J. Toxicol. Pub. Health
Vol. 23, No. 1, pp. 33-38 (2007)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Protective Effect of Carnosine Against Zn-Mediated Toxicity in Cortical Neuronal Cells

Jin-Joo Hue[1], Ah-Ram Lee[1], Yea Eun Lee[1], Min-Hang Cho[1], Ki-Nam Lee[1], Sang Yoon Nam[1], Young Won Yun[1], Jae-Hwang Jeong[2], Sang-Hwa Lee[3] and Beom Jun Lee[1]

[1]College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 361-763
[2]College of Liberal Arts and
[3]Department of Food and Nutrition, Seowon University, Cheongju 361-742, Korea

Received February 14, 2007; Accepted March 15, 2007

Zinc is an endogenous transition metal that can be synaptically released during neuronal activity. However, zinc may contribute to the neuropathology associated with a variety of conditions. Carnosine expressed in glial cells can modulate the effects of zinc on neuronal excitability as a zinc chelator. We hypothesize that carnosine may protect against neurotoxicity of zinc in cortical neuronal cells. The cortical neuronal cells from newborn rats were prepared and exposed to zinc chloride and/or carnosine at various concentrations. Zinc at the doses of 0 to 500 μM decreased neuronal cell viability in a dose-dependent manner. Additionally, at the concentrations of 100 and 200 μM, it significantly decreased cell viability in an exposed time-dependent manner ($p < 0.05$). Treatment with carnosine at the concentrations of 20 and 200 μM significantly increased neuronal cell proliferation by approximately 14% and 20%, respectively, compared to the control ($p < 0.05$). At the concentrations of 100 and 200 μM zinc, 20 μM carnosine significantly increased the viability of neuronal cells by 18.3% and 12.1%, and 200 μM carnosine also increased it by 33.5% and 28.6%, respectively, compared to the normal control group ($p < 0.01$). These results suggest that carnosine at a physiologically relevant level may protect against zinc-mediated toxicity in neuronal cells as an endogenous neuroprotective agent.

Key words: Carnosine, Zinc, Toxicity, Neuronal cells, Cell viability.

INTRODUCTION

Recent study has shown that zinc and copper ions can inhibit cellular proteasome function (Kim *et al.*, 2004), and it has been known for some time that zinc levels are raised in the brains of Alzheimer's disease patients (Danscher *et al.*, 1997; Suh *et al.*, 2000; Bush and Tanzi, 2002). Zinc and copper have also been implicated in diseases with neuropathological components, including Alzheimer's disease, Menkes disease, Wilson's disease, Pick's disease, amyotrophic lateral sclerosis-parkinsonism-dementia, stroke, and seizures (Constantinidis and Tissot 1981; Cuajungco and Lees 1997; DiDonato and Sarkar 1997). Indeed, chelation of zinc and copper is neuroprotective as clioquinol, which chelates both these metal ions, decreases β-amyloid deposition as well as improves learning in transgenic mice (Cherny *et al.*, 2001).

The dipeptide carnosine (β-alanyl-L-histidine) belongs to a group of analogous histidyl dipeptides and normally found in electrically excitable tissues such as brain and skeletal muscle of many vertebrates. The physiological functions of these peptides still remain unknown. In an attempt to examine the role of carnosine in muscle and the central nervous system (CNS), several studies have recently been focused on its cellular localization and biosynthesis (Biffo *et al.*, 1990; Artero *et al.*, 1991; Sassoe-Pognetto *et al.*, 1993; Perroteau *et al.*, 1994). Carnosine synthesis has been studied using several cells including muscle cells, oligodendrocytes, and ensheathing cells of olfactory bulb and it was increased during differentiation of these cells, thereby indicating a role for carnosine in mature tissue (Bakardjiev and Bauer, 1994; Hoffmann *et al.*, 1996 Bakardjiev, 1997). However, elsewhere in the brain carnosine and related peptides are not contained in neu-

