Exp Oncol 2009
31, 3, 163–167

Experimental ONCOLOGY

THE USE OF NANOFERROMAGNETICS TO INCREASE THE CYTOTOXIC EFFECT OF ANTITUMOR DRUGS

V.F. Chekhun, I.N. Todor, N.Yu. Lukyanova, S.I. Shpyleva, L.A. Naleskina, I.K. Khaetsky, G.I. Kulik*

R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kiev, Ukraine

Aim: **To investigate the influence of ferromagnetic nanoparticles on antitumor effect of doxorubicin and mitochondria oxidative phosphorylation.** ***Methods:*** **The study was carried out on the mice-hybrids (C57Bl/6xDBA/2) with intraperitoneally (i/p) transplantated Ehrlich ascitic carcinoma. Single i/p injection of doxorubicin (Dox), stabilized ferromagnetic nanoparticles (Fe_3O_4; 20–40 nm; FM) or their combination were performed 7 days after tumor transplantation. The cytotoxic effect of agents, morphology and cell cycle of tumor cells were studied 24, 48 and 72 h after Dox administration.** ***Results:*** **The investigations showed that ferromagnetic nanoparticles increased the cytotoxic effect of doxorubicin on Ehrlich ascitic carcinoma mainly 48 h after agents' administration. The largest number of apoptotic cells was observed in group of animals in which doxorubicin was administered before ferromagnetic nanoparticles. Moreover, the ferromagnetic nanoparticles at concentration 1.45 µg Fe/ml and, particularly, 7.25 µg Fe/ml decreased mitochondria oxygen consumption in phosphorylation state that may negatively influence their living capability.** ***Conclusions:*** **Obtained data point out the perspective of use of certain sized FM nanoparticles to increase the cytotoixc effect of antitumor drugs.**

Key Words: **nanoferromagnetics, antitumor drugs, cytotoxic effect, mitochondria oxygen consumption.**

Nanoscience is well recognized as a revolutionary step in various field of science and a logical field of study for researchers in the coming years as it is, the study of fundamental principles of molecules and structures between one nanometer (one billionth of a meter) and 100 nanometers in size [1–4]. The biological application of nano-particles is a rapidly developing area of nanotechnology that raises new possibilities in the diagnosis and treatment of various diseases [5–10].

Nanotechnologies and nanostructures are becoming an option in human medical application, including imaging or the delivery of therapeutic drugs to cells, tissues and organs [11–14]. Unique nanoparticles peculiarities such as high surface energy, stable biomolecules absorption, physicochemical peculiarities changes under the influence of physical fields, their small sizes comparable to biomolecules, magnetic peculiarities availability, biocompatibility open new perspectives of nanopreparations using in therapy of various diseases including cancer. Today many nanomedicine studies are devoted to the development of principally new medications using magnetic nanoparticles as a carrier in antitumor drugs to tissues- and target-cells delivery systems. Many types of nanocomposites used in experiments are made of iron particles [9, 12, 13]. However it is not known how ferromagnetic nanoparticles will influence antitumor drugs activity in vivo taking into account their size and concentration.

Therefore the aim of our study was to investigate the influence of ferromagnetic nanoparticles on antitumor effect of doxorubicin in experiment. Moreover as active centers of many complexes of mitochondria respiratory chain contain iron [15], we have investigated the influence of ferromagnetic nanoparticles on tumor cell oxygen consumption and mitochondria oxidative phosphorylation.

Received: September 2, 2009.
*Correspondence: Fax: +38 (044) 258-16-56
E-mail: todor_igor@yahoo.com
Abbreviations used: Dox – doxorubicin; FM – ferromagnetic nanoparticles; i/p – intraperitoneal injection, RCI – respiratory control index.

MATERIALS AND METHODS

Synthesis and characterization of the ferromagnetic nanoparticles. We used magnetite Fe_3O_4 synthesized by the method of electron-beam evaporation, and Fe_3O_4 and NaCl deposition in vacuum. The stabilization of ferromagnetic was carried out by adding 1% polydextrane-70 and phosphatidylcholine during dispersion. Size of stabilized particles was determined using laser correlation spectrometry. Average ferromagnetic nanoparticles (FM) diameter was 20–40 nm (Fig. 1).

