British Journal of Medicine & Medical Research
2(1): 21-30, 2012

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Effect of ^{67}Zn-Nanoparticles on Leukemic Cells and Normal Lymphocytes

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Research Article

Received 20th August 2011
Accepted 21st November 2011
Online Ready 8th December 2011

ABSTRACT

Porphyrin-fulleren-based nanoparticles (NP), containing magnetic isotopes ^{25}Mg, ^{67}Zn and zinc of natural isotope composition (Zn_{total}-NP), have been tested on leukemic cells of patients with T-ALL, B-ALL, AML and lymphocytes of healthy donors. Reliable differences in action of magnetic and non-magnetic zinc isotopes for some types of cells were obtained. Magnetic magnesium isotopes and pure nanoparticles of porphyrinfulleren did not demonstrate any effects. ^{67}Zn-NP induced high cytotoxicity in cells of acute B-lymphoblastic leukemia with LD_{50} almost three times lower, than those for healthy donors, and 4 times lower in comparison with Zn_{total}-NP. Also evaluation of apoptosis process in granulocytes of healthy donors in the case of the preparates were performed by method of flow cytometry.

Keywords: Fullerene-based nanoparticles; Lsotopy; ^{67}Zn; ^{25}Mg; apoptosis; MTT-method; flow cytometry;

ABBREVIATION

ALL – acute lymphoid leukemia; AML – acute myeloid leukemia; NP – nanoparticles; SOD – superoxide dismutase; RPMI – Roswell Park Memorial Institute medium; PSF – Penicillin-Streptomycin-Fungizone; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide; An-FITC – annexin; V-fluorescein isothyocyanate; PI - propidium iodide; $Zn_{comm.}$ – zinc with natural composition of isotopes;

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1. INTRODUCTION

Zinc is an essential microelement that performs multiple functions in the organism. Its action as the apoptosis effector is of particular importance. Apoptotic events may be increased or inhibited (*Colvin et al., 2010*) in dependence on Zn-deficiency or, on the contrary, due to elevation of its intracellular concentration. In particular, it takes place due to participation of zinc in action of antioxidant enzyme superoxide dismutase (SOD) (*Mulligan et al., 2008*). In connection with the fact, that during treatment of cancer diseases a great attention is paid to proliferation, apoptosis and necrosis, than the concentration of zinc in the organism and in a diet becomes an important factor, determining the degree of effects of drugs on cancer cells. However, zinc may itself act as stimulator of apoptosis in the case of cancer in corresponding concentrations and also in combination with other factors (*Donadelli et al., 2009*). Magnetic isotope effect in biochemical reactions, which have been revealed recently (*Buchachenko et al., 2008, 2010*), contributed an additional intrigue to effector action of zinc, because magnetic nuclei of isotope ^{67}Zn constitute 4 % of natural isotope composition of zinc.

Earlier water-soluble nanoparticles of porphyrin-fulleren^{7} (NP), which are able to be accumulated in cardiac muscle, were synthesized in order to deliver magnetic isotope ^{25}Mg to tissues (*Rezayat et al., 2009; Kuznetsov et al., 2007*). In the present work we used these NP to deliver magnetic isotope ^{67}Zn to the organism in order to compare its action with action of zinc of natural isotope composition on cancer cells of the blood. The effect of magnetic isotope of zinc on cells has been studied for the first time.

2. MATERIALS AND METHODS

2.1 Nanoparticles

Nanoparticles (NP), obtained earlier (*Kuznetsov et al., 2007*), were used: Buckminster fullerene (C_{60})- 2 - (butadiene-1-yl) - tetra (o- γ – aminobutyryl- o -phtalyl) porhyrin), into which zinc of natural isotope composition or 96% ^{67}Zn, or (for comparison) magnetic nuclei of ^{25}Mg were introduced. NPs, which lack metal ions, were used as a control.

