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TOXIC EFFECT AND MECHANISMS OF NANOPARTICLES ON FRESHWATER INFUSORIA

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ABSTRACT: Nanoparticle toxicology works toward establishing the hazard of nanoparticles, and therefore their potential risk, in light of the increased use of exposure. The current study was research proper characterization of the nanoparticles for understanding of their toxic effects and mechanisms at the cellular level. Dose and time as a main parameters is essential in hazard identification and risk assessment of nanomaterials. Material for the evaluation of toxic effects was used 20 samples of commercially available and laboratory preparations of metals and nanocarbon, divided into main groups: carbon nanoparticles, metal oxide nanoparticles and metal nanoparticles in different concentration. Fresh water infusoria *Stylonychia mytilus* (wild strain) in exponential growth phase was used as the test object. Analysis of data on oxides of the metals showed higher toxicity than metal nanoparticles. In groups the maximum toxicity was observed in iron oxides (Fe3O4, Fe2O3), copper (CuO) and molybdenum (MoO3) (0,1; 0,025; 0,0125 M) after 24 hours of incubation with the test object Analysis of the effect of metal nanoparticles on the cells of infusoria showed that the maximum toxic effect was observed when exposed to Cu, Fe, Ag (0,025- 0,0015625 M). Statistical analyses showed a high correlation between concentration and time ($P \leq 0,001$). The issue of accessibility of nanomaterials released in the environment for living organisms has been poorly studied. The toxic effects of nanoparticles can be associated with their size and their physicochemical properties.

Keywords: Nanoparticles, Toxic Effect, Stylonychia mytilus, Cytotoxicity, Ecology

1. INTRODUCTION

Active development of researches in nanomaterials leaves open the question of their safety. The safety of nanostructures for environment and human health becomes the top priority. Because of their properties, nanostructures react easier and are able to form complex compounds with unknown properties. This fact adds technological perspective to nanoparticles. At the same time we have to pay special attention for the ecological risks connected with nanostructures [1].

Nanotechnologies represent the convergence of techniques and molecular biology that lead to the development of structures, equipment and systems. Nanoparticles have new functional properties with sizes in range from 1 to 100 nm [2]. At present, there is not enough information for a full understanding of the interaction of nanostructures with biological systems and, thus, it is unclear whether nanostructures have negative effects that cause harmful biological reactions [3], [4].

Some authors point to the risk of carcinogenic effects of nanoparticles. They also note the ability to generate reactive oxygen species (due to the presence of the reaction centers). The nanoparticles are stable and do not undergo biotransformation. They are not removed from the cell that causes stress in cells and their breakage. Also, literature comprises data that nanoparticles may have protective effect on living organisms, increasing the body's resistance to various toxicants [5], [6].

Particle size and surface area are important characteristics of a material with toxicological prospects. When size of particles decreases, the surface area increases, it enables a large number of atoms or molecules to be deposited on the substrate surface. Nanoparticles differ from molecules and ions of the same composition not only in size but also in a higher specific surface and high adsorption and cumulative ability. Their chemical potential increases at the phase interface, thereby changing the solubility, reactivity and catalytic ability [7]. The degree of activity may also depend on the type of nanoparticles (metals, oxides, mixtures, etc.). Thus, the change of physical, chemical and structural properties of nanomaterials due to the decrease in size may cause a number of interactions that might lead to toxicological effects.

2. OBJECTIVES

Therefore, the objective of this work is the assessment of toxic influence of different particles on the cell of test object.

3. MATERIALS AND METHODS

Fresh water infusoria *Stylonychia mytilus* (wild strain) in exponential growth phase was used as the test object. The studied test functions include survival rate, number (biomass). Primary culture of *Stylonychia mytilus* was cultivated in a Lozin-Lozinsky saline solution, (1 g in 1 liter of distilled water). Yeast (*Saccharomyces cerevisiae*) was added: NaCl-0,1%; KCl-0,01%; $CaCl_2$-0,01%; $MgCl_2$-0,01%; $NaHCO_3$-0,02.

The following nanoparticles were used in the study (presented below in Table 1).