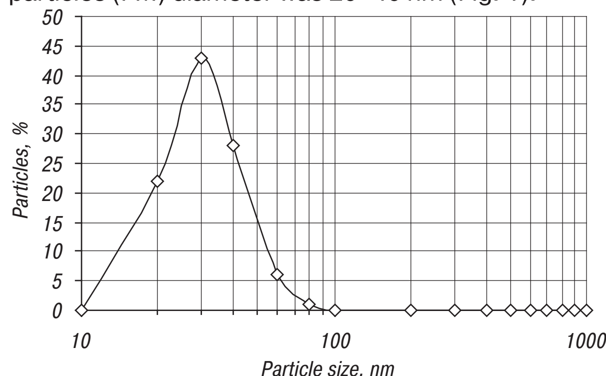

Fig. 1. Distribution of iron oxide particles by size

Determination of the iron content in solution. Iron concentrations (mg/ml) were measured by atomic-absorption spectroscopy [16].

Groups of animals. The study was carried out on the mice-hybrids (C57Bl/6xDBA/2; body weight = 20 g) with intraperitoneally (i/p) transplantated Ehrlich ascitic carcinoma (1.8 x 10^6 cells/mouse). Single i/p injection of doxorubicin (Dox, "EBEWE", Austria), stabilized ferromagnetic nanoparticles or their combination was performed 7 days after tumor transplantation. Mice were divided in 5 groups: 1 — control (i/p 0.3 ml of saline); 2 — doxorubicin administration (i/p; 3 mg/kg body

weight); 3 — ferromagnetic nanoparticles administration (i/p; 3 mg Fe/kg body weight); 4 — FM administration (i/p; 3 mg/kg body weight) and Dox administration (i/p; 3 mg/kg body weight) an hour later; 5 — Dox administration and FM administration an hour later (Table 1). The cytotoxic effect of agents was studied 24, 48 and 72 h after administration. The morphology and cell cycle of tumor cells were examined.

Table 1. Groups of animals

Group Number	Name	Description
1	Control	i/p 0.3 ml of saline
2	Dox	Doxorubicin administration (i/p 3 mg/kg body)
3	FM	Ferromagnetic nanoparticles administration (i/p 3 mg Fe/kg body)
4	FM + Dox	Ferromagnetic nanoparticles administration (i/p 3 mg Fe/kg body) and doxorubicin administration (i/p 3 mg/kg body) 1 h later
5	Dox + FM	Doxorubicin administration (i/p 3 mg/kg body) and ferromagnetic nanoparticles administration (i/p 3 mg Fe/kg body) and 1 h later

The determination of cytotoxic effect. The mean number of dead cells (using trypan blue stain) was the index of cytotoxic effect.

The cell cycle study. The cell cycle was studied by means of flow cytometer ("Coulter Epics XL", Beckman Coulter) with using of propidium iodide. The results were processed in ModFit program. The cell cycle of tumor cells taken from intraperitoneal cavity after i/p injections to mice Dox, FM or their combinations was studied. Moreover the cell cycle of intact Ehrlich ascitic carcinoma cells was studied after these cells incubation *in vitro* with Dox and FM. In this case, the FM concentration was constant (in terms of Fe) at 100 μM. But Dox was added in three different concentrations: 1 μM, 10 μM and 100 μM. 100 μM Dox approximately corresponds to a therapeutic dose of 3 mg/kg *in vivo* conditions.

The determination of oxygen consumption rate. In the first experiments the Ehrlich carcinoma cells were incubated (Medium 199, Sigma, USA; 1.5 h; 26 °C) in presence of 0.73, 1.45, 7.25 and 14.5 μg Fe/ml. After incubation the oxygen consumption rate was determined.