2.2 Studying of Morphology of Biomaterials

Distribution of iron-containing particles, being studied with use of atomic force microscopy (AFM) (*Rezayat et al., 2009*), were used to compare morphology of particles inside membranes of cell organelles. Fuji RX40 films were used for ^{59}Fe-autoradiography of isolated organelles with subsequent microdensitometry of the negative with X-ray electron microscope Farrand XL30 (*Amirshahi$_a$ et al., 2008*). Measurement of radioactivity was made in dioxane (scintillation liquid LKB) at liquid scintillation detector LKB SK260.

2.3 Distribution of Nanoparticles in Components of the Blood

It was detected (Amirshahi**$_b$** et al., 2008) after single injection intravenously of ^{59}Fe-NP (30 mg kg^{-1}, 470-520 Cu kg^{-1}) in the blood components of adult rats, Wistar Albino Glaxo (180-220 g), after certain time periods (3-4 animals at one point).

2.4 Isolation of Mononuclear Cells from Bone Marrow and Periferic Blood

All manipulations were made under sterile conditions in a chamber with laminar flow of air (Flow Lab."Clean Air" Netherland). Bone marrow (BM) or blood cells were diluted by standard medium RPMI (Roswell Park Memorial Institute medium) with PSF (penicillin, streptomycin, fungizone) in relation 1:1, were layered on ficoll (Lymphocyte separation medium, ρ=1.077 r/mL) and centrifuged at 1800 rev/min at 18 C. Interphase ring, containing mononuclear cells, was collected into sterile tube. The cells suspension obtained was diluted by the same standard medium up to reach a volume of 10 mL with subsequent washing and centrifugation.

Cells pellet obtained was re-suspended by the medium and amount of cells was counted in Goryaev chamber according to standard procedure.

2.5 MTT-Method

This method (Veerman et al., 1990; Kaspers et al., 1993) is based on the ability of dehydrogenases of living cells to convert 3-(4,5-dimethylthiazole-2-yl)-2,5-tetrazolium bromide (MTT) of yellow color into insoluble purple-and-blue crystals of MTT-formazan, which were extracted by isopropanol and determined on reader (Microplate Reader 550, Bio-Rad) at λ=550 nm. Intensity of conversion MTT→MTT-formazan reflects the total level of dehydrogenase activity of cells, i.e. a final effect of cytotoxicity of injected preparation (Weisenthal et al., 1985). Mononuclear cells were diluted up to concentration $2x10^6$ mL^{-1} by standard medium. The cell suspension was equally distributed among all wells of 96-well plate 80 mkL in each. Different dilutions of the tested preparations 20 μL were placed into the wells with duplication of each sample. A number of wells contained 20 μL of cultural medium to control cells viability. Extreme concentrations of pure nanoparticle were also used as a control. Six concentrations of each drugs with four fold steps of dilution were prepared.

Next groups were investigated: healthy donors (n=5), blast cells of patients with acute myeloid leukemia – AML (n=5), blast cells of patients with acute B-cell leukemia –B-ALL (n=5) and blast cells of patients with acute T-cell leukemia –T-ALL (n=5).

Plates were incubated in humid incubator with 5% content of CO_2 during 4 days at 37 C. Then 10 μL of MTT solution diluted by 0.9% NaCl up to concentration 5 mg mL^{-1} was added to each well, after what the incubation procedure was repeated during 6 hours.

Crystals of MTT-formazan formed were dissolved in 100 mkL of isopropanol with 2N HCl in rigorous stirring and determined optical density. Viability of cells (CS) was determined according to formulae:

$$CS = (D_{exp.wells} - \text{blank-control}) / (D_{contr.wells} - \text{blank-control}) \, 100\%$$

where $D_{exp.wells}$ – absorbance of sample wells (mean value of two douplets), $D_{contr.wells}$ – absorbance of control wells (mean value of optical density of wells with medium and cells without drugs). Each experiment was multiplied 5 times for each cell line. Statistic treatment of data obtained was performed with the use of U-test of Mann-Whitney (taking into consideration variations at $p<0.05$ as reliable ones). Values of LD_{50} is represented at the Table 1.

