УДК 612.014.46:[546.57+546.56:66-911.38,002.2]

# ASSESSMENT OF $Fe_2O_3$ NANOPARTICLES IMPACT ON FUNCTIONAL ACTIVITY OF RATS' PERITONEAL MACROPHAGES IN EXPERIMENTS *IN VITRO* AND *IN VIVO*

**Dmytrukha N. M., Lugovskoy S. P., Lahutina O. S.**

SI «Institute for Occupational Health of National Academy of Medical Sciences of Ukraine», Kyiv

*Introduction*. Today much attention is paid in the world to studying safety of nanomaterials (NM), which are synthesized and used in different spheres of human activity. Among metallic nanoparticles (NPs), iron and iron oxides are widely used in medicine and pharmacy. An important step in learning, how NPs affect living organisms, is studying their toxicity and biological activity. Macrophages are of a particular interest in this respect, which are available in various tissues and which are key cells in forming an immune response.

*Purpose of the study*. To assess $Fe_2O_3$ NPs influence on viability and functional activity of peritoneal macrophages in rats in experiments *in vitro* and *in vivo*.

*Materials and methods*. $Fe_2O_3$ NPs of 19 nm and 75 nm were obtained by chemical methods. The viability of peritoneal macrophages under the influence of $Fe_2O_3$ NPs of 19 nm and 75 nm was defined by trypan blue and MTT test; the phagocytic activity was assessed by absorption of latex particles and cytochemically; bactericidity – by NBT-test.

*Results*. The studies showed that incubation of macrophages with NPs *in vitro* caused death of 30 % cells, whereas entering NPs into the body *in vivo* had no effect on their viability. NPs activated macrophage phagocytosis, formation of large phagocytic vacuoles, fagosom, lysosomes and fagolizosom as well as production of reactive oxygen forms. NPs activity depends on their size and conditions of experiments.

*Conclusion*. The contact of $Fe_2O_3$ NPs with macrophages activates phagocytosis and formation of reactive oxygen forms, which are aimed at their elimination and neutralization. However, excessive or chronic stimulation of an oxidative stress can lead to cell death, the inflammatory process. Stimulation of the biological activity of macrophages by $Fe_2O_3$ NPs makes it possible to suppose their possible toxic effect on the immune system and requires further immunological studies.

**Key words: nanoparticles of iron oxide, peritoneal macrophages, phagocytosis**

## Introduction

Today a significant attention is paid in the world to development of nanotechnology, directed at obtaining and using materials, formed by nanoparticles (NPs) with the size up to 100 nm. The nanotechnology and nanomaterials (NMs) are used in industry, agriculture, medicine and pharmacy [1, 2].

Among NMs of medical destination iron NPs take the first place. Due to unique magnetic properties iron oxide NPs are used in medicine for contrast enhancement in magnetic resonance imaging; drug delivery; treatment of malignant tumors by magnetic fluid hyperthermia; cell separation, tissue recovery etc. [3]. In addition to ingineered nanomaterials, iron nanoparticles can be a part of a condensation aerosol, produced in the process of iron and steel smelting, welding, cutting and grinding metal surfaces [4].

Taking into account that iron oxide nanoparticles have specific physical and chemical properties and high biological activity, problems of human contacts with them, their entering the body, require studying determination of their toxicity and safety. An important step in learning, how NPs affect a living organism, is to study their effects on cell components of the immune system, which are the first to respond to reaction to foreign agents [5].

Macrophages are of a particular interest in this respect, which are available in various tissues and are the key cells in forming an immune response, and which are involved in iron metabolism in the body. The main function of macrophages is destruction of foreign agents and their own mutated cells by phagocytosis, activation of metabolic pathways in reduction of oxygen forming ($\cdot O_2$, $O_2$, $\cdot OH$, $H_2O_2$). The synthesis of these products is a so-called «oxygen burst», which plays a key role in antimicrobial and anticancer function of monocytes and macrophages [6].

