

Neuroprotective effect of carnosine against salsolinol-induced Parkinson's disease

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Abstract. Carnosine is a dipeptide of β -alanine and histidine amino acids. It is widely present in muscle and brain tissues. Carnosine has been demonstrated to be an antioxidant agent that is beneficial in animals. Reactive oxygen species (ROS) and aldehydes are generated from membrane fatty acid oxidation. The antioxidant potential and toxicity of salsolinol had been extensively studied *in vivo* and *in vitro*. The present study analyzed the protective effect of carnosine against Parkinson's disease in the salsolinol-induced rat brain and rat brain endothelial cells. Antioxidant and biochemical markers were determined *in vitro* and *in vivo*. Histopathological examination was completed in order to evaluate the protective effect of carnosine on the cellular architecture of salsolinol-induced brain tissue. In order to confirm the protective effect of carnosine further, it was also investigated at an *in vitro* level using rat brain endothelial cells. Fluorescence and confocal studies indicated reduced apoptosis in the endothelial cells of the rat brain tissue. Antioxidant enzymes and lipid peroxidation levels were renormalized following treatment with carnosine. In addition, carnosine treatment reduced mitochondria-derived ROS in the rat brain endothelial cells. These findings suggest that carnosine may be a therapeutic agent against salsolinol-induced Parkinson's.

Introduction

Carnosine is a dipeptide composed of β -alanine and histidine amino acids, and is widely abundant in the brain tissues and muscle. It was first identified by a Russian scientist (1) and later in a number of other countries (2-8). Carnosine has been demonstrated to possess antioxidant properties.

Reactive oxygen species (ROS) and aldehydes are produced by fatty acid oxidation and it has been indicated that carnosine is able to scavenge these molecules. Carnosine is a zwitterion with a negative and positive end, and is a well-known compound that reduces advanced glycation end products. End products of advanced glycation may have a critical role in the pathogenesis of a number of diseases, including diabetes mellitus, renal failure, atherosclerosis and neurodegenerative disease (9). Carnosine has also been demonstrated to reduce the development of atherosclerotic plaque (10).

Chronic glycolysis has been reported to accelerate the aging process and the production of carnosine, a crucial therapeutic candidate for neurodegeneration (11). Carnosine is abundant in cerebrospinal fluid, innervated tissues and lenses. Carnosine possesses physiological buffering, wound healing, antioxidant and radioprotectant properties. In addition, it has metal ion chelating, free-radical scavenging, anti-tumour compound, immunomodulator (12) and anti-ageing properties (13,14). However, its proper function remains unknown. The present study investigated the protective effect of carnosine against salsolinol-induced cellular damage.

Free oxygen radicals support the formation of some species that are detrimental to biological molecules. ROS are involved in the aging process (15) and have been indicated to participate in the pathogenesis of joint disease, diabetes, atherosclerosis and Parkinson's disease (16,17). Lipid peroxidation (LPO) generates malondialdehyde (MDA), which may potentially damage the proteins by producing cross-links (18). Previous studies have indicated that carnosine may react with aldehydes to prevent proteins from advanced glycation. This suggests that the high levels of carnosine may be enough to protect against salsolinol induced neurotoxicity.

Salsolinol is a well-known compound that is widely used as a pesticide, piscicide and insecticide. Salsolinol's toxicity has been extensively studied in various *in vitro* (19-21) and *in vivo* (22) systems. Synergistic neurotoxicity may also occur when a small dose of different exogenous factors are applied together. Combination of salsolinol and lipopolysaccharide may result in synergistic toxicity (23). A previous study reported that carnosine may be useful against neurotoxicity (9). The present study analyzes the suppressive effect of carnosine against salsolinol-induced Parkinson's disease in rats and rat brain endothelial cells.

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Materials and methods

Materials. Dulbecco's modified Eagle medium (DMEM), dimethyl sulphoxide (DMSO), sulforhodamine B (SRB), fetal bovine serum (FBS), antibiotics (penicillin-streptomycin) and EDTA were purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). Rat brain endothelial cells (bc3h1) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Animals. A total of 24 healthy, male albino rats were purchased from the Shanghai Animal House (Shanghai Medical College, Shanghai, China), weighing 180-200 g, and were selected for the present study. Rats were maintained in polypropylene cages, under standard condition (relative humidity 62±5% and temperature 25±0.5°C) with a 12-h light dark cycle with access to food and water *ad libitum*. Experimental animal groups were designated as follows: Groups I, II, III and IV (all n=4). All animal experiments were carried out in agreement with the ethical standards of China Medical University, which conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Treatments. Group I received normal saline, group II 100 µg salsolinol, group III 50 µg salsolinol + 50 µg carnosine, and group IV 100 µg salsolinol + 100 µg carnosine. Following 72 h, the animals were sacrificed, and brain tissues were surgically removed. Brain tissue homogenate was prepared and used for the subsequent investigations.

