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Original article

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Comparative analysis of cytology of rat bronchoalveolar lavage fluid after single exposure to metal oxide nanoparticles

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ABSTRACT

Introduction. Human production activities (metallurgical, mining, electronics production and processing, batteries) are related to air pollution of the working area and the environments of complex composition aerosols. Among the aerosol components, ultrathin particles of the nanometer range (including metal nano-oxides) are considered to be the most dangerous. Due to their prevalence, study to assess the cytotoxicity of metal oxide nanoparticles are relevant.

Material and methods. CuO, PbO, CdO, Fe_2O_3, NiO nanoparticle (NP) suspension were obtained by laser ablation. The study was done on white outbred female rats. A single intratracheal instillation of different chemical NPs was performed (in dose 0.5 mg/animal); control animals received a similar amount of deionized water. A day after the NP instillation bronchoalveolar lavage (BAL) was carried out with the subsequent assessment of its cytological indices.

Results. The cytotoxic action of the studied NPs, based on the cytological indices of the BAL fluid, is changed as follows (from greater to lesser): CuO NP > CdO NP > PbO NP > NiO NP > Fe_2O_3 NP.

Limitations. Such physical characteristics of nanoparticles as solubility in water and biological fluids, charge, adsorption capacity, resistance to aggregation, hydrophobicity, adhesion to surfaces, and the ability to generate free radicals have not been studied. Extrapolation of data from rodents to humans shall be done with caution, since cytotoxicity has been characterized only based on the main cellular parameters.

Conclusion. Bronchoalveolar lavage cytology can be used as an effective screening method for the cytotoxic effect of NPs.

Keywords: *metal oxide nanoparticles; cytotoxicity; intratracheal instillation; rats*

Compliance with ethical standards. The animal study protocols were approved by the Institutional Ethics Committee of the Yekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers (Protocol No. 2 of April 20, 2020).

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Contribution of the authors: *Sutunkova M.P., Minigalieva I.A., Privalova L.I.* – the concept and design of the study, editing; *Klinova S.V., Ryabova Yu.V.* – conducting a study, the collection and processing of the material, writing text, editing; *Tazhigulova A.V.* – conducting a study, the collection and processing of the material, editing. *All co-authors* – approval of the final version of the article, responsibility for the integrity of all parts of the article.

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Оригинальная статья

Introduction

Human production activities, such as metallurgy and mining, production and recycling of electronics and batteries, cause pollution of the workplace and ambient air with aerosols of complex chemical composition. Nano-sized particles, including those of metal oxides, are among the components of such aerosols [1]. Airborne nanoparticles are considered a dangerous component of inhaled air, primarily due to their size, which determines particle interaction with the structures of the body [2]. Large particles are retained in the upper airways; smaller ones deposit in the trachea and bronchi, while those smaller than 2 μm can reach alveoli and are most prone to exerting systemic effects [3]. Metal nanoparticles (NPs) exhibit the greatest systemic toxicity and cause severer local reactions of the body following inhalation [4], some of which are determined by specific characteristics of their components and activate certain toxicity mechanisms. In addition, it is also believed that cytotoxicity of nanoparticles increases with the atomic number of their main forming element [5].

Numerous studies conducted both *in vitro* and *in vivo* have demonstrated cytotoxic effects of nanoparticles on bronchi and alveoli.

Studies on the culture of A549 cancer cells from human bronchoalveolar carcinoma showed an increase in the generation of reactive oxygen species (ROS) and a decrease in the level of adenosine triphosphate following the exposure to Al_2O_3, TiO_2, and SiO_2 NPs with the size range of 10 to 60 nm [6]. The exposure to SiO_2 NPs sized 15 and 46 nm induced a dose-dependent decrease in cell viability and a simultaneous increase in the concentration of ROS in the medium [7]. ZnO NPs had a toxic effect on human pulmonary alveolar epithelial cells (HPAEpiC) causing generation and accumulation of ROS in mitochondria by inhibiting the activity of the superoxide dismutase enzyme and reducing glutathione content. ROS, in their turn, open the mitochondrial Ca^{2+} pathway and reduce mitochondrial membrane potentials leading to apoptosis [8]. A comparative assessment of Fe_2O_3 and ZnO nanoparticles on BEAS-2B and A549 cell lines showed that ZnO NPs, but not Fe_2O_3 NPs, cause cell cycle arrest, cell apoptosis, ROS generation, mitochondrial dysfunction, and impaired glucose metabolism, all responsible for cytotoxicity [9]. Fe_3O_4 NPs, however, can also lead to disturbances in mitochondrial activity, increase ROS generation in cells, and cause a significant decrease in ATP levels [10].

