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Effects of Antioxidants on the Viability of the Human Neuroblastoma SH-SY5Y Cell Culture under the Conditions of Heavy-Metal Toxicity

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Abstract

Protective action of antioxidants (mexidol, carnosine, N-acetyl cysteine) and the metal chelator Ca, Na_2-EDTA was studied in the culture of human neuroblastoma SH-SY5Y cells after the addition of salts of heavy metals—lead, cadmium, cobalt, and molybdenum—to the culture medium. Cells were incubated with heavy metals and protectors for 24 h, and cell viability and cell death were evaluated. All the metals lowered cell viability in a concentration-related manner. Different protective agents were studied based on this model. The most pronounced capability of increasing the cell viability in conditions of heavy-metal toxicity was demonstrated by N-acetyl cysteine (the protective effect was demonstrated at the concentrations 0.5-1.0 mM and higher). Protective potential of carnosine was somewhat lower and that of mexidol was minimal.

Keywords: Heavy metals; Lead; Cadmium; Cobalt; Molybdenum, Antioxidants; Carnosine; N-acetyl cysteine; Mexidol; Human neuroblastoma SH-SY5Y cells

Introduction

Environmental pollution by heavy metals presents a risk for human and animal health, being a risk factor for many diseases. Among the most toxic heavy metals are lead (Pb) and cadmium (Cd), the central nervous system (CNS) being one of their main targets. In humans, these metals induce neurotoxic effects accompanied by neurological symptoms [1]. Cadmium can contribute to the development of neurodegenerative conditions including Alzheimer's and Parkinson's diseases [2-4]. Although cobalt (Co) and molybdenum (Mo) are needed for normal cell homeostasis and growth [5,6], high concentrations of these metals induce neurotoxic changes. Cobalt was reported to be genotoxic and carcinogenic [7]. Molybdenum toxicity has been studied insufficiently. Higher concentrations of molybdenum ions in body fluids were reported to inhibit the production of succinate oxidase, sulfite oxidase, glutaminase, cholinesterase, cytochrom oxidase, superoxide dismutase, and other enzymes [8,9] and to interfere with the normal metabolism of copper [10]. The toxicity mechanism of heavy metals is related to their ability to induce the generation of reactive oxygen species (ROS), which can overweigh the antioxidant reserves [11], cause oxidative stress, mitochondrial dysfunction, and cell apoptosis [12-14]. Mechanisms of neurotoxicity by cobalt and molybdenum have been studied insufficiently [14].

Chelating agents are applied for the treatment of heavy-metal poisonings. Different chelating agents are used for particular metals although there are also pluripotent agents such as dimercaprol, aminophenoxyethane-acetic acid (BAPTA), deferoxamine, Ca, Na_2-EDTA, and D-penicillamine. However, the use of chelating agents is limited as they may cause side effects. Another approach to heavy-metal poisonings includes the use of antioxidants such as the vitamins C, E, beta-carotene, alpha-lipoic acid (also used as chelating agent), and melatonin [15]. The antioxidants have been reported to counteract the lead and cadmium accumulation in the body as well as to prevent the oxidative stress [16-20]. Effects of antioxidants under the toxic action of cobalt and molybdenum are insufficiently studied. Further investigation of the toxicity mechanisms as well as the search for antitoxic substances to prevent the negative impact of heavy metals is of current importance.

One of the promising natural antioxidants is carnosine (β-alanyl-L-hystidine), which possesses a membrane protective activity and an ability to bind ions of metals such as copper, cobalt, magnesium, zinc, cadmium, and iron [21,22]. In particular, it is important to further investigate the protective action of carnosine in conditions of heavy-metal toxicity using in vitro cellular systems. Carnosine does efficiently protect the vulnerable membrane-bound enzyme active in the nervous tissue—Na,K-ATPase—one of the key enzymes of antiradical protection [23]. In the human body as well as in other mammals, carnosine is found in skeletal muscles, heart, and specific brain regions (olfactory bulbs). Another biogenic antioxidant—mexidol (ethylmethylhydroxypyridine succinate), possessing antihypoxic, nootropic, and stress-protective action—is used for the treatment of different CNS diseases [24]. At the same time, no data have been found in the literature in favor of its ability to prevent neurotoxic effects of heavy metals.