In vitro studies

Cell culture. Rat brain endothelial cells were cultured in DMEM growth medium containing FBS and 1% penicillin-streptomycin. Cells were maintained under standard conditions in a CO₂ incubator at 37°C with an atmosphere containing 5% CO₂.

Fluorescence microscopy. Rat brain endothelial cells were cultured in a dish. Cells were treated with 100 µg/ml salsolinol, 50 µg/ml salsolinol + 50 µg/ml carnosine or 100 µg salsolinol + 100 µg carnosine, respectively. Following treatment, cells were centrifuged at 500 x g for 5 min at 4°C, and cell volume was adjusted to 10⁴-10⁵ cells/ml. Cells were incubated with acridine orange (AO) and ethidium bromide (EB) dye for 30 min at room temperature. Cells were viewed under a fluorescence microscope (Olympus Corp., Tokyo, Japan), as previously described by Muthuraman *et al* (24).

Determination of ROS production. Rat brain endothelial cells were cultured at 2.5x10⁵ cells/well in a 6-well plate under 37°C and 5% CO₂. Cells were treated with 100 µg/ml salsolinol, 50 µg/ml salsolinol + 50 µg/ml carnosine or 100 µg salsolinol + 100 µg carnosine, respectively. Following treatment, the cells were treated with DCFH-DA for 30 min at 37°C in an atmosphere containing 5% CO₂. Cells were viewed for fluorescence under a fluorescence microscope (Olympus Corp.), as previously described by Muthuraman *et al* (24).

Determination of lipid peroxidation. Cells were seeded in a dish at 2.5x10⁵ cells/well in a 6-well plate. Cells were treated with 100 µg/ml salsolinol, 50 µg/ml salsolinol + 50 µg/ml carnosine or 100 µg salsolinol + 100 µg carnosine, respectively. At the end of all treatment, LPO levels were determined using a kit according to method outlined by Muthuraman *et al* (25). MDA content was measured with a spectrophotometer at 534 nm (Cary 100 UV-Vis; Agilent Technologies, Inc., Santa Clara, CA, USA).

Determination of reduced glutathione. Cells were cultured and grown at 2.5x10⁵ cells per well in a 6-well plate. Cells were treated with 100 µg/ml salsolinol, 50 µg/ml salsolinol + 50 µg/ml carnosine or 100 µg salsolinol + 100 µg carnosine, respectively. GSH levels were measured using a kit according to the method outlined by Muthuraman *et al* (25). The resultant yellow product was measured at 405 nm using a Cary 100 UV-Vis spectrophotometer.

Determination of superoxide dismutase (SOD) and catalase enzyme activities. Cells were seeded in a dish at 2.5x10⁵ cells/well in a 6-well plate. Cells were treated with 100 µg/ml salsolinol, 50 µg/ml salsolinol + 50 µg/ml carnosine or 100 µg salsolinol + 100 µg carnosine, respectively. SOD and catalase enzyme activities were measured using a kit (SOD assay kit, 19160-1KT-F; catalase assay kit, CAT100-1KT; both Sigma-Aldrich; Merck KGaA) according to the method outlined by Muthuraman *et al* (25).

In vivo studies

Determination of lipid peroxidation. Lipid peroxidation was determined using a kit according to the spectrophotometric method of Muthuraman *et al* (25). MDA content was measured by determining the thiobarbituric acid reactive species (TBARS). The resultant product was determined at 534 nm using a Cary 100 UV-Vis spectrophotometer.

Determination of reduced glutathione (GSH). The level of GSH was measured using a kit according to the spectrophotometric method of Muthuraman *et al* (25). The yellow product color was measured using a according to the spectrophotometer at 405 nm.

Determination of SOD and catalase enzyme activities. SOD and catalase enzyme activities were measured using a kit according to the method of Muthuraman *et al* (25).

Histopathological examination. A total of 24 rats were anesthetized with diethyl ether (Sigma-Aldrich; Merck KGaA) and sacrificed by decapitation. Brain tissues were removed and kept in 4% paraformaldehyde at 4°C for 60 min. Hippocampus sections (4-µm thick) were prepared with use of microtome and stained with hematoxylin and eosin. Sections were qualitatively analyzed by light microscopy as previously described (26).

