EXPOSURE TO NANOPARTICLES OF MAGNETITE FE_3O_4 IN THREE DIFFERENT DOSES AND THEIR INFLUENCE ON SELECTED RESPIRATORY PARAMETERS OF BRONCHOALVEOLAR LAVAGE AFTER INTRAVENOUS INSTILLATION

Marta Hurbánková[1], Katarína Volkovová[2], Silvia Černá[1], Soňa Wimmerová[1], Štefánia Moricová[1]
[1]Faculty of Public Health, Slovak Medical University, Bratislava, Slovak Republic
[2]Medical Faculty, Slovak Medical University, Bratislava, Slovak Republic

SUMMARY

Objectives: Due to nano-dimensions (less than 100 nm), can nanoparticles probably penetrate through various membranes and travel from the bloodstream to other organs in the body. The aim of our study was to find out whether NPs Fe_3O_4 (coated with sodium oleate) injected into the tail vein of laboratory Wistar rats pass through the bloodstream to the respiratory tract (in comparison with a control group); and if so whether increasing doses of NPs Fe_3O_4 have an escalating harmful effect on selected bronchoalveolar lavage (BAL) parameters.

Methods: Wistar rats were intravenously given 3 doses of the suspension of NPs Fe_3O_4 (0.1% LD50 = 0.0364, 1.0% = 0.364 and 10.0% = 3.64 mg/kg animal body weight). Seven days later, we sacrificed the animals under anaesthesia, performed bronchoalveolar lavage (BAL), and isolated the collected cells. Many inflammatory and cytotoxic BAL parameters were examined.

Results: Both inflammatory and cytotoxic BAL parameters affected by Fe_3O_4 suspension were changed compared to control results, but not all were statistically significant. Thus, the NPs Fe_3O_4 passed through the bloodstream to the respiratory tract and affected it. The highest concentration of NPs Fe_3O_4 (10%) had the most influence on BAL parameters (7 of 12 parameters). Only 3 parameters showed a pure dose dependence.

Conclusion: We assume that the adverse effect of Fe_3O_4 NPs in our study is probably not correlated with the dose, but rather with the size of the particles or with their surface area.

Key words: nanoparticles, bronchoalveolar lavage, inflammatory and cytotoxic parameters, dose dependence

Address for correspondence: M. Hurbánková, Slovak Medical University, Faculty of Public Health, Limbová 12, 833 01 Bratislava, Slovak Republic. E-mail: marta.hurbankova@szu.sk

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INTRODUCTION

Nanomaterials and nanotechnologies are currently cutting-edge material innovations that are applied in many sectors, such as health care, pharmacy, cosmetics, food, energy, informatics, electrical engineering, electronics, and a full range of high technologies. Due to their diverse and often new properties of nanoparticles (NPs), nanomaterials have a wide range of uses. At present, nanoparticles as well as nanotechnology represent a public health challenge due to their insufficiently known effects on health.

Nanoparticles are particles that are smaller than 100 nm in one or more dimensions. The European Commission defines nanomaterial as a natural, incidental, or manufactured material containing at least 50% nanoparticles. These can be in an unbound state, or in the form of aggregates or agglomerates. The nanoparticles can be spherical, tubular, irregular, or other shapes (1–4).

In recent years, the use of nanotechnologies has been growing exponentially in many areas of production processes, as well as their use in medicine – as clinical tools for the treatment of diseases, delivery of drugs to the diseased area, and diagnostics.

The massive expansion of nanotechnology, together with the production of new nanoparticles that have not yet been in contact with living organisms, maybe a potential problem for human health. Therefore, it is necessary to investigate the effect of NPs on health after experimental and human exposure.

Iron oxide nanoparticles have been approved for drug production and clinical use, such as magnetic resonance imaging (MRI), and are considered a biocompatible material.