Table 1 Characteristics of the used nanoparticles

Name	Size [nm]	Phase and chemical composition	Production method	Surface (m^2/g)
		Metals		
Fe	90	Metallic iron (not less than 99.8% of the mass.) and sorbed gases: CH4, CO2, Ar, N2	Method of electric explosion of a conductor in air	7,7
Cu	97	crystal copper 96,0 ± 4,5%, copper oxide - 4,0 ± 0,4%	High-temperature condensation with subsequent modification of oxygen	24
Zn	90	90%, the rest sorbing gases, zinc oxide and H2O	The electric explosion of wire in an argon atmosphere	5,34
Ag	70	99.99% of metallic silver adsorbed gases to 0,01% - CH4, CO2, Ar, N2	The electric explosion of wire in an argon atmosphere	6,5
Ni	70	Metallic nickel:Ni=99,758%, Mg=0,041%, Al=0,058%, Si=0,049%, S=0,005%, Ti=0,010%, Fe=0,047%, Co=0,032% (electronsand microanalysis)	Method of electric explosion of a conductor in air	4,5-6,0
Mo	50	Mo: 99,7%, O_2: 0,3%	Plasma-chemical method	14
W	50	W: 99.7%;O2: less than 0.3%	Plasma-chemical method	6,5
		Metal oxides		
CuO	90	cupric oxide, CuO 99,6% mass	Plasma-chemical method	14
ZnO	95	ZnO: 96%; Oxides of other metals less than 4%	Plasma-chemical method	9
Fe_3O_4 (I)	65	Fe3O4 at least 99 wt.%, about 1% of the mass. - adsorbed gases: CH4, CO2, O2, N2	Method of electric explosion of a conductor in air	10
Fe_3O_4 (II)	65	Fe3O4 99 % of mass.	Chemical	20
Al_2O_3	54	95% mass. α- Al2O3. 3%, 2% - sorbing gases (nitrogen, hydrocarbons), water	Electrical explosion of aluminum wire in oxygen atmosphere	40
NiO	94	oxide of bivalent nickel NiO: 99,6%	Plasma-chemical method	12
MoO_3	92	MoO_3 : 99,8% mass	Plasma-chemical method	12
		Composite		
FeCo	62,5	70% iron, 30% cobalt	Gas-phase	8,2
CuZn (I)	65	60% copper and 40%zinc	The electric explosion of wire in an argon atmosphere	5-6
CuZn (II)	96,5	60% copper and 40%zinc	Gas-phase	10
		Carbon nanomaterials		
k-SWCNT-90A	1,5	SWCNTs: 90 wt. %	Electric arc evaporation	400

These materials were assessed (particle size, polydispersity, volume, quantitative content of fractions, surface area) by electron scanning, transmission and atomic force microscopy using the following equipment: a LEX T OLS4100, a JSM 7401 F and a JEM-2000 FX("JEOL", Japan).The size distribution of particles was investigated using a Brookhaven 90Plus /BIMAS and ZetaPALS Photocor Compact (Russia) in lysols after dispersing the nanoparticles using an ultrasonic disperser UZDN-2T (Russia) at f-35 kHz, N 300 W, and A-10 μa for 30 min. Toxic effects of the

samples were assessed in a wide range of equimolar concentrations (4M - 6 × 10-6 M). The size of nanoparticles was determined with the help of electronic microscope JSM-740 IF.

Action of toxic substances was studied in a wide range of concentrations (3.2M-6*10^{-6}M).

The sensitivity of *Stylonychia mytilus* to the action of toxicant was determined according to the time of their death. It was registered when protozoa stopped moving, which was accompanied by a violation of the integrity of the cells and lysosomes. The number of cells in 5 ml of medium containing intact infusoria (without nanoparticles) was a control group in all experiments. The total number of cells in 5 ml of medium containing infusoria was counted using a light microscope (MT 5300L). Cells in the stationary phase were incubated at 20 ± 2◦C in medium with toxicants within 24 hours in a concentration range - 3,2-6 × 10-6 M.

ANOVA statistical analysis was utilised and then using the Tukey test (SPSS вер. 17,0). Differences were c onsidered significant if P < 0,05.

4. RESULTS AND DISCUSSION

The results of studies demonstrated that the maximum toxic effect was achieved after the influence of Ag nanoparticles on protozoa. Cell death was observed after 10 minutes of incubation of the test object with the toxicant. The toxicity was observed up to a concentration of 1 * 10-5 M. Nanoparticles of Cu and Fe also caused cell death, but their effect was less potent than that of Ag. Toxic effect caused 100% cell death. Fe toxicity occurred in 24 hours. Cu and Ag toxicity occurred in 10 minutes of incubation. Action other nanometals was characterized by less mortality in comparison with Ag, Cu and Fe. Thus, Zn was less toxic, 100% mortality was observed up to 0.003125 M. When concentration of the element decreased, the number of living cells increased and reached 21% of the total amount in the final concentration (0.0001953125M). Minimal death rate was registered after the influence of Ni and W solutions. Toxicity of Ni was observed at a concentration of 0.005 M and W – at concentration of 0.2 M. The number of dead cells varied from 70 to 100% at other concentrations. This effect can be explained by the small size of Cu, Ag and Fe nanoparticles as compared with Mo, Ni, Zn, W (Fig. 1) It is proven that nanoparticles in size of 2-50 nm will have greater cytotoxicity as compared with larger particles. There are a lot of experimental data are available about nanoparticles of silver and copper. Toxic effects of metal nanoparticles were demonstrated in the studies on other water test objects. For example, Ag and Cu nanoparticles are highly toxic to daphnia (the LD50 over 48 h was 0.06 and 0.04 mg/l) [8].

