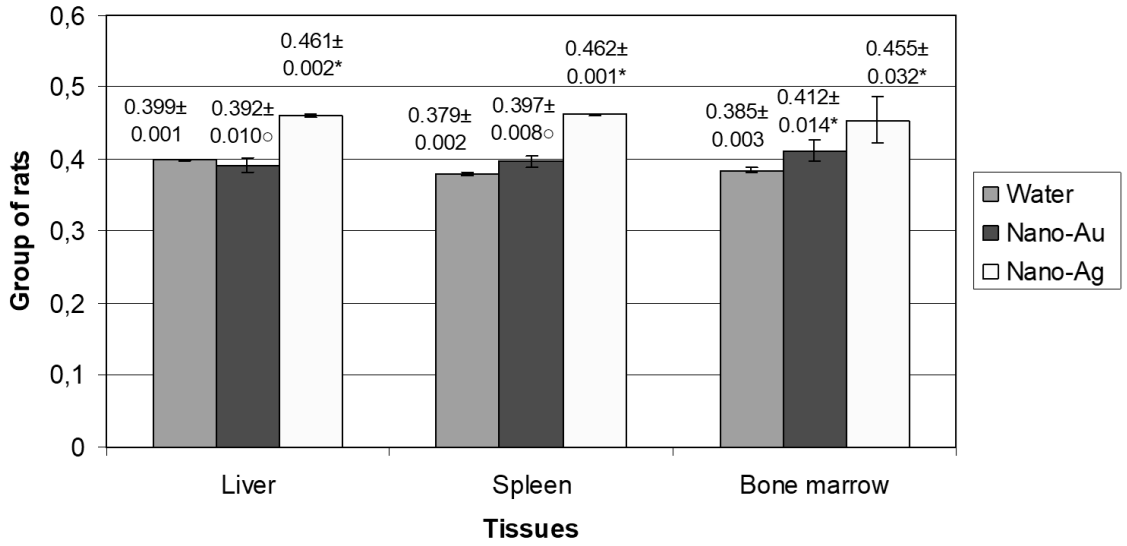

Figure 4. Coefficients of the genomic DNA fragmentation in rats exposed to subchronic administration of gold or silver equidimensional NPs (the RAPD-test), X±s.e Note: statistically significant difference * from the control group; ○ between the group receiving NG and the group receiving NS ($P<0.05$ by Student's t-test)

Speaking about the metal-associated specific differences between patterns of different NPs adverse health effects, we may give as an example a higher damage to the brain striatum (basal nuclei) and the hippocampus exerted by manganese oxide NPs as compared to those of nickel oxide ones (Table 1) [5].

Brain damage was induced also by the subchronic toxicity of copper oxide NPs [10]. Along with such damage to brain basal nuclei neurons, the toxicological syndrome comprised accumulation of CuO in the liver and brain, some decrease in the serum ceruloplasmin level, and anemia. All these phenomena are characteristic of Wilson's disease in humans associated with genetically determined impairment of copper metabolism. We believe that in our animal experiments this syndrome may be considered specific to the chronic toxicity of exogenous copper (in the form of NPs or ultrafine MPs).

However, the NPs neurotoxicity may be associated not only with mechanisms specific for a certain metal (such as manganese or copper) [6,11]. It is well known that any inhaled NPs having the smallest dimensions are able, after deposition in nasal cavity, to be transferred along the olfactory nerve fibres, thus bypassing the blood-brain barrier. Therefore, brain neurons may be damaged by them like any other cells due to the mechanisms common for all metallic NPs.

This statement is illustrated by Figure 5 showing scanning transmission electron microscopy (STEM) image of rat brains after 6 months' inhalation "nose-only" exposure to low concentrations of nickel oxide NPs. Neuron's myelin sheath contains NiO NPs. There are areas of demyelination in axon's sheath, where the electron-dense NPs are detected.

Figure 6 displays a STEM image of a rat alveolocyte containing NiO NPs in its cytoplasm after the type II inhalation exposure for 3 months. The emptied multilamellar bodies and NPs in cytoplasm are evident. The boxed area appears at higher magnification on the right and presents a large accumulation of NPs.

