Experimental assessments of metallic and metal oxide nanoparticles' toxicity

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Abstract. Nanoparticles of metals and their oxides (Me-NPs) are of special interest in the light of health risks assessment and management, because, along with engineered Me-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. The nonspecific responses of the organism to the impact of Me-NP included: changes in the cytological and some biochemical characteristics of the bronchoalveolar lavage fluid caused by the deposition of particles in the lower airways, various manifestations of systemic toxicity including significant damage to the liver and kidneys, moderate neurological disturbances associated with possible penetration of Me-NP into the brain from the blood as well as from the nasal mucous membrane along the olfactory pathway, a paradoxically low manifestation of pulmonary pathology due to low chronic retention of nanoparticles in the lungs, and a genotoxic effect on the organism level. The toxicity and even genotoxicity of Me-NPs can be significantly attenuated by adequately composed combinations of some bioactive agents in innocuous doses.

1. Introduction

The broader the use of nanomaterials in various industries, science, and medicine, the higher the probability that humans would be exposed to the impact of respective nanoparticles (NPs). Some general problems that nanotoxicology faced in relation to NPs of metals and their oxides (Me-NPs) from the very beginning of its development as a special branch of the toxicological science are not only theoretically challenging, but also very urgent for the everyday practice of occupational health risks assessment and management. Our team has been conducting *in vivo* and *in vitro* animal experiments involving Me-NPs and has thus accumulated a certain sum of important data that may be of interest as a contribution to solving this problem.

2. Experimental

The experiments were carried out on the outbred white male and female rats from our breeding colony 3-4 month old with an initial body weight of 200-220 g. Each of exposed and 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 Ekaterinburg Medical Research Centre for Prophylaxis and Health Protection in Industrial Workers.

Suspensions of NPs were produced by laser ablation of metal targets (99.99% purity, 1-mm-thick) placed at 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 and dynamic light scattering. High suspension stability, characterized by zeta potential measured in the Zetasizer Nano ZS analyser (Malvern, UK), allowed increasing the suspensions concentration by partial water evaporation at 50°C.

We assessed toxicity of different metallic particles in nanometre and micrometre ranges mostly using as an experimental model repeated intra-peritoneal injections during 6-7 weeks in non-lethal doses and assessing the thus induced subchronic intoxication with a number of functional, biochemical, and morphological indices. As a rule, a subgroup of rats was similarly exposed to oral administration of innocuous bioactive substances (bioprotective complexes, BPC), presumably increasing the organism's resistance to different mechanisms of toxicity.

Being different in some important details depending on specific toxicodynamic and toxicokinetic mechanisms underlying the toxic action of the different metals, the compositions of all the tested BPCs still have much in common usually comprising: glutamate as an effective cell membrane stabilizer acting through the intensification of ATP synthesis and, at the same time, as one of the precursors of glutathione, which is a powerful cell protector against oxidative stress; the other two glutathione precursors: glycine and cysteine (the latter in a highly active and metabolically well available form of N-acetylcysteine); omega-3 polyunsaturated fatty acids, which intracellular derivatives – eicosanoids – activate DNA replication and thus play an important part in its repair; iodide, taking into consideration the well-known disturbances of the thyroid function caused by some metallic intoxications; essential elements known to be antagonists of the metal that forms MeO-NPs under study; other agents of the organism's anti-oxidant system (vitamins A, E, and C, and selenium); pectin enterosorbent as an agent that hinders the re-absorption of toxic metals excreted into the intestines with bile.

The rats were killed by decapitation under ether anaesthesia and their blood was collected by exsanguination. The liver, spleen, kidneys, and brain were weighed. The assessed blood biochemical indices comprised 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, also thyrotrophic hormone of hypophysis, thyroxin, and 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.

Isolated and combined damaging effects of PbO and CuO NPs were also assessed *in vitro* on an established line of human fibroblasts by a decrease in: the cellular dehydrogenase activity (MTT Assay), the ATP content (Luminescent Cell Viability Assay), the cellular proliferation, viability, spreading, and attachment to substrate evaluated integrally by continuous impedance-based measurement of the Normalized Cell Index.

Table 1. Some functional and biochemical indices of rat organism status following exposure to Al_2O_3, SiO_2, TiO_2 NPs and/or the Bioprotective complex administration (BPC) (x ± s.e.).
NB:*statistically significant difference from the control group;
+ from the group given NPs (without BPC); $p < 0.05$ by Student's t-test with Bonferroni correction.

