Colony-Forming Activity of Unipotent Hemopoietic Precursors under the Effect of Nanosized Ferrites in a Constant Magnetic Field *in Vitro*

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We studied *in vitro* effect of ferrimagnetic nanoparticles in a dose of 3 mg/liter (10 maximum permissible concentrations) on colony-forming capacity of bone marrow granulocytic and monocytic precursors in a constant magnetic field at magnetic field intensity of 200 Oe. We tested powders obtained by the methods of electrical explosion of conductors (magnetite and a mixture of hematite with magnetite) or mechanochemical synthesis (cobalt ferrite). According to electron microscopy, size of particles was within 6-65 nm. Specific effect of nanopowders on functional properties of hemopoietic and stromal cells were demonstrated; this effect was not related to dissolution of these powders, but had a complex nature. It depends on the size and magnetic characteristics of powder particles, the route and dose of administration, and the presence of external magnetic field. It was emphasized that in multicellular systems a reaction of committed hemopoietic precursors mediated via cells (factors) of microenvironment cannot be excluded, the state of this system varying in different individuals and under different conditions. Our data open new vistas for the creation and targeted use of nanosized materials and technologies for individual therapy in the context of personalized medicine.

Key Words: *mouse bone marrow; granulocytic and monocytic precursors; fibroblasts; magnetosensitive iron oxides*

Biological effect of nanosized particles is discussed from the viewpoint of general toxicity of particles formed during implant wear, biocompatibility and intrinsic specific activity of nanoparticles, and properties of nanoparticles as carriers (vehicles) of drugs and biomolecules.

Active search for biocompatible nanomaterials for the construction of systems for targeted delivery of drugs and biomolecules is focused on liposomes and solid particles [1,6]. Magnetic particles should be corrosion-resistant and bioinert, have appropriate magnetic characteristics, and their size should not exceed 1 μ. Magnetic nanoparticles are promising for the use in biology and medicine. They can be used as a vehicle for retention of the drugs in the organism and their controlled release, in experimental cytology and histology for selective sorp-

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tion on cells for their stimulation, destruction, of removal, in hyperthermia of tumors and pathological foci, as a new class of bioactive compounds (magnetic pharmacology) [8].

At the same time, negative data appear on molecular and cellular effects of nanosized particles. For instance, compounds in the form of ultradispersed powders can be potentially dangerous and should be used with caution [11]. In this context, creation of magnetic nanoparticles for biology and medicine is still an urgent problem [8].

The blood system, central nervous system, respiratory and gastrointestinal tract are the targets for nanoparticles. Nanoparticles can escape phagocytosis, circulate in the blood and lymph, cross biological barriers, and distribute in organs and tissues [15].

Here we studied *in vitro* effects of ferrimagnetic nanoparticles and their composites on colony-forming capacity of bone marrow precursors of ganulocytopoiesis and monocytopoiesis in constant magnetic field.

MATERIALS AND METHODS

Ferrimagnetics (ferries) are complex iron oxides combining the properties of ferromagnetic and a semiconductor or a dielectric. Ferrimagnetic were obtained by the methods of electrical explosion of conductors (magnetite, Fe_3O_4, and a mixture of hematite, α-Fe_2O_3, with magnetite) or mechanochemical synthesis (cobalt ferrite, $CoFe_2O_4$).

Powders of cobalt ferrospinel $CoFe_2O_4$ synthesized by the mechanochemical method are loosely bound nanosized particles with a mean diameter of 6-8 nm (range 3-15 nm) packed into aggregates with a diameter of 265±15 nm (Fig. 1). According to the size distribution histogram, particles with a diameter of 4-8 nm constitute ~65%. According to X-ray analysis and electron microscopy, nanopowders apart from the main substance in crystalline state, contain 1-2 volume percent (vol%) α-Fe_2O_3, an insufficient amount of X-ray amorphous phase.

Fe_3O_4 particles obtained by the method of electrical explosion have spherical (in some cases polyhedral shape with crystalline core and thin amorphous shell (Fig. 2), 85% of these particles had a mean diameter of 40±23 nm (in other fields of measurement 67±2 nm) and others had a diameter of 75-113 nm. According to X-ray diffraction analysis, magnetite constitutes 98-99 vol%, while hematite and pure iron in α-modification constitute 1-2 vol%.