Statistical analysis. All experimental data are expressed as the mean ± standard error of the mean. The treated and control groups were compared using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

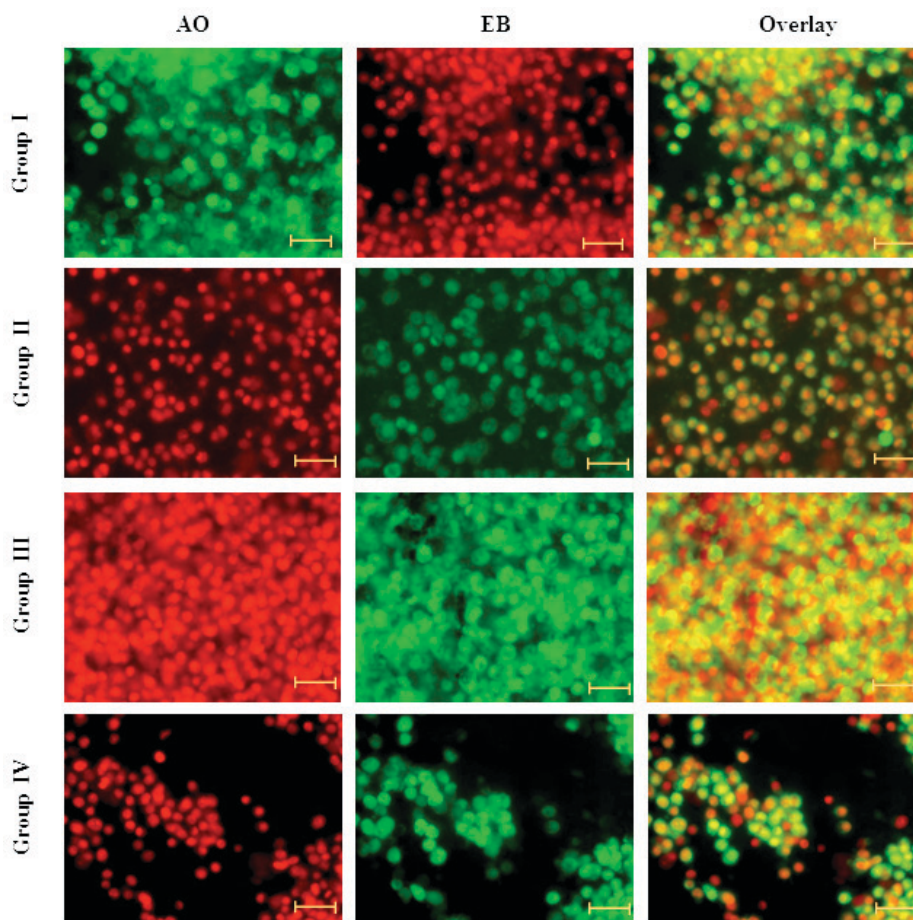


Figure 1. AO/EB double staining by fluorescence microscopy at a magnification of x40. Cells were seeded in 6-well plates and cultured for 24 h to allow adherence, cells were treated as mentioned in the method section. Representative images from three independent experiments. AO, acridine orange; EB, ethidium bromide. Scale bar is 50 μm .

Results

Effect of carnosine on apoptosis. Fluorescence microscopy examination was performed to assess whether the neuroprotective effect of carnosine was associated with the morphological aspect of cell death and apoptosis and the morphological features of cell death. DNA-binding AO and EB dyes were used to differentiate between viable and non-viable cells, as chromatin condensation in the stained nucleus is useful to identify viable, apoptotic and necrotic cells. The neuroprotective effect of carnosine against salsolinol in the rat brain endothelial cells was presented (Fig. 1). Fluorescence analysis indicated normal cell size and morphology in control cells (group I); whereas salsolinol-induced rat brain endothelial cells exhibited altered cell morphology, including apoptosis and necrosis (group II). Administration of 50 $\mu\text{g/ml}$ carnosine and 50 $\mu\text{g/ml}$ salsolinol (group III) markedly reduced the apoptosis and necrosis of rat brain endothelial cells. Administration of 100 $\mu\text{g/ml}$ carnosine and 100 $\mu\text{g/ml}$ rotenone (group IV) markedly reduced the occurrence of apoptosis and necrosis in the endothelial cells towards normal levels (Fig. 1).