An *in vivo* study on rats revealed toxic effects of indium-tin oxide nanoparticles associated with the development of an acute inflammatory reaction after intratracheal administration [11]. Intratracheal instillation of CuO NPs in C57BL/6 mice could induce pulmonary fibrosis by promoting apoptosis of epithelial cells, partly caused by an increase in ROS, in the first place, and exacerbating inflammation in the lung tissue in a dose-dependent manner [12]. The experiment on rats showed that a 10-month chronic inhalation exposure to NiO NPs caused manifestations of systemic toxicity against the background of mild pulmonary pathology associated with a fairly low chronic retention of nanoparticles in the lungs [13]. The similar systemic toxicity following inhalation exposure to lead oxide nanoparticles was reported by Dumkova et al [14].

Intratracheal instillations are no less useful for ranking the toxicity of NPs than inhalation studies [15]. In this regard, it is of interest to compare cytotoxicity of nanoparticles of different chemical composition administered intratracheally.

Material and methods

Synthesis of nanoparticles. The suspensions of nanoparticles (NPs) with a fairly narrow nanoparticle size distribution were prepared at the Ural Multiple Access Center "Modern Nanotechnologies" of the Ural Federal University, Yekaterinburg, Russia, by laser ablation of 1 mm-thick sheet metal targets of 99.99% purity under a <30-mL layer of deionized water. Even though single NPs tend to stick together, the resulting aggregates did not affect the overall pattern of particle diameter distribution in the suspension. The stability of the resulting suspensions was characterized by the zeta potential measured using a Zetasizer Nano ZS analyzer (Malvern, UK). The high stability (up to 42 mV) enabled us to increase the NP concentration in the suspension by partial evaporation of water at 50 °C and obtain the concentration of 0.5 mg/mL without changing the size and chemical identity of the NPs.

All metal oxide nanoparticles had an almost spherical shape. Their shapes and dimensions are shown in Fig. 1.

Experimental animals. We used white outbred female rats with the body weight of about 200 g (±20%) aged ca. 3 months at the beginning of the experiment. The animals were kept in a separate room of the vivarium of our Center; they breathed unfiltered air and were given bottled artesian water and standard balanced feed in accordance with the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and the International Council for Laboratory Animal Science (2012).