Electroexplosive powder (α-Fe_2O_3+Fe_3O_4) consisted of 44 vol% weakly magnetic hematite and 56 vol% magnetite, the mean diameter of particles was 28±9 nm (Fig. 2).

Magnetic characteristics of nanosized powders are presented in Table 1.

For the study of sanitary and chemical properties we used 0.3-3.0 mg/liter nanoparticles, which is equivalent to 1-10 maximally permissible concentrations (MPC) for iron [5]; these concentration were used in biological part of the study. Nanopowders of magnetite or cobalt ferrite (100 g/liter) were dissolved in isotonic NaCl (0.9%) for 3, 7, 8, and 20 days at 37°C under sterile conditions, changes in pH and concentration of Fe^{3+} ions in the solutions were evaluated (method of stripping voltammetry) [2]. We studied solubility of nanosized magnetite under conditions of 5-day exposure to constant magnetic field with an intensity of 1.3 kOe.

For studies on cell cultures, bone marrow cells from 10 CBA/CaLac mice were used. The animals were sacrificed by ether overdose; bone marrow cells were isolated from the femurs and cultured (5×10^5 karyocytes/ml) for 1 h at 37°C in a volume of 5 ml with nanoferrimagnetic iron oxides Fe_3O_4 or $CoFe_2O_4$ (3 mg/liter cell culture, 10 MPC). To control tubes, an equivalent volume (0.5 ml) of the solvent (0.9% NaCl) was added. Culture medium of the following composition was used: 280 mg/liter L-glutamine (Sigma), 40 mg/liter gentamicin sulfate, 15% FCS (ICN), and 85% medium 199 (Vector).

An aliquot (1.5 ml) of the cell suspension treated with a suspension of ferrimagnetic nanoparticles was used for detection of spontaneous (in the absence of growth factors) colony-forming capacity of the bone marrow during cloning of 7.5×10^5 viable karyocytes over 7 days in 35-mm plastic Petri dishes at 37°C, 5% CO_2, and 100% humidity in a CO_2-incubator. A half of all tested cultures were placed on a magnetic mat with a constant magnetic field intensity of 200 Oe.

The remaining part of the suspension was used for evaluation of cytotoxicity of nanoparticles by staining with 0.4% trypan blue before and 24 h after culturing at 37°C.

At stage II, whole fraction of intact myelokaryocytes (1.5×10^5 viable nuclears) was placed in 24-well Corning-Costar plates in 0.9 ml above-described culture medium. After cell adhesion, the volume was brought to 1 ml by adding nanopowders α-Fe_2O_3+Fe_3O_4, $CoFe_2O_4$, or Fe_3O_4 in a final concentration of 3 mg/liter cell culture. To control tubes, an equivalent volume (0.1 ml) of the solvent (0.9% NaCl) was added. The cells were cloned for 7 days in a CO_2 incubator at 37°C and 100% humidity. A half of all tested cultures were placed on a magnetic mat with a constant magnetic field intensity of 200 Oe (0.02 T).

After 7 days, the number of CFU, clones of the progenitor cells, was evaluated. Granulocytic (CFU-G) and monocyte-macrophage precursors (CFU-M) were counted by colonies containing ≥50 and ≥30 nuclear cells, respectively, with typical granulocyte or monocyte morphology after staining with azure II and eosin

Morphological reaction of NIH 3T3 mouse fibroblasts on synthesized nanosized particles of iron oxides was *in vitro* studied. To this end, nanopowders in the specified dose were applied on cell monolayer or cell suspension (5×10^4/cm^2) was applied on the powder placed on the bottom of Petri dishes. Culturing was performed for 1-3 days at 37°C, 5% CO_2, and 100% humidity in 90% medium 199, 10% FCS, 280 mg/liter L-glutamine, 40 mg/liter gentamicin sulfate, and 10^{-6} M dexamethasone [3].

The data were processed statistically using nonparametric Mann—Whitney *U* test (P_U) and Spearman rank correlation coefficient.

RESULTS

According to X-ray diffraction analysis data, magnetite nanopowder contained an iron phase exhibiting well-known effects on the processes of bone marrow hemopoiesis. In light of this, we studied solubility of nanopowders in a modeled biological fluid. It was found that 7-day extracts of magnetite or cobalt ferrite slightly changed proton concentration in the solvent (control); pH of the solvent was about 6.94 (n=4). Mean pH for nanoparticle concentrations of 1-10 MPC was 97.4-98.3% of the control (n=6). No significant relationship between pH and nanoparticle concentration was observed.