Effect of carnosine on intracellular ROS level. ROS are able to facilitate signal transduction processes in the cellular region. Fluorescence studies demonstrated that there was little green

fluorescence in the control cells (group I), whereas green fluorescence was markedly increased in salsolinol-treated cells (group II). Administration of 50 $\mu\text{g/ml}$ carnosine and 50 $\mu\text{g/ml}$ salsolinol (group III) markedly reduced the level of ROS in rat brain endothelial cells. Administration of 100 $\mu\text{g/ml}$ carnosine and 100 $\mu\text{g/ml}$ salsolinol (group IV) markedly reduced the level of ROS in the rat brain endothelial cells, towards normal levels (Fig. 2).

Effect of carnosine on MDA and GSH content in rat brain endothelial cells. The neuroprotective effect of carnosine against salsolinol-induced toxicity in rat brain endothelial cells is presented in Table I. MDA content in control cells was 21.15 ± 1.20 nmol/g, whereas it significantly increased to 36.15 ± 1.0 nmol/g in salsolinol-treated rat brain endothelial cells (group II; $P < 0.05$; Table I). Administration of 50 $\mu\text{g/ml}$ carnosine and 50 $\mu\text{g/ml}$ salsolinol (group III) significantly reduced (31.36 ± 1.2 nmol/g) MDA content in the rat brain endothelial cells, compared with group II ($P < 0.05$; Table I). Administration of 100 $\mu\text{g/ml}$ carnosine and 100 $\mu\text{g/ml}$ salsolinol (group IV) significantly reduced MDA content (23.7 ± 1.1 nmol/g) in the rat brain endothelial cells, compared with group II ($P < 0.05$; Table I).

GSH content in control cells was 73.23 ± 2.2 mg/g (group I), whereas it was significantly reduced to 34.14 ± 1.2 mg/g in

