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# Administration of carnosine in the treatment of acute spinal cord injury

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ABSTRACT

L-Carnosine is an endogenously synthesized dipeptide composed of beta-alanine and L-histidine. It acts as a free radical scavenger and possesses antioxidant properties. L-Carnosine reduces proinflammatory and profibrotic cytokines such as transforming growth factor-beta (TGF-beta), interleukin (IL)-1, and tumor necrosis factor (TNF)-alpha in different experimental settings. In the present study, we investigated the efficacy of L and D-carnosine on the animal model of spinal cord injury (SCI). The spinal cord was exposed via a four-level T5–T8 laminectomy and SCI was produced by extradural compression of the spinal cord at level T6–T7 using an aneurysm clip with a closing force of 24 g. Treatment with D-carnosine (150 mg/kg administered i.p., 1 h and 6 h, after SCI), but not L-carnosine significantly decreased (a) the degree of spinal cord inflammation and tissue injury (histological score), (b) neutrophil infiltration (myeloperoxidase activity), (c) nitrotyrosine formation, inducible NO synthase (iNOS) and Hsp70 expression, (d) proinflammatory cytokines, and (e) apoptosis (TUNEL staining, Fas ligand, Bax, and Bcl-2 expression). Furthermore, D-carnosine (150 mg/kg administered i.p., 1 h and 6 h, after SCI) significantly ameliorated the loss of limb function (evaluated by motor recovery score). Taken together, our results demonstrate the strong difference between L-carnosine and D-carnosine. The result strongly suggests that D-carnosine treatment reduces the development of inflammation and tissue injury associated with spinal cord trauma.



## 1. Introduction

SCI is a highly debilitating pathology, could have traumatic or non-traumatic origin and often could lead to devastating and catastrophic dysfunction. SCI imposes high physical and psychological effects not only to the individual but also to the family and society.

Over the past years much research has been performed on elucidating the mechanisms of SCI. Experimental and clinical studies have suggested that acute SCI is a two-step process involving primary and secondary mechanisms. Primary injury of the spinal cord refers to the initial mechanical damage due to local deformation of the spine. Direct compression and damage of neural elements and blood vessels by fractured and displaced bone fragments or disc material occur after mechanical trauma.

The secondary mechanism includes a cascade of biochemical and cellular processes, such as electrolyte abnormalities, formation free radicals, vascular ischemia, oedema, post-traumatic inflammatory reaction, apoptosis or genetically programmed cell death and other processes [1].

It is known that a progressive neuronal injury results from a combination of secondary injury factors including: ischemia, biochemical alterations, excitotoxicity, neurotransmitter accumulation, protein and lipid oxidation associated with the generation of free radicals [2,3]. Consistent with this idea, neurodegenerative diseases, are characterized by increased production of reactive oxygen molecules (ROS) such as superoxide anions, hydrogen peroxide and peroxynitrite [4]. Free radical-mediated oxidation of lipids, proteins and DNA contribute to enhance adverse mechanisms of neuronal injury, such as spinal cord hypoperfusion, development of oedema, axonal conduction failure and breakdown of energy metabolism [5].

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The importance of free radical mediated cell damage in the SIC is supported by the large number of experimental and clinical studies demonstrating the efficacy of antioxidants agents in improving neuronal damage [6–9].

L-Carnosine is a small molecule composed of the amino acids L-histidine and β-alanine. This dipeptide is found in relatively high concentrations in several body tissues—most notably in skeletal muscle, heart muscle, skin, stomach, nerve tissue and brain. Although the exact biological role is not well understood, numerous studies have demonstrated, both at the tissue and organelle level, that it is endowed with strong and specific antioxidant properties by preventing and reducing the accumulation of oxidised products derived from the lipid peroxidation (LPO) of biological membranes, including amazing anti-aging actions [10].

L-Carnosine and related dipeptides have been shown to prevent peroxidation of model membrane systems leading to the suggestion that they represent water-soluble counterparts to lipid-soluble antioxidants such as α-tocopherol in protecting cell membranes from oxidative damage. Other roles ascribed to these dipeptides include actions as neurotransmitters, modulation of enzymic activities and chelation of heavy metals [11]. Many claims have been made in respect of therapeutic actions of L-carnosine and histidine-containing dipeptides [12]. These include antihypertensive effects, actions as immunomodulating agents, wound healing and antineoplastic effects [11].

L-Carnosine has been recently proposed to act as antioxidant *in vivo* [13,14]. It was demonstrated the neuroprotective effect in astrocytes exposed to LPS- and INF-gamma-induced nitrosative stress [15]. It is important to note that L-carnosine counteracts peroxynitrite-dependent protein alterations such as tyrosine nitration [16]. Recent evidence demonstrated that L-carnosine prevents the up-regulation of iNOS and the induction of both HO-1 and Hsp-70 following nitrosative injury [15]. In addition, a correlation has been found between cell protection and nitric oxide (NO) free-radical scavenging activity of L-carnosine that showed direct NO-trapping ability in cell-free experiments [17]. Such important results can be used to define L-carnosine as an important Heat Shock Response activator, overcoming its considered classical role as multi-target protective compound.

L-Carnosine has been called a longevity nutrient since laboratory studies on tissues indicate that it can delay senescence and provoke cellular rejuvenation in cultured human fibroblasts. Carnosine has been called the anti-aging and anti-oxidant dipeptides [18].

L-Carnosine is, however, known to be rapidly hydrolyzed in human serum by specific hydrolytic enzymes (carnosinases) [19,20], which may limit its therapeutic potential [21]. Such a problem can be overcome by the use of L-carnosine derivatives stable in human serum [22–24]. Specifically, D-carnosine, a non-natural isomer resistant to hydrolysis by carnosinases besides being stable in the plasma, is able to cross the blood–brain barrier, providing therefore, a suitable rationale for systemic interventions in free radical-related diseases [25].

Thus the aim of the present study was to investigate the efficacy of L and D-carnosine on the animal model of SCI.

## 2. Materials and methods

### 2.1. Animals

Male Adult CD1 mice (25–30 g, Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

### 2.2. SCI

Mice were anaesthetized using chloral hydrate (400 mg/kg body weight). A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T5–T8 vertebrae. The spinal cord was exposed via a four-level T5–T8 laminectomy and SCI was produced by extradural compression of the spinal cord at level T6–T7 using an aneurysm clip with a closing force of 24 g. Following surgery, 1.0 cc of saline was administered subcutaneously in order to replace the blood volume lost during the surgery. During recovery from anesthesia, mice were placed on a warm heating pad and covered with a warm towel. Mice were singly housed in a temperature-controlled room at 27 °C for a survival period of 10 days. Food and water were provided to the mice *ad libitum*. During this time period, the animals' bladders were manually voided twice a day until the mice were able to regain normal bladder function. In all injured groups, the spinal cord was compressed for 1 min. Sham animals were only subjected to laminectomy.

### 2.3. Experimental design

Mice were randomly allocated into the following groups:

(i) *SCI + vehicle group*. Mice were subjected to SCI plus administration of saline (administered i.p., 1 h and 6 h after SCI) ($N = 10$);
(ii) *D-carnosine group*. Same as the SCI + vehicle group but in which D-carnosine (150 mg/kg) was administered i.p., 1 h and 6 h after SCI ($N = 10$);
(iii) *L-carnosine group*. Same as the SCI + vehicle group but in which L-carnosine (150 mg/kg) was administered i.p., 1 h and 6 h after SCI ($N = 10$);
(iv) *Sham + vehicle group*. Mice were subjected to the surgical procedures as the above groups except that the aneurysm clip was not applied and to these mice were administered saline (administered i.p., 1 h and 6 h, after SCI) ($N = 10$);
(v) *Sham + D-carnosine group*. Identical to Sham + vehicle group except for the administration of D-carnosine (150 mg/kg administered i.p., 1 h and 6 h, after SCI) ($N = 10$);
(vi) *Sham + L-carnosine group*. Identical to Sham + vehicle group except for the administration of L-carnosine (150 mg/kg administered i.p., 1 h and 6 h, after SCI) ($N = 10$).