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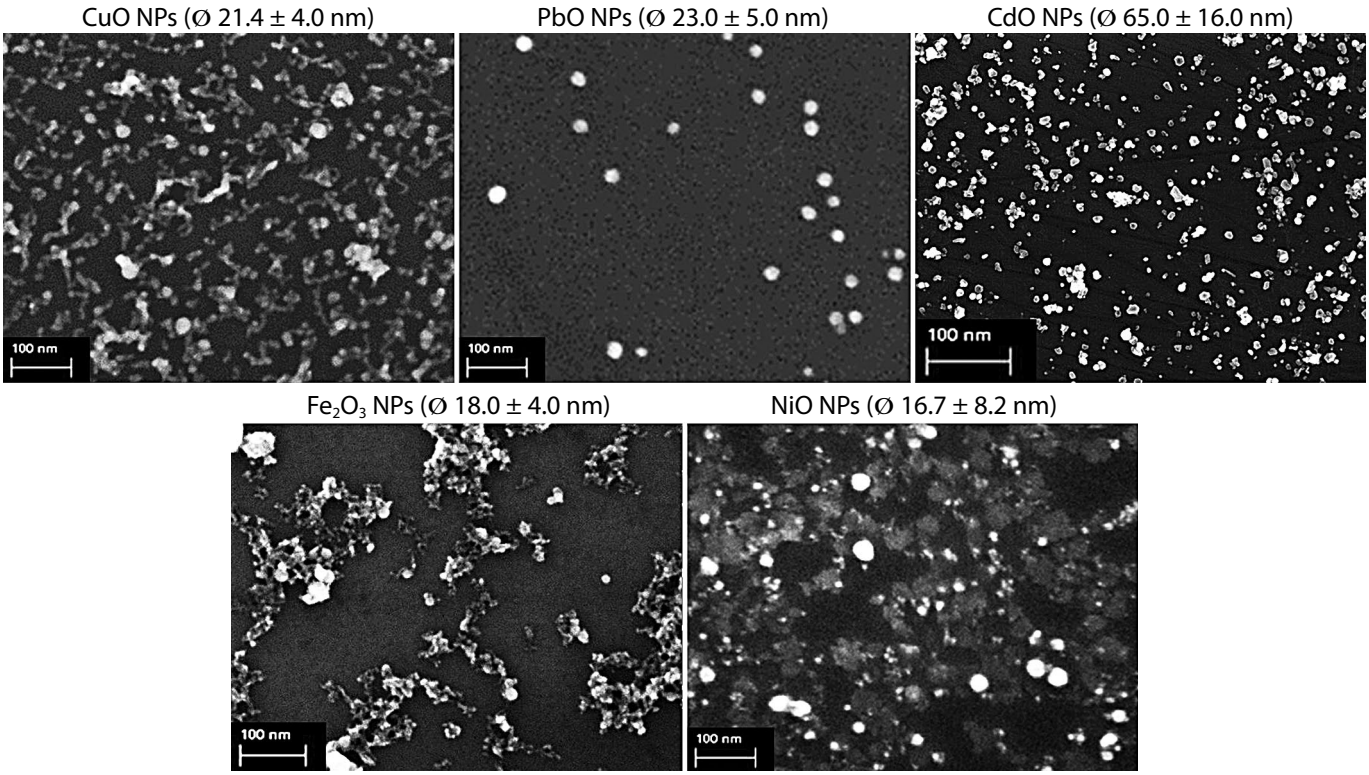

Fig. 1. Visualization of various chemical metal oxide nanoparticles (CuO NPs, PbO NPs, CdO NPs, Fe_2O_3 NPs, NiO NPs) synthesized by laser ablation.
Ø – average diameter ± SE; scanning electron microscopy, magnification 80 000.

The study was approved by the institutional Ethics Committee of the Yekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers (Protocol No. 2 of April 20, 2020).

Cytotoxicity testing. We used ether for rausch-anesthesia of the animals, after which 1 mL of the NP suspension was injected intratracheally at a dose of 0.5 mg per animal; control animals received the same volume of deionized water. The cytotoxic effect was assessed in the bronchoalveolar lavage fluid (BALF) 24 hours after a single intratracheal administration by severity of alveolar phagocytosis. After sampling, the volume of collected BALF was documented and placed in chilled tubes. A BALF aliquot was added to a white blood cell mélanger (a capillary pipette with the ampule dilation) containing 3% acetic acid with methylene blue and the cells were then counted by optical microscopy using the Goryaev chamber grid. The remaining volume of BALF was centrifuged for 4 minutes at 200 g, then the liquid was decanted for the smears to be prepared from the sediment on two glass slides. After drying at the room temperature, the smears were fixed with methyl alcohol, stained with azure-eosin, and examined by immersion microscopy at a 1,000× magnification. Differential counting to determine the percentage of alveolar macrophages, neutrophil leukocytes, lymphocytes, and eosinophils was carried out for at least 100 cells. The conversion to the absolute number of cells was made given the total number of cells in the Goryaev chamber grid.