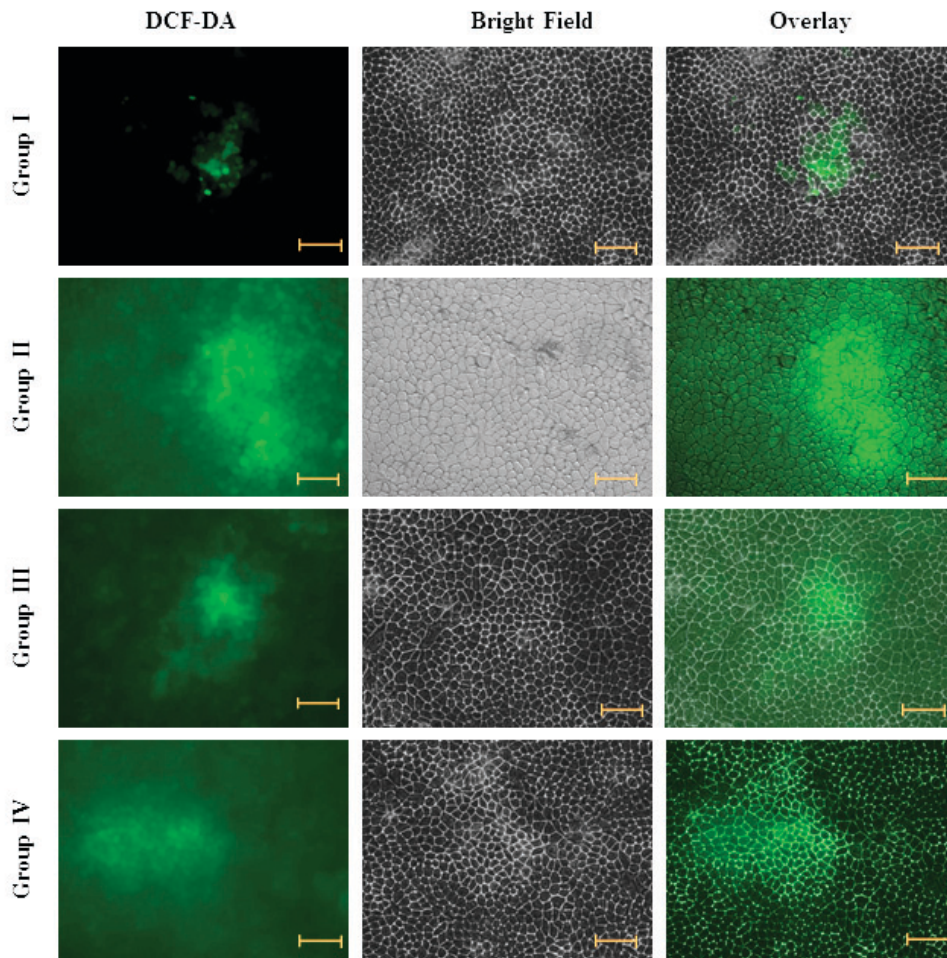


Figure 2. Suppressive effect of carnosine on ROS production. Cells were grown in 6-well plates and allowed to adhere for 24 h. Cells were treated with the carnosine as mentioned in the method. At the end of treatments, cells were stained with 2,7-dichlorodihydro fluorescein diacetate and images were obtained using fluorescence microscopy. Representative images from three independent experiments. ROS, reactive oxygen species. Magnification, x40. Scale bar=50 μ m.

salsolinol-treated rat brain endothelial cells (group II; $P < 0.05$; Table I). Administration of 50 μ g/ml carnosine and 50 μ g/ml salsolinol (group III) significantly increased GSH content to 47.45 ± 1.2 mg/g in the rat brain endothelial cells, compared with group II ($P < 0.05$; Table I). Administration of 100 μ g/ml carnosine and 100 μ g/ml salsolinol (group IV) significantly increased GSH content to 64.8 ± 2.2 mg/g in the rat brain endothelial cells, compared with group II ($P < 0.05$; Table I).

Effect of carnosine on antioxidant enzymes in rat brain endothelial cells. The neuroprotective effect of carnosine against the salsolinol-induced toxicity of rat brain endothelial cells is presented in Table I. SOD activity was identified to be 2.8 ± 0.03 U/mg in the control rat brain endothelial cells (group I), whereas it was significantly reduced to 1.8 ± 0.02 U/g in the salsolinol-induced rat brain endothelial cells (group II; $P < 0.05$; Table I). Administration of 50 μ g/ml carnosine and 50 μ g/ml salsolinol (group III) significantly increased SOD activity to 1.92 ± 0.02 U/g in the rat brain endothelial cells, as compared with group II ($P < 0.05$; Table I). Administration of 100 μ g/ml carnosine and 100 μ g/ml salsolinol (group IV) has significantly increased SOD activity to 2.5 ± 0.03 U/g compared with group II ($P < 0.05$; Table I).

Catalase activity was identified to be 6.7 ± 0.07 U/g in the control rat brain endothelial cells (group I), whereas it was significantly reduced to 3.33 ± 0.05 U/g in the salsolinol-induced rat brain endothelial cells (group II; $P < 0.05$; Table I). Administration of 50 μ g/ml carnosine and 50 μ g/ml salsolinol (group III) significantly increased catalase activity to 3.8 ± 0.02 U/g in the rat brain endothelial cells, as compared with group II ($P < 0.05$; Table I). Administration of 100 μ g/ml carnosine with 100 μ g/ml salsolinol (group IV) significantly increased catalase activity to 5.75 ± 0.05 U/g compared with group II ($P < 0.05$; Table I).

Effect of carnosine on MDA content in rat brain tissue. The neuroprotective effect of carnosine against salsolinol in male albino rats is demonstrated in Fig. 1. MDA content in the control was 26.40 ± 1.1 nmol/g, whereas it significantly increased to 59.55 ± 2.1 nmol/g in salsolinol treated rat brain (group II; $P < 0.05$). Administration of 50 μ g/ml carnosine and 50 μ g/ml salsolinol (group III) significantly reduced compared with group II (48.76 ± 1.6 nmol/g) MDA content in the rat brain ($P < 0.05$). Administration of 100 μ g/ml carnosine and 100 μ g/ml salsolinol (group IV) significantly reduced MDA content (34.11 ± 1.1 nmol/g) in the rat brain ($P < 0.05$; Fig. 3).

Table I. Effect of carnosine against salsolinol induced LPO, GSH, SOD and catalase levels in the rat brain endothelial cells.

Parameter	Group I	Group II	Group III	Group IV
MDA, nmol/g	21.15±1.12	36.15±1.0 ^a	31.36±1.2 ^b	23.70±1.1 ^b
GSH, mg/g	73.23±2.2	34.14±1.2 ^a	47.45±1.2 ^b	64.80±2.2 ^b
SOD, U/mg	2.80±0.03	1.80±0.02 ^a	1.92±0.02 ^b	2.50±0.03 ^b
Catalase, U/g	6.70±0.07	3.33±0.05 ^a	3.80±0.02 ^b	5.75±0.05 ^b

^aP<0.05 vs. group I (control); ^bP<0.05 vs. group II (n=6). Data are expressed as the mean ± standard error of the mean. LPO, lipid peroxidation; GSH, glutathione; SOD, superoxide dismutase.