It can be hypothesized that nanoparticles weakly interact with hydroxyl groups and promote accumulation of protons in the solution, though their effect on the model biological fluid did not exceed allowable limits for medical materials (±1.0 pH units).

Stripping voltammetry detected 42-56 μg/liter Fe^{3+} ions in 3-20-day solutions of nanosized magnetite (the concentration of Fe^{3+} ions in pure solvent was <1μg/liter). This concentration did not exceed 1.4-1.9% of theoretically possible release of pure iron from the solid phase into the solution, which had no appreciable biological effect for nanoparticle concentration range of 1-10 MPC. In the dynamics of dilution process, a decrease in the level of iron ions in the solution (with a rate of 0.89 μg/day) was noted (Fig. 3).

In constant magnetic field, the release of Fe^{3+} ions from the magnetite into the solution also decreased. According to regression equation, substance accumulation was 26 μg/liter (n=4) *vs*. expected value of 54 μg/liter (Fig. 3).

Thus, ferrimagnetic nanopowders are magnet-sensitive weakly soluble materials, which can be used for the creation of nanosystems for the delivery of drugs and biological preparations.

The study of biological inertness of artificial nanosized materials (cytotoxic test with trypan blue), survival of bone marrow cells was 77% (Fe_3O_4)-80% ($CoFe_2O_4$) *vs.* 85% unstained myelokaryocytes in the control. These data suggest that nanosized particles did not induce necrotic changes in cell membranes.

Methods of evaluation of functional activity of cells are highly sensitive to the nature of the test materials [7,14]. For instance, ferrimagnetic pow-

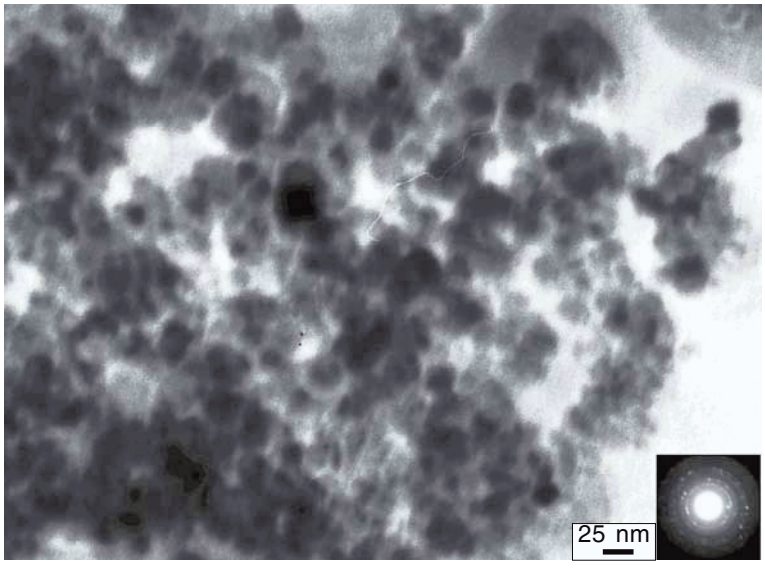

Fig. 1. Transmission electron microscopy of cobalt ferrite powder, ×200,000. Insert: microelectronogram of powder particles.

TABLE 1. Main Magnetic Characteristics of Ferrimagnetic Nanopowders at Room Temperature

Parameter	α-Fe_2O_3+Fe_3O_4	$CoFe_2O_4$	Fe_3O_4
Specific magnetic moment, σ, Gs×cm^3/g	2.7	48	71
Residual magnetization (after removal of magnetic field), Gs×cm^3/g	—	0	10

ders applied on NIH 3T3 fibroblast monolayer did not change the cell morphology and number of dead cells. In other experiment, adhesion of stromal cells to plastic coated with the nanopowder decreased in zones of nanoparticle accumulation. On day 3, the monolayer was formed only in the presence of magnetite, but not cobalt ferrite.

The results of cultural experiments also depended on the route of application of ferrimagnetic nanoparticles (Tables 2 and 3). Preliminary short-term co-culturing of nanoparticles with myelokaryocytes theoretically promotes at least their fixation on cytoplasmic membranes. Nevertheless, no significant reaction of progenitor hemopoietic cells was noted during their further cloning (Table 2).

Nanosized particles of magnetite and hematite added to myelokaryocyte culture on the plastic stimulated the yield of CFU-G by 50% (Table 3). The colony-stimulating effect of nanoparticles directly correlated with their mean size (100%).