Today, an important subject for discussion is a problem of nanoparticle clearence. On the example of silver NPs it was shown that the process of their removing could occur by phagocytosis/macropinocytosis, endocytosis and passive penetration into

cells [7,8]. In paper [9] it was investigated that $Fe_2O_3$ NPs of 10 nm and 50 nm stimulated the phagocytosis process in alveolar macrophages more actively as compared with nanoparticles of 1 micron size.

The purpose of this research was to assess the effect of $Fe_2O_3$ NPs, sized 19nm and 75 nm, on the vitality and functional activity of peritoneal macrophages in rats *in vitro* and *in vivo* experiments.

## Materials and methods

The nanoparticles of $Fe_2O_3$ were obtained chemically in the Department of Photochemistry of L. V. Pisarzhevsky Institute of Physical Chemistry of NAS of Ukraine. Two colloidal solutions have been synthesized: $Fe_2O_3$ NPs with the average size of 19 nm (in 0,5 % gelatin) and 75 nm (in 0,1 % gelatin). The iron concentration in both solutions was similar ($1 \cdot 10^{-3}$ mol/l = 0,1597 g/l).

The effect of $Fe_2O_3$ NPs on the viability and functional activity of rats' peritoneal macrophages was evaluated after 24 hours of cell incubation with NPs *in vitro*. In the experiment *in vitro* the activity of macrophages was determined in 24 hours after a single intraperitoneal administration of NPs to rats.

Wistar rats (160–200 g) were obtained in the vivarium of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine (Kyiv, Ukraine). The animals were kept in standard conditions with food and water ad libitum. All manipulations with animals were made in accordance with the European Convention for Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and the Law of Ukraine «On Protection of Animals from Cruelty» [10].

The macrophages were taken from peritoneal exudates of the control and exposed rats after administration of 15 ml Hanks solution heated to 37 °C. The cells were washed twice by Hanks solution and centrifugated at 1500 r/m for 10 min. The peritoneal macrophages were separated from other cells due to their adhesion to the plastic surface. *In vitro* macrophages were cultured in RPMI 1640, containing 10 % FBS and streptomycin/penicillin (100 IU/mL each) at 37 °C in a humidified incubator with 5 % $CO_2$. The $Fe_2O_3$ nanoparticles were added to cells (iron concentration was 0,1597 g/l) and incubated for 24 hours in the same conditions. The viability of cells was defined by trypan blue and MTT-test (Tetrazolium salts assay), which measured the viability of a cell population relative to control, untreated cells. The cells were treated with nanoparticulates for 24 hours before addition of soluble yellow tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 2-4 hr at 37 °C. During this process, viable cells with active respiratory mitochondrial activity bioreduce MTT into an insoluble purple formazan product, via mitochondrial succinic dehydrogenases, which is subsequently solubilized by dimethyl sulfoxide (DMSO) or detergent, and quantitated on a visible light spectrophotometer. The optic density was measured on the Sunrise Tekan (Austria) at 450 nm [11]. The functional (phagocytic and bactericidal) activity of macrophages was defined by absorption of latex particles and nitroblue tetrazolium (NBT-tes) [12].

The cytochemical study of peritoneal macrophages included determination of cytochemical activity of lysosomal enzymes (acid phosphatase (AP), and Akridin-orange (AO), stained by a classic techniques of light and electronic microscopy [13, 14]. Structural changes in cells were defined by the Transmission electron microscopy (TEM) and the Scanning electron microscopy (SEM) «Tescan MIRA 3») with the system of the local element power-dispersive microanalysis (Oxford Advanced Aztec Energy (IE350)/ X-max 80).

## Results and discussion

The results showed that incubation of peritoneal macrophages *in vitro* with NPs $Fe_2O_3$ 19 nm after 24 hours caused the decrease in the number of viable cells (in the test with trypan blue – up to 65,55 %, and in MTT-test – up to 67,50 %), and with $Fe_2O_3$ 75 nm (to 86,44 % and 85,84 %, respectively) (Fig.1a). *In vivo*, after 24 hours of intraperitoneal administration of $Fe_2O_3$ NPs, the index of viability of peritoneal macrophages did not differ from those of the control (intact) animals (fig. 1b). This can be explained by the interaction of NPs with proteins or other biomolecules, which reduces their impact on cells.