In the field of medicine, many nanoparticles (NPs), including Fe_3O_4 magnetite nanoparticles, are currently considered relatively inert carriers for therapeutic and diagnostic drugs and are expected to be administered intravenously. Because the nanoscale of NPs is so small (less than 100 nm) they can probably penetrate various membranes and get from the blood to other organs in the body (4).

Some NPs may present new potential health risks. In fact, normal human defence mechanisms may not be able to respond

adequately to the newly created particles of unique properties, that the human organism has not met yet. Therefore, the health impact of NPs is becoming a public health issue, leading to an important research requirement (4–7).

"All substances are poisonous – but the dose makes the poison." This famous statement of Paracelsus (1493–1541) is the principle of the basic concept of toxicology. The individual response to chemicals increases in proportion to the dose. The aim of our work also follows from this statement.

The aim of our study was to find out whether NPs of Fe_3O_4 (coated with sodium oleate*) injected into the tail vein of the laboratory Wistar rats pass through the bloodstream to the respiratory tract (in comparison with a control group); and if so whether the increasing dose of Fe_3O_4 NPs has escalating harmful effect on the selected bronchoalveolar lavage (BAL) parameters.

MATERIALS AND METHODS

In this work, we used a suspension of magnetite nanoparticles – Fe_3O_4 in a physiological solution, which contained 10% of rat serum. The size of magnetite NPs was in the range of 14–51 nm. Wistar rats were intravenously given (to a tail vein of an animal) 3 doses of the Fe_3O_4 NPs suspension:

- 0.1% of LD50 = 0.0364 mg/kg animal body weight;
- 1.0% of LD50 = 0.364 mg/kg animal body weight;
- 10.0% z LD50 = 3.64 mg/kg animal body weight.

There were 7–8 animals in each group (control and exposed) Seven days later after i.v. administration of the Fe_3O_4 suspension, we sacrificed the animals under anaesthesia, performed BAL, and isolated the cells from it.

Control animals have been also intravenously given (into the tail vein) saline solution in the same volume as Fe_3O_4 nanoparticles suspension.

We examined:

- number of cells and number of alveolar macrophages (AM) in 1 ml of bronchoalveolar lavage (BAL);
- differential count of BAL cells – AM, polymorphonuclear leukocytes (PMNL), lymphocytes (Ly);
- viability and phagocytic activity of AM;
- proportion of immature and polynuclear cells; and
- cathepsin D (CAT-D), lactate dehydrogenase (LDH), and acid phosphatase (ACP) enzyme activities.

The BAL fluid cells were determined in Bürker chamber and differential count of AM, polymorphonuclears (PMN), lymphocytes and immature cells in the BAL fluid were performed on May-Grünwald Giemsa-Romanowski stained preparations counting 200 cells. Prior to that, the cells were cytocentrifuged on one slide by a Shandon Cytospin centrifuge.

The phagocytic activity of AM was investigated via the modified method by Fornusek et al. (8) using 2-hydroxyethyl methacrylate particles (MSHP) from the Neosys, Prague. Fifty millilitres of particles in PBS (phosphate buffer) was added to 100 ml of BAL fluid, and incubated for 60 min at 37 °C, and shaken at short intervals. Staining was performed by the May-Grünwald Giemsa-Romanowski method. Cells were considered positive (normal phagocyting activity) when they phagocytized three or more particles (8).

To determine the viability of AM, 200 µl of 0.25% erythrosine solution was added to 200 µl aliquots of the cell suspension. The number of viable and non-viable cells was counted using Bürker chamber.

The activities of LDH were measured photometrically with a photometer Eppendorf Geratebau at 366 nm, using LDH-UV (lactate dehydrogenase-ultraviolet) kits.

The AcP measurements were carried out photometrically with a Specol Zeis 1 (Jena, Germany) at 420 nm using acid phosphatase kits.