In the second experiments mitochondria were isolated from tumor cells. The isolation medium contained 0.25 M sucrose, 0.01 M Tris-HCl, 0.001 M EDTA and 0.5% bovine or human albumin, pH 7.4. Homogenate was centrifugated at 600 g for 3 min, then at 1000 g for 7 min without stop. The obtained supernatant was centrifugated for 10 min at 14 000 g. After that supernatant was removed and the fraction of mitochondria was resuspended in 0.35 ml of pre-cooled isolation medium. The mitochondria suspensions were incubated in presence of 1.45 μg Fe/ml and 7.25 μg Fe/ml (1.5 h; 3 °C). Then 0.02 ml of every mitochondria sample was put into polarographic cell 1 ml in volume containing the incubation medium (0.15 M sucrose, 0.05 M KCl, 0.01 M KH_2PO_4, 0.003 M $MgCl_2$, 0.005 M Tris-HCl, 0.005 M succinate, 0.0002 M EDTA, pH 7.4). In 3 min after the beginning of registration, 266 nmoles of ADP were put into the same cell. The temperature of the incubation medium was 26 °C. Oxygen consumption by mitochondria was measured by means of the covered combined platinum electrode of Clark's electrode type. Protein was defined by method of *Lowry*. All reagents were from "SIGMA". For statistical analysis Student's *t*-criterion was used.

RESULTS AND DISCUSSION

As it was mentioned above, for antitumor activity study of FM nanoparticles and doxorubicin against Ehrlich ascitic carcinoma cells, following groups were formed: control (N1); single intraperitoneal doxorubicin injection (N2; Dox); single intraperitoneal FM nanoparticles administration (N3; FM); FM administration (i/p; 3 mg/kg) and Dox administration (i/p; 3 mg/kg) an hour later (N4; FM + Dox); Dox administration and FM administration an hour later (N5; Dox + FM). Injections were performed 7 days after intraperitoneal tumor transplantation. In the last two groups the sequence of intraperitoneal administration of studied agents was specially changed as it wasn't known which combination is optimal. In the literature the doses for administration relative to iron (FM nanoparticles) were equal to 0.8–8 mg/kg [17–19]. But we stopped at an apparently average dose of 3 mg/kg. The results of combined therapy experiments (Fig. 2) showed that the FM nanoparticles stabilized by polydextrane-70, 48 h after the therapeutic injection did not show proliferation stimulation of Ehrlich ascitic carcinoma cells.

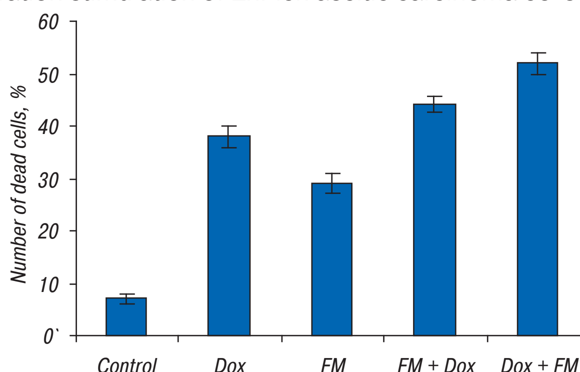

Fig. 2. Percentage of dead cells in ascitic fluid 48 h after administration (n = 10)

At the same time the data that we obtained during dead cells number counting in the ascitic fluid of animals (see Fig. 2) indicated the increase of the dead cells in all experimental groups compared with the control group. Particularly FM nanoparticles and doxorubicin itself increased the index by 22% and 31%, respectively. It could be that if FM nanoparticles of such size would be concentrated in solid tumors with the help of magnetic field, then antitumor therapy could become more effective.

FM nanoparticles administration before doxorubicin injection increased the number of dead cells in ascitic fluid by 6% compared to doxorubicin alone. Moreover, FM administration after the Dox injection increased this number by 14%. The percentage of dead cells in ascitic fluid of animals from groups 1–5 was equal to 7%, 38%, 29%, 44% and 52%, respectively (see Fig. 2).