Nanoparticles of $Fe_2O_3$ during 24 hours of *in vitro* incubation with peritoneal macrophages caused activation of their phagocytic activity and bacterial ability to form reactive types of oxygen as compared with the control cells. Moreover, smaller $Fe_2O_3$ NPs (19 nm)

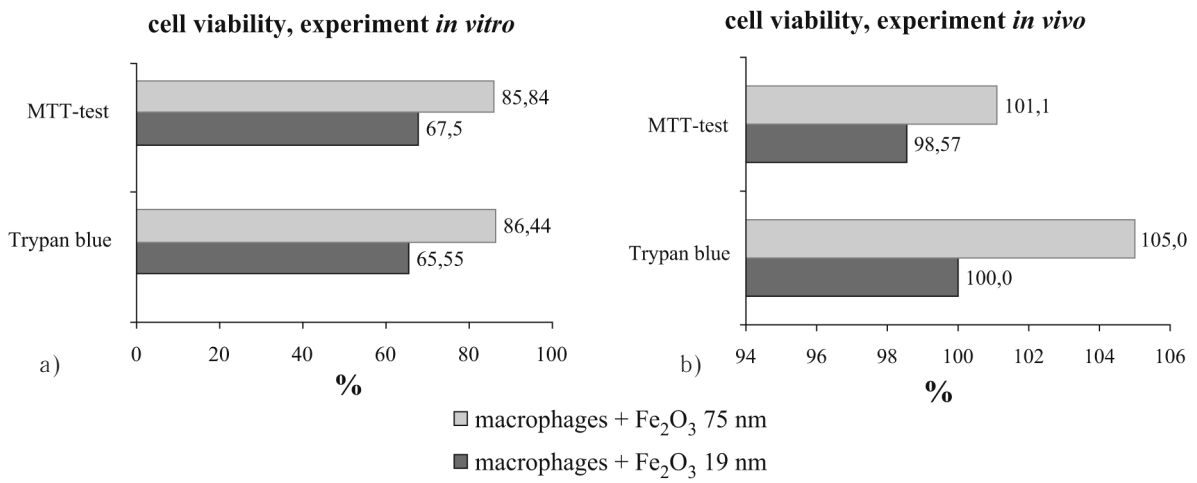


*Fig. 1. Cytotoxic effect of $Fe_2O_3$ NPs of 19 nm and 75 nm in rats on peritoneal macrophages (Tripan blue and MTT- test): a) after 24 hours in vitro incubation, b) after 24 hours of intraperitoneal administration to rats*

showed more stimulating effects than large NPs (75 nm) (table 1).

The phagocytic activity of peritoneal macrophages in the exposed rats after intraperitoneal administration of iron oxide NPs did not change significantly as compared to the control animals. However, NPs of both size (19 nm and 75 nm) stimulated development of reactive oxygen forms in macrophages both in spontaneous and stimulated NBT-test. It should be noted that as it was *in vitro* experiments small $Fe_2O_3$ NPs were more active than NPs of a large size (table 2).

The data suggest that a contact macrophages with $Fe_2O_3$ nanoparticles *in vitro* as well as *in vivo* leads to activation of phagocytosis and production of reactive oxygen forms, directed at elimination and neutralization of NPs as foreign agents.

Cytochemical studies, using acridine orange, showed the increased formation of large phagocytic vacuoles, phagosomes, lyzosomes and phagolyzosomes in the cytoplasm of rats' peritoneal macrophages (fig. 2) after 24 hours effect of $Fe_2O_3$ of NPs of both size (fig. 2.a). The activity of acid phosphatase in cells' lyzosomes and phagolizosomes was increased (fig. 2. b, c).