As described below mice ($N = 10$ from each group for each parameters) were sacrificed at 24 h after SCI in order to proceed with the different analyses and parameter evaluations.

In a separate set of experiments other 10 animals for each group were observed until 10 days after SCI in order to evaluate the motor score.

In another set of experiments other 10 animals for each group were observed in order to evaluate the dose-effect curve.

### 2.4. Light microscopy

Spinal cord biopsies were taken at 24 h following trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5-μm-thick sections. Tissue longitudinal sections (thickness 5 μm) were deparaffinized with xylene, stained with Haematoxylin/Eosin (H&E), with silver impregnation for reticulum and studied using light microscopy (Dialux 22 Leitz).

The segments of each spinal cord were evaluated in the rostral/caudal perilesional area by an experienced histopathologist (RO). Damaged neurons were counted and the histopathologic changes of the gray matter were scored on a 6-point scale [26]: 0, no lesion observed; 1, gray matter contained 1–5 eosinophilic neurons; 2, gray

matter contained 5–10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction; (one third to one half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for an individual mouse. All the histological studies were performed in a blinded fashion.

### 2.5. Myeloperoxidase activity

To test whether spinal cord damage was associated with polymorphonuclear infiltration, we measured myeloperoxidase activity in the perilesional spinal cord tissue. MPO activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in the spinal cord tissues as previously described [27] at 24 h after SCI. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide $min^{-1}$ at 37 °C and was expressed in units/g of wet tissue.

### 2.6. ELISA measurement of TNF-α and IL-1β

To test whether spinal cord damage was associated with pro-inflammatory cytokines formation, we measured TNF-α and IL-1β levels in the perilesional spinal cord tissue. For the measurement of cytokines levels, a 1 cm sample containing the lesion site (or comparable region of sham operated animals) was rapidly dissected and homogenized in 1 ml PBS containing protease inhibitors (Complete protease inhibitor tablets, Roche). TNF-α and IL-1β levels were assayed using DuoSet ELISA Development System (R&D Systems). All assays were carried out in duplicate using recommended buffers, diluents and substrates. Absorbency was determined using a microplate reader at 450 nm (Thermo Scientific, Multiskan FC Microplate Photometer). The intra-assay coefficient of variations for both assays was less than 10%. The concentration of the cytokines in the tissue was mentioned as pg/100 mg wet tissue.

### 2.7. Immunohistochemical localization of iNOS, nitrotyrosine, PAR, FasL, Bax and Bcl-2, S-100

To test whether carnosine may modulate the inflammatory process, we analyzed the spinal cord tissue levels of iNOS, nitrotyrosine, PAR, FasL, Bax and Bcl-2, S-100.

At 24 h after SCI, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin, respectively. Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (Upstate, 1:500 in PBS, v/v), anti-PAR antibody (BioMol, 1:200 in PBS, v/v), anti-FasL antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), anti-Bax antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v) or with anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), or with anti-S100 polyclonal antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), or with anti-iNOS polyclonal antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vector Laboratories, DBA). To verify the binding specificity for nitrotyrosine, PAR, FasL, S-100, Bax and Bcl-2, iNOS, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections indicating that the immunoreactions were positive in all the experiments carried out. Immunocytochemistry photographs ($n = 5$ photos from each samples collected from all rats in each experimental group) were assessed by densitometry using Imaging Densitometer (AxioVision, Zeiss, Milan, Italy) and a computer program.

### 2.8. Western blot analysis for Bax, Bcl-2 and Hsp70

To test whether spinal cord damage was associated with cell death by apoptosis, we measured Bax, Bcl-2 in the perilesional spinal cord tissue. Cytosolic and nuclear extracts were prepared as previously described [28] with slight modifications. Briefly, spinal cord tissues from each mouse were suspended in extraction Buffer A containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 0.15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM ethylene diamine-tetra-acetic acid (EDTA), 0.2 mM PMSF, 20 μM leupeptin, 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000 × *g* at 4 °C, the supernatants containing the nuclear protein were stored at −80 °C for further analysis. The levels of Bax, Bcl-2 and Hsp70 were quantified in cytosolic fractions from spinal cord tissue collected 24 h after SCI. The filters were blocked with 1× PBS, 5% (w/v) non fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs anti-Bax (1:500; Santa Cruz Biotechnology), anti-Bcl-2 (1:500; Santa Cruz Biotechnology) or anti-Hsp70 (1:1000; Stressgen, Ann Arbor, MI, USA), in 1× PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 °C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson Immuno Research, West Grove, PA) for 1 h at room temperature.

To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin (1:10,000 Sigma–Aldrich Corp.) The relative expression of the protein bands of Bax (~23 kDa), Bcl-2 (~29 kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM).

### 2.9. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay

To test whether spinal cord damage was associated with cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit DBA, Milan, Italy). Briefly, tissue segments containing the lesion (1 cm on each side of the lesion, rostrally/caudally to the perilesional area) were cut into longitudinal 5-μm-thick sections. Tissue were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% $H_2O_2$ for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

### 2.10. Grading of motor disturbance

Motor function of the hindlimbs was evaluated by open-field testing using the methodology of the Basso Mouse Scale (BMS) score on postoperative days as described by Basso et al. [29].

### 2.11. Materials

The primary antibodies directed at FasL, Bax, Bcl-2 and inducible Hsp70 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The secondary antibody was obtained from Jackson Immuno Research, Laboratories, Inc. (Jackson, USA). Unless otherwise stated, all compounds were obtained from Sigma–Aldrich Company Ltd. (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

### 2.12. Statistical evaluation

All values in the figures and texts are expressed as mean ± standard error of the mean (SEM) of N observations. For the *in vivo* studies N represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. Statistical analysis was performed by a two-way analysis of variance and the post hoc Bonferroni-corrected *t*-tests to compare differences between Sham and injured animals in the absence and presence of the D-carnosine and/or L-carnosine. Other data were analyzed by one-way ANOVA or Kruskal–Wallis test followed by post-hoc test for multiple comparisons. In all cases, a level of 5% was considered statistically significant. BMS scale data were analyzed by the Mann–Whitney test and considered significant when *P*-value was $<0.05$.