Statistical analysis. The Student's *t*-test with Bonferronni correction was used to identify statistically significant differences between the groups. The results are presented as the mean ± standard error (SE).

Results

In the series of metal oxide nanoparticles (CuO, PbO, CdO, Fe_2O_3, and NiO NPs) studied at the dose of 0.5 mg/animal, we observed the greatest increase in the total cell count caused by exposure to CuO and CdO NPs (Fig. 2). The smallest cellular influx in the lung was observed following the exposure to Fe_2O_3 NPs.

The highest increase in the neutrophil leukocyte count was observed after the exposure to CuO NPs, and, to a slightly lesser extent, to CdO NPs (Fig. 3). No changes were registered after instillation of Fe_2O_3 NPs. Following the exposure to PbO and NiO NPs, we found no statistically significant changes in the neutrophil leukocyte count but noticed its rising trend.

The alveolar macrophage count increased almost equally following the exposure to CuO, NiO, and CdO NPs, while a statistically significant increase was noted after the exposure to NiO NPs only (Fig. 4).

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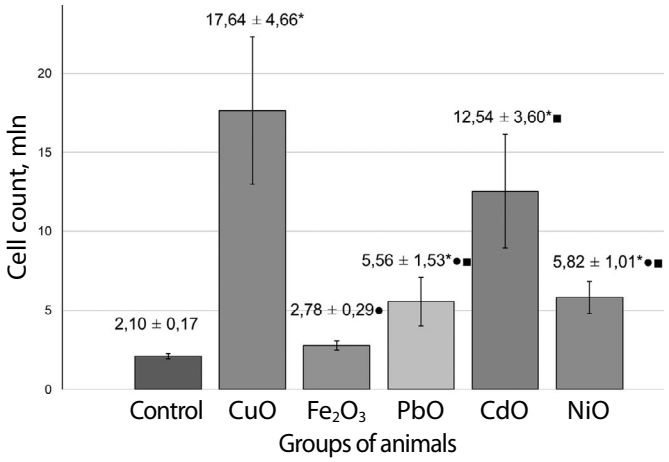

Fig. 2. The total number of cells in the rat BALF after exposure to metal oxide nanoparticles.
Symbols show a statistically significant difference * – from control; • – from the CuO group; ■ – from the Fe_2O_3 group (p<0.05 according to Student's t-test).

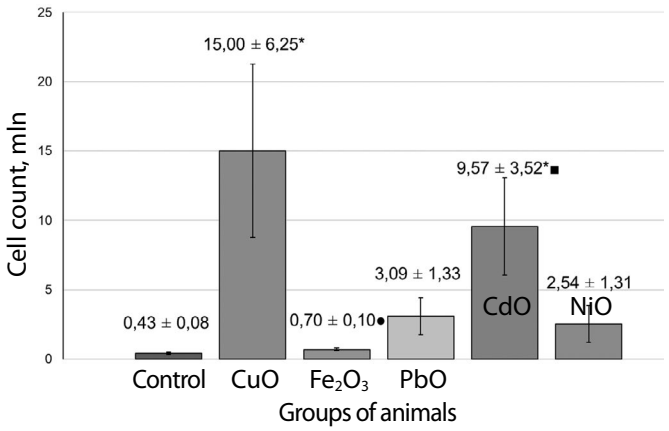

Fig. 3. The number of neutrophilic leukocytes in rat BALF after exposure to metal oxide nanoparticles.
Symbols show a statistically significant difference: * – from control; • – from the CuO group; ■ – from the Fe_2O_3 group (p<0.05 according to Student's t-test).