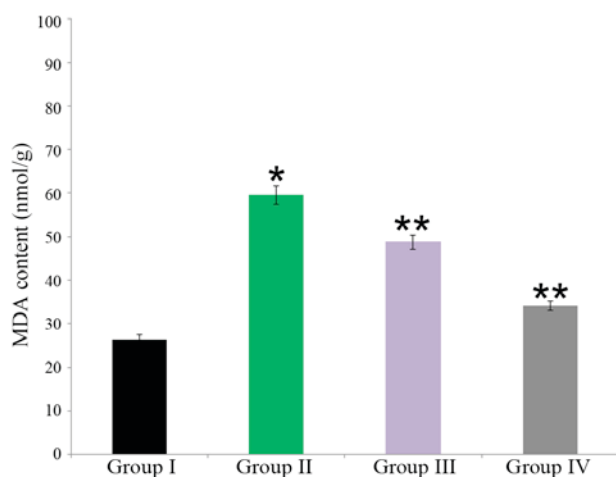


Figure 3. Suppressive effect of carnosine on lipid peroxidation in the male albino rat brain at 48 h. Results are presented as nmol/g compared with the control. *P<0.05 vs. group I (control); **P<0.05 vs. group II. Data are expressed as mean ± standard deviation. MDA, malondialdehyde.

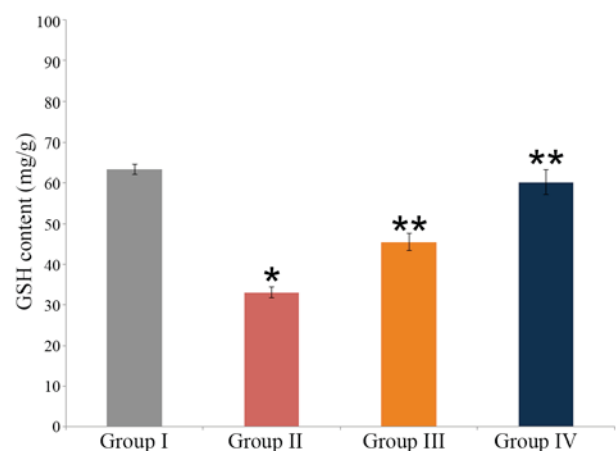


Figure 4. Protective effect of carnosine on GSH content in the male albino rat brain at 48 h. Results are presented as mg/g compared with the control. *P<0.05 vs. group I (control); **P<0.05 vs. group II. Data are expressed as mean ± standard error of the mean. GSH, glutathione.

Effect of carnosine on GSH content in rat brain tissue. The neuroprotective effect of carnosine against salsolinol in male albino rats is indicated in Fig. 4. The GSH content in the control was 63.3±1.2 mg/g (group I), whereas it was significantly reduced to 33.10±1.2 mg/g in salsolinol treated rat brain

(group II; P<0.05). Administration of 50 µg/ml carnosine and 50 µg/ml salsolinol (group III) significantly increased GSH content to 45.45±2.1 mg/g in the rat brain, as compared with the content in group II. Administration of 100 µg/ml carnosine and 100 µg/ml salsolinol (group IV) significantly increased GSH content to 60.21±3.0 mg/g in the rat brain, as compared with group II (P<0.05; Fig. 4).

Effect of carnosine on SOD activity in rat brain tissue. The neuroprotective effect of carnosine against salsolinol in male albino rats is presented in Fig. 5. SOD activity was identified to be 2.53±0.11 U/mg in the control rat brain (group I), whereas it was significantly reduced to 1.35±0.01 U/mg in the salsolinol treated rat brain (group II; P<0.05). Administration of 50 µg/ml carnosine and 50 µg/ml salsolinol (group III) significantly increased SOD activity to 2.01±0.04 U/mg in the rat brain compared with group II (P<0.05). Administration of 100 µg/ml carnosine and 100 µg/ml salsolinol (group IV) significantly increased SOD activity to 2.47±0.12 U/mg in the rat brain, as compared with group II (P<0.05; Fig. 5).

