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Estimation of iron nanoparticles' substance biosafety *in vitro* and *in vivo*

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Abstract. The aim of the work was to estimate biosafety of iron nanoparticles' substance according to *in vitro* and *in vivo* tests. **Materials and Methods:** biosafety of the substance of 40 nm sized spherical zero-valent iron nanoparticles has been established *in vitro* using cytotoxicity and genotoxicity tests. Biosafety *in vivo* has been determined according to the acute toxicity parameter (LD₅₀) and the effect on cardiac function and state of rabbits' hemodynamics. **Results:** there were no cytotoxic and genotoxic effects of iron nanoparticles' substance on the test culture of eukaryotic cells. In case of intravenous injection of nanoparticles the average LD₅₀ for BALB/c mice was 220.3 mg/kg. Iron nanoparticles' substance injected intravenously was safe according to hemodynamic parameters. **Conclusion:** obtained data on biosafety estimation denotes low level of potential risks associated with iron nanoparticles' substance.

Keywords: iron nanoparticles, biosafety, genotoxicity, cytotoxicity, hemodynamics.

Significant progress in the area of new nanomaterials' creation and nanotechnology opens perspectives in effective treatment of socially important diseases, such as anemia [2, 8–10]. According to the World Health Organization anemia affects one-quarter of the world's population. The main cause of anemia is iron deficiency [1, 15].

Varieties of iron medications are widely prescribed as a part of medical care for patients with iron deficiency anemia in order to recover iron pool in the body. However, existing antianemic drugs are not usually effective and safe because of insufficient iron bioavailability, as well as a large number and high frequency of adverse reactions [13]. Therefore, synthesis and development of new classes of biosafe and biocompatible antianemic drugs is an urgent task. Iron nanoparticles are promising in this direction, due to their enhanced biological activity at the molecular level. However, data on the biosafety of iron nanoparticles' impact on both humans and environment are not adequately detailed. Subsequently, versatile study is required to avoid possible risks [12, 14].

In this context, **the objective of the study** was to evaluate biosafety of iron nanoparticles' substance *in vitro* and *in vivo*.

Materials and methods

Substance of 40 nm sized spherical zero-valent iron nanoparticles used in this study have been synthesized by chemical condensation method in aqueous medium by iron (III) chloride restoration at the F.D. Ovcharenko Institute of Colloidal Chemistry of NAS of Ukraine according to the original protocols.

Biosafety of the iron nanoparticles' substance has been determined *in vitro* using cytotoxicity and genotoxicity tests according to the Methodical recommendations "Safety assessment of medical nanopreparations" approved by the Scientific Expert Council of the State Expert Centre of the Ministry of Health of Ukraine (protocol №8, 26.09.2013) [4].

Eukaryotic cell line CHO-K1 (Chinese hamster ovary) from the collection of the State Scientific Control Institute of Biotechnology and Strains of Microorganisms (Kyiv) has been used in cytotoxicity and genotoxicity tests. The number of living cells during the experiments was not less than 90% that had been determined using 0.4% trypan blue aqueous solution.

Cytotoxicity was assessed by two methods: 1) recording of living cells number was performed after staining with crystal violet and determining the percentage of living cells in relation to untreated control ones according to the formula: $(P_{\text{experiment}}/P_{\text{control}}) \times 100$; 2) evaluation of changes in the intensity of cellular absorption of supravital stain neutral red (NR). Recording of cytotoxicity was performed by determining the percentage of living cells ($P_{\text{experiment}}$) relative to the control (P_{control}). The percentage of the cells that actively absorbed NR was defined via the formula: $(P_{\text{experiment}}/P_{\text{control}}) \times 100$. The IC₁₀₀ and IC₅₀ indicators, which are the concentrations that cause respectively 100% and 50% reduction in cellular NR absorption activity, served as the cytotoxicity criteria.

Evaluation of iron nanoparticles' genotoxicity was performed using the conventional scheme of comet assay in alkaline modification [4].