Index	Control	Al_2O_3+SiO_2 +TiO_2	Al_2O_3+SiO_2 +TiO_2 and BPC	BPC
Ceruloplasmin in blood serum, mg%	33.14± 1.13	**42.61± 1.88***	38.36± 2.71	30.54± 1.82
Reduced glutathione in whole blood, μmol/L	26.82± 1.19	**22.55± 1.41***	**26.39± 1.36**$^{+}$	28.17± 1.35
Albumin content of blood serum, g/L	44.34± 0.61	**41.91± 0.88***	43.38± 0.94	44.91± 0.90
A/G index	1.24± 0.04	**1.11± 0.04***	1.17± 0.05	1.27± 0.05
ALT activity in blood serum, IU/L	70.82± 3.24	66.75± 3.55	**83.09± 5.13***$^{+}$	84.98± 4.69

Table 2. Coefficient of DNA Fragmentation (Cfr) of Nucleated Blood Cells after exposure to Al_2O_3, SiO_2, TiO_2 NPs and/or the BPC administration (x ± s.e.).
NB: statistically significant difference *from the control group; $^{+}$from that exposed to Me-NPs without the BPC.

Exposure to	C_{fr}
Al_2O_3-NP +TiO_2-NP+SiO_2-NP (full doses)	0.6430±0.0189*
Al_2O_3-NP+TiO_2-NP+SiO_2-NP (half doses)	0.4849±0.0068*$^{+}$
Al_2O_3-NP +TiO_2-NP+SiO_2-NP(full doses)+BPC	0.4742±0.0067^{+}
BPC	0.4143±0.0047
Control	0.4023±0.0064

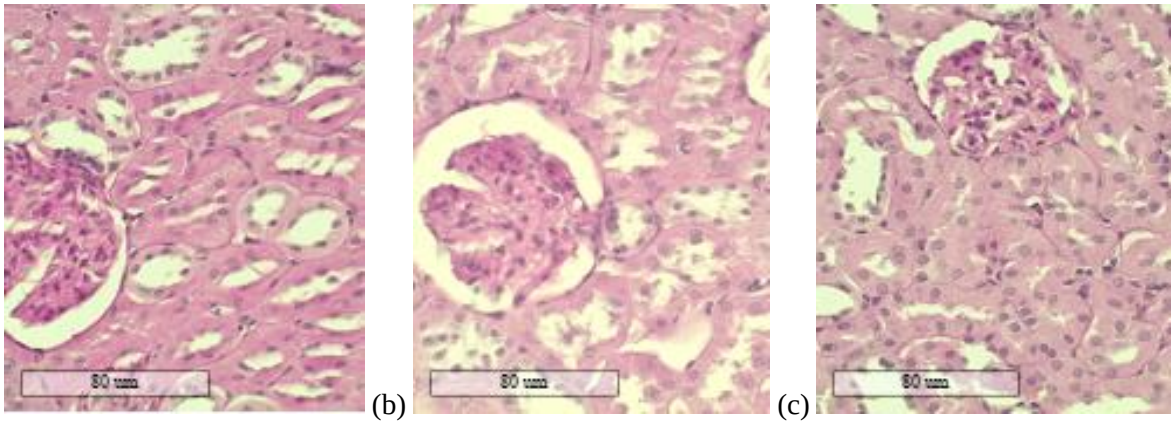
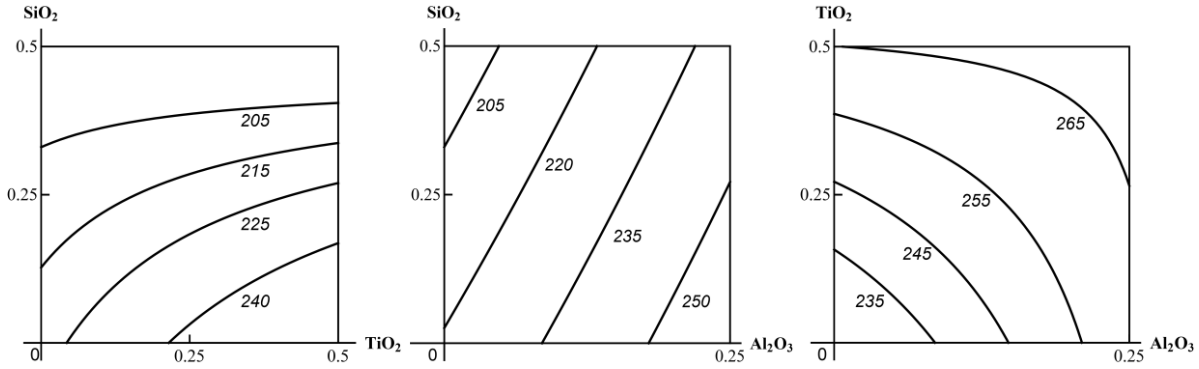