The external magnetic field in the used doses was biologically intert and had no effect on the growth of granulocytic and monocytic precursors. At the same time, nanosized ferrimagnetics enhanced or inhibited proliferation capacity of CFU-G acting as modulators of the effect of constant magnetic field (Tables 2 and 3). In turn, the modulating effect of nanoparticles depended on the application route, phase composition, size, magnetic characteristics, and probably, irregular distribution in the cell culture (Fig. 4). In contrast to magnetite and its mixture with hematite, cobalt ferrospinel characterized by the least particle diameter and medium magnetic characteristics had little effect on the growth of CFU-G and CFU-M in the magnetic field (Table 2, 3). This phenomenon can be explained by considerable differences in magnetic characteristics of the ferrimagnetic nanopowders used in our study (Table 1).

The size can considerably modulate magnetic characteristics of particles in the nanodispersed state [10]. The decrease in the diameter of metal particles to a certain critical state is accompanied by violation of domen structure, which is associated with an increase in coercitive force, while further decrease in the particle size can lead to the appear-

TABLE 2. Content of Hemopoietic Precursors (per 10^5 Nuclears/cm^2) after Preliminary Mixing of Myelokaryocytes with Ferrimagnetic Nanoparticles

Sample (n=4)	Without constant magnetic field		Constant magnetic field	
	CFU-G	CFU-M	CFU-G	CFU-M
Control	2.03	0.94	1.43	0.88
Fe_3O_4	1.08	1.2	2.85*	1.90
$CoFe_2O_4$	2.08	2.37	2.19	2.24*

Note. *p=0.04 compared to the control.

TABLE 3. Content of Hemopoietic Precursors (per 10^5 Nuclears/cm^2) after Addition of Ferrimagnetic Nanoparticles to Myelokaryocyte Cultured on Plastic

Sample (n=4)	Without constant magnetic field		Constant magnetic field	
	CFU-G	CFU-M	CFU-G	CFU-M
Control	5.27	3.86	6.18	3.20
Fe_3O_4	8.29*	5.65	5.08	3.58
α-Fe_2O_3+Fe_3O_4	7.91*	5.27	4.14$^+$	3.21
$CoFe_2O_4$	4.52	3.01	5.84	3.95

Note. *p=0.02 compared to the control; ^+p=0.03 compared to values without magnetic exposure.