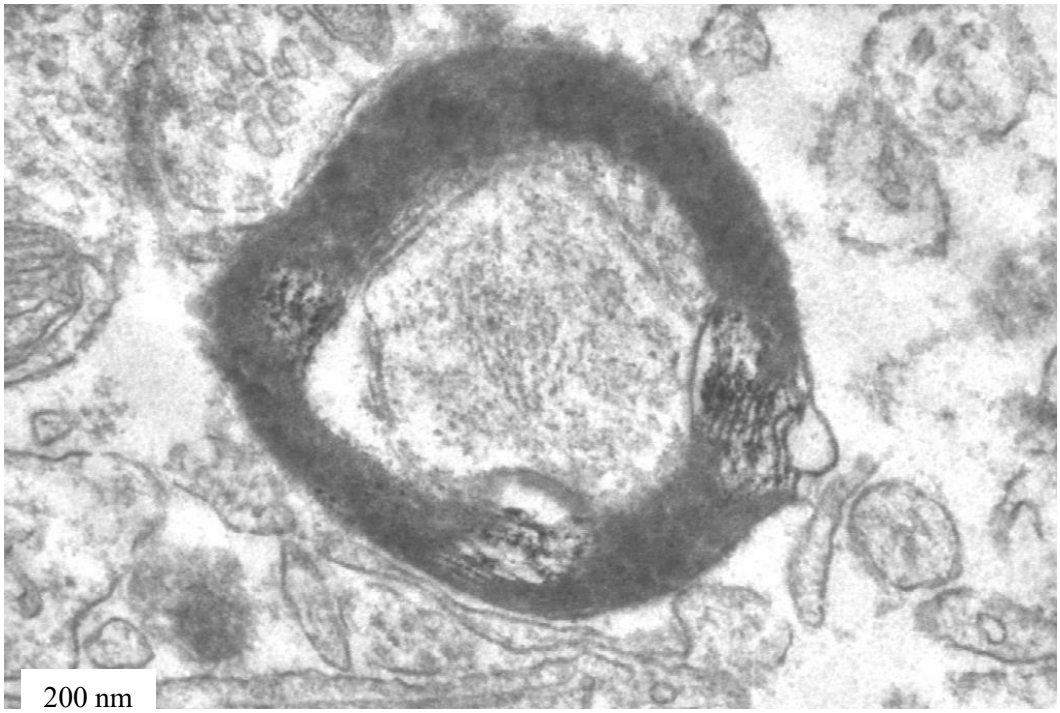

Figure 5. Brain by STEM. A neuron's myelin sheath containing NiO NPs.

