doi: 10.56761/EFRE2024.C5-O-010603

Biological impact of Fe_xO_y nanoparticles

A.S. Gerasimov[1,2,], V.G. Ilves[2], S. Yu. Sokovnin[1,2], T.R. Sultanova[3], M.V. Ulitko[1,4], V.D. Volochneva[1]*

[1]*Ural Federal University, Yekaterinburg, Russia*
[2]*Institute of Electrophysics UB RAS, Yekaterinburg, Russia*
[3]*Institute of Immunology and Physiology UB RAS, Yekaterinburg, Russia*
[4]*Institute Of Medica Cell Technology, Yekaterinburg, Russia*
[*]*andrei.gerasimov@urfu.ru*

Abstract. This paper presents a study of the biomedical application of iron oxide Fe_xO_y nanoparticles (NPs) obtained by radiation-chemical method, also after annealing and coating with nanosilver. The effect of NPs on organs and blood preparations of laboratory mice has been studied. The cytotoxicity against rat macrophages, HeLa and Hep-2 tumor lines was investigated. In addition, the antioxidant activity against healthy monkey kidney cells Vero was described. An increase in cytotoxic and enzymatic effects was found after nanosilver coating. Significant cytotoxicity of Fe_xO_y and Fe_xO_y+Ag S400 NPs against tumor cultures was revealed.
Keywords: Nanoparticles, iron oxide, nanosilver, cytotoxicity

1. Introduction

Currently, the development of nanotechnology is taking place in many industries, especially in the biomedical field. Nanoparticles (NPs), in particular of metal oxides, are of particular interest. NPs are mostly characterized by a large specific surface area, various defects and pronounced non-stoichiometry. Together, these factors open up the possibility for them to exhibit unique magnetic, catalytic, redox and other properties applicable in biomedicine [1]. NPs can be used for targeted drug delivery [2], as MRI contrast agents [3], magnetic hyperthermia agents [3], anti- and pro-oxidant compounds [4]. At the same time, such materials must be stable in physiological media, non-toxic, dispersible in water, biocompatible and non-immunogenic [5].

One of the most promising candidates for use in biomedicine are NPs of iron oxide. In addition to its pronounced oxidative and magnetic properties, this material is relatively affordable and stable. FexOy NPs have already been approved for clinical use as MRI contrast agents [3], however, the cytotoxic effect on different cells is still not clear. In addition, various methods of obtaining, as well as NPs treatment, can greatly change their properties, which makes the task of studying the biological effects of NPs iron oxide relevant for research.

In this work, the analysis of the biological effects of iron oxide Fe_xO_y NPs, obtained by radiation-chemical method, including those coated with nanosilver and annealed at various temperatures (0–500 °C) was carried out. The effect of different doses of NPs on *in vivo* models in laboratory rats with a single administration by various methods was evaluated. Histological studies of the liver, kidneys, and spleen were performed, as well as analysis of blood smears after administration of NPs. Additionally, the cytotoxicity and antioxidant activity of NPs were analyzed in an *in vitro* model using Vero monkey kidney cell lines, the cytotoxicity of HeLa tumor line and human laryngeal carcinoma Hep-2, rat macrophages.

2. Materials and methods

2.1. Characteristics of the NPs

NPs were obtained by radiation-chemical method using iron (II) sulfate heptahydrate as a precursor. The synthesis technique and the description of the initial sample were described in detail earlier [6]. A pulsed periodic nanosecond electron accelerator URT-0.5 was used as a source of ionizing radiation [7]. The repetition rate of the accelerator was 10 pulses/s, the irradiation time was 2700 seconds, and the absorption dose was 2317 kGy.

A radiation-chemical method was used for the nanosilver-coated iron oxide Fe_xO_y composites preparation and has been described earlier [10]. A suspension based on an $AgNO_3$ sorbitol solution with the addition of iron oxide NPs was irradiated in Petri dishes using a nanosecond electron accelerator URT-0.5 [7]. The irradiated suspensions were kept for 96 hours, then the solutions were drained, and the resulting powders were washed with distilled water three times and dried.