Table 1: LD_{50} of drugs containing ^{67}Zn and Zn_{total} in different cell types.

	LD_{50}, µg/mL			
	T-ALL	**B-ALL**	**AML**	**Healthy donors**
Zn-67	81 ±9	21 ±3	85 ±9	60 ±6
$Zn_{comm..}$	>100	81 ±8	67 ±7	56 ±5

2.6 Assessment of Apoptosis Induction

Assessment of effects of the preparations on apoptosis of periferal blood granulocytes in healthy donors was performed with the use of reagent kit FITC Annexin V Apoptosis Detection Kit 1 (BD Pharmingen). Staining of cells by annexin–V conjugated with fluorochrome (An-FITC) and propidium iodide (PI) represents an optimal method adopted for apoptosis documentation, which is performed by stress-oxidation on cytometer, because living cells do not bind annexin and are not permeable for cationic dyes (Omerod, 1994). Oxidative stress in granulocytes was induced by Ca-ionophore ionomycin (0.1 µmole L^{-1}).

Granulocytes from the blood of healthy donors were prepared with the use of gradient centrifugation on ficoll with density 1,050 g/mL. Then 10 mln. of cells was diluted up to 1 mL with the medium. 200 µL of suspension obtained was transferred to tubes for cytometry, 2 mL of phosphate buffered solution (PBS) was added to each tube and sedimented on centrifuge at 1000 rev/min during 5 min. at 18 C. The pellet was re-suspended in 200 µL of the buffer and suspension was transferred into two tubes. 5 µL of An-FITC and 10 µL PI were added to one of them. Another tube contained non-stained cells and served as negative control and for choosing amplification parameters. The samples were shaken on vortex and incubated in dark conditions during 15 min at room temperature. After that 400 µL of the same buffer was added to samples and analysis was performed on cytometer FACScan (Becton Dickinson, USA) with excitation of fluorescence at λ=488 nm and registration of emission in green band at channel FL1 (525 nm), and red band – at channel FL2 (585 nm).

Another part of suspension was transferred by 100 µL into wells of 24-well plate, 100 µL of preparations with concentration of 10 mg mL^{-1} was added to each well and then the medium was added to each well up to total volume of 1 mL. Control well contained 100 µL of cell suspension and 900 µL of medium. Incubation was performed during 24 hours in humid incubator with 5% of CO_2 at 37C, after that the cells were washed by phosphate buffered solution and then assessment of spontaneous apoptosis was performed. To assess the results CELLQuest program was used with next parameters: living (intact) cells do not bind both An-FITC or PI, earlier apoptotic cells are stained by An-FITC only, late apoptotic cells are stained by An-FITC and PI, necrotic cells are stained by PI only.

Concentration of 10 µg/mL was used, because in concentration of preparation equal to 100 µg mL^{-1} viability of cells was close to zero. Selection constituted 10 samples.

3. RESULTS AND DISCUSSION

The distribution of NPs in blood showed (*Rezayat et al., 2009*) that the NP accumulation was observed in lymphocytes (of rats) during 120 h, that is interesting for medical purposes, because it makes them to be used.

On Fig.1 it was shown, that penetration of NPs into human lymphocytes through cell membrane, that was detected by the method of electrophoretic perfusion with subsequent autoradiography. This gives a basis for delivering of zinc ions to blood cells by nanoparticles.

Results of MMT-test in the form of dose-effect dependence on different types of cells for drugs studied are presented on Fig. 2-5.

