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EXPERIMENTAL ARTICLES

The Protective Effect of (S)-Trolox–Carnosine on a Human Neuroblastoma SH-SY5Y Cell Culture under the Impact of Heavy Metals

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Abstract—Evaluation of the dose-dependent effects of heavy metals on the viability of a human neuroblastoma SH-SY5Y cell culture showed that 50% cell death was observed in the presence of 5×10^{-4} M lead, 5×10^{-6} M cadmium, 5×10^{-5} M cobalt, and 10^{-5} M molybdenum. The presence of these metals led to an increase in the level of reactive oxygen species (ROS) (from 39% to 74% in the cases of lead and cobalt, respectively). We revealed a cytoprotective effect against toxic heavy metals (HMs) of a new synthetic compound, (S)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl-β-alanyl-L-hystidine. This compound is a combination of carnosine with a water-soluble vitamin E analog, trolox (S-trolox–carnosine, S-TC). S-TC efficiently increased the cell viability in the presence of any of the studied metals, which correlated with a decrease in the proportion of necrotic cells and with efficient inhibition of ROS formation. Trolox also had a large cytoprotective effect under toxic conditions caused by lead, cadmium, and cobalt. The protective activity of carnosine under these conditions was significantly lower than the effects of trolox or trolox–carnosine. In general, these results revealed the greater cytoprotective effect of S-trolox–carnosine in the presence of heavy metals as compared to its precursors, trolox and carnosine.

Keywords: lead, cadmium, cobalt, molybdenum, heavy metals, reactive oxygen species, oxidative stress, carnosine, trolox, trolox–carnosine, antioxidants

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INTRODUCTION

The problem of environmental pollution with heavy metals (HMs) from natural and anthropogenic sources remains important throughout the world. Currently, HMs (cadmium, lead, cobalt, molybdenum) are considered as serious environmental and industrial pollutants, which may pose a serious risk to human and animal health [1]. One of the most toxic and carcinogenic HMs is cadmium; it is present in the air of industrial enterprises and is a food contaminant and a component of cigarette smoke [2]. Cadmium and lead can accumulate in the body and show toxic effects even at low doses [3]. These metals can adversely affect the nervous, immune, reproductive, and cardiovascular systems; the central nervous system (CNS) is one of the main targets of HMs. Lead is able to penetrate the blood–brain barrier and accumulate in the brain, which may change its metabolism and, thereby, lead to physiological disorders [4]. Exposure to lead causes neurotoxic effects, which results in behavioral disorders and reduced cognitive functions of the human brain and experimental animals [5]. The toxic effects of cadmium and lead vary from insignificant influence on cognitive brain functions at low concentrations to severe encephalopathy under acute poisoning [6]. In contrast to cadmium and lead, cobalt and molybdenum are essential trace elements that are necessary for normal growth, development, cell homeostasis, many enzymatic reactions, and for the normal functioning of the brain [7, 8]. However, at high doses these elements can be toxic [9]. Cobalt is a genotoxic and carcinogenic metal [10] and can cause neurotoxic effects, which are manifested in the deterioration of memory and suppression of learning in experimental animals [6, 11].Molybdenum can inhibit the activity of a number of enzymes, disrupt metabolism of calcium, phosphorus, and copper, and induce osteoporosis, limp, and alterations of connective tissue [12, 13].

The mechanisms of the toxic effects of HMs on the body remain poorly understood. It has been shown in a number of works that the earliest manifestation of the toxic effects of HMs (cadmium, cobalt, and lead) on the body is the development of oxidative stress

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(OS) in the tissues of experimental animals. This process is accompanied by the formation of reactive oxygen species (ROS), accumulation of lipid, protein, and DNA oxidation products, and a simultaneous decrease in the activities of Na^+/K^+-ATPase and Mg^{2+}-ATPase in the brain and key enzymes of antioxidant defense [9, 14–16]. Further development of OS may lead to mitochondrial dysfunction and cell death caused by reduced expression of the antiapoptotic Bcl-xl protein [17].