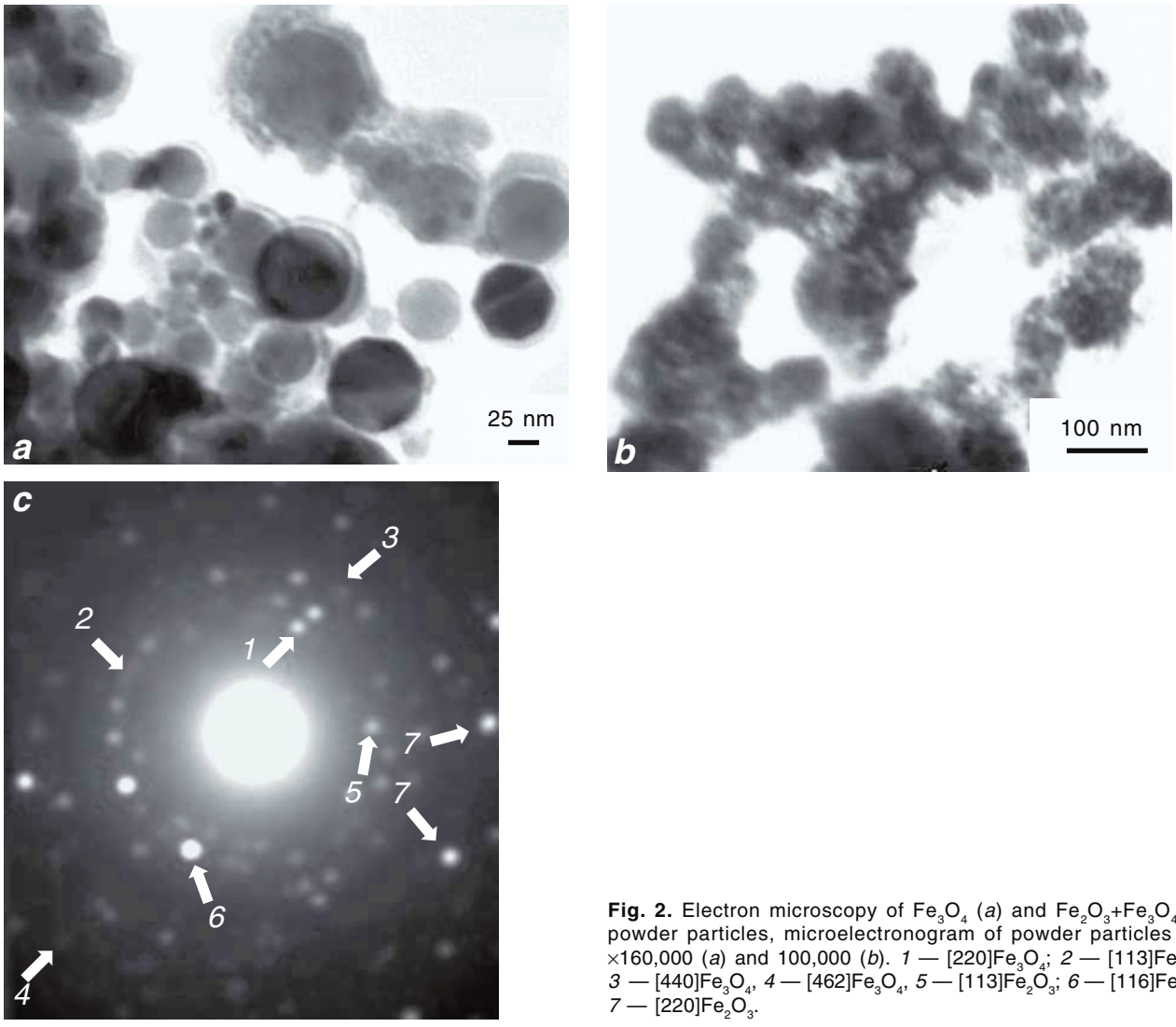

Fig. 2. Electron microscopy of Fe_3O_4 (*a*) and Fe_2O_3+Fe_3O_4 (*b*) powder particles, microelectronogram of powder particles (*c*). ×160,000 (*a*) and 100,000 (*b*). *1* — [220]Fe_3O_4; *2* — [113]Fe_3O_4, *3* — [440]Fe_3O_4, *4* — [462]Fe_3O_4, *5* — [113]Fe_2O_3; *6* — [116]Fe_2O_3; *7* — [220]Fe_2O_3.

ance of supermagnetic properties and reduction of their capacity to respond to external stationary magnetic field.

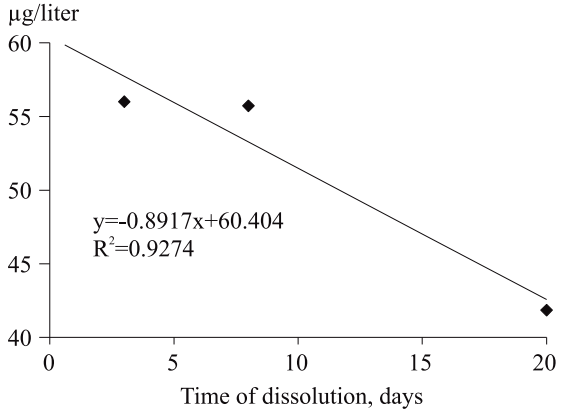

Fig. 3. Dynamics of dissolution of nanosized magnetite particles. Ordinate: concentration of iron ions in the solution, µg/liter. Each point represents a result of 4 measurements.

Fundamental magnetic properties of nanosized powders of ferrimagnetic oxides Fe_3O_4 and $CoFe_2O_4$ differ considerably from the properties of these compounds in the solid state [9]. Reduction of the size of the structural element from 10^5 to 3-15 nm led to the appearance of the properties of clustered spin glass in these compounds. In this state, spins are frozen in certain, but varying from one point to another directions, which leads to the loss of long-range order in the system. The specific magnetic moment σ of magnetite and cobalt ferrospinel considerably decreases to 71 and 48 Gs×cm^3/g, respectively (Table 1), but remains high enough for biomedical purposes, *e.g.* for immobilization and delivery of drugs or for hyperthermia therapy.

Large nanoparticles (mean diameter 28-67 nm) with extreme magnetic parameters produced appreciable functional effect on progenitor cells of granulocytopoiesis both in the magnetic field and without it (Table 1). Hence, the size of particles can be

more important than magnetic properties for the choice of further trends in the use of ferrimagnetic nanoparticles in biology and medicine.