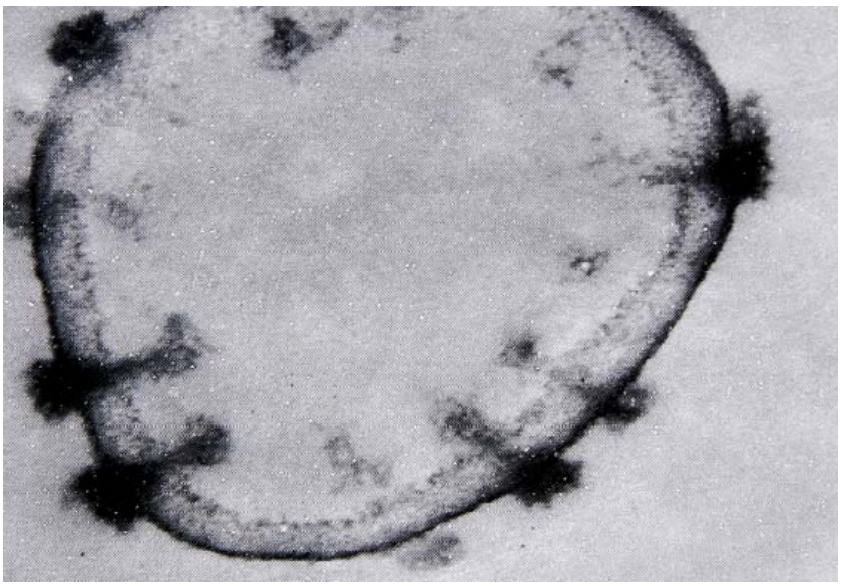

Figure 1: Penetration of nanoparticles into the human lymphocyte at pH 8.7 (7.5 mA/cm^2, 120-140 mV/cm^2).

It is obvious, that there is no dependence on concentration of ^{25}Mg-NP for any type of leukemic cells, when more than 80% of cells survive in their presence up to concentration of preparation equal to 100 μg mL^{-1}. The same picture was observed in the case of pure NP (data not shown).

In case of healthy donors blood (Fig.2) zinc-containing nanoparticles expresses cytotoxic activity starting at concentration ~10 μg mL^{-1}, however, there is no difference between effects of the isotope ^{67}Zn and zinc of total isotope composition. Almost equal results (i.e. without reliable differences) were observed in case of cells T-ALL (Fig.3).

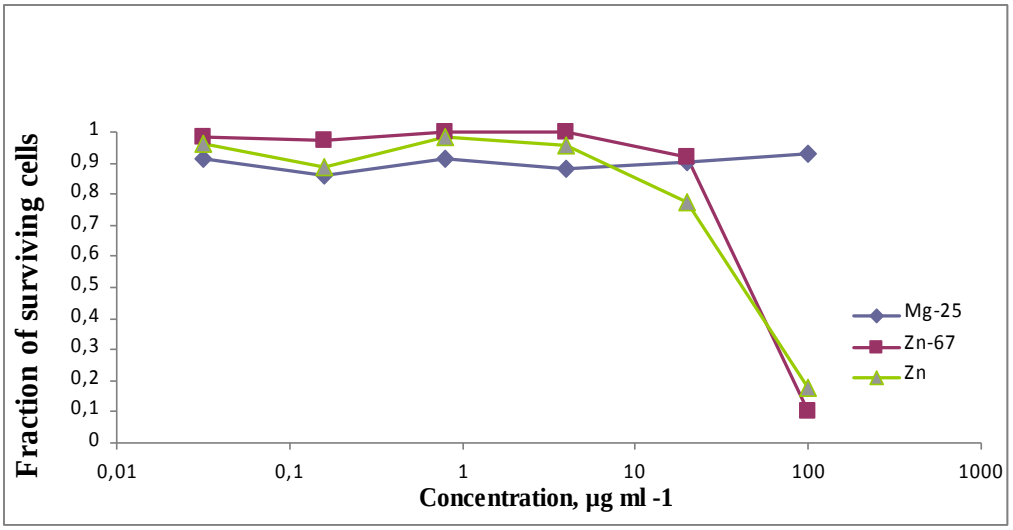

Figure 2: The dependence of the surviving cell fraction from healthy donors on concentration of injected drugs.