We recently showed that the cell death and the decrease in the cell viability under the action of HMs in the human neuroblastoma SH-SY5Y cell culture may be prevented by antioxidants (AOs). One of the most efficient was the natural dipeptide carnosine (β-alanyl-L-histidine).

The protective effects of carnosine are due to its antioxidant and proton buffer properties, its ability to chelate metals with variable valences and prevent the formation of glycated proteins and lipid peroxidation products (so-called AGE and ALE, advanced glycation and advanced lipoxidation end products) [18]. However, the presence of specific enzymes in human blood and tissues, i.e., tissue and serum carnosinases, which lead to its rapid hydrolysis [19–22], is a significant limiting factor in using carnosine as a drug. To protect carnosine from these enzymes, researchers created a new synthetic compound, which is a combination of carnosine with a water-soluble vitamin E analog, trolox. We previously showed using in vitro models that a new synthetic carnosine derivative, (S)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl-β-alanyl-L-hystidine (S-Trolox–carnosine, S-TC), has high antioxidant and membrane-stabilizing activities and is resistant to hydrolysis by serum carnosinase. S-TC protected blood serum lipoproteins from Fe^{2+}-induced oxidation more efficiently than trolox or carnosine. ROS formation was prevented by S-TC more efficiently than by trolox or carnosine in a suspension of cerebellar granule cells under oxidative stress induced by N-methyl-D-aspartate (NMDA) or hydrogen peroxide [23]. It was shown using in vivo experiments that S-TC enhanced the resistance of rats to acute hypobaric hypoxia and protected the brain from the oxidative damage by increasing the endogenous antioxidant activity [24]. Introduction of this compound in the diet of *Drosophila melanogaster* led to a greater increase in their lifespan compared to the addition of carnosine, which indicates the efficient geroprotective action of S-TC [25].

However, there is no literature data on the ability of S-TC to protect neuronal cells from the toxic actions of HMs. Therefore, the goal of this study was to evaluate the protective effects of trolox–carnosine on SH-SY5Y human neuroblastoma cells under the impacts of heavy metals.

MATERIALS AND METHODS

Cell cultivation. A fast-growing human neuroblastoma SH-SY5Y cell culture (ATCC®, United States) was used in this work. Cells were cultured in a medium containing a 1 : 1 mixture of Dulbecco MEM with Earle's salts without glutamine (PanEco, Russia) and F-12 without glutamine (PanEco, Russia) supplemented with 1% penicillin-streptomycin (PanEco, Russia) and 10% fetal calf serum (PAA Laboratories, Austria). The cells were cultured in a cell incubator (ShelLab) at 37°C, 90% humidity, and 5% CO_2 for 6–7 days. The medium was replaced by a new one every 3 days. The cells were passaged every 7–8 days depending on the growth rate.

Evaluation of cell viability using the MTT test. Cells were passaged in 96-well plates (15000 cells per well), kept for 3 days, followed by the addition of OS inductors (HMs salts, i.e., lead diacetate, cadmium chloride, cobalt chloride, and ammonium molybdate) and OS protectors (carnosine, trolox, and S-trolox–carnosine) at corresponding concentrations (eight wells per each group). The cells were incubated for 24 h, followed by the addition of MTT (3-(4,5-dimethyl-thiasol-2-yl)-2,5-diphenyl-tetrasolium bromide, Dia M, Russia) in the culture medium at a final concentration of 0.5 mg/mL. After incubation of cells with MTT for 3 h, the medium was removed from the wells and the dry formazan residue was dissolved in dimethylsulfoxide (100 μL) to obtain a purple solution.

The plates were placed into a Synergy H4 microplate spectrophotometer (BioTek), the solutions were stirred in wells for 10 min, and optical absorption was measured at 570 nm and 660 nm. The optical absorption values at 660 nm were subtracted from those at 570 nm [26, 27]. The results were presented as a percentage of the optical absorption value in control wells, which was taken as 100%. The Microsoft Excel program was used to process the results.