N-acetyl cysteine (NAC) is a derivative of the sulfur-containing amino acid methionine which is the main source of sulfhydryl groups inactivating ROS [25]. It has neuroprotective properties increasing the activity of antioxidant enzymes [26]. In this study, we investigated protective action of the antioxidants mexidol and carnosine in comparison with N-acetyl cysteine (NAC) and the chelating agent Ca, Na_2-EDTA (hereinafter EDTA) in conditions of heavy-metal toxicity (lead, cadmium, cobalt, and molybdenum) using a culture of human neuroblastoma SH-SY5Y cells.

Materials and Methods

Cell culture

In this study, a fast-growing human neuroblastoma SH-SY5Y cell culture (ATCC®) was used. The cells were cultured in the mixture of Minimum Essential Medium (MEM) Eagle with Earle's salts and glutamine (PanEco, Russia) and F-12 without glutamine (PanEco, Russia) in 1:1 ratio with the addition of 1% penicillin-streptomycin solution (PanEco, Russia) and 10% fetal bovine serum (PAA Laboratories, USA).

The cell culture was kept in an incubator (ShelLab, USA) at 37°C, 90% relative humidity, and 5% CO_2 for 6-7 days. The medium was changed every 3 days.

Cell viability determination using MTT assay

The cells were seeded into 96-well plates with 1.5×10^3 cells per well. For the study of dose-dependent effects of heavy-metal toxicity, the cells were subdivided into 8 groups of 12 wells each: in the fi st group, intact cells were used without adding the heavy metal compounds, while in groups 2-7 the concentration of each heavy metal was 10^{-2} to10^{-8} M. To study the protective effect of antioxidants 3 days after seeding the salts of heavy metals (lead diacetate, cadmium chloride, cobalt chloride and ammonium molybdate), protectors (carnosine, mexidol, EDTA and N-acetyl cysteine) were added in different concentrations (8 wells per group). Incubation time in all cases was 24 h.

Next a solution of the MTT reagent—3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Dia-m, Russia)—prepared using the culture medium at the fi al concentration of 0.5 mg/ml was added. After the incubation of cells with the MTT reagent for 3 h, the medium was removed from the wells. In each well, dried formazan residue was reconstituted in 100 µl dimethyl sulfoxide (DMSO). After that the plates were placed into a Synergy H4 Microplate Reader (spectrophotometer) (BioTek, USA), where the solution in wells was stirred for 10 min, and then the absorbance was measured using light with wavelengths $\lambda = 570$ nm and $\lambda = 660$ nm [27,28]. The value of absorbance measured at $\lambda = 570$ nm was extracted from the value obtained at $\lambda = 660$ nm. The data are presented as percentages of the absorbance values of the control solution in the control wells, which were taken for 100%. The processing of the results was performed using Microsoft xcel software.

Determining the amount of dead cells

The cells were seeded into the 24-well plates, 6×10^4 cells per well. Three days after the passage, the salts of heavy metals (lead diacetate, cadmium chloride, cobalt chloride, and ammonium molybdate) and protective substances (carnosine, mexidol, EDTA and N-acetyl cysteine) were added simultaneously (4 wells per each group). After 24 h of incubation, the cells were detached from the medium using Trypsin-EDTA (PanEco). The cell death rates were determined by staining with the fluorescent dye propidium iodide (PI). PI was added to the cell suspension at the fi al concentration of 10 µM 3 min before the data reading ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 610$ nm). The obtained cell suspension stained with PI was analyzed using FACS Calibur flow cytometer (BD Biosciences, USA). For each measurement of fluorescence intensity, 10,000 events were registered. The results obtained were analyzed using WinMDI 2.8 software (Scripps Institute, LaJolla, USA). The data were processed using Cell Quest Pro (BD, USA) and Microsoft Excel softwares.

Figure 1 shows the population of human neuroblastoma SH-SY5Y cells with the X axis representing the fluorescence intensity distribution of PI. The Y axis represents the amount of events; the two lower quadrants represent living cells; the two upper quadrants represent dead cells characterized by intense PI fluorescence. The proportion of dead cells was estimated as the percentage of the whole quantity of cells.