**Table 1**

**The functional activity of peritoneal macrophages in control rats after 24 hour of *in vitro* incubation with $Fe_2O_3$ NPs**

| Rats cells | Indices, M±m | | |
|---|---|---|---|
| | NBT- spontaneous, % | NBT-stimulated, % | Phagocytic activity, % |
| Macrophages + 0,5 % gelatin | 8,6 ± 0,3 | 12,2 ± 0,8 | 17,6 ± 0,4 |
| Macrophages +$Fe_2O_3$ NPs,19 nm | 18,2 ± 0,9* | 21,2 ± 0,2* | 30,3 ± 0,2* |
| Macrophages +$Fe_2O_3$ NPs,75 nm | 13,1 ± 0,3* | 18,8 ± 0,7* | 20,4 ± 0,1* |

**Indicate significant differences as compared with the control (P < 0,05).*

**Table 2**

**The functional activity of rats' peritoneal macrophages after 24 hours of intraperitoneal administration of $Fe_2O_3$ NPs**

| Group of animals, n = 6 | Indices, M±m | | |
|---|---|---|---|
| | NBT- spontaneous, % | NBT-stimulated, % | Phagocytic activity, % |
| Control | 16,8 ± 0,1 | 24,3 ± 0,9 | 36,0 ± 1,7 |
| $Fe_2O_3$ NPs,19 nm | 21,0 ± 0,1* | 32,2 ± 0,8* | 35,7 ± 1,8 |
| $Fe_2O_3$ NPs, 75 nm | 20,6 ± 0,9* | 28,2 ± 0,7* | 34,7 ± 0,4 |