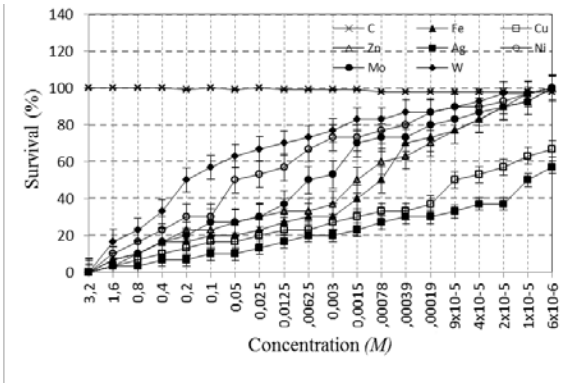

Fig. 1. Influence of metal nanoparticles at different concentration on *Stylonychia mytilus* survival

Studies of Ag and Cu on Danio rerio demonstrated the increased mortality and disease process [9], [10]. The effect of colloidal nanosilver on growth and structure of laboratory populations of *Scenedesmus quadricauda* (Turp.) *Bréb*. and *Monoraphidium arcuatum* (Korsch.) *Hind*. at concentrations ranging from 0.0001 to 1 mg/l.

The toxicity of colloidal silver was expressed at a concentration of 0.1 mg / l and above. Moreover, algostatic effect was observed, its duration was directly dependent on the concentration of silver in the environment [11], [12].

Rather different data were obtained by a toxicity analysis of metal oxide nanoparticles. Maximum toxicity was registered in oxides of iron (Fe3O4 (I), Fe3O4 (II)), copper (CuO) and molybdenum (MoO3). 100% mortality of infusorias was observed under the influence of iron oxide. It is also possible to ascertain the negative chemotaxis (movement of the attractant), because most of the dead cells were located around the perimeter of the main nanoparticle concentrations.

No changes in the cells of infusorias were identified in the earlier periods. The influence of copper oxide in comparison with iron oxides was less pronounced. The toxic effect (100% death) was observed in 6 hours.

In 24 hours it attained a maximum at 0.1, 0.025 and 0.0125 M in nanoparticle solutions. When concentration decreased, the number of surviving cells increased from 3% to 10% (at concentration of 0,0001953125M).

The assessment of MoO_3 toxicity demonstrated that cell death occurred in 10 minutes of contact with a solution of nanoparticles (at a concentration of 0.0125 M). This situation remained throughout the whole time period. ZnO, TiO_2 and NiO possessed minimal toxicity. Their action was manifested only in the initial concentration (0.1 M) (Fig. 2)

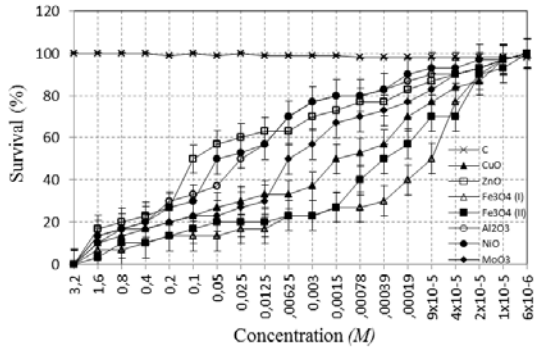

Fig. 2. Influence of metal oxide nanoparticles at different concentrations on survival of *Stylonychia mytilus*

Toxicity of metal oxides (TiO_2, ZrO_2, Al_2O_3, and CeO_2) was assessed on green algae (*Pseudokirchneriella subcapitata*) and the effect on the photosynthetic activity was demonstrated [13]. It is also noted that the zinc oxide inhibits the growth of test object at a concentration of 600 μg / l [14]. The negative impact of oxides of zinc, aluminum and titanium was shown on embryos of *Danio rerio*. It was revealed that ZnO has the maximum influence [15]. The lethal dose (LD50) of zinc oxide nanoparticles was 1.8 mg / l after 96 h of incubation.