Statistical analysis

All experiments were performed three times, and results are presented as mean ± SME (M ± m). The data were processed using Statistica 7.0 software. The cell survival rates and the proportion of dead cells in intact cultures were taken for 100% of the cultures. Statistical significance was determined between untreated groups and treated groups, using one-way analysis of variance (ANOVA) and the Student's t-test. A value of $p < 0.05$ was considered to be significant.

Results and Discussion

Different concentrations of the heavy-metal salts were used to evaluate the protective action of the tested substances added to the culture medium. The percentage of living cells in the cell cultures was around 70-75%. As shown in Figure 2, a 25% cell death level was detected after an addition of 10^{-4} M lead diacetate solution. Cobalt chloride induced 30% cell death when added at 10^{-5} M concentration. Cadmium and molybdenum salts had higher toxicity compared to those of lead and cobalt, so the 30% cell death level was reached at a lower concentration of the metal salts (5×10^{-5} M). Further

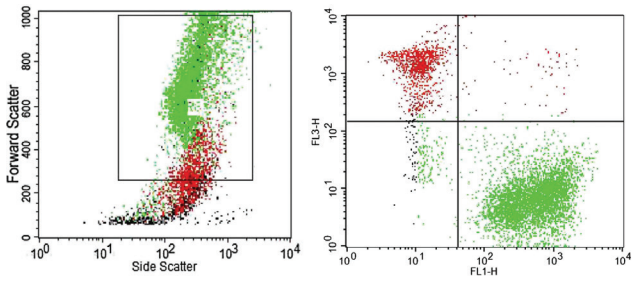

Figure 1: Human neuroblastoma SH-SY5Y cell population in PI coordinates (red FL3-H)

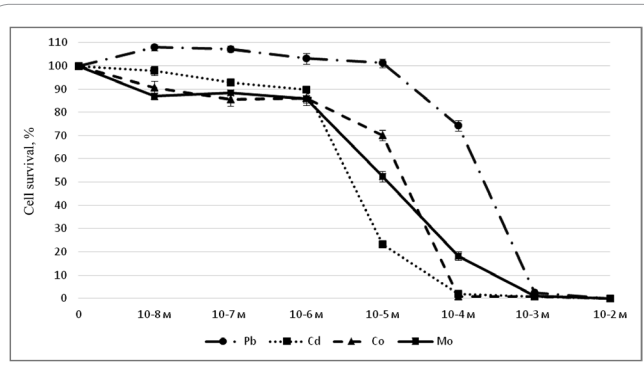

Figure 2: Dose-dependent effects of lead, cadmium, cobalt, and molybdenum toxicities. The intact-cell viability level was taken as 100%

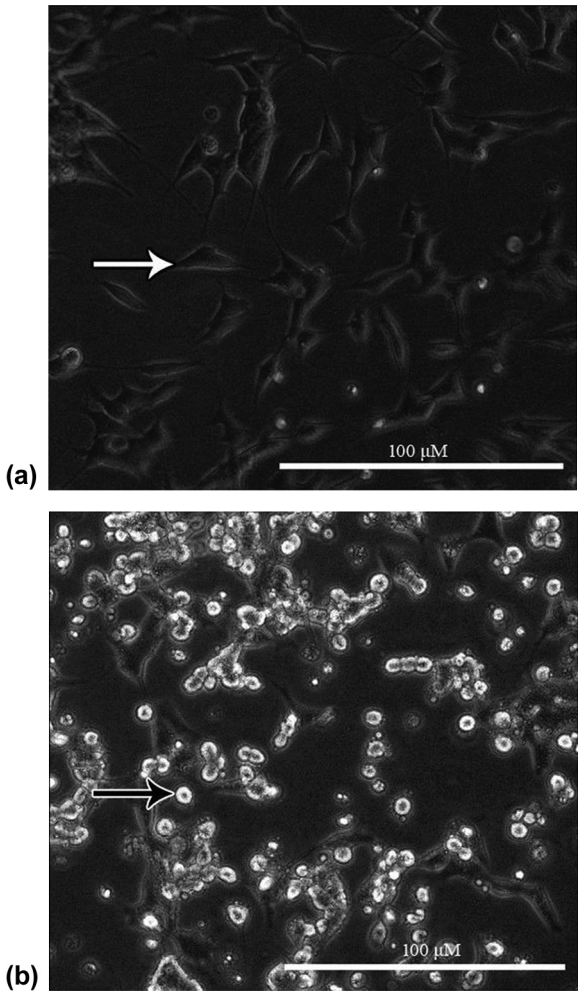

Figure 3: Human neuroblastoma SH-SY5Y cell culture phase contrast microphotographs. A—intact neuroblastoma cells, B—after 1 h of incubation with 10^{-3} M lead. Light arrows indicate living cells; dark arrows indicate dead cells

increase in the concentration of all studied metals resulted in 100% cell death.