Microscopic examination of preparations was carried out using a fluorescent microscope ("JIOMAM P8", Russia) with 490 nm excitation filter, 510 dichroic mirror, 530 nm cutting filter, and 200-400X magnifying power. At least 100 "DNA-comets" with no "tails" overlapping were analyzed for each specimen. Analysis of "DNA-comets" was performed visually. In this case, "DNA comets" were distributed into five types with corresponding numeric value from 0 to 4. The DNA damage level was expressed as "DNA-comet" index (I_{DNA}), which was calculated according to the formula:

$$I_{\text{DNA}} = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) / \Sigma, \text{ where}$$

$n - n_0 - n_4$ – the number of “DNA-comets” of each type, Σ – total number of “DNA-comets”.

Statistical analysis of the results was performed for each experimental point by comparing the DNA damage indices in the experimental and control groups. The native CHO-K1 cells served as a negative control (C⁻), whereas N-nitrosomethylurea-treated cells were used as a positive control (C⁺), due to well-known genotoxic properties of this compound. Statistically significant reproducible effect was considered to be the criterion for a positive result.

Biosafety of the iron nanoparticles' substance was assessed *in vivo* according to acute toxicity parameters and the influence on animal's heart activity and hemodynamics [11].

Animal studies were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (18.03.1986), Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (24.11.1986), the Order of the Ministry of Health of Ukraine №66 (13.02.2006), the Law of Ukraine “On the Protection of Animals from Cruelty” (2006).

Animals used in the experiment were kept under standard vivarium conditions of the O.O. Bogomolets National Medical University [11].

Acute intravenous toxicity of iron nanoparticles' substance was studied on 64 females and 56 males of white BALB/c mice with weight 18–22 g and age 2–2.5 months. Animals in the control group were injected the solvent (water for injections). Registration and counting of dead animals in each experimental dose levels were carried out during 14 days. Based on the obtained data probit analysis of dose-effect correlation was performed according to D.J. Finney [3] using computer software BioStat 2009 for Windows (v5.8.4).

Hemodynamic changes under the influence of iron nanoparticles' experimental antianemic substance were determined on 9 Chinchilla rabbits of both sexes weighing 3.0–4.3 kg. The animals were anesthetized via intramuscular injection of 50% urethane solution in the dose of 1 g/kg body weight. After attachment of three standard electrodes for electrocardiogram registration, as well as left ventricle and femoral artery catheterization in the acute experiment the following parameters were recorded using HP Viridia Component Monitoring System of “Hewlett Packard” (USA): heart rate (HR, min⁻¹), left ventricle maximum pressure (LVP_{max}, mm Hg), and average arterial pressure (AP_{mean}, mm Hg). Solution of iron nanoparticles' substance (10 mg/ml) was slowly injected into rabbits' ear vein three times with an interval of 30 minutes in the following doses: first dose – 6 mg/kg, second dose – 12 mg/kg, third dose – 24 mg/kg.

The above-mentioned indices of systemic hemodynamics were recorded before the first injection of solution of iron nanoparticles' substance and during 5, 10, 20, and 30 min. after each of three injections of nanoparticles.

Statistical analysis of the data was performed using computer software BioStat 2009 for Windows (v5.8.4) and Microsoft Office Excel 2007. Detection of single outliers was performed using Q-test. The value of arithmetic means (M) and statistical confidence intervals (m) were calculated.

Significance of differences between the arithmetic means was determined using Student's t-test. Changes of the indices were considered to be reliable when the level of significance exceeded 95% (p<0.05).

Results and discussion

High biological safety and biocompatibility of a nanomaterial-based potential pharmaceutical substance is a prerequisite for its further preclinical and clinical trials. However, early detection of highly predictive markers of nanoparticles-induced disturbances in biological systems is a key challenge in prevision and preventing of their potential toxic effects on the human body as well as on the environment [6].

Modern toxicology tests are largely based on the use of cell lines according to the well-known fact that the effect of chemicals on living organisms is manifested at the cellular level [6].