Figure 1. Kidneys of (a) control rats, (b) rats exposed to Al_2O_3+TiO_2+SiO_2 NPs, (c) rats exposed to Al_2O_3+TiO_2+SiO_2 NPs with background oral administration of BPC. PAS stain, magnification 400.



(a) (b) (c)

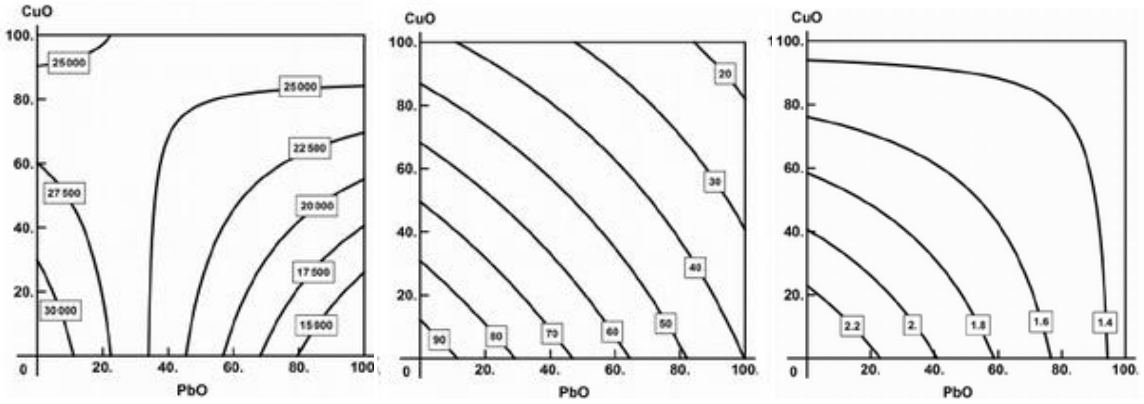
Figure 2. Isobolograms of combined subchronic toxicity assessed by an increase in the concentration of AST in blood serum under exposure to (a) SiO_2-NP + TiO_2-NP (opposite action), (b) SiO_2-NP + Al_2O_3-NP (opposite action), (c) TiO_2-NP + Al_2O_3-NP (subadditivity of unidirectional action). The axes represent doses of corresponding MeO-NPs in mg per rat; the numbers at the isoboles denote the magnitude of the effect (in IU/L).

For mathematical analysis of the combined toxicity, we used the Response Surface Method. The two-dimensional image of such a surface (usually, a hyperbolic paraboloid) is graphically presented by Loeve isoboles obtained when it is sectioned on different levels.

3. Results and discussion

Table 1 presents some results related to functional and biochemical indices for the organism's status assessed in an experiment with a ternary combination of Me-NPs (Al_2O_3-NP+SiO_2-NP +TiO_2-NP) that was in detail described in Ref. [1]. For readers' convenience, we present only those indices, which were statistically significant different from controls. Coefficient or DNA fragmentation values obtained in the same research are given in Table 2.

It can be seen that several of these indices tended toward normalization under the influence of the BPC (particularly those relating to the genotoxic effect). This experiment and many other similar ones [2-5] proved that the biological protection against the Me-NP's toxicity was the most effective when we used not a single bio-protector, but a combination of those having different main mechanisms of protective action. Some examples of the BPC's protective efficiency against the combined action of Me-NPs are demonstrated in the Tables 1, 2 and Figure 1.



(a) (b) (c)

Figure 3. Isobolograms characterizing the combined toxic action of CuO-NP and PbO-NPs in experiments on the fibroblast culture as estimated by increase in (a) the intensity of the luminescent signal, (b) the formazan formation, (c) Normalized Cell Index. Numbers on axes are MeO-NP concentrations in μg/mL, numbers on isoboles are corresponding effect values.