## 3. Results

### 3.1. Effects of carnosine on the severity of spinal cord trauma

The severity of the trauma at the level of the perilesional area, assessed by the presence of oedema as well as alteration of the white matter and infiltration of leukocytes (Fig. 1B, see histological score I), was evaluated at 24 h after injury. Significant damage to

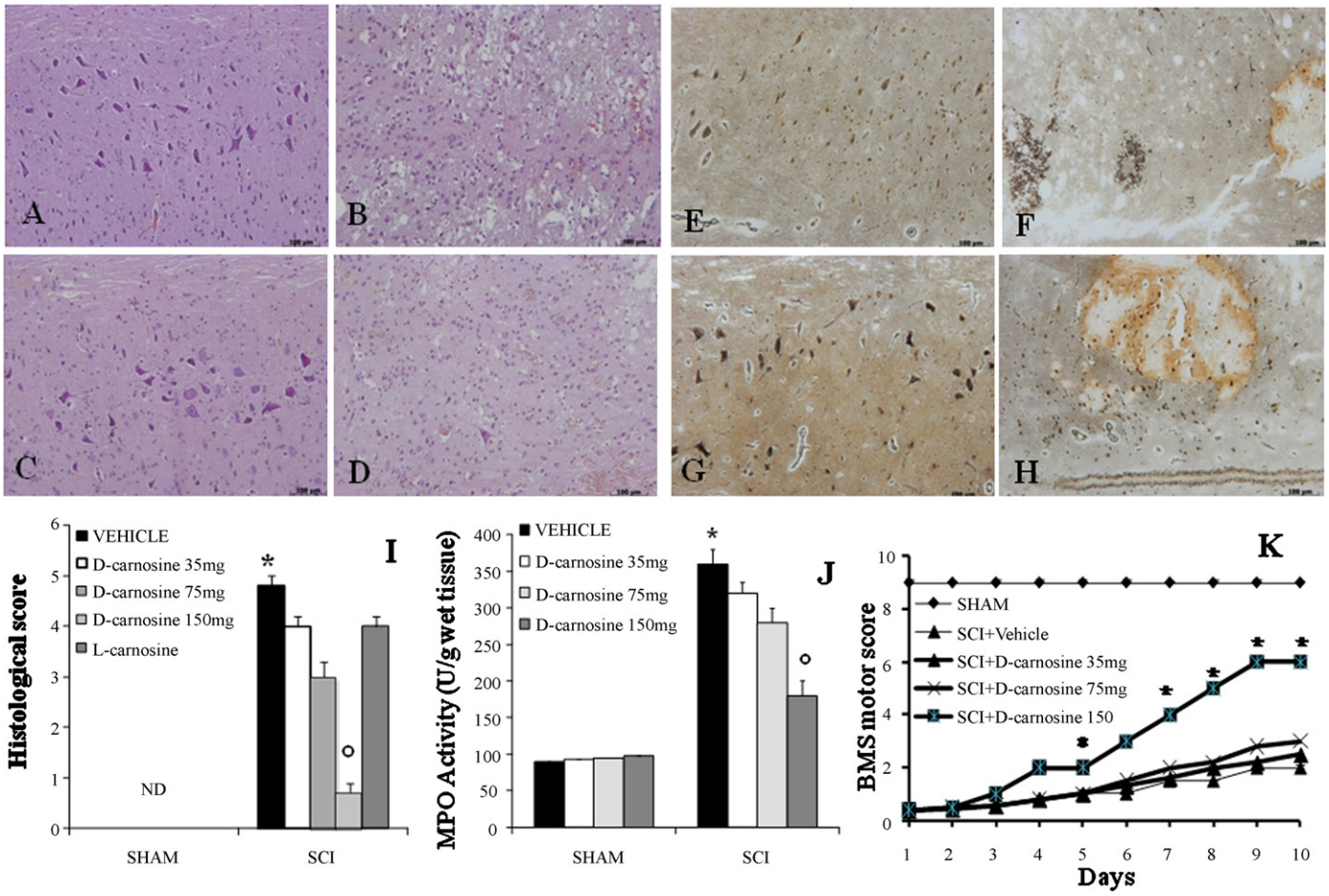


**Fig. 1.** Effect of D and L-carnosine treatments on histological alterations of the spinal cord tissue 24 h after injury, MPO and motor function. Significant damage to the spinal cord in mice subjected to SCI, at the perilesional area, was apparent, as evidenced by the presence of oedema as well as alteration of the white matter 24 h after injury (B) when compared with sham-operated mice (A). Notably, a significant protection from SCI-associated damage was observed in the tissue samples collected from D-carnosine treated mice (C). On the contrary no protection against the SCI was observed in L-carnosine-treated mice (D). The histological score (I) was made by an independent observer. In sham-treated mice a normal presence of reticular and nervous fibers was observed (E). On the contrary in the spinal cord tissues collected at 24 h after SCI (F), was observed a significant alteration of reticular and nervous fibers. D-Carnosine treatment significantly reduced the alteration of reticular and nervous fibers associated with SCI (G). On the contrary L-carnosine treatment no reduced the alteration (H). Following the injury, MPO activity in spinal cord from SCI mice was significantly increased at 24 h after the damage in comparison to sham mice. Treatment with D-carnosine attenuated neutrophil infiltration into the spinal cord in a dose-dependent fashion (J). The degree of motor disturbance was assessed every day until 10 days after SCI using the methodology of the Basso Mouse Scale (BMS) score (K). Treatments with D-carnosine enhanced the recovery after SCI in a dose-dependent fashion. This figure is representative of at least 3 experiments performed on different experimental days. Data are means ± s.e. of 10 mice for each group. $^{*}P < 0.01$ vs. Sham. $^{\circ}P < 0.01$ vs SCI + vehicle.

the spinal cord was observed in the spinal cord tissue from SCI mice when compared with sham-operated mice (Fig. 1A, see histological score I). Notably, significant protection against the SCI was observed in D-carnosine-treated mice (Fig. 1C, see histological score I; Two-way ANOVA, degree of freedom for treatment (D-carnosine and L-carnosine) is 1.0, for animals (sham and SCI) is 4.0. Treatment accounts for 72.89% of the total variance. $F_{1,90}$ = 54,450.00; $P$ value is <0.0001), on the contrary no protection against the SCI was observed in L-carnosine-treated mice (Fig. 1D, see histological score I). Reticular and nervous fibers tissues structure were observed by silver impregnation. In sham-treated mice a normal presence of reticular and nervous fibers was observed (Fig. 1E). A significant alteration of reticular and nervous fibers was observed in the spinal cord tissues collected at 24 h after SCI (Fig. 1F). D-Carnosine (150 mg/kg) treatment significantly reduced the alteration of reticular and nervous fibers associated with SCI (Fig. 1G). On the contrary L-carnosine treatment no reduced the reticular and nervous fibers alteration (Fig. 1H).

The abovementioned histological pattern of SCI appeared to be correlated with the influx of leukocytes into the spinal cord. Therefore, we investigated the effect of D-carnosine on the neutrophil infiltration by measuring tissue MPO activity. MPO activity was significantly elevated in the spinal cord at 24 h after injury in mice subjected to SCI when compared with sham-operated mice (Fig. 1J). Treatment with D-carnosine attenuated neutrophil infiltration into the spinal cord at 24 h after injury in dose-dependent fashion (Fig. 1J; Two-way ANOVA, degree of freedom for treatment [D-carnosine and L-carnosine] is 1.0, for animals [sham and SCI] is 3.0; Treatment accounts for 80.27% of the total variance. $F_{1,72}$ = 145,924,000. The $P$ value is < 0.0001).

In order to evaluate if histological damage to the spinal cord was associated with a loss of motor function, the BMS hind limb locomotor rating scale score was evaluated. While motor function was only slightly impaired in sham mice, mice subjected to SCI had significant deficits in hind limb movement (Fig. 1K). D-Carnosine reduced the functional deficits induced by SCI in dose-dependent fashion (Fig. 1K; Mann Whitney test SCI + D-carnosine35 vs SCI + vehicle, $P$ = 0.6217, $U$ = 43 ns; SCI + D-carnosine75 vs SCI + vehicle, $P$ = 0.4471, $U$ = 39.50 ns; SCI + D-carnosine150 vs SCI + vehicle, $P$ = 0.0480, $U$ = 23.50, $^{*}P$ = 0.05).