It can be summarized that cadmium, cobalt, and molybdenum lowered the cell viability in a concentration-related manner, while 30% cell death rate was observed in the presence of heavy-metal salts at concentrations from 10^{-4} to 5×10^{-5} M. As for lead, a slight elevation of the cell survival curve (from 100 to almost 110 %) within the concentration range of lead diacetate from 0 to 10^{-5} M was noticed (Figure 2). It means that low concentrations of lead elevated the cell survival rate over the level of intact cells, which can be regarded as a hormetic effect.

The obtained results served as the basis for choosing optimal concentrations of heavy metal salts, that significantly reduced viability (lead—10^{-4} M; cadmium—5×10^{-5} M; cobalt—10^{-5} M; molybdenum—5×10^{-5} M) for the purpose of evaluation of the protective action of the tested substances.

Figure 3 represents the cell population of human neuroblastoma before (Figure 3A) and 1 h after an addition of 10^{-3} M lead diacetate solution (Figure 3B). It can be seen on the microphotographs that addition of lead to the medium increases the cell death rate.

Comparative assessment of the protective action of studied substances

Figures 4-7 illustrate the increase in cell viability (%) caused by the protective action of carnosine, mexidol, NAC, and EDTA (at 0.25, 0.5,

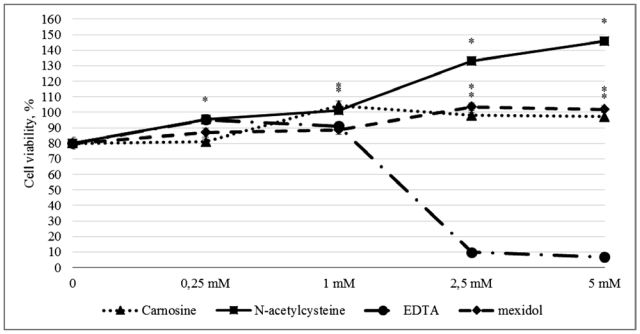

Figure 4: The effect of carnosine, N-acetylcysteine, EDTA, and mexidol at concentrations from 0.25 to 5 mM on cell viability after the addition of lead diacetate solution with a concentration of 10^{-4} M. Note: $*p < 0.05$; the level of significance (p) was calculated for the difference between the treated and untreated (intact) cells

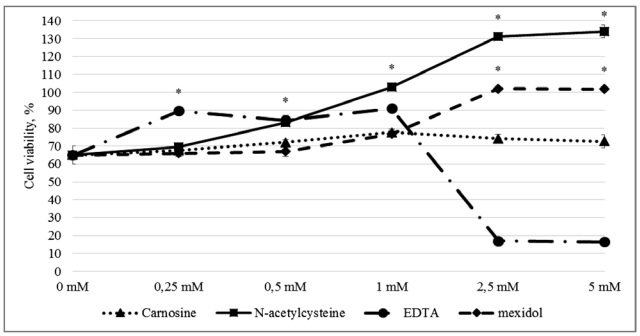

Figure 5: The effects of carnosine, N-acetylcysteine, EDTA, and mexidol at concentrations from 0.25 to 5 mM on cell viability after the addition of 5×10^{-5} M cadmium chloride solution; $*p < 0.05$

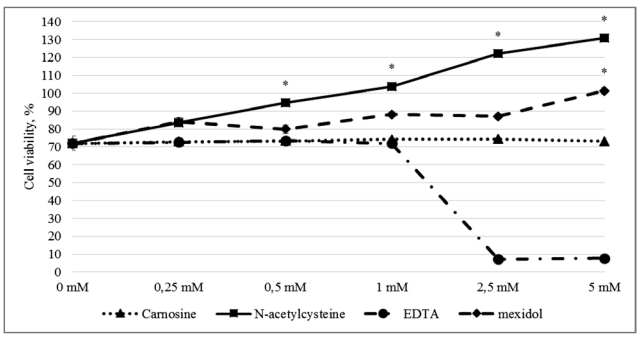

Figure 6: The effect of carnosine, N-acetylcysteine, EDTA and mexidol at concentrations from 0.25 to 5 mM on cell viability after an addition of 10^{-5} M cobalt chloride solution; $*p < 0.05$