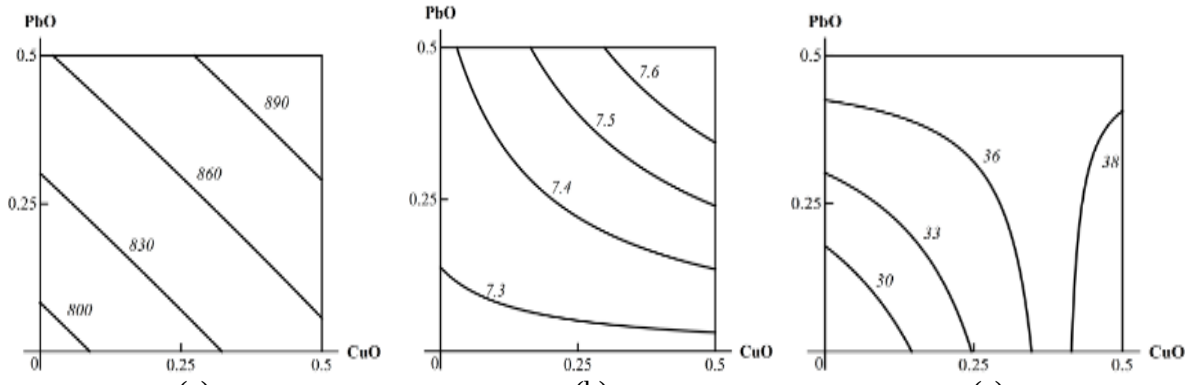


Figure 4. CuO-NP+PbO-NP combined subchronic toxicity for (a) thrombocyte count (additivity), (b) erythrocytes count (superadditivity at low effect levels and additivity at high effect levels), (c) diuresis (subadditivity at low effect levels and oppositely directed action at high effect levels).

The organism's response to a simultaneous exposure to any two of the NP species under study was not of a unique type. In fact, it was characterized by a complex interaction between different types of combined toxicity (additivity, subadditivity, or superadditivity of unidirectional action and different variants of opposite action) depending on many factors. Thus, comparison of Figures 2a and 2b shows that the combination SiO_2-NP + TiO_2-NP displays subadditivity of unidirectional action for one effect (increase in the concentration of ceruloplasmin in the blood serum) and contra-directional action for another one (increase in AST concentration); the combination SiO_2-NP + Al_2O_3-NP) demonstrates additive and opposite actions, respectively; and the combination TiO_2-NP + Al_2O_3-NP – additivity and subadditivity of unidirectional action. An example of how the dependence of the type of combined toxicity varies for one and the same effect at different levels of it and different Me-NP doses is illustrated in Figure 2c.

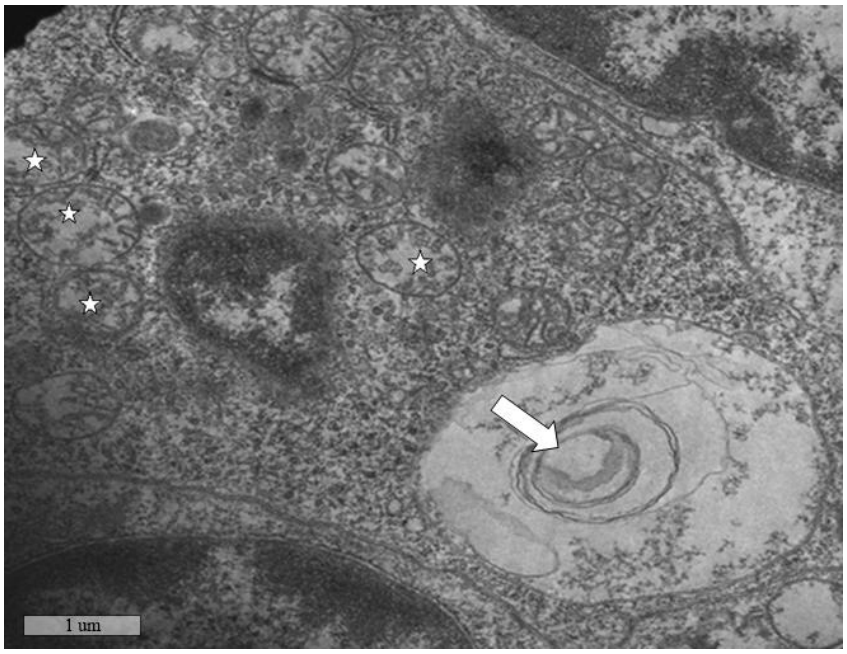


Figure 5. Concentric membranous formation and cytoplasmic vacuolization (arrow), and marked damage to mitochondria (asterisks) in a spleen cell from a rat exposed to ZnO-NPs. Scanning transmitted electron microscopy.