For the determination of cathepsin D levels, the cell suspension was diluted with Triton X 100 in PBS (final concentration of Triton: 0.1%), mixed, three times frozen and thawed and centrifuged at 14000 x g for 20 min. The activity of cathepsin D was measured spectrophotometrically.

A detailed description of the mentioned methodology can be found in papers by Hurbankova et al. (9–11) and Dziedzic et al. (12).

We used IBM SPSS 19 software for statistical evaluation, and characterized the observed variable by arithmetic mean, standard deviation, median, minimum, and maximum value. Due to the small number of measured values in all 4 groups, we used non-parametric tests.

In the first step we used the Kruskal-Wallis test, in the second step, we used the Mann-Whitney test to compare the exposed groups to the control group. In figures, we used medians and expressed the degree of variability by 95% confidence intervals for the mean. For statistical significance, we chose the level $\alpha = 0.05$.

RESULTS

The results of the differential count of BAL cells (percentage share of AM, Ly, PMN) compared to the control results are statistically significantly reduced in percentage share of AM and statistically significantly increased in percentage share of PMNL and Ly.

All three concentrations (0.1, 1.0, 10.0%) of Fe_3O_4 nanoparticle suspensions compared to the control are statistically significantly reduced in phagocytic activity and viability of AM and significantly increased in CAT-D enzyme levels, and after 1% and 10% concentrations of Fe_3O_4 nanoparticle suspension in ACP enzyme levels.

Magnetite – nanoparticles Fe_3O_4 i.v. injected into the tail vein pass into the respiratory tract affecting some BAL parameters. Of the 12 parameters examined by us, the highest dose of 10% Fe_3O_4 suspension affected 10 parameters, 7 statistically significantly.

Statistically significantly affected parameters by dose 10% Fe_3O_4 were: viability of AM; percentage share of AM, PMN, Ly – from differential count cells; percentage of phagocytic activity of AM; ACP enzyme level; and CAT-D enzyme level.

Statistically non-significantly affected parameters by dose 10% Fe_3O_4 were: number of AM in 1 ml BAL; number of cells in 1 ml BAL; and percentage of immature AM.

*Nanoparticles are coated with sodium oleate for the reason that their lower reactivity than uncoated particles is assumed.

Table 1. *Selected inflammatory parameters comparing three concentrations of Fe_3O_4 nanoparticles to the control group*

BAL parameters	0.1% Fe_3O_4	1% Fe_3O_4	10% Fe_3O_4
	vs. control		
Number of cells/ml BAL	NS ↑	NS ↑	NS ↑
	0.116	0.116	0.14
Number of AM/ml BAL	NS ↑	NS	NS ↑
	0.725	0.725	0.316
Differential count of BAL cells – AM (%)	S ↓	S ↓	S ↓
	0.001	**0.001**	**0.001**
Differential count of BAL cells – Ly (%)	**S ↑**	**S ↑**	**S ↑**
	0.001	**0.001**	**0.001**
Differential count of BAL cells – PMN (%)	**S ↑**	**S ↑**	**S ↑**
	0.015	**0.001**	**0.001**
Proportion of polynuclear cells – AM (%)	NS	NS	NS
	0.336	0.724	0.396
Proportion of immature – AM (%)	NS ↑	NS ↑	NS ↑
	0.079	0.198	0.081

NS – statistically non-significant; S – statistically significant; ↓ – decrease of values; ↑ – increase of values; AM – alveolar macrophages; Ly – lymphocytes; PMN – polymorphonuclear leukocytes; BAL – bronchoalveolar lavage