Correspondence to: Beom Jun Lee, Department of Veterinary Public Health College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea
E-mail: beomjun@cbu.ac.kr

rons while are confined to various popular glial cells (Biffo *et al.*, 1990). The latter findings are in agreement with previous studies on the biosynthesis of carnosine by primary cultures of glial cells obtained from rodent brain (Bauer *et al.*, 1982) which demonstrated not only these cells but also that the peptides is released into the culture medium. The functions of oligodendrocytes in the CNS, with the exception of myelin production, are not well defined. This macro glial type of cells expresses neuro-ligand receptors and so additional neuron-oligodendrocyte and astrocyte-oligodendrocyte interactions may exist unrelated to the process of myelination. Indeed, carnosine has the potential to suppress many of the biochemical changes (e.g., protein oxidation, glycation, AGE formation, and cross-linking) that accompany aging and associated pathologies. Glycation, generation of advanced glycosylation end-products (AGEs), and formation of protein carbonyl groups play important roles in aging, diabetes and its secondary complications, and neurodegenerative conditions. In *in vitro* experiment, carnosine protects brain neurons (Boldyrev *et al.*, 1999; Tabakman *et al.*, 2002) and non-neuronal cells (Kang *et al.*, 2002) against oxidative injury. In the olfactory system, carnosine is expressed in high concentrations (2~5 mM) in the olfactory sensory neurons and their terminals in the glomerular layer of the olfactory bulbs (Ferriero and Marogolis, 1975).

Carnosine can chelate transitional metals such as copper, zinc, cobalt, and nickel. Zinc ion produce weak complexes with carnosine without changing the conformation of the dipeptide (Brown and Antholine, 1979). Meanwhile, zinc-carnosine complex can be applied for treatment of ischemia-reperfusion injury and gastric damage, due to free radical scavenging activity (Cho *et al.*, 1991; Yoshikawa *et al.*, 1991). We have tested the hypothesis that carnosine may provide protection against zinc-mediated neurotoxicity in cortical neuronal cells from newborn Spraque-Dawley, as determined by cell viability. This study might imply a neuroprotective function of carnosine at physiological levels.

MATERIALS AND METHODS

Materials. L-Carnosine, poly-L-lysin and zinc chloride were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Trysin, trysin-EDTA, serum-free Neurobasal medium, glutamine, penicillin, and streptomycin were purchased from Gibco Co. (Gaithersburg, MD, USA).

Cell culture. Cortical neuronal cells were obtained from 15-day-old Spraque-Dawley rat fetuses. After removal of meninges, the cortices were dissociated with trypsin. Proteolysis was stopped by adding soybean trypsin inhibitor. All cell suspensions were made in serum-free Neurobasal (Nb) medium supplemented with B27, 0.5 mM glutamine, and penicillin/streptomycin. Cells were plated at a 60-mm dish and kept in a poly-L-lysine-coated 96-well at 37°C with a 5%CO_2/95% air atmosphere. Cells were plated at 2×10^4 cells/well in a poly-L-lysine-coated 96-well and plated at 5×10^5 cells/well in a poly-L-lysine-coated 60-mm dish.

Exposure to carnosine and/or zinc. Toxicity experiments were carried out after the neuronal cells were cultured for 8 days. Neuronal counts were made at randomly marked portions of 96-well plate before the application of zinc chloride salt and/or L-carnosine. Exposure of cells in serum-free neurobasal (Nb) culture medium to zinc chloride salt and/or an agent combination of both zinc and carnosine was accomplished for 0~4 hr. The culture medium was then replaced with a new serum-free Neurobasal (Nb) medium (Gibco, Gaithersburg, MD) supplemented with B27 (Gibco), 0.5 mM glutamine, and penicillin/streptomycin and the cells were incubated for 24 hr. The employed concentrations for zinc chloride salt were 0, 5, 10, 20, 50, 100, 200, and 500 μM. Whereas that of carnosine were 2, 20, and 200 μM. The viability of the cells was evaluated 24 hr after the beginning of treatment via the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (WST-8; Dojindo Molecular Technologies Inc., Gaithersburg, MA, U.S.A.) reduction assay using a microplate reader (Bio-TEK Instruments Inc., Winooski, Vermont, U.S.A.) at 450 nm.

Statistical analysis. Data were statistically analyzed by one-way ANOVA using a SPSS program (*SPSS* base 10.0 User's Guide, USA). A significant difference between treated and non-treated control groups was determined by the Student's t-test at the level of $p < 0.05$.