### 3.2. Effects of carnosine on modulation of cytokines and iNOS immunoreactivity after SCI

To test whether carnosine may modulate the inflammatory process, we analyzed the spinal cord tissue levels of pro-inflammatory cytokines. A substantial increase in TNF-α and IL-1β productions was found in spinal cord tissues samples collected from SCI mice 24 h after SCI (Fig. 2A and B, respectively). Spinal cord levels of TNF-α and IL-1β were significantly attenuated by the intraperitoneal injection of D-carnosine (150 mg/kg) (Fig. 2A and B, respectively). For TNF-α $F_{3,26}$ = 138.7 and $P < 0.0001$; for IL-1β $F_{3,16}$ = 326.3 and $P < 0.0001$. In addition to determine the role of nitric oxide (NO) produced during SCI, iNOS immunoreactivity was evaluated. A significant increase of iNOS (Fig. 2D, see densitometry analysis F) immunoreactivity was observed in the spinal cord from mice subjected to SCI when compared with sham-operated mice (Fig. 2C, see densitometry analysis F). D-Carnosine (150 mg/kg) treatment prevented the SCI-induced iNOS immunoreactivity (Fig. 2E, see densitometry analysis F; $F_{3,8}$ = 109.6 and $P < 0.0001$).

### 3.3. Effects of carnosine on nitrotyrosine and PAR formation after SCI

Twenty-four hours after SCI, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the spinal cord sections to determine the localization of various reactive nitrogen species produced during SCI. Spinal cord sections from sham-operated mice did not stain for nitrotyrosine (Fig. 3A, see densitometry analysis G), whereas spinal cord sections obtained from SCI mice exhibited positive

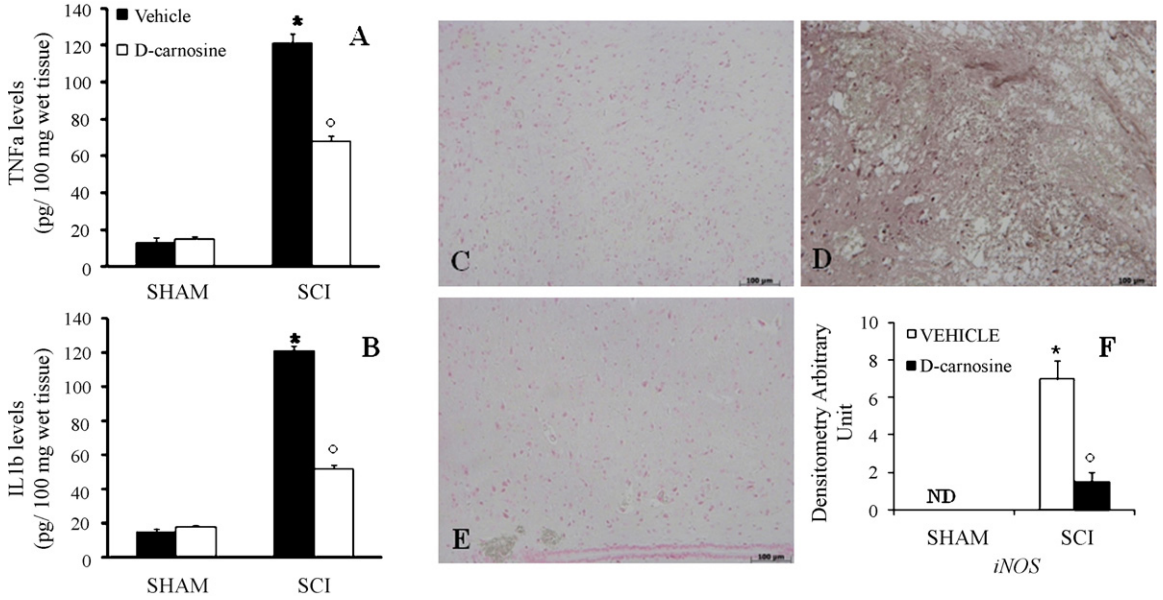


**Fig. 2.** Effects of D-carnosine treatment cytokines and iNOS immunoreactivity in spinal cord tissue. By ELISA measurement, TNF-α (A) and IL-1β (B) levels were significantly increased in the spinal cord from SCI mice. On the contrary, D-carnosine treatment prevented the SCI-induced levels of these cytokines (A and B). In addition, spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for iNOS. A positive staining for iNOS was significantly increased in the spinal cord from SCI mice (D) when compared with sham-treated mice (C). On the contrary, D-carnosine treatment significantly reduced the SCI-induced immunoreactivity for iNOS (E). Densitometry analysis of immunocytochemistry photographs ($n$ = 5 photos from each sample collected from all mice in each experimental group) for iNOS (F) from spinal cord tissues was assessed. Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. Data are means ± s.e. of 10 mice for each group. $^{*}P < 0.01$ vs. Sham. $^{\circ}P < 0.01$ vs SCI + vehicle.