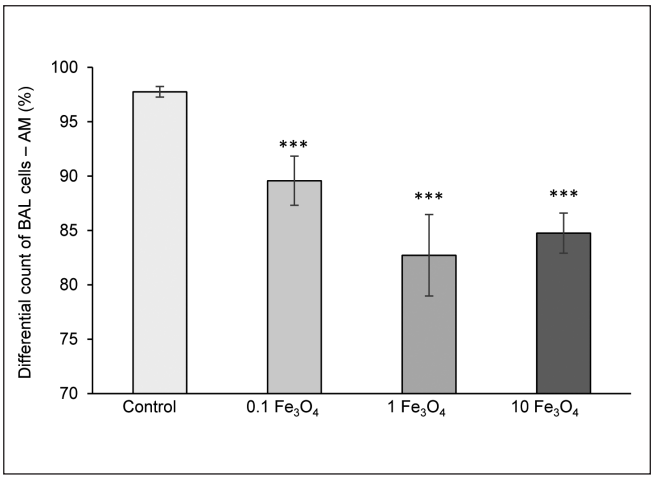

Fig. 1. *Percentage of AM from differential count of BAL cells.*

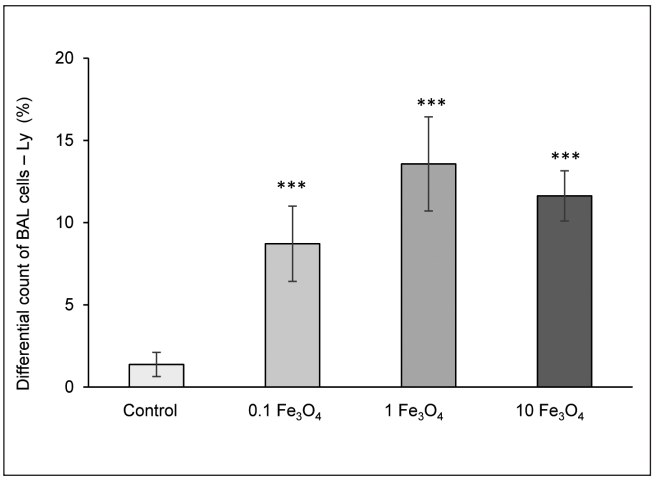

Fig. 3. *Percentage of Ly from differential count of BAL.*

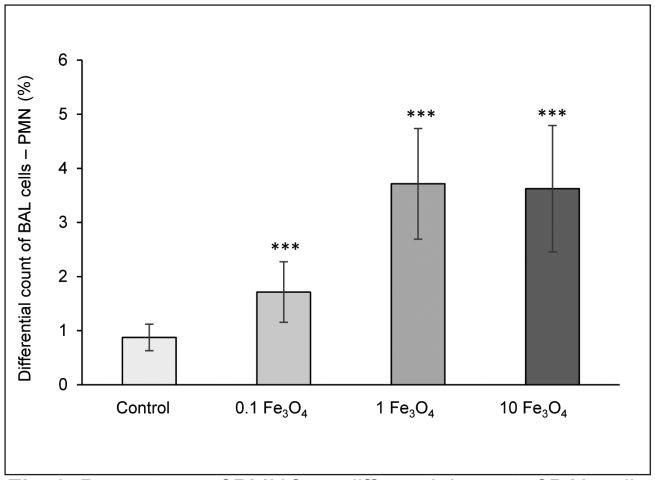

Fig. 2. *Percentage of PMN from differential count of BAL cells.*

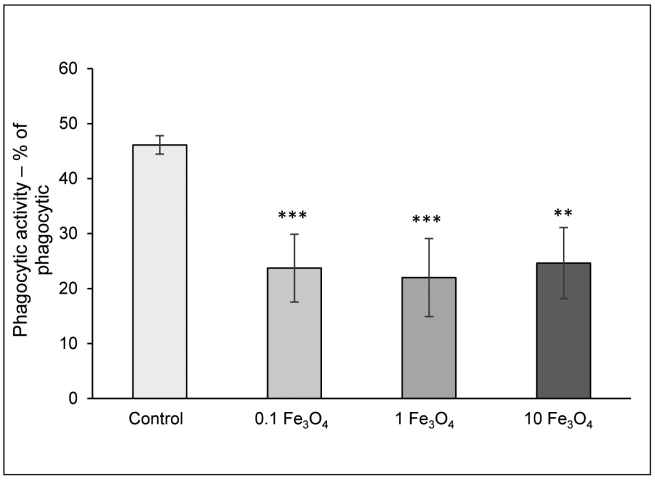

Fig .4. *Percentage of phagocytizing AM.*

Table 2. *Selected cytotoxic parameters comparing three concentrations of Fe_3O_4 nanoparticles to the control group*