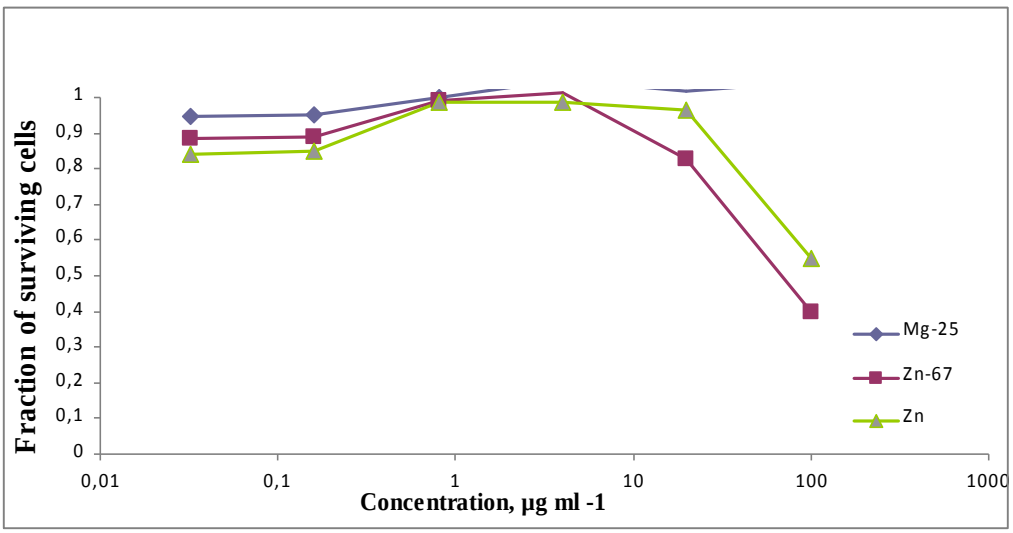

Figure 3: The dependence of surviving leukemic cell fraction from T-ALL patients on administered drugs concentration.

The most interesting results were obtained for AML and B-ALL cells.

In the case of AML reliable differences between action of ^{67}Zn and Zn_{total} have already seen at the smallest concentration of the preparations (Fig.4). ^{67}Zn-NP acts similar to ^{25}Mg and pure NP till concentrations equal to ~15 µg mL^{-1} (cells viability exceeds 85%), but at more high concentrations behaves as in the case of healthy donors. Effects of $Zn_{total.}$-NP, being less dependent on the preparation concentration, exhibits more great toxic action (viability of cells 50-60%). Reliable differences between effects of these two preparations disappear at

concentration >15 μg mL^{-1}, i.e. due to standart increasing of zinc ions cytotoxicity. This strange behavior of zinc ions may be due to parallel disorders in homeostasis of zinc against AML. In the future it is necessary to pay attention to this when diagnosing patients. The behavior of the magnetic isotope becomes clear if one involve the signaling pathways associated with the depletion of ATP. Zn-67 in this case is able to cancel these disturbances.