**Indicate significant differences as compared with the control (P < 0,05).*

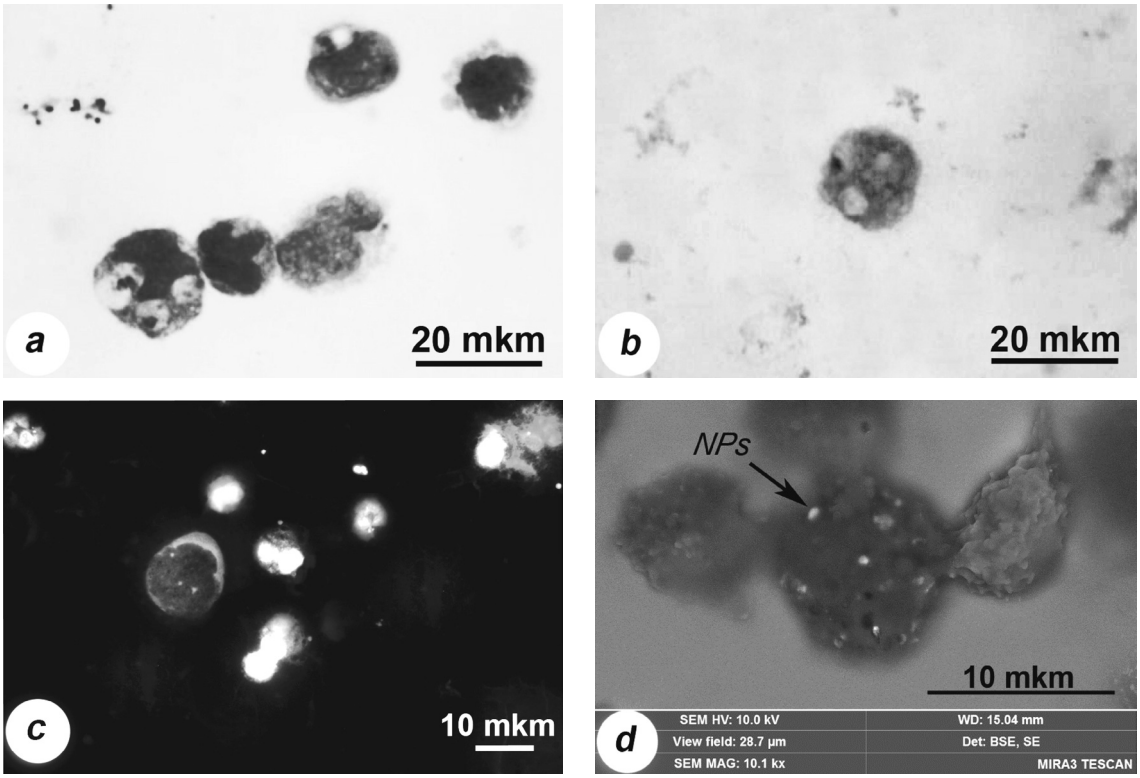


*Fig. 2. The morphological features of peritoneal macrophages in rats after 24 hours in vivo exposition of $Fe_2O_3$ NPs: a) azur-eosin staining; b) cytochemical reaction of naphthol AS-BS phosphate (intracellular acid phosphatase activity); c) AO- positive intracellular inclusions in cytoplasm peritoneal macrophages. Akridin-orange staining; d) scanning electron microscopy (SEM) micrographs of peritoneal macrophages. Detection of $Fe_2O_3$ NPs (of secondary electrons) the method of local element power-dispersive microanalysis (→).*

These data can point to activation of $Fe_2O_3$ NPs phagocytic function of macrophages.

The results of the electron microscopy showed that main pathway of $Fe_2O_3$ NPs (19 nm and 75 nm) in the macrophages' cytoplasm is phagocytosis. This is confirmed by a large number of phagosomes and phagolizosomes found on the surface of cells, containing amorphous and electron dense materials and numerous small crystalline dense inclusions. The latter, according to the local element power-dispersive microanalysis, were presented as iron NPs (fig. 2. d).

## Conclusion

The obtained data make it possible to conclude that one of the pathways of penetration of iron oxide NPs into macrophages is phagocytosis, directed at neutralization and elimination of foreign substances. Activation of phagocytosis was confirmed by histochemical markers: formation of large phagocytic vacuoles, phagosoms, phagolizosoms, high activity of acid phosphatase and synthesis of reactive oxygen forms. NPs in experiments *in vivo* were less active, probably due to their interaction with proteins and other biomolecules. The activity of NPs depended on their size. $Fe_2O_3$ NPs of 19 nm, in particular, have a greater impact on the functional activity of macrophages, including the synthesis of free radicals, which can cause cell death. Our data corresponds to the results of other authors, which have shown that a free radical formation can be triggered by NPs [15]. The resulting overproduction or chronic production of "reactive oxygen forms" can damage DNA, proteins and lipids, which then affect cellular processes [6]. The oxidative stress reaction can be a signal of a cell damage and activation of an inflammatory reaction [16].

Thus, high biological activity of $Fe_2O_3$ NPs in interaction with peritoneal macrophages suppose their possible toxic effect, calling for further extended immunological studies.

## References

1. Yokel, R. A., Macphail, R. C. 2011. "Engineered nanomaterials: exposures, hazards, and risk prevention", J. Occup Med Toxicol., no. 6, pp. 7–13.

2. Buzea, C., Blandino, I. I. P., Robbie, K. 2001. "Nanomaterials and nanoparticles: sources and toxicity", Biointerphases, v. 2, no. 4, pp. 17–71.

3. Faraji, M., Yamini, Y., Rezaee, M. 2010. "Magnetic nanoparticles: synthesis, stabilization, functionalization, characterization, and applications", J. Iran. Chem. Soc., v. 7, no. 1, pp. 1–37.

4. Katsnelson, B. A., Privalova, L. I., Sutunkova, M. P. et al. 2012,"Uptake of some metallic nanoparticles by and their impact on pulmonary macrophages *in vivo* as viewed by optical, atomic force and transmission electron microscopy", J. Nanomed. & Nanotech., v. 3, no. 1, pp. 1–9.

5. Dobrovolskaia, M. 2007, "Immunological properties of engineered nanomaterials", Nat. Nanotechnol., no. 2, pp. 469–478.

6. Park, J. B. Phagocytosis induced superoxide formation and apoptosis in macrophages, 2003, Exp. Mol. Med., v. 35, no. 5, pp. 325–335.

7. Carlson, C., Hussain, S. M., Schrand, A. M. [et al.]. 2008, "Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species", J. Phys. Chem. B., v. 112, no. 43, pp. 13608–13619.