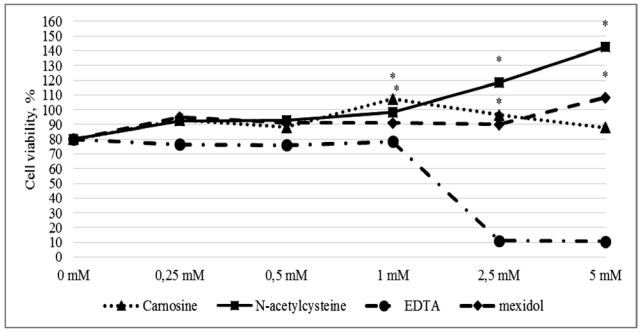

Figure 7: The effect of carnosine, N-acetylcysteine, EDTA and mexidol at concentrations from 0.25 to 5 mM on cell viability after an addition of 5×10^{-5} M ammonium molybdate solution; *$p < 0.05$

1.0, 2.5, and 5.0 mM concentrations) in conditions of lead (Figure 4), cadmium (Figure 5), cobalt (Figure 6), and molybdenum (Figure 7) toxicities.

Under the influence of a lead concentration of 10^{-4} M (Figure 4), the most pronounced protective action was demonstrated for NAC: it started from the concentration of 0.5 mM NAC (100% survived cells) and increased with the increasing concentration of lead diacetate in the culture medium: 1 mM—100%, 2.5 mM—133%, 5 mM—145%. Carnosine enhanced the cell viability up to 100% (the level of intact cells), starting from the concentration of 1 mM, while mexidol did this starting only from the concentration 2.5 mM. EDTA had no significant effect when used in low concentrations, whereas high concentrations (2.5 and 5 mM) resulted in approximately 90% cell death.

Under the impact of 5×10^{-5} M cadmium chloride solution (Figure 5), the most pronounced protective action was noticed for NAC, which started to appear in the presence of 1 mM NAC (100% survived cells) and increased with increasing concentration. The addition of mexidol to the culture medium resulted in 100% cell survival starting from the concentration of 2.5 mM. EDTA used at 0.25, 0.5, and 1 mM concentrations significantly increased cell survival in conditions of heavy-metal toxicity (up to 90%), but the cell survival rate did not reach the intact-cell level. Carnosine did not increase the viability of cells exposed to cadmium.

In conditions of the toxic impact of 10^{-5} M cobalt chloride solution (Figure 6), the most pronounced protective effect was noticed for 1 mM NAC, which under these conditions elevated the cell survival rate up to 100% (intact-cell level). The increase in concentration resulted in further improvement of cell viability: 2.5 mM—120%; 5 mM—130%. Mexidol increased cell survival to 88% when used at 1 and 2.5 mM concentrations reaching 100% level of intact cells when used at the concentration of 5 mM. For carnosin, no protective action was observed.

Under the impact of molybdenum (Figure 7), the most effective protective action was noticed for NAC, which increased the survival to the 100% level starting from the concentration of 1 mM. Addition of carnosine to the culture medium resulted in 100% cell survival at the concentrations of 1 and 2.5 mM, while mexidol only exhibited a similar activity when used at the concentration of 5 mM. EDTA used at low concentrations did not affect cell viability whereas high concentrations resulted in 90% cell death.

An addition of 1 mM NAC, 1 mM carnosine, and 5 mM mexidol to intact cells resulted in approximately 20% increase in cell viability, while 1 mM EDTA resulted in 15% decrease in cell viability (data not illustrated).

Antioxidant protective action and the cell death rate

The concentration of heavy metals used in the MTT assay resulted in a 1.5-time increase in the cell death rate registered on the basis of PI fluorescence intensity (Table 1).