BAL parameters	0.1% Fe_3O_4	1% Fe_3O_4	10% Fe_3O_4
	vs. control		
Phagocytic activity – % of phagocytising AM	**S ↓**	**S ↓**	**S ↓**
	0.001	**0.001**	**0.002**
Viability – % of living cells	**S ↓**	**S ↓**	**S ↓**
	0.001	**0.001**	**0.001**
LDH in BAL µkat/g of proteins^{-1}	NS ↑	NS	NS
	0.916	0.487	0.462
ACP in BAL nkat/g of proteins^{-1}	NS ↑	**S ↑**	**S ↑**
	0.6	**0.001**	**0.001**
CATD in BAL Utyr.mg of proteins^{-1}	**S ↑**	**S ↑**	**S ↑**
	0.012	**0.001**	**0.001**

NS – statistically non-significant; S – statistically significant; ↓ – decrease of values; ↑ – increase of values; AM – alveolar macrophages; LDH – lactate dehydrogenase; AcP – acid phosphatase; CAT-D – cathepsin D; BAL – bronchoalveolar lavage

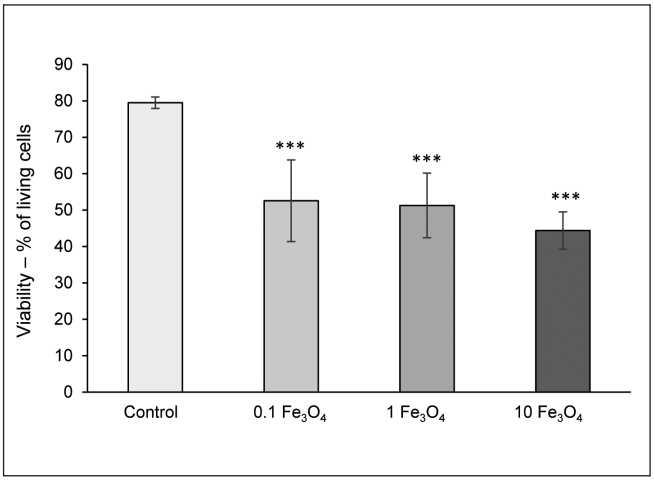

Fig. 5. *Viability – percentage of living cells (AM).*

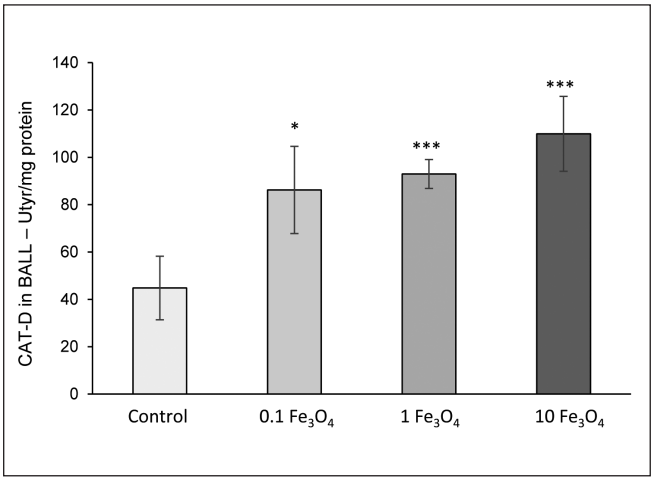

Fig. 7. *Cathepsin D (CAT-D) enzyme level in BAL.*

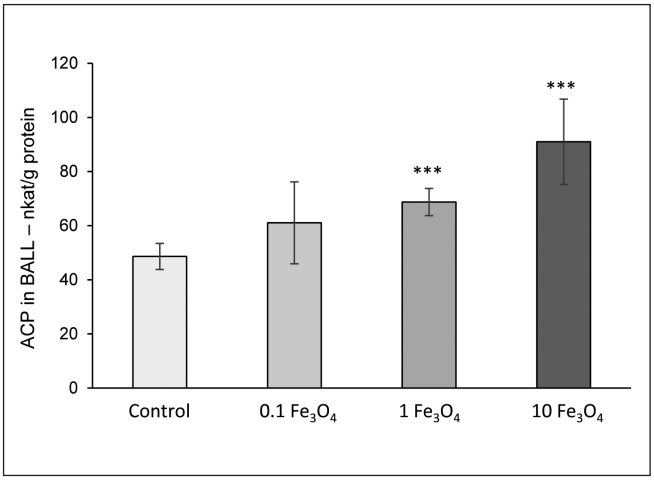

Fig. 6. *Acid phosphatase (ACP) enzyme level in BAL.*

Statistically significant – clear dose dependence – after 10% Fe_3O_4 was found only in the viability of AM, ACP enzyme level, and CAT-D enzyme level.

DISCUSSION

While nanotechnologies are advancing rapidly in development, nano-safety tends to lag behind, as general knowledge of studies of the mechanisms of effect of nanoparticle-cell interaction is still insufficient. For this reason, many scientific teams are dealing with this topic – especially the impact of nanoparticles on health.

Magnetite nanoparticles are considered relatively biocompatible – and their properties are being investigated because they should be used – for i.v. administration in diagnostics and as drug carriers in therapy.

In our study, we wanted to find out whether NPs Fe_3O_4 injected into the tail vein of the laboratory Wistar rats pass through the bloodstream to the respiratory tract (comparison with a control group), and if so, whether the increasing dose of NPs Fe_3O_4 has escalating harmful effect (more toxic effects) on the selected bronchoalveolar lavage (BAL) parameters.