Effect of carnosine on catalase activity in rat brain tissue. The neuroprotective effect of carnosine against salsolinol in male albino rats is presented in Fig. 6. Catalase activity was identified to be 10.14±0.1 U/g in the control rat brain (group I), this was significantly reduced to 5.45±0.13 U/g in the salsolinol treated rat brain (P<0.05; group II). Administration of 50 µg/ml carnosine and 50 µg/ml salsolinol (group III) significantly increased catalase activity to 6.4±0.11 U/g in the rat brain, as compared with group II (P<0.05). Administration of 100 µg/ml carnosine and 100 µg/ml salsolinol (group IV) significantly increased catalase activity to 8.5±0.21 U/g in the rat brain, as compared with group II (P<0.05; Fig. 6).

Effect of carnosine on rat brain histopathology. The neuroprotective effect of carnosine against salsolinol in male albino rats is presented in Fig. 7. Histopathological analysis demonstrated a normal cellular architecture in control rats (group I), whereas salsolinol-induced rat brain exhibited altered cellular structure, including apoptosis and necrosis of cells (group II). Administration of 50 µg/ml carnosine and 50 µg/ml salsolinol (group III) markedly improved the rat brain cells. Administration of 100 µg/ml carnosine and 100 µg/ml salsolinol (group IV) markedly improved the rat brain cell architecture, returning to normal (Fig. 7).

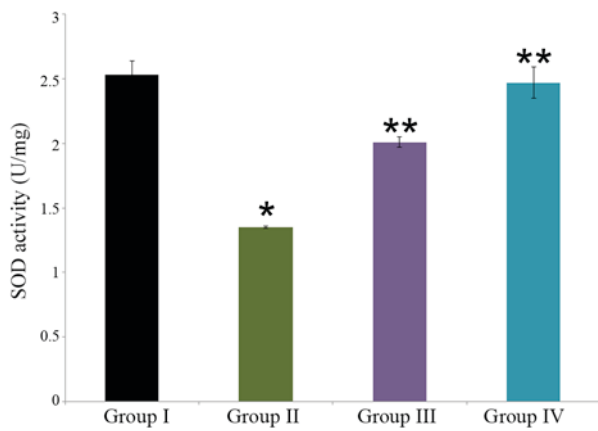


Figure 5. Protective effect of carnosine on SOD activity in the male albino rat brain at 48 h. Results are presented as U/g compared with the control. * $P < 0.05$ vs. group I (control); ** $P < 0.05$ vs. group II. Data are expressed as mean \pm standard error of the mean.

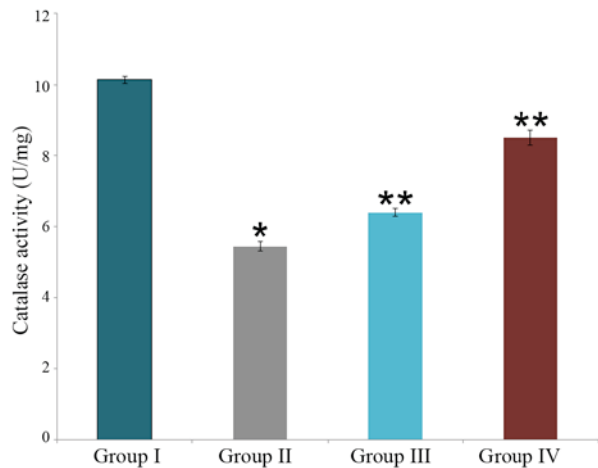


Figure 6. Protective effect of carnosine on catalase activity in the male albino rat brain at 48 h. Results are presented as U/g compared with the control. * $P < 0.05$ vs. group I (control); ** $P < 0.05$ vs. group II. Data are expressed as the mean \pm standard error of the mean. SOD, superoxide dismutase.

Discussion

The experimental results in the present study demonstrated that carnosine had neuroprotective effects on the male albino rats, rat brain endothelial cells and appear to exhibit a clear dose-dependence when exposing cells to higher concentrations. Induction of tumor cell apoptosis is an essential property of anti-cancer therapeutics (27). Apoptosis is defined as a morphological and biochemical alteration of cells and therefore, a morphological study is vital for apoptosis investigations. In the current study, carnosine treatment exerted increasing suppressive effects on the male albino rats and rat brain endothelial cells with increasing concentrations.