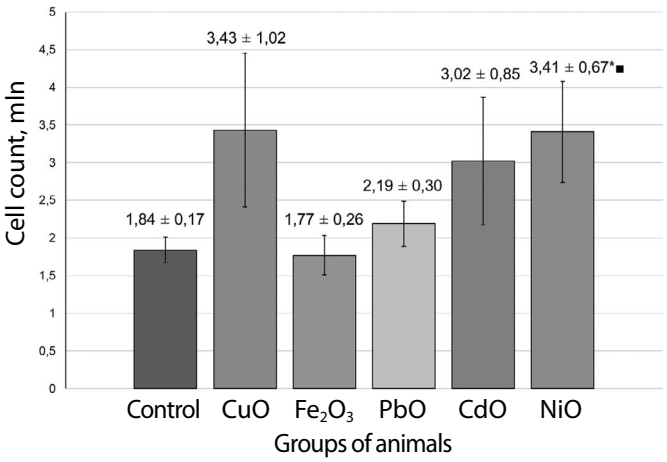

Fig. 4. Number of alveolar macrophages in rat BALF after exposure to metal oxide nanoparticles.
Symbols show a statistically significant difference * – from control; ■ – from the Fe_2O_3 group (p<0.05 according to Student's t-test).

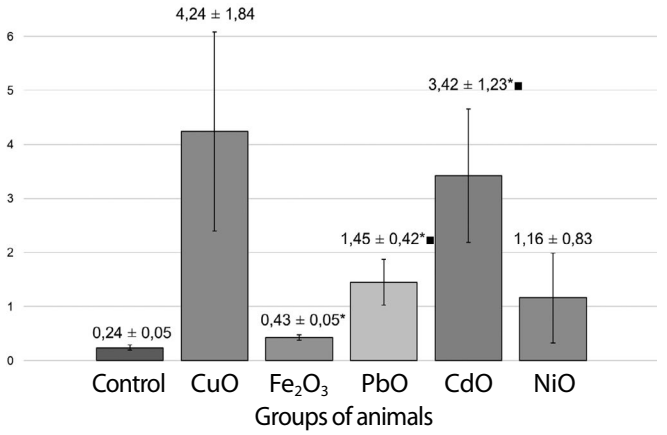

Fig. 5. The ratio of the number of neutrophilic leukocytes to the number of alveolar macrophages in rat BALF after exposure to metal oxide nanoparticles.
Symbols show a statistically significant difference * – from control; ■ – from the Fe_2O_3 group (p<0.05 according to Student's t-test).

The main criterion for comparative assessment of the cytotoxic effect is the neutrophil leukocyte to alveolar macrophage ratio. Its value was found to be the highest after CuO NP exposure (Fig. 5) and somewhat lower after CdO NP exposure, while Fe_2O_3 NP exposure caused minimal changes in this ratio.

Discussion

When foreign particles, including nanoparticles, enter the lower airways, phagocytic activity is mobilized. Alveolar macrophages are the first to phagocytize foreign particles while the products of their destruction stimulate the influx of neutrophil leukocytes. The more cytotoxic the foreign particles are, that is, the more damage they cause to the pulmonary tissue or population of phagocytic cells, the greater is the influx of neutrophil leukocytes [16]. The cellular shift towards neutrophil leukocytes assessed by the neutrophil leukocyte to alveolar macrophage ratio is therefore a key indicator for comparing cytotoxicity [17].

As for the impact of the chemical composition of metal oxide NPs, the largest influx of neutrophil leukocytes was observed following the exposure to CuO NPs (Fig. 2). It is important to note that among the elements examined in the form of oxide nanoparticles, copper is an essential element; yet, CuO NPs exerted an even greater cytotoxic effect than nano-sized particles of non-essential elements, such as lead and cadmium. This might be related to the fact that CuO NPs possess high redox activity. They induce intensive generation of reactive oxygen species by rapid dissolution and an easy change in the oxidation state of dissolved copper; besides, like all nanoparticles, they