Annealing of Fe_xO_y NP, including Fe_xO_y+Ag composite, was carried out in electrocorundum crucibles at temperatures from 0 to 500 °C. Annealed samples will be further designated S0 – S500 depending on the annealing temperature. The isothermal exposure time was 10 minutes, the samples were cooled in an oven to temperatures of 100–150 °C [6]. It is widely known that the phase transformation of magnetite (Fe_3O_4) when heated in air follows the scheme: magnetite-maghemite-hematite [8]. The effect of annealing on the structural, textural, and magnetic properties of the studied NPs was presented earlier [9].

2.2. In vivo assay

The study of the biological effects of Fe_xO_y NPs was conducted on 30 white mice kept in standard vivarium conditions. The assessment of the damaging effect was carried out for the Fe_xO_y S400 NPs sample at concentrations of 5 mg/kg and 10 mg/kg. The NPs was diluted in saline solution. The damaging effect was evaluated with different methods of administration: intravenous, subcutaneous, intramuscular. The mice were divided into 10 equal experimental groups, including intact and control. The animals were injected with 0.2 ml of a suspension of NPs, once, with a concentration corresponding to the experimental group. The animals were removed from the experiment by an overdose of diethyl ether. Peripheral blood was taken from the caudal vein for analysis on a hemanalyzer and Romanovsky-Giemse staining to count the leukoformula.

Organs (liver, kidney, spleen) were also taken out for further histological examination. A small fragment of the organ was placed in a histological cassette and fixed in formalin for 24 hours. Next, with the help of a closed-type histological wiring machine Shandon Excelsior, the organ was wired in alcohols of ascending concentration, after which the organ was enclosed in paraffin with the help of a paraffin filling station EG1160 (Leica). Sections of 5–7 microns thick were obtained from paraffin blocks using a semi-automatic sledge microtome HM 450 (Thermo FS), which were then dried on a heating table at a temperature of 52 °C for an hour. Hematoxylin and eosin staining was used to visualize the cellular structures.

2.3. In vitro assay

The cytotoxic activity of Fe_xO_y NPs was studied on the HeLa tumor line and human laryngeal carcinoma Hep-2, rat macrophages. For the Vero monkey kidney cell line also the antioxidant activity was investigated. NPs suspensions were introduced into cell cultures in three final concentrations: 0.1 mg/ml, 0.5 mg/ml and 1 mg/ml. Oxidative stress was modeled by introducing hydrogen peroxide into cell cultures at the concentration of 0.5 mM.

To assess the viability of cells, the MTT test was used, a colorimetric test to assess the metabolic activity of cells. The cells were seeded into a 96-well tablet at a dose of $1 \cdot 10^5$ cl/ml. The volume of the suspension was 100 µl. The cells were cultured 24 h at 37 °C in an Igla MEM ("Biolot") medium containing glutamine (1%), 10% embryonic veal serum and gentamicin (50 mg/l), in a humidified atmosphere of 5% CO_2. After that, suspensions of NPs were added to the wells in appropriate concentrations. 15 holes were used as a control sample. In the case of assessing the viability of rat macrophages, cell counting in a hemocytometer was used after staining with vital dye trypan blue.

3. Results and discussion

3.1. Investigation of the biological effect of Fe_xO_y NPs on laboratory rats

Visual analysis of micro-preparations of the mice's liver in the control groups in a light microscope showed that the liver tissue does not differ from the orthodox picture. Were found no visible pathological changes.

Histological analysis of the liver in the experimental groups treated with iron oxide nanoparticles, showed the development of pathological changes. At the NPs' concentration of 5 mg/kg, areas of hepatocytes with cloudy, granule-rich cytoplasm are detected, which indicates the occurrence of granular dystrophy. Hepatocytes overflowing with vacuoles are observed for the group, injected with concentration 10 mg/kg, which indicates the onset of hydropic (balloon) dystrophy. The boundaries of the cells are blurred. The sinusoidal capillaries are narrow and barely distinguishable.