Assessment of mixtures toxicity demonstrated that CuZn mixture causes the maximum cell death. At all concentrations 100% cell death was detected. In comparison with this mixture, brass was characterized by less toxicity; total death of infusoria was observed up to a concentration of 0.0015625 M. Moreover, this effect was expressed in 10 minutes of incubation and remained until the end of the period. The number of surviving cells varied from 10 to 25% of the total number. FeCo was less toxic, total cell death was observed in 24 hours and up to a concentration of 0.05 M, no toxic effect was observed in other concentrations (Fig. 3).

Toxic effect at concentrations up to 0.0125 M was observed for the whole time period after the analysis of the first group. Test objects were still be able to move in further dilutions, 100% of infusoria were alive. The relative resistance to carbon nanomaterials can be explained by the fact that protozoa used them as food [16], [17] and [18]. In a recent study, the toxicity of fullerenes C60 for two aquatic species (*Daphnia* and *Pimephales*) caused lipid peroxidation (LPO) in brain.

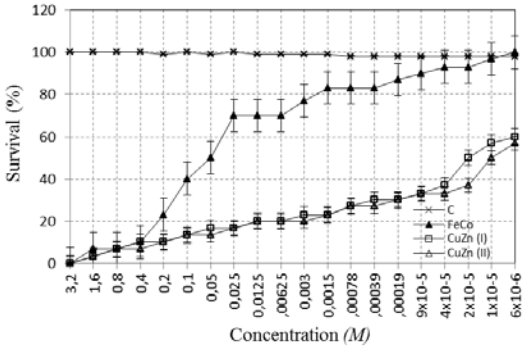

Fig. 3. Influence of mixtures of metal nanoparticles at different concentrations on survival of *Stylonychia mytilus*

LPO significantly increased in the gills. And the result is a significant increase in the expression of genes associated with the inflammatory response and metabolism. In contact with water, C60 spontaneously forms stable set (nanoC60) with dimensions D = 25-500 nm. Prokaryotic effect of these aggregates inhibits the growth (0.4 ppm) even at relatively low concentrations and reduces the rate of aerobic respiration (4 ppm) [19]. In addition, chronic effects of carbon nanoparticles, fullerenes C60 were studied using midges in *Chironomus riparius* at different periods of life. The influence of fullerenes C60 on growth of 10-day and 42-day species was studied at a nominal concentration of 0.0004-80 mg / kg of dry weight.

The body length decreased at a concentration of 0.0025-20 mg / kg, but no effect occurred at higher concentrations. Stunt was observed at a concentration of 0.5 mg / kg. The observed effects correlate with the analyzed sizes of particles in sediment indicating that small agglomerates of fullerene cause more serious consequences for *C. Riparius*, than larger agglomerates that was observed at higher doses of C60. The results have demonstrated that fullerenes can be dangerous for sediment dwellers; it is manifested in changing ecotoxic parameters that influence the survival of water organisms [20].

The study of nanoparticle toxic action demonstrated that the studied samples had different toxic action towards test cells (Table 2).

Table 2 Biological effect of nanoparticles on *Stylonychia mytilus*

Name	Concentration (M)			
	Tox	LC50	LOEC	NOEC
Metals				
Fe	3,2 - 0,0015	0,00075	0,00039	0,00019 - 6×10^{-6}
Cu	3,2 - 0,00019	9×10^{-5}	4×10^{-5} - 2×10^{-5}	1×10^{-5} - 6×10^{-6}
Zn	3,2 - 0,003	0,00015	0,00078 - 0,00039	0,00019 - 6×10^{-6}
Ag	3,2 - 2×10^{-5}	1×10^{-5}	6×10^{-6}	-
Ni	3,2 - 0,1	0,05	0,025 - 0,0125	0,00625 - 6×10^{-6}
Mo	3,2 - 0,0125	0,00625	0,003	0,00015 - 6×10^{-6}
W	3,2 - 0,4	0,2	0,1	0,05 - 6×10^{-6}
Metal oxides				
CuO	3,2 - 0,003	0,00015	0,00078 - 0,00019	9×10^{-5} - 6×10^{-6}
ZnO	3,2 - 0,2	0,1	0,05	0,025 - 6×10^{-6}
Fe_3O_4 (I)	3,2 - 0,00019	9×10^{-5}	4×10^{-5}	2×10^{-5} - 6×10^{-6}
Fe_3O_4 (II)	3,2 - 0,00078	0,00039	0,00019	9×10^{-5} - 6×10^{-6}
Al_2O_3	3,2 - 0,05	0,025	0,0125	0,00625 - 6×10^{-6}
NiO	3,2 - 0,1	0,05	0,025 - 0,0125	0,00625 - 6×10^{-6}
MoO_3	3,2 - 0,00625	0,003	0,0015	0,00078 - 6×10^{-6}
Composite				
FeCo	3,2 - 0,1	0,05	0,025	0,0125 - 6×10^{-6}
CuZn (I)	3,2 - 4×10^{-5}	2×10^{-5}	1×10^{-5}- 6×10^{-6}	-
CuZn (II)	3,2 - 2×10^{-5}	1×10^{-5}	6×10^{-6}	-
Carbon nanomaterials				
k-SWCNT-90A	3,2 - 0,0125	0,00625	0,003	0,00015 - 6×10^{-6}