Therefore, taking into account the known expediency and efficacy of cell culture models for estimation of nanomaterials' general toxic effects, the first step in iron nanoparticles' substance safety determination was *in vitro* cytotoxicity and genotoxicity tests.

The recorded data on live cells number after exposure to iron nanoparticles' substance showed no cytotoxic effect of nanoparticles on the test culture of eukaryotic cells.

Biosafety evaluation of iron nanoparticles' substance in terms of genotoxicity showed the following. There was no primary DNA damage in the samples of eukaryotic cells of CHO-K1 cell line treated with iron nanoparticles in the investigated concentration range compared with the influence of N-nitrosomethylurea which is a well-known genotoxicity agent [4].

The indicator of DNA damage (“DNA-comet” index, I_{DNA}) under the influence of the studied iron nanoparticles in different concentrations reached the values that were close to I_{DNA} of the negative control (Table 1).

Evaluation of genotoxicity of iron nanoparticles' substance

Iron nanoparticles	"DNA-comet" index (I_{DNA})
C=10.0·10 ⁻⁵ mg/ml	0.012±0.001
C= 0.01 mg/ml	0.013±0.001
C= 0.1 mg/ml	0.013±0.001
Negative control	0.014±0.001
Positive control	3.01±0.1

Thus, the data obtained *in vitro* have demonstrated that the investigated iron nanoparticles' substance is biosafe according to cytotoxicity and genotoxicity indices.

The next stage of research was to determine biosafety of the iron nanoparticles' substance *in vivo*.

Dose-dependent lethality of mice was found in the study of iron nanoparticles' toxicity after a single intravenous injection (Table 2).

Table 2

Mice lethality after a single intravenous injection of iron nanoparticles' substance depending on the dose level during 14-days assessment period

Dose level, mg/kg	Lethality in groups		General mice lethality
	Males, n=56	Females, n=64	
0 (control)	0/8	0/8	0/16
130	-	0/8	-
155	-	1/8	-
180	0/8	3/8	3/16
205	2/8	3/8	5/16
230	3/8	5/8	8/16
255	7/8	6/8	13/16
280	7/8	8/8	15/16
305	8/8	-	-
LD ₅₀ , mg/kg	231.4±8,1	207.5±10,6	220.3±7,1

The signs of intoxication developed immediately after the injection of iron nanoparticles' solution in toxic (lethal) doses. The leading symptoms of intoxication included depression, muscle weakness, seizures, sings of pulmonary edema indicating a primary lesion of the cardiovascular and respiratory systems and, consequently, the nervous system. Weight gain violations were also observed during the first 4 days after injection. As a result, some animals died at the height of above-mentioned symptoms (Table 2).

The calculated LD₅₀ value and its standard error (Table 2) indicate the presence of sexual sensitivity to iron nanoparticles' toxic effects. In this case, the females are more sensitive, since LD₅₀ for the males is about 11.5% higher. Therefore, according to the LD₅₀ values iron nanoparticles' substance can be attributed to the class of low-toxic materials (toxicity class IV) according to H.C. Hodge and L.H. Sterner [5].

The assessment of changes in cardiac activity and hemodynamic status of experimental animals (rabbits) after intravenous injection of iron nanoparticles' substance showed the following. Hemodynamic parameters that had been registered before the injection of the substance were equal to the next values: HR – 265.8±12.8 min⁻¹, LVP_{max} – 115.8±8.9 mm Hg, AP_{mean} – 97.3±5.8 mm Hg. Injection of iron nanoparticles' solution in the dose of 6 mg/kg did not lead to significant changes of hemodynamics' parameters compared with initial values. The next administration of iron nanoparticles in the dose of 12 mg/kg (total dose 18 mg/kg) also did not induce changes of registered indices.

The third injection of iron nanoparticles' substance in the dose of 24 mg/kg (total dose 42 mg/kg) caused a significant decrease in LVP_{max} by 12.2–29.8%, and AP_{mean} by 23.6–32.9% starting from 0–5 minutes, compared with the initial data (before the first injection).