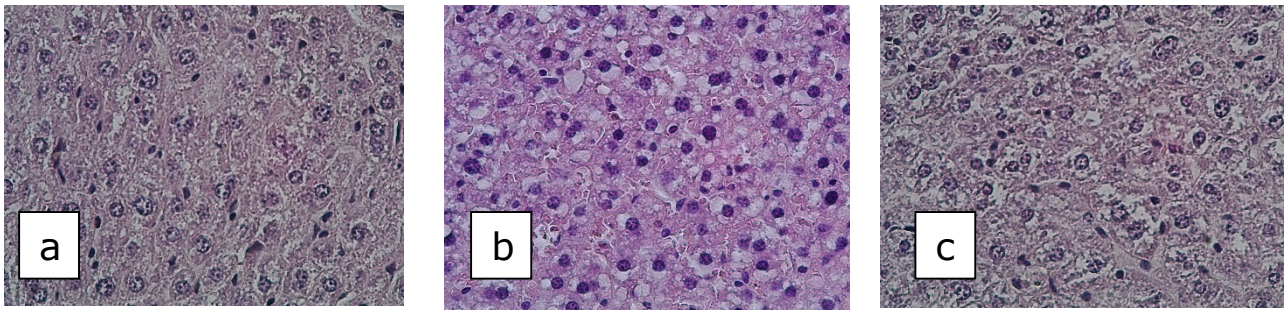

Fig. 1. Micro–preparations of the mice's liver treated with Fe_xO_y S400 NPs at a concentration of 10 mg/kg: a – intramuscular administration, b –subcutaneous administration, c – intravenous administration. Hematoxylin-eosin staining, zoom x400

Histological analysis of the kidneys and spleen showed no significant changes after administration of the Fe_xO_y S400 NPs. An increase in the number of red blood cells in the spleen tissue is observed after NPs injection. The kidney tissue in the control groups and the experimental groups did not differ from the orthodox picture.

A study of the effect on internal organs after administration of Fe_xO_y S400 NPs to experimental animals showed that intramuscular injection is the preferred method. The studied iron oxide nanoparticles had the greatest effect on the liver, which allows us to conclude that their predominant accumulation is in this organ.

Table 1. Blood smear analysis.

Blood cells population	The reference value, %	Intact, %	Fe_xO_y S400 (5 mg/kg), %	Fe_xO_y+Ag S400 (5 mg/kg), %
Rod-shaped neutrophils	2–5	5	2	4
Segmented neutrophils	55–68	58	40	36
Eosinophils	2–4	4	6	4
Basophils	0–1	0	4	2
Lymphocytes	23–35	26	40	44
Monocytes	5–8	7	8	10

The blood cell analysis did not show significant pathological changes in the control group. According to the data from Table 1, in the groups of animals that were treated with Fe_xO_y S400 and Fe_xO_y+Ag S400 NPs at a concentration of 5 mg /kg, was found a reduced content of segmented neutrophils, and an increased content of basophils and lymphocytes. When Fe_xO_y S400 is

administered, an increased content of eosinophils is also observed, and when Fe_xO_y+Ag S400 – monocytes.

In addition to changes in the ratio of blood cell populations, pathologically altered forms of red blood cells were discovered. When introducing Fe_xO_y S400 nanoparticles, acanthocytes were detected, and when introducing Fe_xO_y+Ag S400 nanoparticles, in addition to acanthocytes, a coin-shaped association of erythrocytes and their granularity were observed.

3.2. Cytotoxicity of Fe_xO_y NPs against rat macrophage cells

The administration of metal oxides NPs into living organisms inevitably leads to an immune system response. In this case, a safety application study was conducted on the use of Fe_xO_y and Fe_xO_y+Ag NPs against cells of the rat immune system. The results of the macrophage viability study are shown in Figure 2.