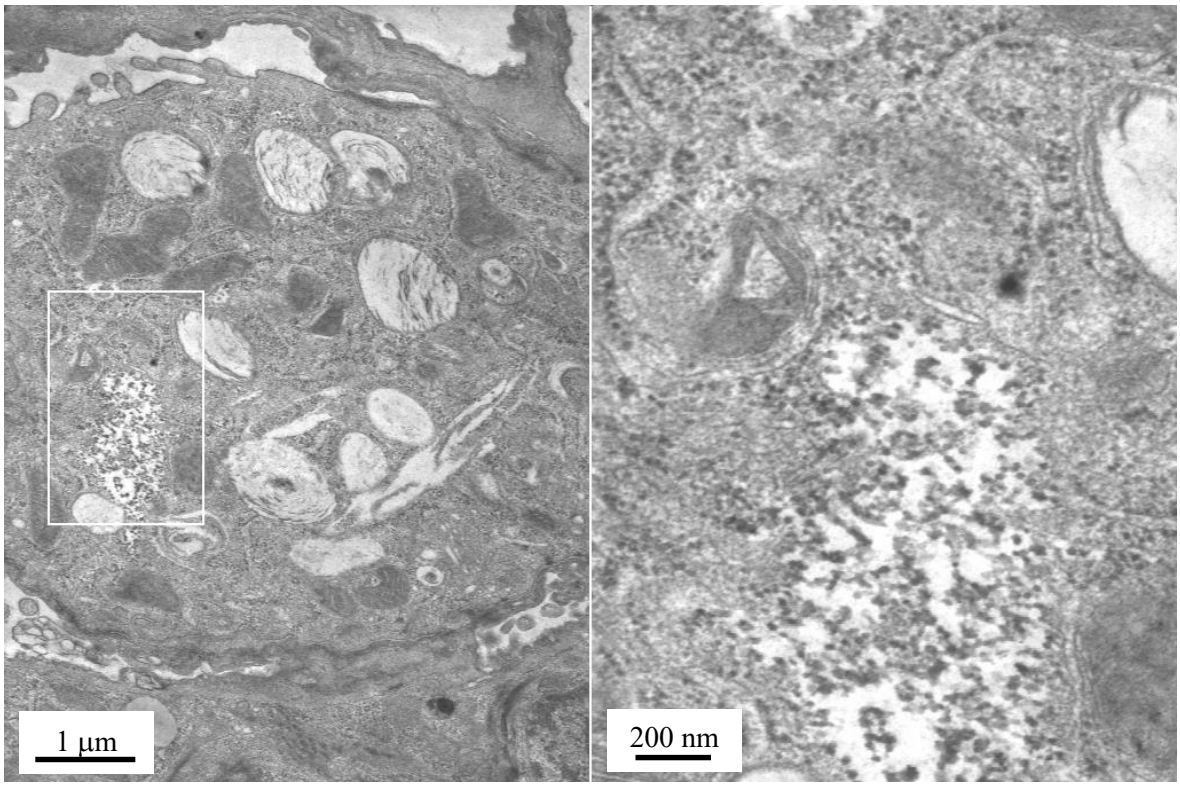

Figure 6. STEM image of type II alveolocyte with NiO NPs in its cytoplasm.