To evaluate the dose dependence of HMs toxic effects, the cells were divided into eight groups by twelve wells. Group 1 contained intact cells without HMs and groups 2–8 contained HMs at concentrations of 10^{-2}–10^{-8} M. The concentrations corresponding to cell viability of 75–80% were chosen to evaluate the neuroprotective effect of the studied compounds.

Evaluation of the proportion of dead cells. Cells were passaged into 24-well plates (60000 per well), kept for 3 days, followed by the addition of OS inductors (HMs salts, i.e., lead diacetate, cadmium chloride, cobalt chloride, and ammonium molybdate) and OS protectors (carnosine, trolox, and S-trolox–carnosine) at corresponding concentrations (four wells per each group). The proportion of dead cells was evaluated by staining with fluorescent dye, propidium iodide (PI, Invitrogen, United States). Three minutes before measurement, PI (λ_{ex} = 485 nm, λ_{em} = 610 nm) was

added in the suspension of cells at a final concentration of 10 μM.

The ROS level in the cell population was evaluated using the fluorescent dye, 2',7'-dichlorodihydrofluoresceine diacetate $DCFH_2$-DA (λ_{ex} = 495 nm; λ_{em} = 520 nm, Biotium, United States), which was enzymatically converted into the fluorescent product, 2',7'-dichlorofluoresceine (DCF), in the presence of ROS. After incubation of cells with OS inductors and protectors for 24 h, $DCFH_2$-DA was added into wells at a final concentration of 100 μM, and the reaction mixtures were kept for 40 min in the dark. Cells were then removed from the substrate with Trypsin-EDTA (PanEco, Russia).

The resultant cell suspensions stained with PI and $DCFH_2$-DA were analyzed on a FACS Calibur flow cytometer (BD Biosciences, United States). Each measurement registered 10000 events. The results were analyzed using the WinMDI 2.8 program (Scripps Institute, La Jolla, United States). The results were processed in the Cell Quest Pro (BD, United States) and Microsoft Excel programs.

S-trolox–carnosine was synthesized and submitted by Hamari Chemicals LTD (Japan).

Statistical processing of the results was performed using the nonparametric *U* Mann–Whitney test.

RESULTS

The dose-dependence effect of heavy metals on cell viability. Figure 1 shows that 50% cell death occurred in the presence of 5×10^{-4} M lead, 5×10^{-6} M of cadmium, 5×10^{-5} M of cobalt, and 10^{-5} M of molybdenum. The further increase in the concentration of cobalt and cadmium to 10^{-4} M and of lead and molybdenum to 10^{-3} M and higher led to 100% cell death.

Evaluation of the protective effects of trolox–carnosine, carnosine, and trolox on cell viability under HM intoxication. To evaluate the protective effects of antioxidants, we chose the HM concentration that caused not more than 20–25% cell death as evaluated by the MTT test (Fig. 1). These concentrations were 10^{-4} M for lead, 10^{-5} M for cobalt, and 5×10^{-5} M for cadmium and molybdenum.

The protective effect of S-TC was evaluated in comparison with carnosine and trolox at three concentrations, i.e., 0.25 mM, 0.5 mM, and 1.0 mM (Fig. 2).

The efficiency of S-TC under lead intoxication (Fig. 2a) was comparable with that of trolox because 100% cell viability was observed in the presence of the minimal concentrations (0.25 mM) of these compounds in the culture. With an increase in their concentrations from 0.5 mM to 1 mM, the cell viability remained within the target values (95–105%). Carnosine provided 100% cell viability only at the highest concentration of 1 mM.

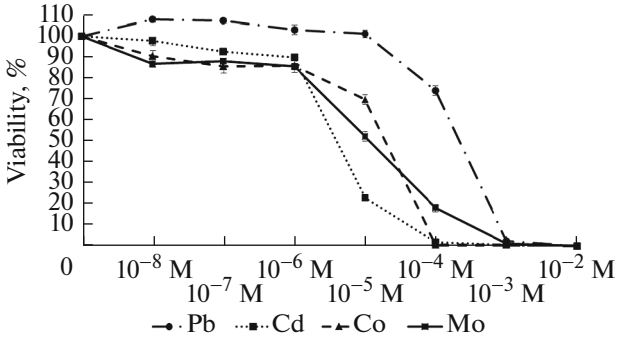

Fig. 1. The dose-dependent toxicity of lead, cadmium, cobalt, and molybdenum salts in the range of concentrations of 0–10^{-2} M. The viability of intact cells was taken as 100%.