Morphological studies are important to understand the cytotoxic impact of the carnosine with apoptosis. Carnosine has been demonstrated to protect rat brain endothelial cells against the toxic effects of amyloid peptides. Carnosine protects cells by scavenging MDA and 4-hydroxynonenal, which usually react with macromolecules (28). Therefore, ROS production and involvement are one of the potential mechanisms. Interference

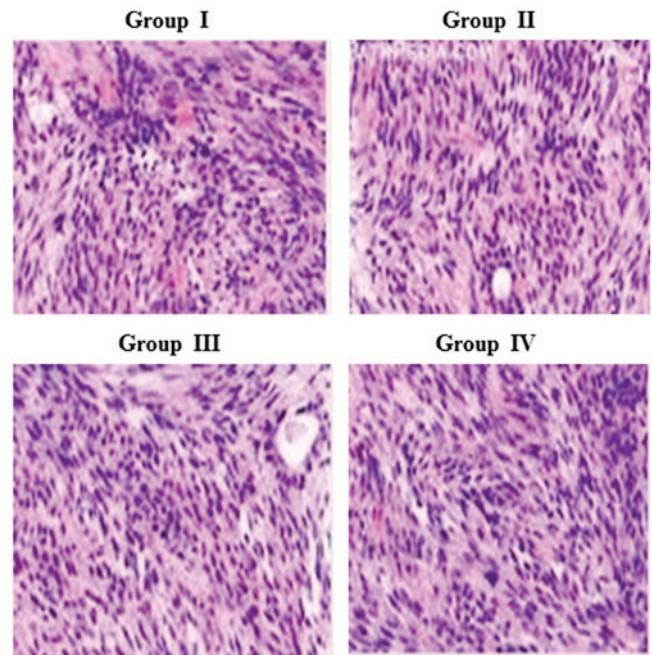


Figure 7. Protective effect of carnosine on the cellular architecture of the male albino rat brain at 48 h. Hippocampus sections were prepared (5- μ m thick) and stained with hematoxylin and eosin. Stained sections were analyzed qualitatively by light microscope at a magnification of $\times 40$. Scale bar is 50 μ m.

using stimulating peptide catabolism, scavenging superoxide radicals and effects on second messenger processes are considered to be further explanations. The results of the current study indicate that carnosine treatment reduced lipid peroxidation in the rat brain and endothelial cells. Carnosine also significantly increased GSH and antioxidant enzyme activities, which further confirms the protective effects of carnosine.

Carnosine significantly normalized cell morphology at an *in vivo* and *in vitro* level. It has been demonstrated that accumulation of glycated and damaged proteins occurs during normal aging (29) and in larger quantities during Alzheimer's disease (30,31). Carnosine protects against age-related macromolecular damage via the production of ROS (1). Carnosine is an anti-glycating compound (13) and previous studies have indicated that carnosine may delay senescence (14) and inhibit DNA oxidation in human fibroblasts (32). The present study demonstrated that carnosine acts against lipid peroxidation and antioxidant markers by altering its toxicity and inhibiting protein damage. Brain, muscle and lens tissues in animals contain high amount of carnosine (33).

The results of the current study indicated that salsolinol may induce neurotoxicity in the male albino rat brain and rat brain endothelial cells (34,35). Treatment with carnosine exhibited a significant improvement in neurotoxicity. The lower concentration (50 μ g/ml) of carnosine used in the present study was able to significantly protect against neurotoxicity induced by salsolinol in the rat brain and endothelial cells. Salsolinol is known to cause neurotoxicity via the inhibition of mitochondrial complex II and by initiating apoptosis through the increased production of free radicals (36). Carnosine has been demonstrated to possess neuroprotective effects through the inhibition of apoptosis with a consequent reduction in the

production of ROS. Although, even at a high concentration, carnosine may partially recover or inhibit the toxicity induced by salsolinol in the rat brain and endothelial cells. Investigation of carnosine in combination with other drugs for their synergistic action would be worthwhile and notable as it may be helpful in determining the therapeutic effect of the carnosine as a monotherapy and in combination with other drugs.

In conclusion, salsolinol exerted neurotoxicity in the male albino rat brain tissues and rat brain endothelial cells. This cytotoxicity was reversed following treatment with carnosine, as demonstrated by the results of the current study. The *in vivo* levels of MDA, GSH, SOD and catalase were renormalized following carnosine treatment, and the cellular architecture of the rat brain also began to return to normal. Morphological and apoptotic changes were evaluated by fluorescence microscopy, which confirmed that there was a reduction of apoptosis following carnosine treatment. The level of ROS was reduced in the current study due to a decrease in the oxidative stress. MDA, GSH, SOD and catalase levels were also measured *in vitro*, and these findings matched the *in vivo* measurements. The experimental results of the present study may conclude that salsolinol exerts neurotoxicity, and treatment with carnosine may significantly reverse this toxicity.

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