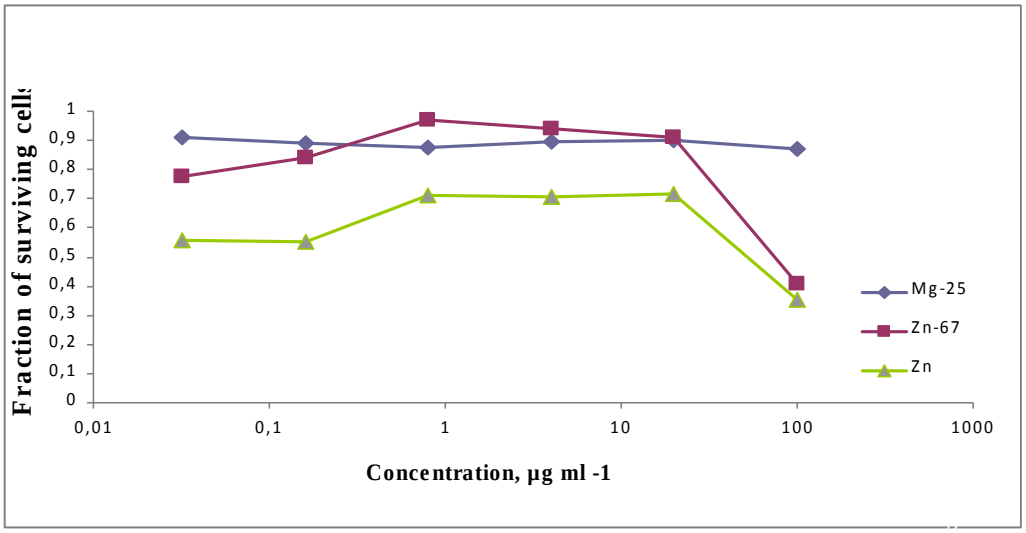

Figure 4: The dependence of surviving leukemic cell fraction from AML patients on administered drugs concentration.

In the case of B-ALL more high toxicity of ^{67}Zn-Np is observed starting from 1 μg mL^{-1} and these differences are increased with growth of the drug concentration (Fig.5).

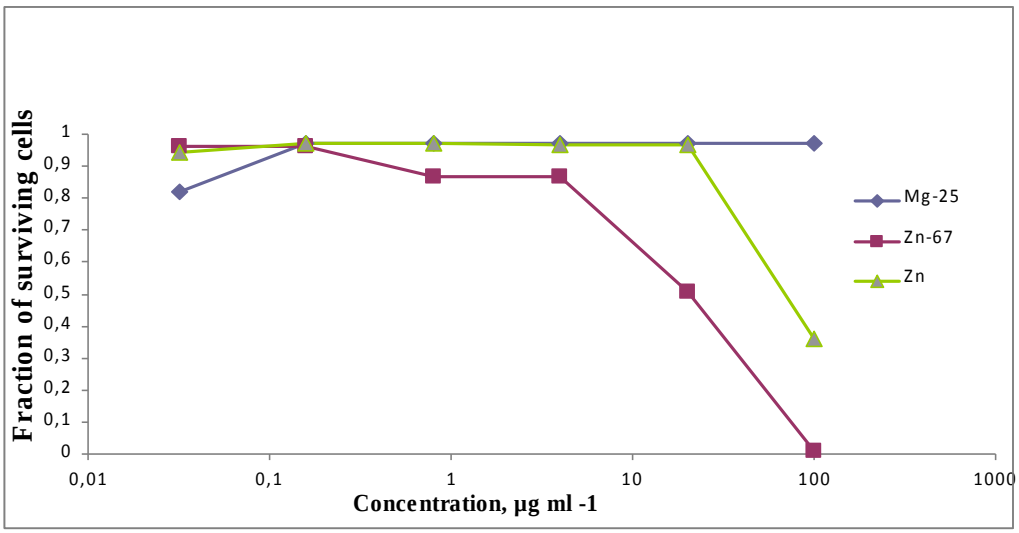

Figure 5: The dependence of surviving leukemic cell fraction from B-ALL patients on administered drugs concentration.