RESULTS

Neuronal cell toxicity by zinc. The rate of cell death was increased by increasing the zinc concentration to which the cells were exposed for 2 hr (Fig. 1). Zinc decreased the viability of the neuronal cells in a dose-dependent manner. At a concentration of 10 μM zinc, the neuronal cells were died by 22%, which correspond to 78% viability, compared with the normal control group. At a concentration of 50 μM zinc, the neuronal cell count was significantly decreased by 40%, compared with the normal control ($p < 0.05$). The concentration which of zinc took for 50% of the cells to die was located between the concentrations of 100 and 200 μM

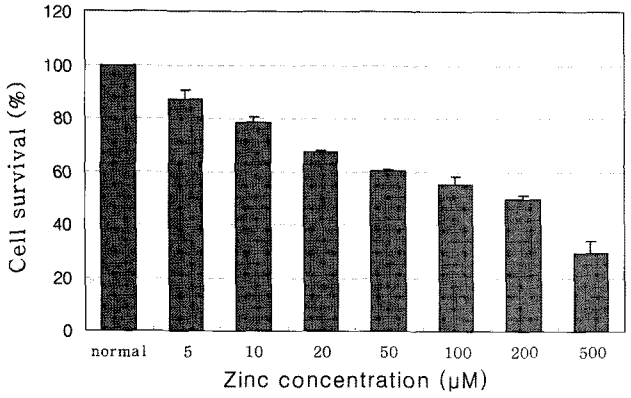

Fig. 1. The effects of various zinc concentrations on the viability of cortical neuronal cells. Data obtained from three series of experiments were expressed as % of survival of neuronal cells. Zinc significantly decreased the neuronal cell viability at all concentrations, compared with normal control group ($p < 0.05$).

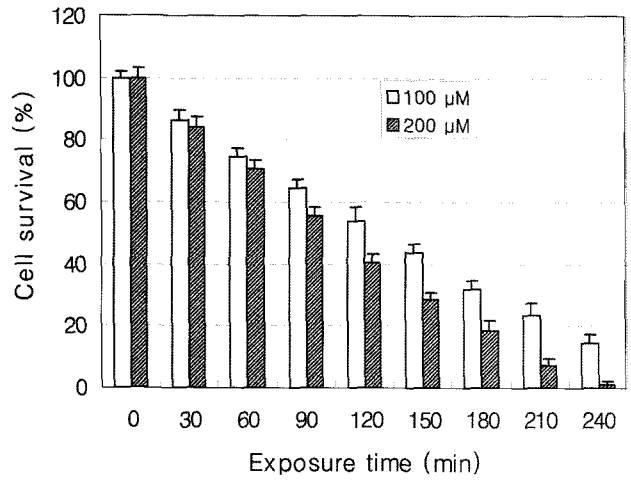

Fig. 2. The toxic effects of 100 and 200 μM zinc in relation to various exposure periods in cortical neuronal cells. Data obtained from three series of experiments are expressed as % of survival of neuronal cells. Zinc significantly decreased the neuronal cell viability in an exposed time-dependent manner, compared with normal control group ($p < 0.05$).

of zinc. In addition, zinc at the concentrations of 100 and 200 μM significantly decreased the cell viability in an exposed time-dependent manner (Fig. 2). From this figure, it is shown that the exposure of the neuronal cells to 200 mM zinc for over 4 hr caused the cells to die completely.

Effects of carnosine on the proliferation of the neuronal cells. The neuronal cells were exposed to carnosine for 2 hr and then the culture medium was replaced with the same new medium and incubated for

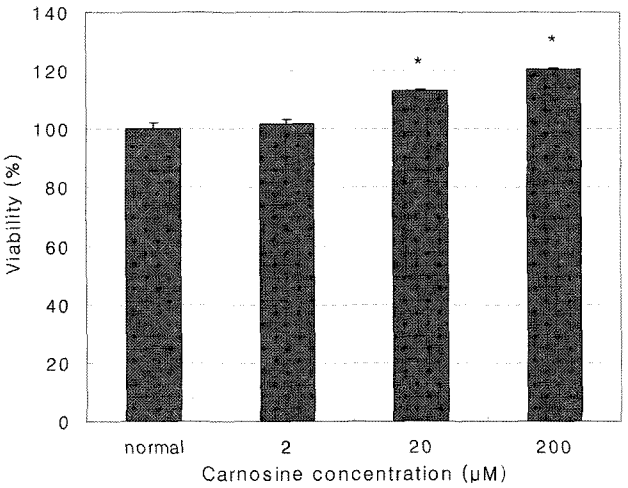

Fig. 3. Effect of carnosine on the proliferation of neuronal cells. Data obtained from three series of experiments are expressed as % of viability of neuronal cell treated with carnosine without exposure to zinc. *Significantly different from normal control at the level of $p < 0.05$.