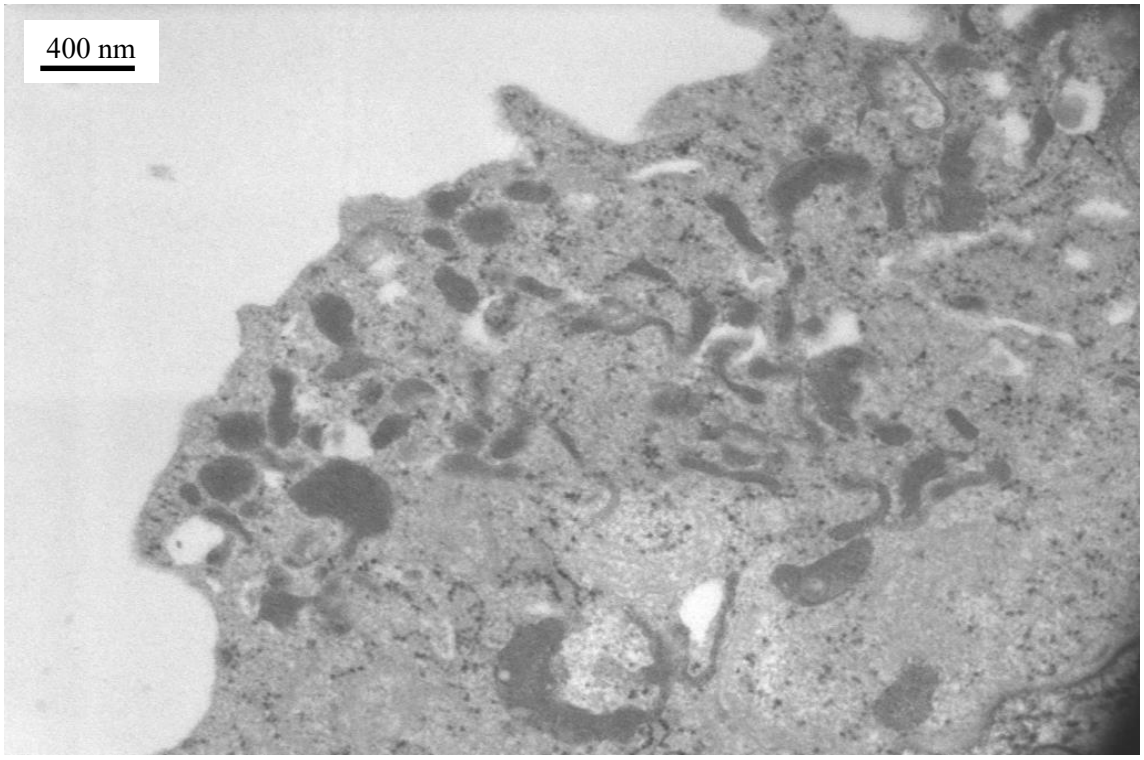

Figure 7. STEM image of an alveolar macrophage with NPs within cytoplasm.

Figure 7 displays a STEM image of a rat alveolar macrophage with NPs in its cytoplasm following the 6 months of inhalation exposure. NiO NPs are scattered in cytoplasm.

The coefficient of genomic DNA fragmentation in the blood nucleated cells (Table 2) is proved to be statistically significant and virtually equally increased in the rats exposed to all the NPs tested, but the background BPC administration significantly reduced the effect of the combined toxic exposure, while the BPC alone did not produce any significant influence on the control value.

NiO NPs show a distinct genotoxicity within every experimental timeline (of 3, 6, and 9 months), which later deepens as the exposure duration increases (Table 2). 6 months fragmentation coefficient is statistically significantly increased compared to that after 3 months of the exposure. 9 months fragmentation coefficient is statistically significantly higher than the 6 months one, while there is no increasing in the control group.

Table 2. Fragmentation coefficients of genomic DNA in nucleated blood cells of rats being chronically exposed to NiO NPs via inhalation, ($X \pm S_x$). *NB*: * marks the estimated values that statistically significant ($P<0,05$) differ from the control group.

The nucleated cells of peripheral blood	**The duration of inhalational exposure**					
	3 months		**6 months**		**9 months**	
	Control	**NiO NPs**	**Control**	**NiO NPs**	**Control**	**NiO NPs**
	0,4229± 0,0008	0,4480± 0,0017*****	0,4247± 0,0006	0,5332± 0,0031*****	0,4244± 0,0005	0,5447± 0,0036*****

Table 3. Fragmentation coefficients of genomic DNA in rats following their chronic inhalation exposure to NiO NPs and / or the Bioprotective complex administration (BPC), (X±Sx). * marks the estimated values that statistically significant (P<0,05) differ from the control group; $^{+}$ marks the estimated values that statistically significant (P<0,05) differ from the NiO NPs group.

Nucleated cells of peripheral blood	**Duration of inhalational exposure - 3 months**			
	Control	**NiO NPs**	**NiO NPs + BPC**	**BPC**
	0,4229±0,0008	0,4480±0,0017*	0,4264±0,0008*$^{+}$	0,4219±0,0003

As is shown in the Table 3, BPC administration in NiO NPs inhalation exposure has taken the fragmentation coefficient down (to about the normal values).

Our research has demonstrated that metallic NPs are much more noxious than their fine micrometric or even submicron counterparts. At the same time, cytotoxicity, organ-systemic toxicity, and in vivo genotoxicity of NPs having a given geometry strongly depend on their chemical nature and on the properties associated with the latter (the solubility included) both quantitatively and qualitatively due to specific mechanisms of action characteristic of a given metal in any chemical form.

Even with the high activity of defence mechanisms, metallic NPs are one of the most dangerous occupational and environmental hazards due to their extremely high toxicity and an apparently imminent genotoxicity. However, even if the remarkably low levels are actually harmless, they are too difficult to maintain to guarantee workers' occupational safety. That is why we believe an additional protective system is needed, the so-called biological prophylaxis designed to enhance the organism's resistance to general and specific mechanisms of nanotoxicity. The idea emerged from our long-term experience with a successful bioprotection of the organism against a number of other toxicants, including some mineral microparticles.