Under cadmium intoxication (Fig. 2b), 100% cell viability was observed in the presence of 0.25 mM S-TC. A further increase in its concentration in the culture led to a decrease in cell viability to 82–81%. Carnosine was also effective only at the low concentration (0.25 mM), which resulted in the cell viability of 86–88%. In contrast to carnosine and S-TC, trolox provided 100% cell viability only at its highest concentration of 1 mM.

Under cobalt intoxication (Fig. 2c), the cell viability was 105%, 100%, and 90% in the presence of S-TC at 0.25, 0.5, and 1 mM concentration, respectively. Trolox increased the cell viability in the culture to 86% regardless of its concentration. Carnosine had no significant effect on the cell viability.

Under molybdenum intoxication (Fig. 2d), 100% cell viability was achieved in the presence of 0.25 mM S-TC. A further increase in its concentration led to a decrease in its efficiency. Trolox was more efficient at 0.5 mM, which resulted in an increase in the cell viability to 108%. The cell viability in the presence of 1 mM trolox was 95%. Carnosine increased the cell viability to 106% only at the highest concentration of 1 mM.

The cytoprotective effects of carnosine, trolox, and S-TC at the minimal concentration of 0.25 mM were compared using the MTT test (table). S-TC protected cells from the toxic actions of all of the studied metals; trolox was efficient in the presence of lead, cobalt, and molybdenum, and carnosine was efficient in the presence of cadmium and molybdenum.

Evaluation of the protective effects of trolox–carnosine, carnosine, and trolox on cell death and ROS level under HM intoxication. The cells were stained with propidium bromide (PI) to evaluate the effects of trolox–carnosine, carnosine, and trolox on the cell death rate. The proportion of dead cells increased on average by 50% in the presence of all of the studied metals (table). Only 0.25 mM S-TC, in contrast to carnosine and trolox at the same concentration, effi-