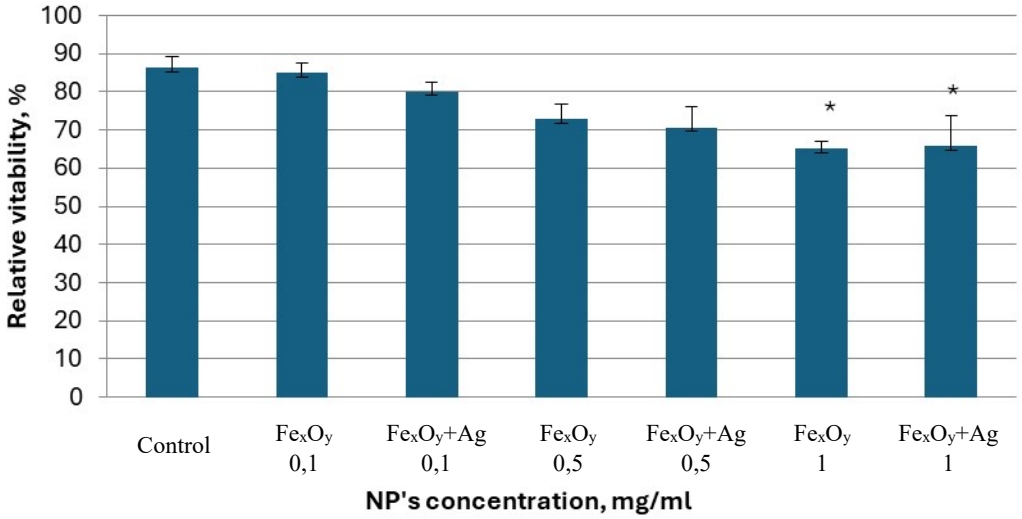

Fig. 2. Relative viability of macrophage culture after different concentrations of Fe_xO_y NPs administration (* – the difference with the control is valid ($p < 0.05$)).

Low concentrations of iron oxide Fe_xO_y and Fe_xO_y+Ag NPs (0.1–0.5 mg/ml) had no toxic effect on rat macrophages. However, with an increase in the dose of NP to 1 mg/ml, a decrease in the viability of macrophages is observed.

3.3. Cytotoxicity and antioxidant activity of Fe_xO_y NPs against Vero cell culture

The result of the viability investigation of Vero monkey kidney cell lines, after administration of Fe_xO_y and Fe_xO_y+Ag S0, S200, S400, S500 NPs in various concentrations are shown in Table 2.

It is noted that the Fe_xO_y S0 sample did not have a cytotoxic effect on the cell culture. At the same time, this sample coated with nanosilver reduced cell viability to 40% and 45% at concentrations of 0.5 and 1 mg/ml, respectively. Considering the dynamics of changes in the cytotoxic effect of NPs with a change in the annealing temperature, a slight decrease in the cytotoxicity for S400 samples was observed. It should also be noted that for the undoped S400 and S500 samples, the greatest cytotoxic effect is observed for a minimum concentration of 0.1 mg/ml. At the same time, the relative survival rate of the cells increased in proportion to the concentration of the NPs samples. This behavior is atypical for NPs and may indicate that these samples can catalyze the repair mechanisms of these cells.

Table 2. Relative viability of Vero cells, after Fe_xO_y NPs administration.

Sample	NP's concentration, mg/ml		
	0.1	**0.5**	**1**
Fe_xO_y S0	96.36	95.96	130.39
Fe_xO_y S200	85.73	55.44	63.16
Fe_xO_y S400	74.86	91.02	100.03
Fe_xO_y S500	69.98	72.87	93.60
Fe_xO_y+Ag S0	83.59	38.93	45.36
Fe_xO_y+Ag S200	81.36	50.24	92.04
Fe_xO_y+Ag S400	71.20	80.08	66.77
Fe_xO_y+Ag S500	88.56	73.79	65.00

The results of the antioxidant activity investigation of Fe_xO_y and Fe_xO_y+Ag NPs S0, S200, S400, S500 in various concentrations on the Vero monkey kidney cell line are shown in the table 3. For all samples, an antioxidant effect is observed, increasing in proportion to the concentration of NPs. When comparing the cell viability after administration of Fe_xO_y and Fe_xO_y+Ag NPs at appropriate annealing temperatures, it was found that the antioxidant activity of the undoped S0 sample was higher. This observation is consistent with the high cytotoxicity of Fe_xO_y+Ag S0 NPs demonstrated earlier. At the same time, the greatest antioxidant effect was obtained for the Fe_xO_y+Ag S200 sample, at a concentration of 1 mg/ml. In this case, cell viability increased by 4 times compared to the peroxide control.