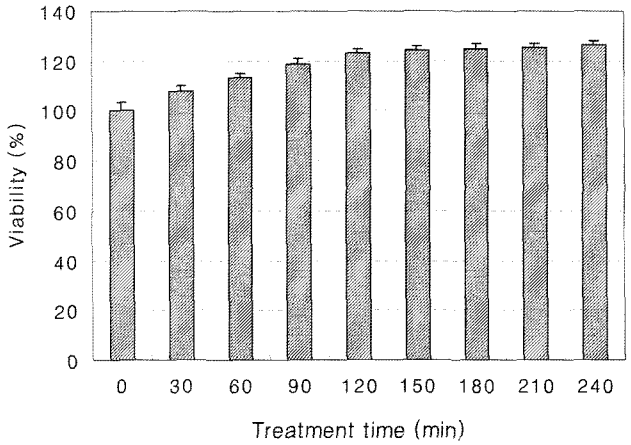

Fig. 4. Effect of 200 μM carnosine on the proliferation of neuronal cells. Data obtained from three series of experiments are expressed as % of viability of neuronal cell treated with carnosine. Carnosine at the concentration of 200 μM significantly increased the neuronal cell proliferation compared with normal control group ($p < 0.05$).

24 hr. The rate of cell proliferation was increased by increasing the concentrations of carnosine in a concentration-dependent manner (Fig. 3). Carnosine at the concentrations of 20 and 200 μM, significantly increased the neuronal cell proliferation by approximately 14% and 20%, respectively, compared to the normal control ($p < 0.05$). In addition, the proliferation of neuronal cells by 200 μM carnosine was increased by time (Fig. 4). The increase in cell proliferation by 200 μM carnosine became steady following treatment over than 2 hr (Fig. 4).

Protection by carnosine against zinc-mediated neurotoxicity. Carnosine and zinc were co-applied to cultured cortical neuronal cells for 2 hr. At a concentration of 20 μM, carnosine protected the neuronal cells against neurotoxicity caused by 100 μM or 200 μM zinc (Fig. 5). Carnosine significantly increased the viability of the neuronal cells by 18.3% and 12.1%, respectively, in the presence of 100 μM and 200 μM zinc ($p < 0.05$).

At a concentration of 200 μM, carnosine prevented the toxic effects caused by 100 μM and 200 μM zinc (Fig. 6). Carnosine significantly increased the viability of neuronal cells by 33.5% and 28.6%, respectively, at the concentrations of 100 μM and 200 μM zinc ($p < 0.05$). The protective effect of carnosine against zinc-induced neurotoxicity was stronger by increasing carnosine concentration.

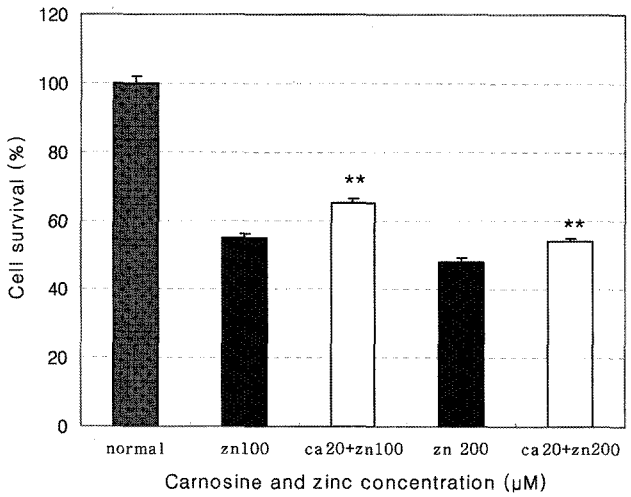

Fig. 5. The protective effect of 20 μM carnosine against zinc-mediated neurotoxicity. Carnosine and zinc were co-exposed to the cortical neuronal cells for 2 hr. Data obtained from three series of experiments were expressed as % of survival of neuronal cell. **Significantly different from the respective zinc control at the level of $p < 0.01$.

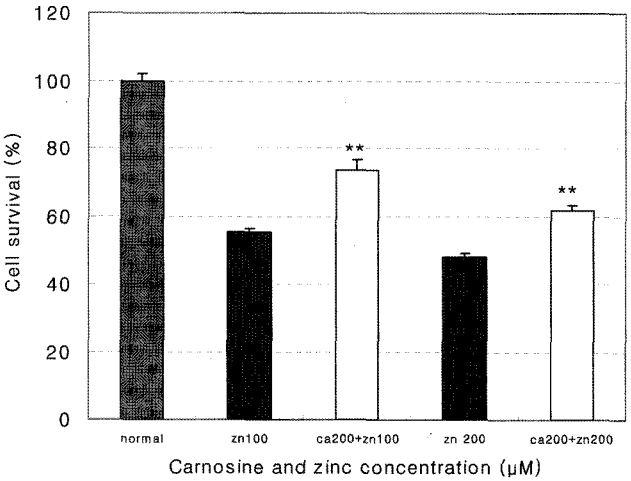

Fig. 6. The protective effect of 200 μM carnosine against zinc-mediated neurotoxicity. Carnosine and zinc were co-exposed to the cortical neuronal cells for 2 hr. Data obtained from three series of experiments were expressed as % of survival of neuronal cell. **Significantly different from the respective zinc control at the level of $p < 0.01$.