Addition of 1 mM NAC to the culture medium, in conditions of toxicity by all studied heavy metals, lowered the cell death rate (lead, to 46%; cadmium, 69%; cobalt, 34%; and molybdenum, 45%) compared to the control. The similar effect under the same experimental conditions was observed for 1 mM carnosine (lead, to 41%; cadmium, 46%; cobalt, 21%; and molybdenum, 48%). 1 mM mexidol solution lowered the cell death rate in the cultures treated by molybdenum (44%) and cobalt (37%). EDTA used at the concentration of 1 mM had no influence on cell death after the impacts of all studied metals. Adding of NAC, carnosine, and mexidol to the intact-cell cultures did not influence the cell death rate, whereas the addition of EDTA resulted in 14.9% increase in the cell death rate under the same experimental conditions.

The results of this study indicate that addition of each of the four studied metals to the cultured human neuroblastoma SH-SY5 cells produces a decrease in the cell viability and increase in the cell death rate. It is known that the mechanism of toxic action of heavy metals can include the oxidative stress [14]. We have chosen natural (carnosine) and synthetic (mexidol) antioxidants as protectors to be tested. NAC and the chelating agent EDTA, possessing proven antitoxic action in conditions of heavy-metal toxicity [15], were used for comparison.

The highest potential of increasing cell viability in conditions of heavy metal (lead, molybdenum, cobalt, cadmium) toxicity was demonstrated by NAC; the protective effect was detected from concentrations as low as 0.5-1.0 mM. The signal elevation above 100%, registered in the course of MTT assay after the addition of NAC, can be explained by the fact that NAC as an antioxidant increases the ability of the cells to reduce tetrazolium. Accordingly, an increase in the amount of the survived cells in the culture was observed [29]. Mexidol was also effective against the toxic action of all heavy metals, although 100% cell survival was reached at higher concentrations (2.5-5 mM) of the protective agents. Carnosine (concentrations of 1.0-2.5 mM) elevated the cell viability under the impact of lead and molybdenum. Ethylenediaminetetraacetic acid (EDTA) is used in practice to eliminate radioactive and toxic metals from the body, thanks to its ability to form chelate complexes with metals [30]. In our experiments, EDTA was effective only at relatively low concentrations of cadmium (0.25, 0.5, and 1 mM).

Studied compounds, 1 mM	Lead	Cadmium	Cobalt	Molybdenum	Intact cells
Control	149.9 ± 2.5	147 ± 3.6	140.5 ± 2.9	153.6 ± 2.2	100 ± 1.5
NAC	103.5 ± 0.8*	77.6 ± 2.2*	106.7 ± 0.7*	109.2 ± 2*	96.5 ± 2.5
Carnosine	109.1 ± 3*	101.4 ± 2.4*	120.5 ± 2*	106.4 ± 1.8*	95 ± 1.9
Mexidol	148.5 ± 1.3	146.8 ± 0.8	114.2 ± 2.9*	109.9 ± 1.3*	108 ± 2
EDTA	148.6 ± 1.6	135.2 ± 2.4	145.7 ± 1.9	152.3 ± 2.8	114.9 ± 2.2*

Table 1: Protective action of the studied substances on the cell death rate. The effect of carnosine, N-acetylcysteine, EDTA, and mexidol at a concentration of 1 mM after additions of 10^{-4} lead, 5×10^{-5} cadmium, 10^{-5} cobalt and 5×10^{-5} M molybdenum salts. The proportion of dead intact cells was taken as 100%. *$p < 0.05$

According to the degree of the protective effect, i.e., lowering of the cell death rate, all the studied substances can be placed in the following sequence: NAC > carnosine > mexidol.

Apart from antioxidant capacity, the decrease in the cell death rate upon carnosine administration jointly with heavy metals can be explained by the ability of carnosine to form complexes with metal ions—copper, magnesium, zinc, cadmium, iron, etc. [22].

The ability of mexidol to increase cell viability and lower cell death rate in a culture can be related to its direct antioxidant potential, in particular, to its abilities to bind superoxide anion radicals [31], react with lipid peroxide radicals and hydroxyl peptide radicals, lower nitric oxide (NO) level in tissues, and increase the activity of antioxidant enzymes [32].

Conclusion

In conclusion, the data obtained in this study demonstrated that the studied antioxidants possess a capability to enhance the resistance of cultured cells in conditions of heavy metal toxicity, which may be indicative of their protective potential in vivo, in particular, for nervous tissues. This should give an impulse for further studies with the goal to attain practical significance.

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