Table 3. Relative viability of Vero cells, after administration of hydrogen peroxide and Fe_xO_y NPs.

Sample	NP's concentration, mg/ml			
	H_2O_2	**0.1**	**0.5**	**1**
Fe_xO_y S0	29.71	37.21	52.20	91.18
Fe_xO_y S200	8.30	9.85	17.17	25.01
Fe_xO_y S400	14.73	18.87	40.15	56.63
Fe_xO_y S500	12.61	19.63	23.10	48.84
Fe_xO_y+Ag S0	13.06	19.75	28.13	38.64
Fe_xO_y+Ag S200	21.15	25.43	42.95	80.99
Fe_xO_y+Ag S400	20.24	23.83	48.10	61.70
Fe_xO_y+Ag S500	14.05	19.31	23.20	47.46

3.4. Cytotoxicity of Fe_xO_y NPs aganist tumor cells

During the experiment, a decrease in the viability of HeLa tumor culture was observed with after Fe_xO_y S0 and S200 NPs treatment at a concentration of 1 mg/ml by 68% and 29%, respectively. Also, the addition of the S100 sample at concentrations of 0.5 and 1 mg/ml led to a decrease in cell viability by 48–75%. When S400 and S500 were administrated, viability decreased at all concentrations by 93% and 30–40%, respectively.

In general, moderate cytotoxicity of Fe_xO_y NPs is observed with respect to the human cervical carcinoma HeLa tumor culture, apart from the S400 sample, which showed high cytotoxicity.

Fe_xO_y S400 and Fe_xO_y+Ag S400 samples (Figure 3) had a cytotoxic effect on Hep-2 cell culture, reducing the relative cell viability by 42–48% and 30–74%, respectively, at concentrations of 0.5 and 1 mg/ml.

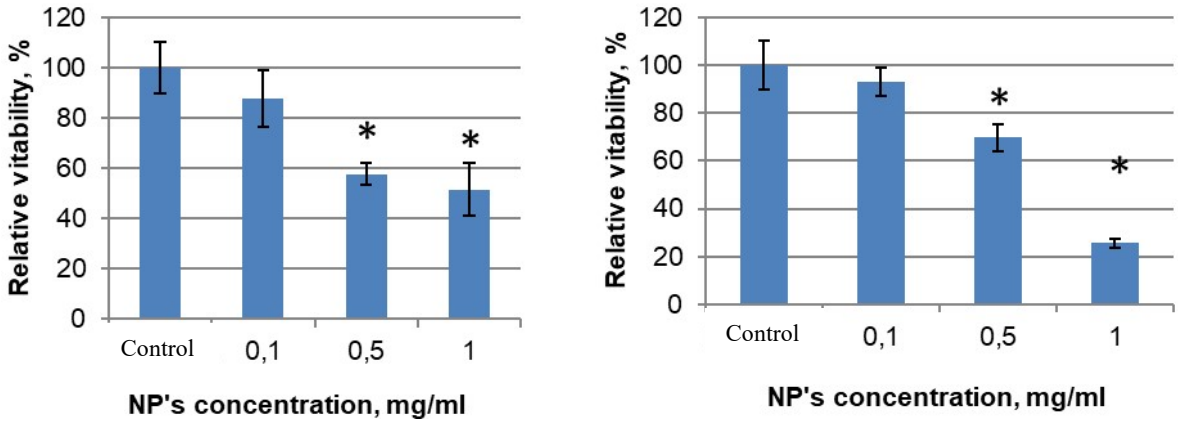

Fig. 3. Relative viability of Hep-2 cell culture after Fe_xO_y and Fe_xO_y+Ag NPs administration (* – the difference with the control is valid ($p < 0.05$)).