It should be noted that the similar data were presented in [13]. However authors used entirely different magnet-controlled nanoparticles. The cytotoxic effect

of Dox, FM and their combination was less expressed in 24 and 72 h after administration of agents than in 48 h. Thereby, we studied apoptosis induction in tumor cells. The number of apoptotic cells in the fifth group (Dox + FM) 48 h after agents administration reached 65% (Table 2), while the number of tumor apoptotic cells in animals treated with doxorubicin alone was 45%.

Table 2. Number of cells in apoptosis (*in vivo*)

Group	Number of cells in apoptosis, %
1 (Control)	0–1
2 (Dox)	45
3 (FM)	3–5
4 (FM + Dox)	58
5 (Dox + FM)	65

Since doxorubicin is a phase-specific preparation and the biggest sensitivity to it is observed in S-phase [20], we studied influence of doxorubicin, FM nanoparticles and their combinations on the cell distribution by cell cycle phases. In groups where animals were administrated with Dox (N2, N4 and N5) we observed significant decrease in number of cells in S-phase. In group 5 the cells number in $G_0 + G_1$-phase was increased (Fig. 3, Table 3).

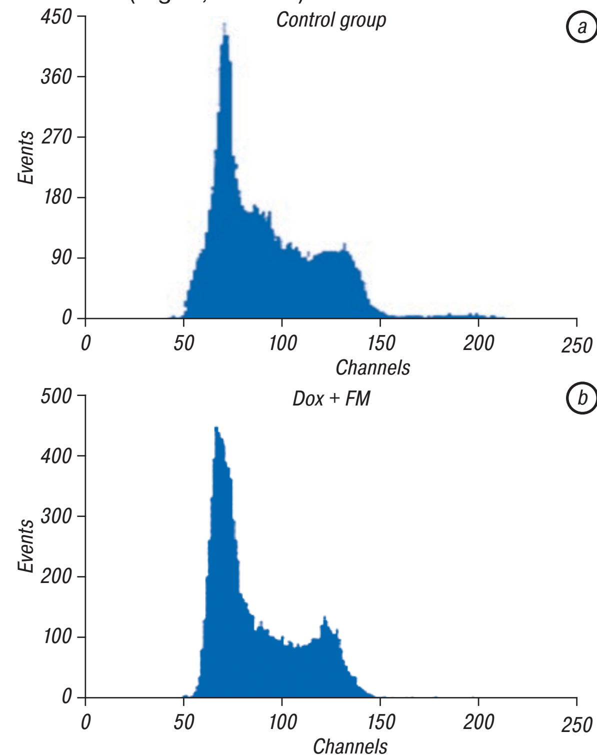

Fig. 3. The cell cycle analysis (*in vivo*)

Table 3. Distribution of Ehrlich ascitic carcinoma cells treated by FM nanoparticles and Dox by cell cycle phases (*in vivo*)

Cell cycle phases	Number of cells, % (control group)	Number of cells, % (Dox + FM)
$G_0 + G_1$	25.55	41.79
S-phase	61.27	48.65
$G_2 + M$	13.18	9.56

The results of cell cycle study of Ehrlich ascitic tumor cells are presented in Fig. 4 and Fig. 5. Incubation of tumor cells with two agents for 48 h resulted in the decrease of cells number in S-phase and the increase of cells population in $G_0 + G_1$ phase. However, it should be mentioned that Dox concentration used during incubation was varied, but FM concentration was constant. In this case though the picture of cells distribution in cell cycle has changed but was not proportional to the Dox concentration. It apparently indicates that the cell cycle is mainly influenced by FM.