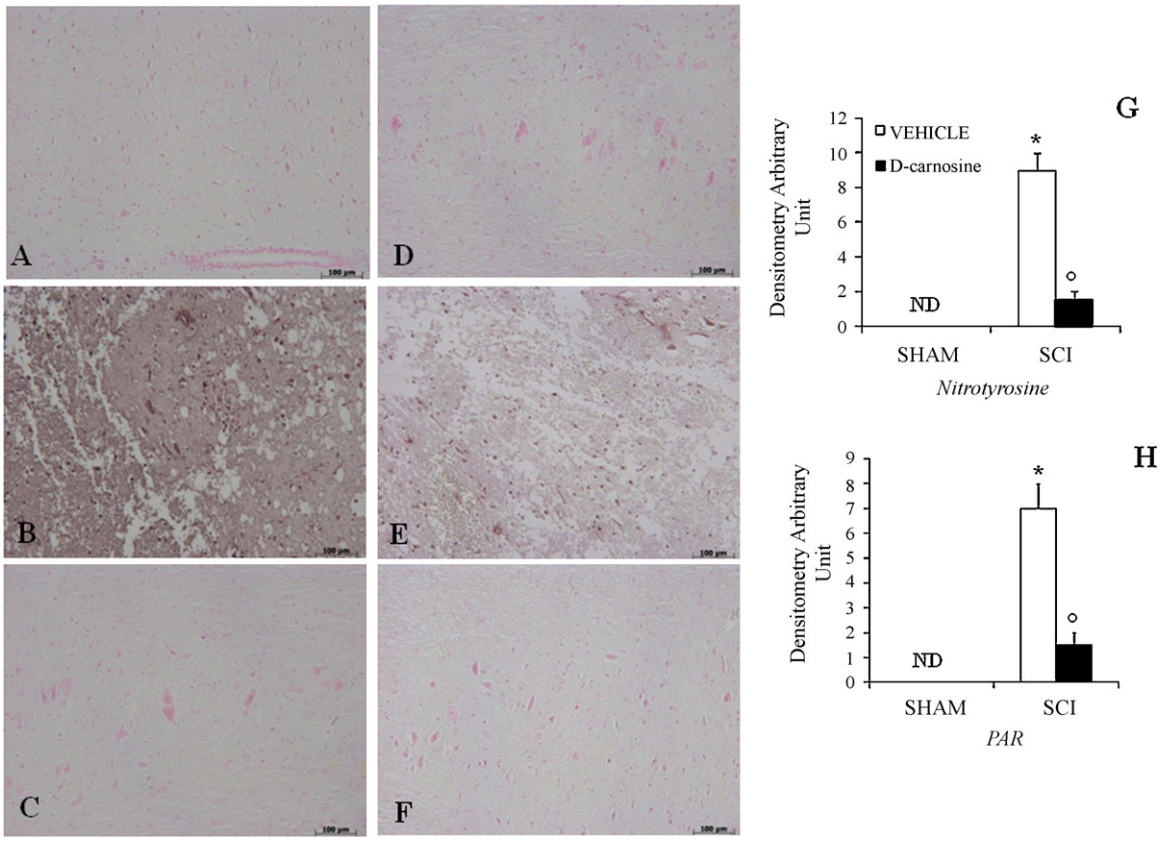


**Fig. 3.** Effects of D-carnosine on nitrotyrosine and PAR. In tissue sections obtained from vehicle-treated animals after SCI demonstrate positive staining for nitrotyrosine mainly localized in inflammatory, in nuclei of Schwann cells in the white and gray matter (B). D-Carnosine treatment reduced the degree of positive staining for nitrotyrosine (C) in the spinal cord. Immunohistochemistry for PAR, an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR localized in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice (E). D-Carnosine treatment reduced the degree of positive staining for PAR (F) in the spinal cord. No positive staining was observed for nitrotyrosine and PAR in spinal cord section of sham-treated mice (A and D, respectively). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for nitrotyrosine (G) and PAR (H) from spinal cord tissues was assessed. Data are expressed as % of total tissue area. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). This figure is representative of at least 3 experiments performed on different experimental days. Data are means ± s.e. of 10 mice for each group. $^{*}P < 0.01$ vs. Sham. $^{\circ}P < 0.01$ vs SCI + vehicle.

staining for nitrotyrosine (Fig. 3B, see densitometry analysis G). The positive staining was mainly localized in inflammatory cells as well as in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues. D-Carnosine (150 mg/kg) reduced the degree of positive staining for nitrotyrosine (Fig. 3C, see densitometry analysis G; $F_{3,12} = 199.8$ and $P < 0.0001$) in the spinal cord.

In addition, in our study, immunohistochemistry for PAR, as an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR localized in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues from mice subjected to SCI (Fig. 3E, see densitometry analysis H). D-Carnosine (150 mg/kg) treatment reduced the degree of positive staining for PAR (Fig. 3F, see densitometry analysis H; $F_{3,12} = 49.37$ and $< 0.0001$) in the spinal cord. Spinal cord sections from sham-operated mice did not stain for PAR (Fig. 3D, see densitometry analysis H).

### 3.4. Effects of carnosine on immunoreactivity for FasL after SCI

Immunohistological staining for FasL in the spinal cord was also determined 24 h after injury. Spinal cord sections from sham-operated mice did not stain for FasL (Fig. 4A, see densitometry analysis D), whereas spinal cord sections obtained from SCI mice exhibited positive staining for FasL (Fig. 4B, see densitometry analysis D) mainly localized in inflammatory cells as well as in nuclei of Schwann cells. D-Carnosine (150 mg/kg) reduced the degree of positive staining for FasL in the spinal cord (Fig. 4C, see densitometry analysis D; $F_{3,12} = 88.08$ and $P < 0.0001$).

### 3.5. Effects of carnosine on expression of Bax and Bcl-2

The appearance of Bax in homogenates of spinal cord was investigated by Western blot at 24 h after SCI. A basal level of Bax was detected in the spinal cord from sham-operated animals (Fig. 5A and A1). Bax levels were substantially increased in the spinal cord from saline treated mice subjected to SCI (Fig. 5A and A1). The treatment with D-carnosine (150 mg/kg) decreased the SCI-induced Bax expression (Fig. 5A and A1; $F_{2,6} = 359.7$ and $P < 0.0001$). To detect Bcl-2 expression, whole extracts from spinal cord of each mouse were also analyzed by Western blot analysis. A low basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Fig. 5B and B1). 24 h after SCI, the Bcl-2 expression was significantly reduced in whole extracts obtained from spinal cord of SCI + saline mice group (Fig. 5B and B1). Treatment with D-carnosine decreased significantly the SCI-induced inhibition of Bcl-2 expression (Fig. 5B and B1; $F_{2,6} = 10.42$ and $P = 0.0112$).

Moreover, samples of spinal cord tissue were taken at 24 h after SCI also to determine the immunohistological staining for Bax and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (Fig. 5C) whereas spinal cord sections obtained from SCI mice exhibited a positive staining for Bax (Fig. 5D). D-Carnosine (150 mg/kg) reduced the degree of positive staining for Bax in the

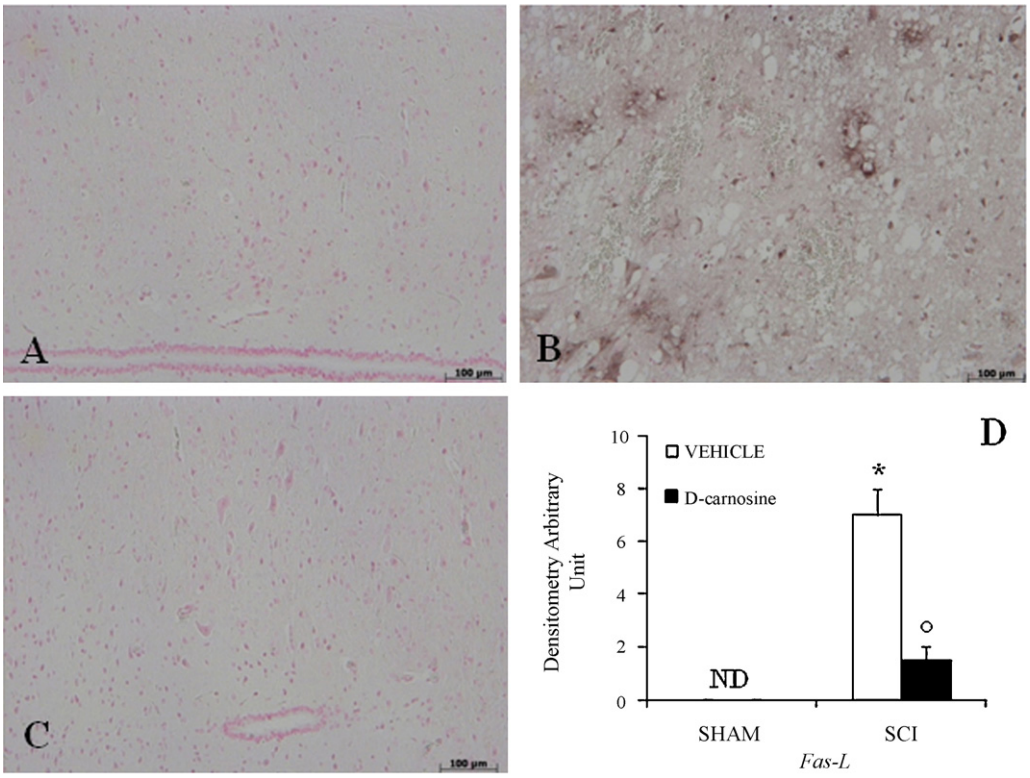


**Fig. 4.** Effect of D-carnosine on immunohistochemical localization of FasL. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for FasL. A substantial increase in FasL (B) expression was found in inflammatory cells, in nuclei of Schwann cells in the spinal cord tissues from SCI mice when compared with sham-treated mice (A). Spinal cord levels of FasL (C) were significantly attenuated in D-carnosine-SCI treated mice in comparison to SCI animals. Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for FasL (D) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). This figure is representative of at least 3 experiments performed on different experimental days. $^{*}P < 0.01$ vs. Sham. $^{\circ}P < 0.01$ vs SCI + vehicle.

spinal cord of mice subjected to SCI (Fig. 5E). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Fig. 5F) while in SCI mice the staining significantly reduced (Fig. 5G). D-Carnosine (150 mg/kg) attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (Fig. 5H).