4. Conclusion

The analysis showed the possibility of biomedical application of iron oxide Fe_xO_y NPs as antioxidant and antitumor agents. It has been found that intramuscular injection is the preferred and safest way to administer NPs to experimental animals. NPs had no significant toxic effects on the kidneys and spleen of mice. However, dose-dependent pathological changes in the liver were detected with the administration of Fe_xO_y S400 NPs at concentrations of 5 mg/kg and 10 mg/kg, which narrows the possibilities of using this sample.

The low concentration of Fe_xO_y and Fe_xO_y+Ag NPs (0.1 mg/ml) had no toxic effect on the culture of normal Vero and the rat immune system cells. With an increase in the dose, almost all the samples studied had a cytotoxic effect to one degree or another. At the same time, the nanosilver coating generally increases the cytotoxicity and the antioxidant properties of NPs.

The cytotoxic effect of the studied samples of Fe_xO_y iron oxide nanopowders on tumor cells of human laryngeal carcinoma Hep-2 and human cervical carcinoma HeLa has been established. The greatest damaging effect was found in Fe_xO_y S400 samples. The Ag coating enhances the cytotoxic effect.

Further research is needed to increase the potential of the investigated NPs in the biomedical field. The effect of reducing cytotoxicity with an increase in the dose of administered NPs deserves more careful study.

Acknowledgement

This work was financially supported by the Russian Science Foundation (project No 22-19-00239)

5. References

[1] M. Nikzamir, A. Akbarzadeh, Y. Panahi, An overview on nanoparticles used in biomedicine and their cytotoxicity, *Journal of Drug Delivery Science and Technology*, **61**, 102316, 2021, doi:10.1016/j.jddst.2020.102316

[2] R. Liu, et al., Advances of nanoparticles as drug delivery systems for disease diagnosis and treatment, *Chinese chemical letters*, **34**(2), 107518, 2023, doi:10.1016/j.cclet.2022.05.032

[3] B.M. Galeano-Villar, et al., Core-shell Fe@ Fe_xO_y nanoring system: a versatile platform for biomedical applications, *Materials & Design*, **213**, 110303, 2022, doi:10.1016/j.matdes.2021.110303

[4] X. Ge, Z. Cao, L. Chu, The antioxidant effect of the metal and metal-oxide nanoparticles, *Antioxidants*, **11**(4), 791, 2022, doi:10.3390/antiox11040791

[5] M. González-Béjar, L. Francés-Soriano, J. Pérez-Prieto, Upconversion nanoparticles for bioimaging and regenerative medicine, *Frontiers in Bioengineering and Biotechnology*, **4**, 47, 2016, doi:10.3389/fbioe.2016.00047
[6] M.E. Balezin, S.Y. Sokovnin, M. Uimin, Preparation of iron oxide nanopowders by the radiation-chemical method, *Journal of Physics: Conference Series*, **2064**(1), 012086, 2021, doi.org/10.1088/1742-6596/2064/1/012086
[7] S.Y. Sokovnin, M.E. Balezin, Improving the Operating Characteristics of an УРТ-0.5 Accelerator, *Instruments and Experimental Techniques*, **48**, 392, 2005, doi:10.1007/s10786-005-0068-0
[8] P.K. Gallagher, E.M. Gyorgy, H.E. Bair, An anomaly in the oxidation rate of magnetite at its Curie temperature, *The Journal of Chemical Physics*, **71,** 830, 1979, doi:10.1063/1.438373
[9] V.G. Ilves, et al., Effect of air annealing on properties of maghemite nanoparticles produced by radiation-chemical method, *Ceramics International*, **49**(15), 25414, 2023, doi:10.1016/j.ceramint.2023.05.080
[10] S.Y. Sokovnin, M.E. Balezin, M.A. Kiseleva, Preparation and properties of silver nanopowders and composites based on a radiation-chemical method, *Radiation Physics and Chemistry*, **179**, 109218, 2021, 10.1016/j.radphyschem.2020.109218