Investigated indices of cardiovascular system activity were significantly decreased for 30 min. with a tendency to a partial recovery to baseline values.

After injection of the iron nanoparticles' substance in studied doses heart rate of the experimental animals did not significantly change.

These data indicate safety of the iron nanoparticles' substance in case of slow intravenous injection according to the studied hemodynamic parameters. Thus, compared to the influence of the iron nanoparticles' substance in the total dose of 42 mg/kg on experimental animals' cardiovascular system, the maximum single dose of one of the most commonly used antianemic drugs – iron sucrose – in case of intravenous infusion to anemic patients is about 7 mg/kg [7].

Thus, studied substance of 40 nm sized spherical zero-valent iron nanoparticles is characterized by a low level of potential hazard. There were no cytotoxic and genotoxic effects of the substance *in vitro*. Nanoparticles' substance belongs to low-toxic compounds (IV class of toxicity by Hodge H.C. and Sterner L.H.) in terms of LD₅₀ level in case of intravenous injection and is safe according to the impact on experimental animals' cardiovascular system parameters in condition of slow intravenous injection.

Prospects for future research

The observed safety of investigated iron nanoparticles' substance that have been established in experiments *in vitro* and *in vivo* indicates the expediency of further preclinical studies and determination of the molecular and biochemical mechanisms of iron nanoparticles' effect on biological systems of different levels of organization.

References

1. Stefanov O. Preclinical studies of drugs (Guidelines). Avitsenna. 2002; 527.
2. Guidelines "Safety assessment of medical nanopreparations" approved by the Scientific Expert Council of the State Expert Centre of the Ministry of Health of Ukraine (protocol №8, 09.26.2013). Kyiv. 2013; 108.
3. Moskalenko V.F., Chekman I.S., Chernykh V.P., et al. Nanoscience, nanopharmacology, nanopharmacy: perspectives of research, implementation in medical practice. *Klinichna farmatsiia*. 2010; 14, 1: 1-5.
4. Moskalenko V.F., Lisovyi V.M., Chekman I.S., et al. Scientific basis of nanomedicine, nanopharmacology, and nanopharmacy. *Visnyk NMU im. O.O.Bohomoltsia*. 2009; 2: 17-31.
5. Paton B., Moskalenko V., Chekman I., Movchan B. Nanoscience and nanotechnology: technological, medical, and social aspects. *Visn. NAN Ukrainy*. 2009; 6: 18–26.
6. Kundiev Yu.I., Ulberg Z.R., Trakhtenberg I.M., et al. Problem of assessing the potential risks of nanomaterials and its solving. *Dopovidi NAN Ukrainy*. 2013; 1: 177 – 184.
7. Chekman I.S. Nanopharmacology. Kyiv. Zadruga. 2011; 424.
8. Balarajan Y., Ramakrishnan U., Ozaltin E., et al. Anaemia in low-income and middle-income countries. *Lancet* 2011; 378(9809): 2123-2135.
9. Finney D.J. Probit Analysis. – 3rd edition, chapt. 3 and 4. Cambridge: Cambridge University Press, 1971: 333.
10. Hodge H.C., Sterner L.H. Tabulation of toxicity classes. *Am Industr Hyg Ass Quart* 1943; 10: 93.
11. Macdougall I.C., Geisser P. Use of intravenous iron supplementation in chronic kidney disease: an update. *Iran J Kidney Dis* 2013; 7: 9-22.
12. Pumera M. Nanotoxicology: the molecular science point of view. *Chem Asian J* 2011, 6: 340.
13. Qunibi W.Y. The efficacy and safety of current intravenous iron preparations for the management of iron-deficiency anaemia: a review. *Arzneimittelforschung* 2010; 60: 399.
14. Santamaria A. Historical overview of nanotechnology and nanotoxicology. *Methods Mol Biol* 2012; 926: 1.
15. Worldwide prevalence of anaemia 1993–2005: WHO global database on anaemia / Ed. by B. de Benoist, E. McLean, I. Egli and M. Cogswell, 2008; 48.