The differential count of cells (in our case BAL cells) is an important indicator indicating the state of the organism after exposure to nanoparticles. We recorded a statistically increased percentage of polymorphonuclear leukocytes compared to the

control results after i.v. administration of NPs Fe_3O_4 suspension – but there was no dose dependence (Fig. 2).

Similar results to PMNL we also observed in the case of a statistically increased percentage of lymphocytes from the differential count of BAL cells after exposure to magnetite – Fe_3O_4 nanoparticles – but we did not detect a dose dependence (Fig. 3).

The third type of cells from the differential count of BAL are alveolar macrophages, whose percentage was statistically significantly reduced in our study, after being affected by Fe_3O_4nanoparticles, compared to the control results, but there was no dose dependence (Fig. 1).

A decrease in the number of macrophages, cell viability, and phagocytic activity may result in a weakened clearance of inhaled nanoparticles, which may subsequently lead to an increase in the effective dose of a potentially harmful substance (Fig. 1, 4, and 5).

The main role of AM is the removal of foreign particles from the lungs, which happens by triggering an inflammatory reaction and inducing defence and immunomodulating mechanisms. Alveolar macrophages act as defence cells in the process of exposure to foreign substances, but under certain conditions, they can cause unwanted effects (10, 12, 13).

In addition to the ability of phagocytosis, AMs can play an important role by producing inflammatory mediators, various cytokines, growth factors, and reactive oxygen intermediates.

The mentioned pathomechanisms were investigated by many researchers on various industrial dusts (14–16).

Currently, there is a lack of elucidation of similar defence mechanisms through AMs after exposure to nano-sized particles.