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have a large active surface area [18]. Another study also revealed higher cytotoxicity and DNA damage after exposure to CuO NPs compared to TiO_2, ZnO, $CuZnFe_2O_4$, Fe3O4, and Fe_2O_3 nanoparticles, carbon and nanotubes [19]. A recent review [20] provides data on pulmonary effects of copper nanoparticles administered intranasally in mice and rats, including inflammation, ROS generation, development of alveolitis, broncheolitis, and fibrosis, and epithelial damage. Intratracheal exposure to CuO NPs causes acute bronchioloalveolar inflammation with diffuse pulmonary edema, which indicates pulmonary toxicity of CuO NPs [21]. Abundance of free copper ions leads to a cascade of redox reactions causing generation of reactive oxygen species, which destroy the cell both internally and externally. It is noteworthy that ROS generation under the influence of NPs, additionally induced by membrane lipid peroxidation, can lead to a loss of membrane elasticity, which, like abnormally high fluidity, inevitably results in cell death [2]. In addition, we cannot rule out contribution of the mechanisms of entry into cells to the toxic effect of essential elements, including copper. Cuillel et al (2014) in their experiments on hepatocytes of the HepG2 line showed that NPs penetrated inside (most likely by endocytosis), bypassing the cellular mechanisms of protection from excess copper [22].

PbO NPs also exerted a cytotoxic effect (we observed an increase in total cellularity and the neutrophil leukocyte to alveolar macrophage ratio (Figs. 2 and 5)), but to a significantly lesser extent than CuO or CdO NPs, which was most likely related to the lower cytotoxicity of this element. Median lethal doses of lead and its compounds are known to be higher than those of cadmium [23].

NiO NPs caused the largest influx of alveolar macrophages compared to other NPs (Fig. 4). Neutrophilic and lymphocytic inflammatory responses appeared 24 hours after intratracheal administration of NiO NPs in the study by Jeong et al [24]. Other researchers have demonstrated that nickel NPs induce oxidative stress, severe and persistent pulmonary inflammation and fibrosis [25, 26]. In our study, administration of NiO NPs caused the greatest eosinophilic response compared to other NPs. Similar results were reported elsewhere [27].

A number of studies have shown that iron NPs have low pulmonary toxicity both *in vitro* and *in vivo* [28–30]. In our experiment, Fe_2O_3 NPs caused a minimal influx of neutrophil leukocytes and a decrease in the number of alveolar macrophages, both leading to a significant increase in the neutrophil leukocyte to pulmonary macrophage ratio. It is interesting that in this case, the ratio indicates the cytotoxic effect of Fe_2O_3 NPs, although they did not induce an intensive macrophage influx into the lungs. The latter may indirectly indicate that Fe_2O_3 NPs caused less damage to the cells that have absorbed them compared to other metal oxide NPs under study.

Summarizing the above, we can conclude that the chemical composition of nanoparticles has a great impact on their cytotoxic potential. The same dose of different metal oxide nanoparticles can have both a strong (CuO NPs) and weak cytotoxic effect (Fe_2O_3 NPs).

Study limitations. Such physical characteristics of nanoparticles as solubility in water and biological fluids, charge, adsorption capacity, resistance to aggregation, hydrophobicity, adhesion to surfaces, and the ability to generate free radicals have not been studied. Extrapolation of data from rodents to humans should be done with caution, since cytotoxicity has been characterized only on the basis of cytological parameters.

Conclusions

The comparison of cytological parameters of the rat bronchoalveolar lavage fluid following a single intratracheal administration of CuO, PbO, CdO, Fe_2O_3, and NiO nanoparticles showed that their chemical composition determined their cytotoxic properties. This can be explained by the known ability of NPs to dissolve in biological fluids. We established that the same dose of different metal oxide nanoparticles can have a strong (like CuO NPs) or weak cytotoxic effect (F_2O_3 NPs). In the descending order, cytotoxicity of the nanoparticles tested was as follows: CuO NPs > PbO NPs > CdO NPs > NiO NPs > Fe_2O_3 NPs.

Changes in cytological parameters of bronchoalveolar lavage can be used as an effective method of screening for cytotoxic effects of nanoparticles.

Compliance with ethical standards. The study was conducted in compliance with the International Guiding Principles for Biomedical Research Involving Animals by the Council for International Organizations of Medical Sciences and the International Council for Laboratory Animal Science, 2012. Study approval was provided by the institutional Ethics Committee of the Yekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers (Protocol No. 2 of April 20, 2020).

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