Bioprotectors acting by different toxicokinetic and toxicodynamic mechanisms proved to be most effective when administered not separately but in combinations (the so-called "bioprophylactic complexes" or, abbreviated, BPC). In all the studies we have found that, as expected, the toxicity and even genotoxicity of metallic NPs could be really decreased against the use of certain BPCs compositions. A lot of bioprophylactic complexes are of a similar composition and, we believe, similar BPCs should comprise most of the same components when further looking for bioprotection against other metallic NPs.

Concerning bioprotection against the adverse effects of metallic NPs, up to now we have substantiated theoretically and tested experimentally four BPCs protecting against:

- nano silver,
- nano copper oxide [4],
- a combination of nano nickel oxide and nano manganese oxide [6],
- a combination of nano copper oxide, nano zinc oxide, and nano lead oxide [3],
- a combination of nano titanium dioxide, nano aluminum oxide, and nano silicon dioxide [12].

The above-described results demonstrate the attenuating effects of the bioprotectors on the subchronic systemic toxicity of Me-NPs. In all the studies we found that, as expected, the toxicity and even genotoxicity of metallic NPs could be really decreased against the background of adequately composed BPCs (Fig. 8) [10].

Based on our results, we have obtained the Patents Rights Documents, related to the Bioprotection against toxicity of different NPs: silver, copper oxide, nickel and manganese oxides.

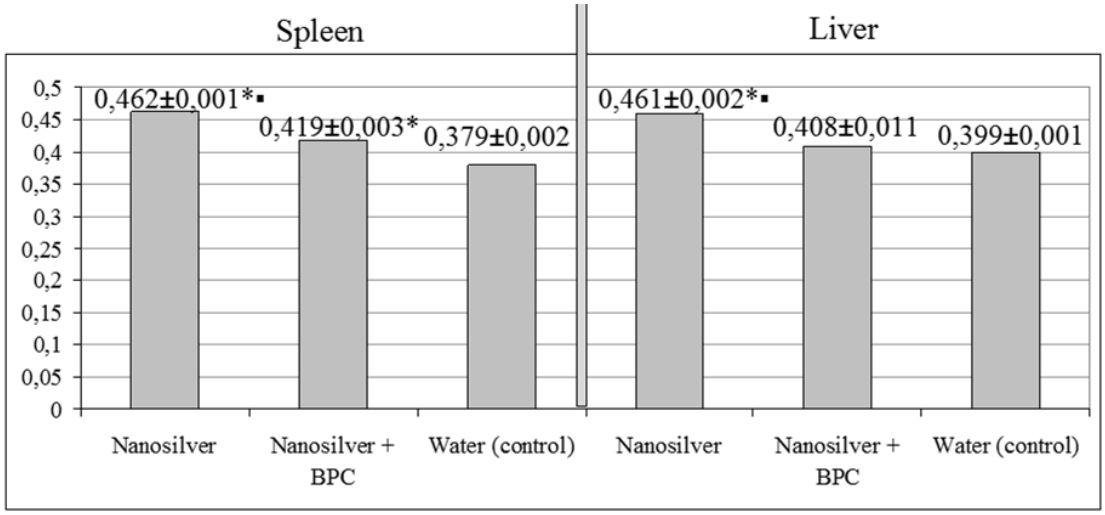

Figure 8. Coefficient of fragmentation of the genomic DNA in spleen (left) and liver (right) cells of rats exposed to nanosilver without (1st columns) or with (2nd columns) bioprotectors; 3rd columns – control (X±Sx). There is statistically significant difference from the control group (P<0.05) and from the group nAg+bioprotectors. * statistically significant (P < 0,05) difference from controls; ▪ - the same from nanoAg + BPC.

4. Conclusion

The toxic effects of metal and metal oxide NPs are much higher than those of their micrometric counterparts, given the same exposure pathways and similar chemistry, even of minimal (including submicron) sizes. For a given size, the toxicity of NPs depends on their chemical nature and the related properties, including solubility.

It was assumed that at some low level of potentially dangerous exposure, a balance between a toxic's adverse biological activity and the organism's natural defensive mechanisms could prevent the development of any identifiable disease or condition or even mild subclinical anomalies.

It was shown that the toxicity and even genotoxicity of metallic NPs could be significantly decreased using adequately composed Bioprotective Complexes.

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