If nanoparticles are not effectively eliminated by phagocytosis, they can gradually reach any tissue of the organ through the circulatory and lymphatic systems, where they can cause irreversible damage or DNA mutations (17).

By the term cell viability, we mean that cells are alive (active, living, able to reproduce) – they are viable. If the cells are not proliferating or cannot reproduce, their viability is suppressed – the cells are dead. The decreased viability of alveolar macrophages after exposure to magnetite nanoparticles in our case may be due to their functional overload (Fig. 5).

According to Chlap et al. the increase in immature forms of AM can also occur as a result of a pathological reaction after exposure to inorganic particles, intense cigarette smoking, as well as in certain interstitial lung diseases (sarcoidosis, silicosis, asbestosis) (18).

In our case, exposure to Fe_3O_4 nanoparticles did not significantly affect the immature form of AM – compared to the control. Compared to the control, we noted an increase, but it was not statistically significant. It was probably not necessary to strengthen the defence – an increase in immature forms of AM within 7 days of i.v. administration of a suspension of magnetite nanoparticles.

Multinucleated cells are also an indicator of inflammation, but 7 days after i.v. administration of NP suspension may not yet manifest, rather, their increase in chronic inflammation is assumed.

Binucleated and multinucleated cells are a reflection of accelerated mitotic cell division. They normally occur in BAL around 1% (19). In our study, we did not detect a significant increase in multinucleated cells.

Damage of cells by the surface of nanoparticles can lead to functional and structural changes, including impaired enzyme function.

In a study focusing on the cytotoxicity, oxidative stress and genotoxicity of nanoparticles of iron oxides (Fe_3O_4) on two human cell lines, skin epithelial cells A431 and lung epithelial cells A549, Ahamed et al. found dose-dependent cytotoxicity in both cell types as demonstrated by decreased cell viability and increased enzyme levels (20). We observed similar results in our study (Fig. 5, 6, 7).

The expression of genes is involved in the antioxidant response, manifested by an increase in the levels of catalase (CAT-D), glutathione peroxidase, superoxide dismutase, glutathione S-transferase and thioredoxin reductase, among other mechanisms, also by destabilization of the lysosomal membrane (21).

Nanoparticles can interact with proteins and enzymes in mammalian cells and can interfere with antioxidant defence mechanisms that lead to the production of reactive oxygen species causing an inflammatory response, perturbation, and damage to mitochondria, causing apoptosis or necrosis (22, 23).

Regarding the dose dependence in our study, not all BAL parameters examined by us showed it, but all parameters after i.v. injection of Fe_3O_4 nanoparticles were changed compared to the control results. The highest concentration – 10% Fe_3O_4 proved to be the most influencing – (7 of 12 parameters). For this reason, we partially agree with Duffin et al. (24) and with other authors (6, 25), that nanoparticles do not correlate with mass dose, but with particle size, i.e., the smaller the NPs, the larger the surface area.

Some NPs may represent new potential health risks. In fact, the normal human defence mechanisms may be unable to respond adequately to the newly created particles with unique features, because the human organism has not yet encountered them. Therefore, the impact of NPs on health has become a public health issue, resulting in an important need for further research.

CONCLUSION

Magnetite – nanoparticles Fe_3O_4 (coated with sodium oleate) i.v. injected into the tail vein pass into the respiratory tract and affect some BAL parameters (compared to the control). Of the 12 parameters examined by us, the highest dose of 10% Fe_3O_4 suspension affected ten parameters, seven of them were statistically significant, but only three parameters showed real dose dependence.

We confirmed dose dependence only partially. Questions remain as to why some of the BAL parameters in our study are dose-dependent, while others are not. Therefore, we assume that the adverse effect of NPs probably does not correlate with the dose, but more with the size of the particles – with their surface area, as published by some authors.

Smaller NPs have a larger surface area and therefore may be more reactive. We believe that the results of our study also contributed in part to the expected knowledge.

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Conflicts of Interest

None declared

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