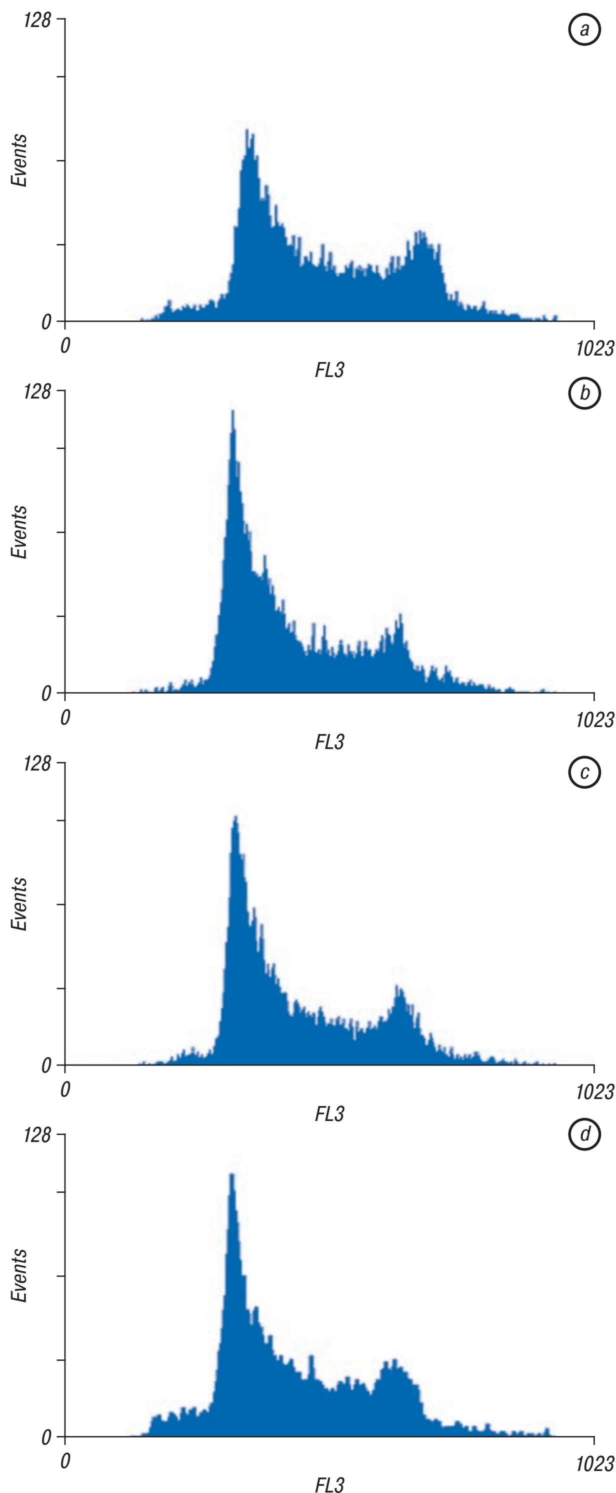

Fig. 4. The cell cycle of Ehrlich carcinoma cells 48 h after incubation with Dox and FM (*in vitro*). *a* — control; *b* — 1 μM Dox + FM; *c* — 10 μM Dox + FM; *d* — 100 μM Dox + FM

Taking into account that many complexes of mitochondria respiratory chain contain iron [15], we have investigated the influence of ferromagnetic nanoparticles on tumor cell oxygen consumption and mitochondria oxidative phosphorylation. Ferromagnetic concentration (Fe content, μg/ml) in experiments

in vitro was choiced according to isoeffectiveness of cytotoxic preparation effect *in vivo*. It was found that 1.45 µg Fe/ml, 7.25 µg Fe/ml and 14.5 µg Fe/ml resulted in the decrease of oxygen consumption rate by tumor cells (by 6%, 51%, 68%, respectively; Fig. 6). The 7.25 µg Fe/ml resulted in the decrease of mitochondria oxygen consumption in state 2 (V_2; $P < 0.05$). But mitochondria oxygen consumption rate in phosphorylation state (V_3; at ATP synthesis) decreased essentially: at 1.45 µg Fe/ml — by 25% ($P < 0.01$), at 7.25 µg Fe/ml — by 37% ($P < 0.001$; Table 4). In turn, it resulted in the decrease of respiratory control and ATP production. But decoupling of mitochondria oxidative phosphorylation was not observed.