### 3.6. Effects of carnosine on expression of Hsp70 after injury

In addition, levels of inducible Hsp70 expression were also measured as markers of oxidative stress in animals exposed to SCI in the absence or in the presence of treatment with D-carnosine or L-carnosine and compared to control sham operated mice. Compared to control mice (Fig. 6A and B), Hsp70 expression significantly increased in the SCI + saline mice group, whereas treatment with D-carnosine (150 mg/kg), but not L-carnosine, decreased significantly the SCI-induced increase of Hsp70 expression (Fig. 6A and B; $F_{3,8} = 16.39$ and $P = 0.0009$).

### 3.7. Effects of carnosine on apoptosis in spinal cord after injury

To test whether the tissue damage was associated with the induction of apoptosis, we evaluated TUNEL-like staining in the perilesional spinal cord tissue at 24 h after injury. No apoptotic cells were detected in the spinal cord from sham-operated mice (Fig. 7A, see positive cell count D). At 24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 7B, see positive cell count D). In contrast, tissues obtained from mice treated with D-carnosine (150 mg/kg) demonstrated no apoptotic cells or fragments (Fig. 7C, see positive cell count D; $F_{3,8} = 79.26$ and $P < 0.0001$).

### 3.8. Effects of carnosine S100β immunoreactivity after injury

S100β immunoreactive quiescent astrocytes with a small size and thin processes were seen distributed throughout the white and gray matters of the studied levels of the sham-operated mice (Fig. 8A, see densitometry analysis D). The SCI in the saline mice group increased the number of S100β immunoreactive astrocytes with a large cytoplasm and thick processes, in the white and gray matters of the spinal cord at cranial and caudal levels adjacent to the injury (Fig. 8B, see densitometry analysis D). This number of S100β immunoreactive astrocytes is decreased in the spinal cord obtained from SCI + D-carnosine (150 mg/kg) treated mice (Fig. 8C, see densitometry analysis D; $F_{3,8} = 129.1$ and $P < 0.0001$).

## 4. Discussion

In the last decade, several studies have been performed to understand the biological functions played by carnosine-related dipeptides in the nervous system.

Carnosine is found in brain, heart, skin, muscles, kidneys, gut, and other tissues. It has been suggested to be potentially useful for treating Alzheimer's disease [30], autism [31], brain ischemia [32] Parkinson's disease [33], Down's syndrome [34], epilepsy [35], and aging [36].

Despite the abundance of data suggesting that carnosine and related compounds have antioxidant properties and cellular protective effects, several investigators have suggested that

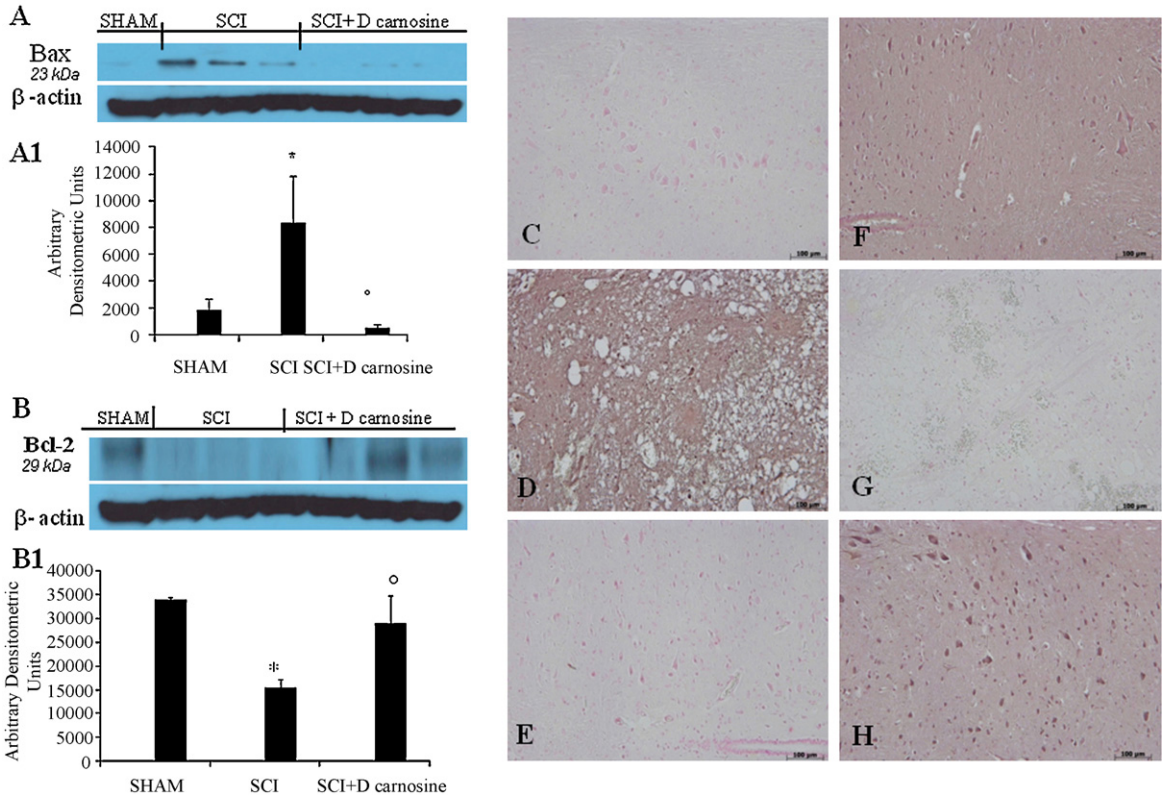


**Fig. 5.** Western blot and immunohistochemical analyses for Bax and Bcl-2. By Western blot analysis, Bax levels were appreciably increased in the spinal cord from SCI mice (A and A1). On the contrary, D-carnosine treatment prevented SCI-induced Bax expression (A and A1). Moreover, a basal level of Bcl-2 expression was detected in spinal cord samples from sham-operated mice. Bcl-2 expression was significantly reduced in spinal cord samples from SCI mice (B and B1). D-Carnosine treatment significantly reduced the SCI-induced inhibition of Bcl-2 expression (B and B1). The results in panel A1 and B1 are expressed as mean ± s.e.m. from $n = 5/6$ spinal cord for each group. $^{*}P < 0.01$ versus sham, $^{\circ}P < 0.01$ versus SCI + vehicle. Sections of spinal cord from SCI mice exhibited positive staining for Bax (D). D-Carnosine treatment reduced the degree of positive staining for Bax in the spinal cord of mice subjected to SCI (E). In addition, in the spinal cord sections from SCI control mice, the staining for Bcl-2 significantly reduced (G). D-Carnosine treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (H). No positive staining for Bax and Bcl-2 was observed in spinal cord sections of sham-treated mice (C and F, respectively). This figure is representative of at least 3 experiments performed on different experimental days. $^{*}P < 0.01$ vs. Sham. $^{\circ}P < 0.01$ vs SCI + vehicle.

carnosine is an endogenous neuroprotector [32], Pubill et al. [37] reported that carnosine prevents methamphetamine-induced gliosis. Gallant et al. [38] gave rats dietary carnosine and found that it reduced mortality and improved behavioural recovery of rats subjected to common carotid artery occlusion. Carnosine has not been tested in any model of SCI, whether chronic or acute. The complex pathophysiology of SCI may explain the difficulty for finding a suitable therapy [39]. To mimic the majority of mechanical events that lead to various forms of human SCI, several experimental models have been developed [40]; one of the most commonly used model of SCI is the compression model.