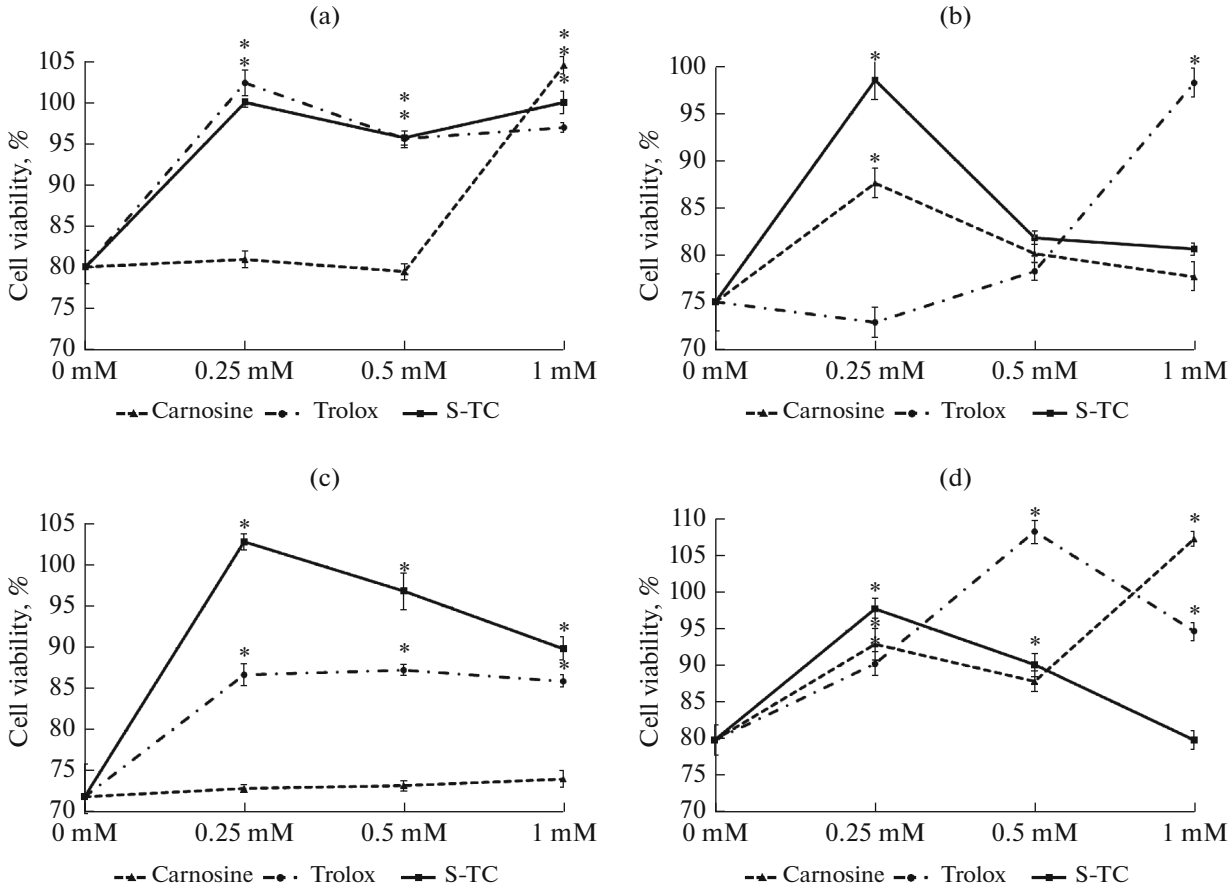

Fig. 2. The effects of carnosine, trolox, and S-TC in the range of concentrations of 0.25–1 mM on cell viability in the presence of 10^{-4} M lead (a); 5×10^{-5} M cadmium (b); 10^{-5} M cobalt (c); 5×10^{-5} M molybdenum (d). (*) $p < 0.05$ relative to control cells with the addition of only studied metal.

ciently prevented the development of necrotic damage in cells under the action of each of the metals. The protective action of trolox was observed only in the case of molybdenum, while carnosine was efficient in the presence of lead and cadmium but not cobalt and molybdenum.

When studying the influence of heavy metals on the ROS formation in neuroblastoma cells, we found that all of the studied heavy metals induced oxidative stress in the cell culture. The ROS level increased from 39% to 74% in the cases of lead and cobalt, respectively (table). S-TC significantly decreased the ROS formation in the presence of cobalt and molybdenum and was relatively efficient in the presence of cadmium. Trolox significantly decreased the ROS formation in the presence of lead, cadmium, and cobalt and was relatively efficient in the presence of molybdenum. Carnosine inhibited the ROS formation in the presence of molybdenum but was ineffective in the presence of cobalt. In the case of lead and cadmium, carnosine showed prooxidant activity and increased the ROS formation up to 216.1 ± 10.3% and 218.4 ± 4.0%, respectively.

DISCUSSION

The results indicate the differences in the cytoprotective properties of carnosine, trolox, and trolox–carnosine under intoxication with heavy metals. The differences may be due to not only to the protectors themselves but also due to the metals. The differences in the mechanisms of the toxic action of the studied metals on the cellular level should be taken into account. Cobalt (like iron, copper, chromium, and vanadium) is involved in redox reactions that are associated with ROS formation, whereas the toxic action of lead and cadmium (like that of nickel, mercury and arsenic) is based on the depletion of thiol-containing antioxidants (glutathione, lipoic acid, cysteine, and N-acetylcysteine) and antioxidant enzymes [28, 29]. In both cases, the result is an increase in the ROS formation in cells and tissues and the development of oxidative stress. Therefore, the use of antioxidants prevents the toxic actions of heavy metals, although the protective mechanisms may be different [30, 31]. The effects of molybdenum should be considered separately. They may be associated with its involvement in the activation of a number of redox enzymes, i.e., xan-