8. Park, J., Lim, D. H., Lim, H. J. [et al.]. 2011, "Size dependent macrophage responses and toxicological effects of Ag nanoparticles", Chem.Commun. (Camb), no. 47, pp. 4382–4384.

9. Metz, S., Bonaterra, G., Rudelius, M. [et al.]. 2004, "Capacity of human monocytes to phagocytose approved iron oxide MR contrast agents *in vitro*", Eur. Radio., v. 14, no. 10, pp. 1851–1858.

10. European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. 1986. Strasbourg, http://conventions.coe.int/treaty/en/treaties/html/123.htm

11. Basic cell culture protocols. Third edition. 2005. Ed. by Cheryl D. Helgason, Cindy L. Miller / Human Press Inc., Totowa New Jersey, v. 290, 371 p.

12. Freeman R., King B., 1972. "Technique for the performance of the nitro-blue tetrazolium (NBT) test", J. of Clinical Pathology, v. 25, no. 10, pp. 912–914.

13. Lojda, Z., Gossrau, R., Schiebler, T.H. 1982, "Enzyme Histochemistry. A Laboratory manual", Moskva : Mir, 259 p.

14. De Brauwer, E., Jacobs, J., Nieman, F. [et al.]. 1999,"Test Characteristics of Acridine Orange, Gram, and May-Grünwald-Giemsa Stains for Enumeration of Intracellular Organisms in Bronchoalveolar Lavage Fluid", J. Clinical microbiology, v. 37, no. 2, pp. 427–429.

15. Sioutas, Li. N., Cho, C., Schmitz, A. [et al.]. 2003, "Ultrafine particulate polutants induced oxidative stress and mitochondrial damage", Environ. Health Perspect., v. 111, no. 4, pp. 455–460.

16. Brown, D. M., Donaldson, K., Borm, P. J. [et al.]. 2004, "Calcium and ROS-mediated activation of transcription factors and TNF-alpha cytokine gene exptession in macrophages exposed to ultrafine particles", Am. J. Physiol. Lung Cell Mol. Physiol., v. 286, no. 24, pp. 344–353.


**Дмитруха Н. М., Луговський С. П., Лагутіна О. С.**

## ОЦІНКА ВПЛИВУ НАНОЧАСТИНОК $Fe_2O_3$ НА ФУНКЦІОНАЛЬНУ АКТИВНІСТЬ ПЕРИТОНЕАЛЬНИХ МАКРОФАГІВ ЩУРІВ У ДОСЛІДАХ *IN VITRO* ТА *IN VIVO*

ДУ «Інститут медицини праці НАМН України», м. Київ

*Вступ*. Сьогодні в світі значна увага приділяється дослідженню безпечності наноматеріалів (НМ), які синтизують і використовують у різних сферах діяльності людини. Серед наночастинок (НЧ) металів, які активно застосовуються в медицині, фармації, є НЧ заліза та його оксидів, Важливим кроком для вивчення того, як вони впливають на живий організм, є дослідження їхньої токсичності та біологічної активності. Особливий інтерес у цьому аспекті представляють макрофаги, які присутні в різних тканинах, і є ключовими клітинами у формуванні імунної відповіді.

*Мета дослідження* – оцінка впливу НЧ $Fe_2O_3$ на життєздатність та функціональну активність перитонеальних макрофагів щурів у дослідах *in vitro* та *in vivo*.

*Матеріали та методи дослідження*. Наночастинки $Fe_2O_3$ 19 нм і 75 нм були отримані хімічним способом. Життєздатність перитонеальних макрофагів за впливу НЧ $Fe_2O_3$ визначали фарбуванням трипановим синім та в МТТ-тесті, фагоцитарну активність клітин оцінювали за поглинанням часточок латексу та цитохімічно, бактерицидність – у тесті з нітросинім тетразолієм (НСТ-тест).

*Результати*. Встановлено, що інкубація в умовах *in vitro* спричиняла загибель 30 % макрофагів, тоді як надходження в організм не впливало на життєздатність цих клітин. НЧ $Fe_2O_3$ стимулювали процес фагоцитозу, утворення великих фагоцитарних вакуолей, фагосом, лізосом і фаголізосом, а також продукцію активних форм кисню. Більшу активність проявляли НЧ $Fe_2O_3$ 19 нм.