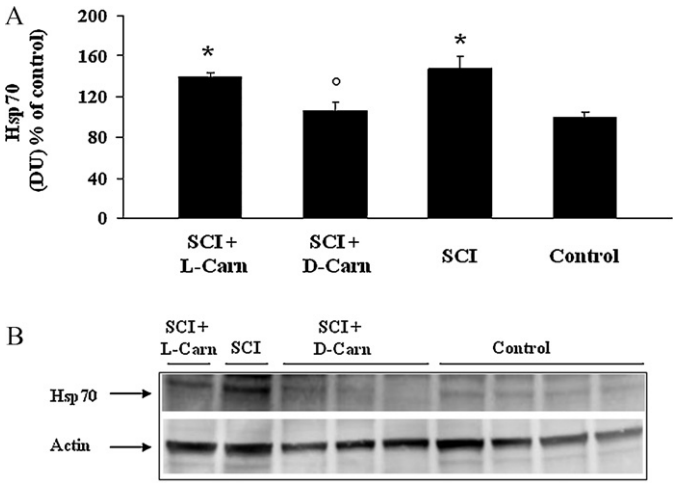


**Fig. 6.** Analysis of Hsp70. Hsp70, protein levels were measured by western blot using specific antibodies as described in Section 2. (A) Hsp70 expression 24 h after SCI, in absence and presence of treatment with D-carnosine or L-carnosine. Bar graphs represent the densitometric evaluation of specific bands after normalization with the densitometric values of β-actin, used as a loading control. Values are expressed as mean ± s.e.m. of six separate experiments. A representative immunoblot is shown (B). $^{*}P < 0.01$ versus control. $^{\circ}P < 0.05$ vs SCI operated animal group.

In this model, injury is induced by applying either a weight or an aneurysm clip to the spinal cord [41] to replicate the persistence of cord compression that is commonly observed in human SCI [42]. Inflammation is a universal defence and reparative response to tissue injury and the spinal cord is no exception.

After SCI, blood-borne monocytes/macrophages are recruited, as are locally activated resident microglia, both of which subsequently invade to phagocytose the injured tissue [43].

Recent evidence, however, suggests that leukocytes, especially neutrophils which are the first leukocytes to arrive within the injured spinal cord [3] may also be directly involved in the pathogenesis and extension of SCI in rats. Several authors have demonstrated that neutrophils are especially prominent in a 'marginal zone' around the main area of injury and infarction at 24 h [44]. This study provides the first evidence that D-carnosine attenuates at 24 h after SCI: the degree of spinal cord damage and the infiltration of the spinal cord with PMNs.

These and other reactive cells produce cytokines, such as tumor necrosis factor (TNF-α), interleukins and interferons, that mediate the inflammatory response and can contribute to further tissue damage [45,46]. Among the various cytokines involved in secondary CNS injury, TNF-α is perhaps the most extensively

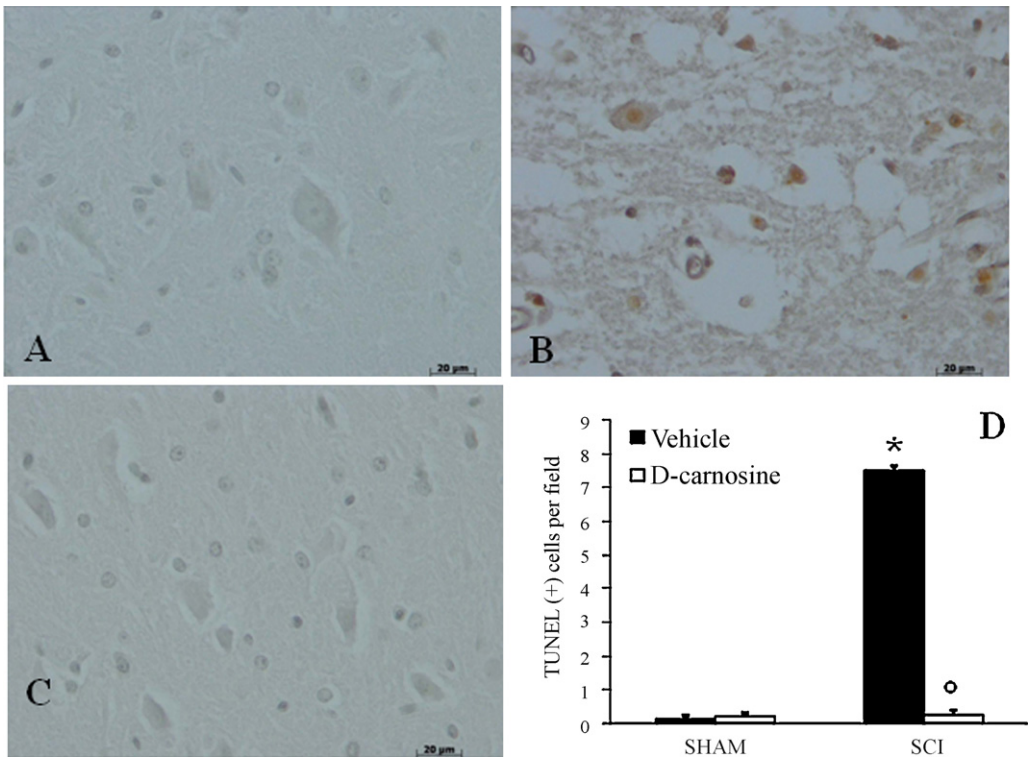


**Fig. 7.** Effects of D-carnosine on TUNEL-like staining in the perilesional spinal cord tissue. At 24 h after the trauma, SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (B). In contrast, tissues obtained from mice treated with D-carnosine no apoptotic cells or fragments (C). No apoptotic cells were detected in the spinal cord from sham-operated mice (A). The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide (D). Figure is representative of at least 3 experiments performed on different experimental days.