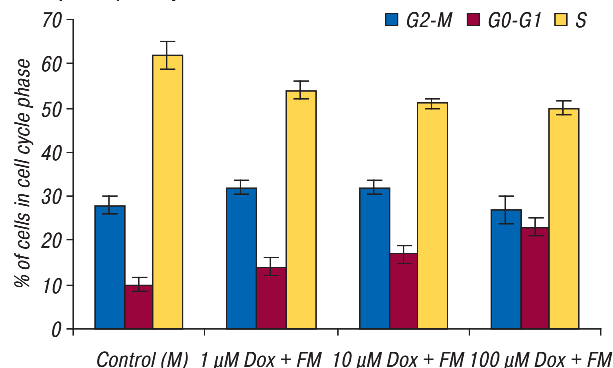

Fig. 5. The distribution of Ehrlich carcinoma cells by cell cycle phases 48 h after incubation with Dox and FM (*in vitro*)

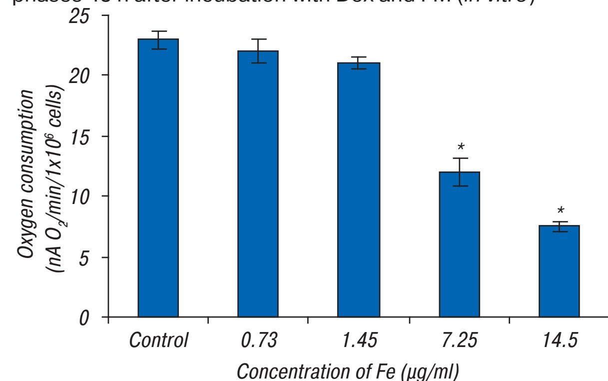

Fig. 6. The rate of oxygen consumption by Ehrlich carcinoma cells incubated with FM nanoparticles
*Significantly different from control (n = 7).

Table 4. The respiration of mitochondria from tumor cells treated by FM nanoparticles (M ± m; n = 7)

Fe concentration	V_2	V_3	V_4	RCI	ADP/O
0 (control)	23.2 ± 0.8	105.6 ± 2.3	23.2 ± 0.8	4.6 ± 0.2	1.9 ± 0.01
1.45 µg Fe/ml	21.0 ± 0.6	79.0 ± 1.6*	21.0 ± 0.6	3.8 ± 0.1*	1.84 ± 0.02*
7.25 µg Fe/ml	20.6 ± 0.7	66.4 ± 1.5*	20.6 ± 0.7	3.2 ± 0.1*	1.83 ± 0.02*

*Significantly different from control; V_2, V_3, V_4 – [(nanoatoms O_2/min) / mg protein]. V_2 – the rate of oxygen consumption by mitochondria in the presence of substrate for oxidation (succinate). V_3 – the rate of oxygen consumption during phosphorylation of ADP added into a polarographic cell. V_4 – the rate of oxygen consumption after ADP transformation into ATP. RCI – the respiratory control index. ADP/O – the index showing the number of ATP molecules synthesized per 1 absorbed O_2 atom.

Thus, the FM nanoparticles (20–40 nm) increase the cytotoxic effect of Dox against Ehrlich ascitic carcinoma. The largest number of apoptotic cells was observed in group of animals in which doxorubicin was administered before FM nanoparticles. Moreover, the FM nanoparticles at concentration 1.45 µg Fe/ml and, particularly, 7.25 µg Fe/ml decreased the mitochondria oxygen consumption in phosphorylation state that may negatively influence their living capability. Obtained results point out the perspective of use of FM nanoparticles to increase the cytotoixc effect of antitumor drugs. However, future research should be also devoted to the study of FM nanoparticles toxic effects on organism.

REFERENCES

1. **Losic D, Rosengarten G, Mitchell JG, *et al.*** Pore architecture of diatom frustules: potential nanostructured membranes for molecular and particle separations. J Nanosci Nanotechnol 2006; **6**: 982–9.

2. **Köger N.** Prescribing diatom morphology: towards genetic engineering of biological nanomaterials. Curr Opin Chem Biol 2007; **11**: 662–9.