Note: Tox – the concentration causing 0-39 % survival object; LC50 – the concentration causing 50% survival of object; LOEC – the concentration causing 40-69 % survival of object; NOEC – the concentration causing 70-100 % survival of object [21]

Hydrophobic properties and zeta potential (ζ-potential) of particles in solution are the main parameters that help to assess the toxicity (except for the size of nanoparticles). Smaller particles and positively charged particles have a pronounced toxic effect. The zeta potential, electrical potential that is produced by the motion of particles between the adsorption layer of ions located on the surface of the particles and the diffusion layer of ions surrounding the particles determine the interaction of nanoparticles with membrane of cell, its damage and lethal effect. For example, chitosan nanoparticles and chitosan nanoparticles containing copper ions inhibit bacterial growth. The toxic effect is expressed in violation of membrane structure and cell aggregation. Aggregation of bacteria occurs in the presence of chitosan nanoparticles with a positive zeta potential, but when there is no copper [22].

Studies on microorganisms showed that in the case of substances, which have a bactericidal effect, such as silver or zinc, the increase of surface plays an important role when the material is presented in the form of nanoparticles. Nanoparticles also release copper ions more actively than the usual surface. But this does not exclude other mechanisms of action. Silver nanoparticles penetrate into cells; interact with proteins, particularly with proteins containing sulfur and DNA. They inhibit the fission process and cause cell death. At the same time, membranes damage under the action of silver ions. Free radicals that evolve under their action can damage DNA. The antibacterial effect of ZnO is also associated with the release of hydrogen peroxide and membrane damage. And it is the main reason of their toxicity. The main mechanism of action for silver nanoparticles is DNA damage. In both cases, the nanoparticles disrupt the structure of the membrane by physical interaction. ZnO inhibits the growth of bacteria, primarily Gram-positive. In contrast, the silver nanoparticles are more active against Gram-negative bacteria [23].

The iron oxide is non-toxic for the bacteria. Iron oxide nanoparticles are able to penetrate into the cell and cause the formation of reactive oxygen

species, so they can lead to death of bacteria. In the case of iron oxide nanoparticles not only to their anti-bacterial properties can be used for combating microorganisms, but also their ability to affect the movement of body due to their charge. Antimicrobial agents may be bound with these particles, and their delivery will be the main function of these nanoparticles.

Thus, studies have shown that the toxicity of nanoparticles of metals, oxides and mixtures varies and depends on physical and chemical properties. Size of particles also influences on it. It concerns metal nanoparticles and cationic properties in case of oxides and mixtures.

5. CONCLUSIONS

Processes that control transport and removal of nanoparticles in an aqueous medium are not clearly understood. The future of nanomaterials in aquatic ecosystems is controlled by a number of biotic / abiotic processes such as dissolution, dispersion, interaction between nanomaterials and natural chemicals, human impact in the ecosystem. Moreover, assessing the environmental risk, it is important to understand the environmental consequences of the impact of nanomaterials. Before unconscious dumping of huge amounts of hazardous nanomaterials in the environment, the questions of solubility and degradation of nanomaterials in water needs to be studied. The initial information on the safety, toxicity and adaptation of aquatic environment shall be gained.

Due to the growth of nanotechnology, regardless of the potential benefits, the researchers need to anticipate and characterize the potential risks associated with the new technology. Despite this, currently there are not enough convincing data that indicate that these effects will become a serious problem, and that they cannot be solved by a rational scientific approach. At the same time, it is impossible to ignore the safety assessment of nanomaterials.

6. ACKNOWLEDGEMENTS

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