Protective effects of trolox–carnosine, carnosine, and trolox on cell death and ROS level under HMs intoxication

Group	Viability (MTT test)	Cell death (PI)	ROS level
LEAD 10^{-4} M	80.0 ± 2.1	149.9 ± 2.5	139.0 ± 4.9
Carnosine 0.25 mM	80.9 ± 1.0	128.8 ± 4.3*	216.1 ± 10.3*
Trolox 0.25 mM	102.3 ± 1.6*	151.3 ± 5.0	97.6± 9.4*
Trolox–carnosine 0.25 mM	100.0 ± 0.5*	90.7 ± 4.5*	133.5 ± 4.2
CADMIUM 5×10^{-5} M	74.9 ± 3.2	147.0 ± 3.6	162.4 ± 6.5
Carnosine 0.25 mM	87.6 ± 0.9*	125.4 ± 6.9*	218.4 ± 4.0*
Trolox 0.25 mM	72.8 ± 1.6	150.2 ± 7.2	112.6 ± 3.4*
Trolox–carnosine 0.25 mM	99.4 ± 2.1*	115.7 ± 4.7*	141.7 ± 7.3
COBALT 10^{-5} M	72.2 ± 3.9	140.5 ± 2.9	173.6 ± 9.2
Carnosine 0.25 mM	73.0 ± 0.5	138.1 ± 5.3	153.9 ± 2.1
Trolox 0.25 mM	86.8 ± 1.3*	133.7 ± 3.2	110.2 ± 5.0*
Trolox–carnosine 0.25 mM	102.8 ± 1.0*	106.4 ± 3.5*	114.5 ± 3.4*
MOLYBDENUM 5×10^{-5} M	79.7 ± 2.0	153.6 ± 2.2	143.8 ± 7.5
Carnosine 0.25 mM	93.0 ± 2.2*	150.9 ± 3.3	124,7 ± 6.1*
Trolox 0.25 mM	90.3 ± 1.6*	100.3 ± 3.9*	129.3 ± 6.8
Trolox–carnosine 0.25 mM	97.9 ± 1.3*	97.3 ± 5.1*	120.6 ± 5.6*

thine oxidase/dehydrogenase, sulfite oxidase, and aldehyde oxidase [32].

The results showed that S-TC was efficient in the enhancement of cell viability in the presence of any of the studied metals (to 97.9 ± 1.3% for molybdenum and 102.8 ± 1.0% for cobalt). This parameter correlated with a decrease in the proportion of necrotic cells (from 115.7 ± 4.7% for cadmium to 90.7 ± 4.5% for lead) (table). S-TC efficiently inhibited the ROS formation, including the case of the maximal increase in the level of these species upon the toxic action of cobalt from 173.6 ± 9.2% to 114.5 ± 3.4% (table). It should be noted that the cytoprotective effect of S-TC (an increase in the cell viability and a decrease in the proportion of the dead cells) in the presence of lead was not associated with the inhibition of ROS formation. Trolox also exhibited a large antioxidant activity under oxidative stress induced by lead, cadmium, and cobalt. As an example, it reduced the cobalt-induced ROS formation from 173.6 ± 9.2% to 110.2 ± 5.0% and enhanced the cell viability from 72.2 ± 3.9% to 86.8 ± 1.3, although a decrease in the proportion of necrotic cells was not observed. The results suggest that the cytoprotective action of S-TC and trolox is due to their ability to neutralize ROS, which was clearly evident in the case of the toxic action of cobalt. The protective effect of S-TC in the presence of lead and cadmium and the same but more pronounced effect of trolox may be due to the involvement of these compounds in redox reactions, which maintain the level of thiol-containing antioxidants [33, 34]. In general, the cytoprotective effect of trolox–carnosine in the presence of heavy metals significantly exceeds that of trolox. The activity of carnosine under the same conditions is considerably lower as compared to trolox and trolox–carnosine. The prooxidant effect of carnosine that was found in the presence of lead and cadmium requires further investigation.

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