3. **Vo-Dinh T, eds.** Protein Nanotechnology, Protocols, Instrumentation, and Applications. Series: Methods in Molecular Biology; Totowa: Humana Press Inc, 2008; 452 p.

4. **Zschornack G, Grossmann F, Ovsyannikov VP, *et al.*** Highly charged ions for high-tech applications. German-Ukrainian Symposium on Nanoscience and Nanotechnology, abstract book; Essen, 2008: 80.

5. **Kentsch J, Durr M, Schnelle T, *et al.*** Microdevices for separation, accumulation and analysis of biological micro- and nanoparticles. IEE Proc Nanobiotechnol 2003; **150**: 82–9.

6. **Neugebauer S, Müller U, Lochmüller T, *et al.*** Characterization of nanopore electrode structures as basis for amplified electrochemical assays. Electroanalysis 2006; **18**: 1929–36.

7. **Greulich C, Kittler S, Epple M, *et al.*** Studies on the biocompatibility and the interaction of silver nanoparticles with human mesenchymal stem cells (hMSCs). Langenbecks Arch Surg 2009; **394**: 495–502.

8. **Saleem M, Meyer MC, Breitenstein D, *et al.*** The surfactant peptide KL4 in lipid monolayers: phase behavior, topography and chemical distribution. J Biol Chem 2008; **283**: 5195–207.

9. **Chekhun VF, Kulik GI, Todor IN, *et al.*** The influence of ferromagnetic nanoparticles on antitumor effect of doxorubicin in Ehrlich ascitic carcinoma-bearing mice. German-Ukrainian Symposium on Nanoscience and Nanotechnology, abstract book; Essen, 2008: 72.

10. **Bilyy R, Podhorodecki A, Nyk M, *et al.*** Utilization of GaN:Eu3+ nanocrystals for the detection of programmed cell death. Physica E: Low-Dimentional Systems and Nanostructures 2008; **40**: 2096–9.

11. **Arruebo M, Galan M, Navascues N, *et al.*** Development of magnetic nanostructured silica-based materials as potential vector for drug-delivery application. Chem Materials 2006; **18**: 1911–9.

12. **Fernandez-Pacheco R, Marquina C, Valdivia JG, *et al.*** Magnetic nanoparticles for local drug delivery using magnetic implants. J Magn Magn Mater 2007; **311**: 318–22.

13. **Galanov AI, Yurmasova TA, Savelyev GG, *et al.*** Development of nanostructured iron-based materials as potential vectors for drug-delivery application. Sib Onkol Zhurn 2008; **3**: 50–7 (In Russian).

14. **Darton NJ, Hallmark B, Han X, *et al.*** The in-flow capture of superparamagnetic nanoparticles for targeting therapeutics. Nanomedicine 2008; **4**: 19–29.

15. **Gautheron DC.** Mitochondrial oxydative phosphorylation and respiratory chain: review. J Inher Metab Dis 1984; **7**: 57–61.

16. **Beilby JP, Prins AW, Swanson NR.** Determination of hepatic iron concentration in fresh and paraffin-embedded tissue. Clin Chem 1999; **45**: 573–4.

17. **Okon E, Pouliquen D, Okon P, *et al.*** Biodegradation of magnetite dextran nanoparticles in the rat. A histologic and biophysical study. Lab Invest 1994; **71**: 895–903.

18. **Van Beers BE, Sempoux C, Materne R, *et al.*** Biodistribution of ultrasmall iron oxide particles in the rat. J Magn Reson Imaging 2001; **13**: 594–9.

19. **Briley-Saebo K, Bjornerud A, Grant D, *et al.*** Hepatic cellular distribution and degradation of iron oxide nanoparticles following single intravenous injection in rats: implications for magnetic resonance imaging. Cell Tissue Res 2004; **316**: 315–23.

20. **Potter AJ, Gollahon KA, Palanca BJA, *et al.*** Flow cytometric analysis of the cell cycle phase specificity of DNA damage induced by radiation, hydrogen peroxide and doxorubicin. Carcinogenesis 2002; **23**: 389–401.

Copyright © Experimental Oncology, 2009