*Висновок.* НЧ $Fe_2O_3$ при контакті з макрофагами викликають активацію фагоцитозу й утворення реактивних форм кисню, які спрямовані на їхню елімінацію та знешкодження. Однак надмірна або хронічна стимуляція оксидативного стресу може призвести до загибелі клітин, розвитку запального процесу. Отримані дані дозволяють припустити про можливий токсичний вплив НЧ $Fe_2O_3$ на функціонування інших ланок імунної системи організму, що вимагає проведення розширених імунологічних досліджень.

**Ключові слова: наночастинки оксиду заліза, макрофаги, фагоцитоз, імунотоксичність**

**Дмитруха Н. Н., Луговской С. П., Лагутина О. С.**

## ОЦЕНКА ВЛИЯНИЯ НАНОЧАСТИЦ $Fe_2O_3$ НА ФУНКЦИОНАЛЬНУЮ АКТИВНОСТЬ ПЕРИТОНЕАЛЬНЫХ МАКРОФАГОВ КРЫС В ОПЫТАХ *IN VITRO* И *IN VIVO*

ГУ «Институт медицины труда НАМН Украины», г. Киев

*Вступление.* Сегодня в мире большое внимание уделяется исследованию безопасности наноматериалов (НМ), которые синтезируются и используются в различных сферах деятельности человека. Среди наночастиц (НЧ) металлов, которые активно применяются в медицине, фармации, интерес представляют НЧ железа и его оксидов. Важным для изучения того, как НЧ влияют на живой организм, является исследование их токсичности и биологической активности. Особый интерес в этом аспекте представляют макрофаги, которые присутствуют в различных тканях, и являются ключевыми клетками в формировании иммунного ответа.
*Цель исследования.* Оценка влияния НЧ $Fe_2O_3$ на жизнеспособность и функциональную активность перитонеальных макрофагов крыс в опытах *in vitro* и *in vivo*.
*Материалы и методы исследования.* Наночастицы $Fe_2O_3$ 19 нм и 75 нм синтезированы химическим способом. Жизнеспособность перитонеальных макрофагов при воздействии НЧ $Fe_2O_3$ определяли окраской трипановым синим и в МТТ-тесте, фагоцитарную активность клеток оценивали по поглощению частиц латекса и цитохимически, бактерицидность – в тесте с нитросиним тетразолием (НСТ-тест).
*Результаты.* Установлено, что инкубация в условиях *in vitro* с НЧ $Fe_2O_3$ вызывала гибель 30 % макрофагов, тогда как поступление их в организм не влияло на жизнеспособность этих клеток. НЧ $Fe_2O_3$ стимулировали процесс фагоцитоза, образование крупных фагоцитарных вакуолей, фагосом, лизосом и фаголизосом, а также продукцию активных форм кислорода. Большую активность проявляли НЧ $Fe_2O_3$ 19 нм.
*Вывод.* НЧ $Fe_2O_3$ при контакте с макрофагами вызывает активацию фагоцитоза и образование реактивных форм кислорода, которые направлены на их элиминацию и обезвреживание. Однако чрезмерная или хроническая стимуляция оксидативного стресса может привести к гибели клеток, развитию воспалительного процесса. Полученные данные позволяют предположить о возможном токсическом влиянии НЧ $Fe_2O_3$ на функционирование других звеньев иммунной системы организма, что требует проведения расширенных иммунологических исследований.

**Ключевые слова: наночастицы оксида железа, макрофаги, фагоцитоз, иммунотоксичность**

*Надійшла: 03.09.2015 р.*

**Контактна особа:** Дмитруха Наталія Миколаївна, доктор біологічних наук, лабораторія промислової токсикології та гігієни праці при використанні хімічних речовин, ДУ «Інститут медицини праці НАМН України», буд. 75, вул. Саксаганського, м. Київ, 01033. Тел.: + 38 0 44 289 51 85.
Електронна пошта: dmytrukha@ukr.net