DISCUSSION

Carnosine is a naturally-occurring dipeptide (β-alanyl-L-histidine) normally found in brain and muscle, sometimes at surprisingly large concentrations (up to 20 mM). Biochemically, the dipeptide seems to possess a number of protective functions including anti-oxidant, free-radical scavenger and anti-glycating agent (Lee *et al.*, 1999a, b, c), as well as being able to bind to protein carbonyls and suppress their cross-linking activity (Hipkiss and Brownson, 2000; Lee *et al.*, 1999c). Importantly, carnosine is also a zinc chelator (Quinn *et al.*, 1992; Baran 2000) and it therefore is not surprising that the dipeptide suppresses zinc-induced neurotoxicity in cultured olfactory neurones (Horning *et al.*, 2000). Furthermore, our results showed that zinc is a very neurotoxic compound at various concentrations of 5, 10, 20, 50, 100, 200, and 500 μM. The present findings were in consistent with that previously reported by Gallant *et al.* (2000). In his *in vivo* and *in vitro* studies, he demonstrated that synaptic concentrations of zinc can be considered as neurotoxic in certain circumstances, such as ischemia or seizures (Gallant *et al.*, 2000). Within the cell, zinc can trigger widespread disruptions of normal cellular functions. It has been proposed that zinc induces an increase in membrane calcium permeability, thereby disrupting calcium homeostasis (Weiss *et al.*, 1993). This metal is also known to inhibit mitochondrial electron transport, resulting in pump failure, and thus, energy depletion (Donaldson *et al.*, 1973). Other toxic effects that zinc could potentially induce include the disruption of normal tubulin assembly (Kress *et al.*, 1981) and the overactivation of calcium-mediated enzymes (Csermely *et al.*, 1988). Furthermore, zinc reacts with the thiol and imidazole moieties of many proteins (Chvapil *et al.*, 1972). Therefore, it can disrupt structure and function by binding proteins.

There are many reasons for the present study to confirm how effectively carnosine could protect the damage on neuronal cells occurred by zinc chloride salt. The present study shows that 200 μM carnosine increased the viability of neuronal cells by 33.5% and 28.6%, respectively, with the concentrations of 100 and 200 μM zinc, compared with the respective control

groups. Nevertheless, carnosine could not protect against zinc above 300 μM in our study (data not shown). Although there is a little evidence to either directly support or refute this idea, there are a few studies which can be interpreted positively. When autistic children were fed carnosine, their autistic behaviour was suppressed (Chez *et al.*, 2002). Whilst the mechanisms involved in these effects are entirely obscure. This study indicated that feeding of carnosine to humans can affect the brain function, despite the presence of tissue carnosinases. Antonini *et al.* (2002) showed that oral administration of carnosine to adult humans led to a rise in serum total anti-oxidant capacity, as well as the behaviour of chicks was also altered by carnosine (Tomonaga *et al.*, 2004). Furthermore, there is much evidence showing that the dipeptide also protects cells in neuroexcitatory and hyperoxic conditions (Boldyrev *et al.*, 1997, 2004), and that more than one protective mechanism may be involved (Boldyrev *et al.*, 1999). Proteasomes could be subject to inactivation by lipid peroxidation products (malondialdehyde, hydroxynonenal and acrolein), protein carbonyls and crosslinked peptides (Carrard *et al.*, 2003). If carnosine behaves as a zinc chelator and suppresses the metal ions' inhibitory effects towards proteasomes, then one would predict that the dipeptide could stimulate intracellular proteolysis under some circumstances. Carnosine's apparent ability to act as an aldehyde/carbonyl scavenger could be additionally beneficial (Hipkiss and Chana, 1998; Hipkiss, 2002). In conclusion, our results show that physiologically relevant concentrations of carnosine provide a protective effect against toxicity induced by zinc. The chelating ability of the histidine residue of this dipeptide may contribute to the attenuation of zinc toxicity, thereby reducing the incidence of neuropathy.

ACKNOWLEDGMENT

This work was supported by the Korea research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-205-E00033).

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