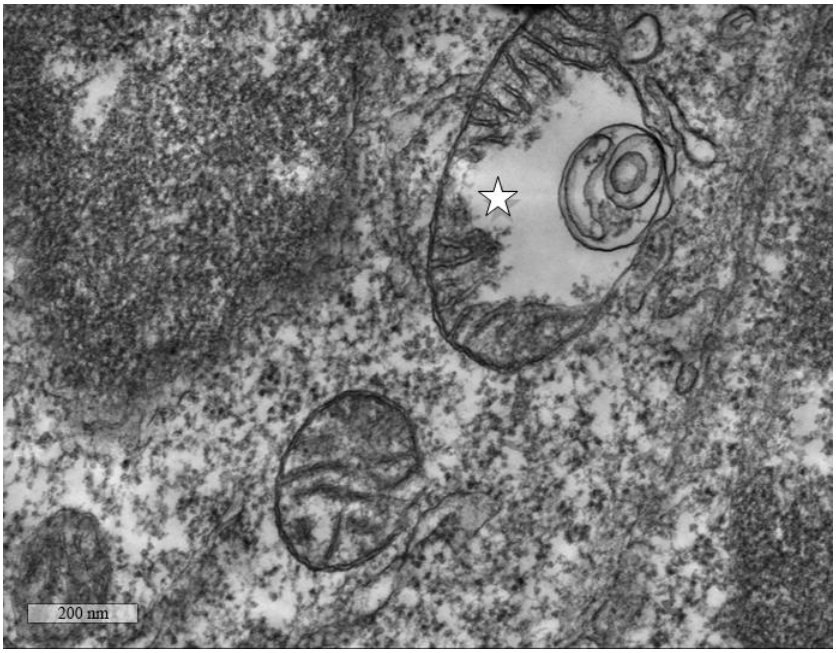


Figure 6. A partially destroyed mitochondrion (marked by asterisk) in a thymus rat cell exposed to PbO-NPs and ZnO-NPs. Scanning transmitted electron microscopy.

The variability of the typology of combined cytotoxic action displayed by CuO-NP and PbO-NP *in vitro* is presented in Figure 3 [6], in general, being similar to its variability *in vivo* on system and organism levels (Fig. 4). The subadditive type of unidirectional combined toxicity manifested itself only at the lowest doses of both agents, while a higher dosage of at least one of them resulted in different variants of contra-directional action. The same feature was observed previously for some non-specific *in vivo* toxicity measures.

In rats exposed to repeated intraperitoneal injections of CuO-NPs, PbO-NPs, and/or ZnO-NPs [7], transmission electron microscopy of liver, spleen, kidney, myocardium, brain, thymus, and testicle tissues revealed uniform ultrastructural changes, the most frequent being vacuolization of the cytoplasm with concentric membranous inclusions in it, demyelinizations of nervous fibres in the brain and especially damage to mitochondria with partial or complete loss of cristae (Figs. 5,6).

4. Conclusion

Metallic nanoparticles are one of the most dangerous occupational and environmental hazards due to their especially high toxicity and virtually obligatory genotoxicity. Adverse effects of metallic nanoparticles on all levels from molecular to organo-systemic can be markedly attenuated by background administration of combinations of some bioactive agents (bioprotectors) in innocuous doses. Along with decreasing exposures to nanoparticles, enhancing the organism's resistance to their adverse effects with the help of such bioprotectors can be an efficient auxiliary tool of health risk management.

A mathematical analysis has shown that for all the Me-NP combinations studied by us there exist not merely three traditionally acknowledged types of binary combined toxicity (additivity, subadditivity and superadditivity or synergism) but different associated assessments depending on exactly which effect is considered, on its level, as well as on dose levels and their ratio.

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