The results of our experiments attest to possible dose-response relationship of the biological effect of nanoparticles in the absence and presence of a magnetic field (Fig. 5). According to study protocol, when ferrites were added to myelokaryocytes before their seeding onto plastic, the final concentration of artificial nanosized particles was 0.9 mg/liter (3 MPC), *i.e.* 30% of the initial dose.

Moreover, fibroblasts, elements of the hemopoiesis-inducing environment essential for proliferation and differentiation of progenitor and mature granulocytes [4], also can be a target of magnet-sensitive ferrites. Similarly to NIH 3T3 fibroblast culture, addition of nanosized magnetite, but not cobalt ferrite to whole bone marrow cell culture promoted adhesion of stromal cells, formation of cell-cell contacts along the magnetic field lines (Fig. 4).

Iron oxide nanoparticles (mean diameter 15-60 nm) can be passive or active agents for biological targets [12], which implies their use in various spheres of nanomedicine, including biomarkers, molecular diagnostics, systems of drug delivery and release, and cell technologies [15].

High specific surface area of the powders determines their high activity in cell environment [15]. Specific effect of nanopowders on functional properties of hemopoietic and stromal cells were demonstrated; this effect was not related to dissolution of these powders, but had a complex nature. It depends on the physicochemical properties of powders, route and dose of administration, and the presence of external magnetic field. Moreover, in multicellular systems a reaction of committed hemopoietic precursors mediated via cells (factors) of microenvironment cannot be excluded, the state of microenvironment varying in different individuals and under different conditions.

Our data open new vistas for the creation and targeted use of nanosized materials and technologies for individual therapy in the context of personalized medicine [13]. For instance, cobalt ferrit due to its magnetic properties and the absence of residual magnetization (Table 1), small size of particles and biological inertness towards the pool of hemopoietic stem cells is a promising compound for the creation of a vehicle for targeted delivery of drugs and biomolecules. Functionally active nanosized magnetite can be used in cell technologies.

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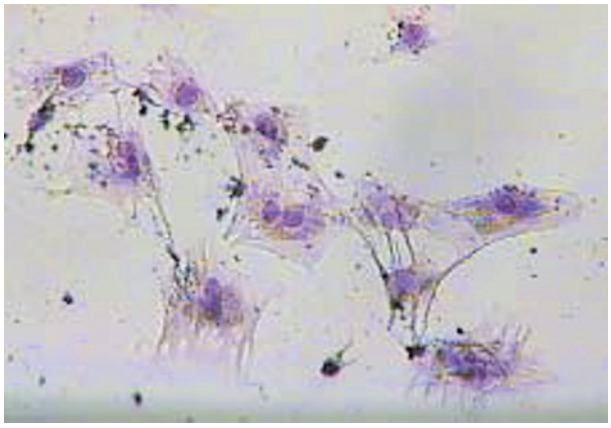

Fig. 4. Stromal mechanocytes in bone marrow culture exposed to nanosized magnetite and constant magnetic field (×700).

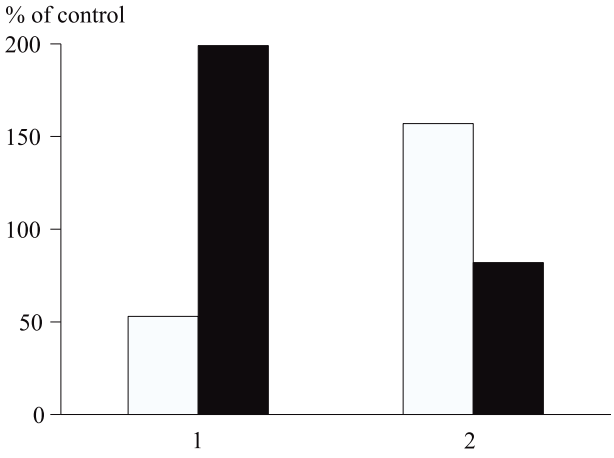

Fig. 5. Dependence of the growth of unipotent granulocytic precursors on the dose of magnetite under intact conditions (light bars) and during exposure to external magnetic field (dark bars). Abscissa: dose of nanoparticles, maximum permissible concentration 3 (*1*) and 10 μg/liter (*2*); ordinate: amplitude of the effect.

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