The method of flow cytometry was used on granulocytes of healthy donors in order to reveal the link between the difference in action of magnetic and non-magnetic zinc nuclei and oxidative stress and, consequently, with participation of zinc ions in the activity of superoxide dismutase and other antioxidant structures. The level of viability of intact cells till introduction of the drugs is considered as 1.00 on Fig.6. This level was unchanged in the case of ^{25}Mg-NP addition. This result evidences that the energetic component is not principal for viability of healthy cells under action of oxidative stress, taking into account the fact, that ^{25}Mg plays an important role in the energetic processes in a cell, increasing yield of ATP (*(Buchachenko et al., 2008*). In the presence of zinc-containing nanoparticles viability of healthy cells was increased by 3- (^{67}Zn-NP) and 4-fold ($Zn_{gen.}$-NP). Thus, the differences in action of zinc isotopes on the process are not sufficient. More fine discrimination between "zones of influence" of zinc isotopes is to be a goal for future investigations. At the moment, it is possible to conclude the next:

- Effects of magnetic and non-magnetic isotopes of zinc are differed in the case of some types of leukemic cells and these differences are not directly linked with energetic processes (because there is no effect of ^{25}Mg);
- There are no facts confirming the different influence of magnetic and non-magnetic isotopes on the lymphocytes of healthy donors;
- The differences in effects of magnetic and non-magnetic zinc isotopes on apoptosis, induced by oxidative stress, are not sufficient and require further and more precise investigation.

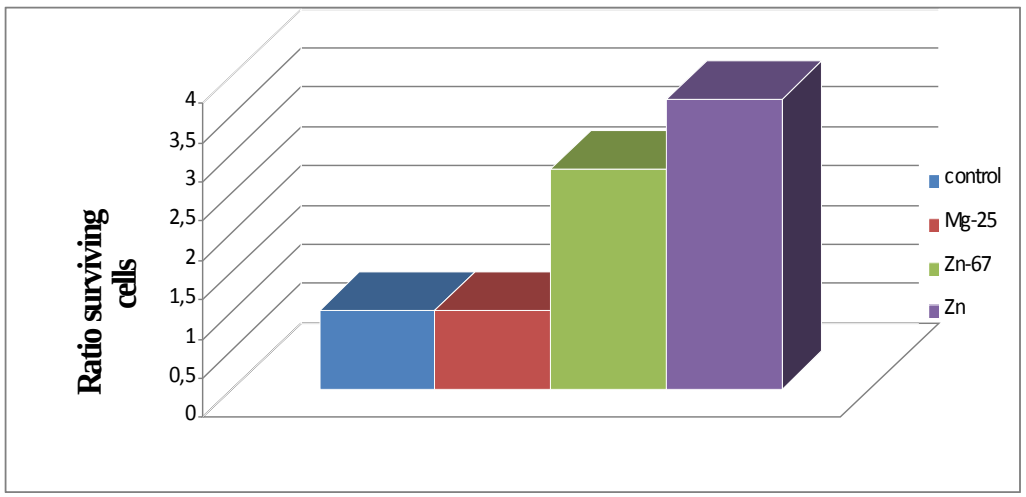

Figure 6: Fraction of viable granulocyte cells from healthy donors under oxidative stress-induced apoptosis in the presence of drugs (10 mg/mL) compared to control.
Fraction of viable cells in the control (incubation without drugs) is taken as 1 (blue column).

4. CONCLUSION

It is obvious, that the alterations, taking place in cells in the case of acute leukemia of different type, are significantly differed by degree of response to zinc ions action. The

smallest differences in comparison to healthy donors are observed in the case of T-ALL. Moreover, the B-ALL and T-ALL cases are differed principally in their responses to action of magnetic nuclei isotope ^{67}Zn in comparison to common pool of zinc. This makes the magnetic isotope of zinc potentially possible therapeutic agent in the case of B-ALL, especially if take into account the possibility of its targeting delivery by fulleren based nanoparticles.

For the present there are no reasons to tell about statistically meaningful differences between action of magnetic and non-magnetic zinc isotopes in suppression of apoptosis induced by oxidative stress. This question requires additional, more precise methods of investigation.

ACKNOWLEDGEMENTS

This work was supported by RFBR grant № 10-08-00680. We are grateful to Prof. Kuznetsov D.A for help in the work, and to Kuznetsov V.I. for kindly providing of isotopes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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