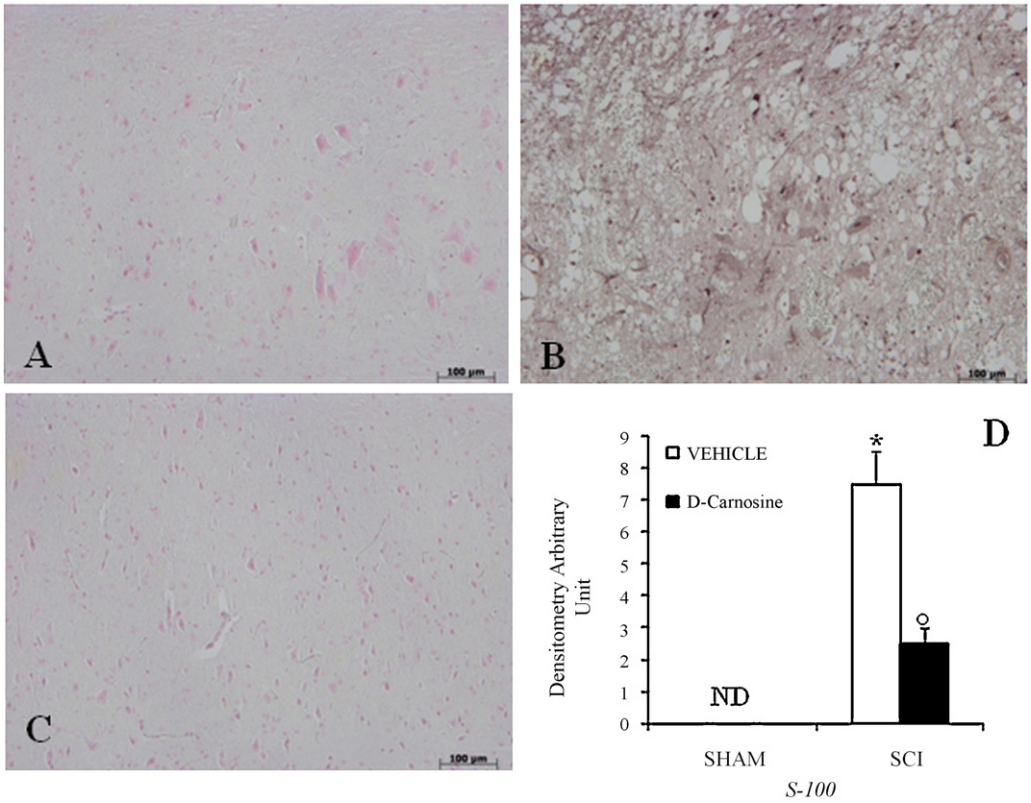


**Fig. 8.** Effect of D-carnosine on S-100 immunoreactivity. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for S-100. No positive staining for S-100 was observed in sham-treated mice (A). A substantial increase in S-100 (B) expression was found in inflammatory cells, in nuclei of Schwann cells in the spinal cord tissues from SCI mice. Spinal cord immunoreactivity of S-100 (C) were significantly attenuated in D-carnosine-SCI treated mice in comparison to SCI animals. Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for S-100 (D) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). This figure is representative of at least 3 experiments performed on different experimental days. $^{*}P < 0.01$ vs. Sham. $^{\circ}P < 0.01$ vs SCI + vehicle.

studied. It has been shown to accumulate quickly at the site of SCI [47]. Its release soon after injury promotes the further migration of blood borne activated leukocytes into the spinal cord and can stimulate additional cytokine production.

In this study, we documented increased production of both TNF-α and IL-1β after SCI. Remarkably, there was no increase in the expression of either TNF-α or IL-1β in the spinal cord section obtained from mice subject to SCI and treated with D-carnosine.

Previous in vitro studies have shown that cytokines such as interferon-γ and tumor necrosis factor-α induce iNOS mRNA in glial cells [48] In the case of SCI, Hamada et al. [49] reported that in the compression model NO produced by iNOS is neurotoxic, while NO produced by the constitutive forms such as eNOS or nNOS is neuroprotective. NO cytotoxicity emerges, in part, by reaction with superoxide anion ($O_2^-$) to generate peroxynitrite ($ONOO^-$) [50]. This study demonstrates that D-carnosine attenuates the expression of iNOS in the tissue from SCI treated mice when compared with injured mice.

In addition, several studies have implicated the formation of reactive species of oxygen and nitrogen in the secondary neuronal damage of SCI [51]. In particular, it has been demonstrated that peroxynitrite probably contributes to secondary neuronal damage through pathways resulting from the chemical modification of cellular proteins and lipids [51]. To confirm the pathological contributions of peroxynitrite to secondary damage after SCI, we have evaluated nitrotyrosine formation, Hsp70 activation and PARP activity in the injured tissue. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation "footprint" of peroxynitrite [52]. However, there is recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine [53]. Therefore, increased nitrotyrosine staining is considered as an indication of "increased nitrosative stress" rather than a specific marker of the peroxynitrite generation. This finding is also corroborated by elevated expression of Hsp70 which we found in SCI operated mice, in agreement with the notion that Hsp70 expression is triggered by signals of oxidative and/or nitrosative stress. Recent studies have demonstrated the induction of apoptosis in a different cell lines in response to reactive oxygen species, peroxynitrite, and nitric oxide [54]. Results of our study reveal that the immunostaining for nitrotyrosine, as well as induction of Hsp70 are reduced in SCI-operated mice treated with D-carnosine. Notwithstanding its properties of scavenging the NO-free radical [17] and preventing the protein tyrosine nitration [17], the up-regulation of iNOS and the induction of Hsp70 following strong nitrosative conditions [16], L-carnosine do not show these effects in SCI-operated mice. The reason of such different behaviour between L and D-carnosine in our experimental model might be ascribed to their different hydrolysis rate *in vivo*. Because D-carnosine is not hydrolyzed by the carnosinases, the enzymatic action *in vivo* would prevent L-carnosine, but not D-carnosine, from showing their protective effects after SCI.

PARP activity is believed to contribute to neuronal cell death in a variety of neurological conditions [55], including traumatic brain injury [56] and SCI [57], as a consequence of energy failure [58] or through modification of the activity of various proteins by poly(ADP-ribosylation) [59]. Continuous or excessive activation of PARP results in a substantial depletion of intracellular $NAD^+$ and subsequently, adenosine triphosphate (ATP), leading to cellular dysfunction and ultimately, cell death [60]. Additionally, it has been shown that various PARP inhibitors exerts protective effects in models of SCI [61]. On the contrary, treatment with carnosine, increased a positive staining of PAR localized in nuclei in the white matter of spinal cord tissue.

Several studies suggest that glial cells in neurodegenerative diseases are affected more than neurons by apoptotic cell death [40]. Apoptosis is an important mediator of secondary damage after SCI. It incurs its effects through at least two phases: an initial phase, in which apoptosis accompanies necrosis in the degeneration of multiple cell types, and a later phase which is predominantly confined to white matter and involves oligodendrocytes and microglia [62]. Chronologically, apoptosis initially occurs 6 h post-injury at the lesion centre and lasts for several days in association with a steadily increased number of apoptotic cells in this region.

In an effort to prevent or diminish levels of apoptosis, in this study by using the Tunel coloration kit, we clearly confirmed in induction of apoptosis during SCI and that the treatment with D-carnosine, decreased the degree of apoptosis.

Moreover, it is well known that Bax, a pro-apoptotic gene, plays an important role in developmental cell death [62] and in CNS injury [63]. Similarly, it has been shown that the administration of Bcl-xL fusion protein (Bcl-2 is the most expressed anti-apoptotic molecule in adult central nervous system) into injured spinal cords significantly increased neuronal survival, suggesting that SCI-induced changes in Bcl-xL contribute considerably to neuronal death [64]. Based on this evidence, by using western blot assay and immunohistochemical staining, we have identified proapoptotic transcriptional changes, such as up-regulation Bax and down regulation of Bcl-2 in the SCI-operated mice. Consistent to this, some authors have also shown that FasL and p75 receptors are expressed on oligodendrocytes, astrocytes and microglia in the spinal cord following SCI. FasL and p75 co-localize on many TUNEL-positive cells, suggesting that the FasL- and p75-initiated cell death cascades may participate in the demise of some glia following SCI. Thus, it has been recently pointed out that FasL signaling can play a central role in SCI [65]. In agreement with all this, we confirm here that SCI leads to a substantial activation of FasL in the spinal cord, and this most likely may sustain the progression of tissue injury. In the present study, we also found that treatment with D-carnosine, decreased the lost of the antiapoptotic way and the proapoptotic pathway activation, although the exact mechanism remain elusive. These data are also supported by the observation of a significant more expression of FAS-L on section of spinal cord obtained SCI-operated mice when compared with tissues sections obtained by D-carnosine-operated mice.

Finally, in this study, we demonstrate the strong difference between L-carnosine and D-carnosine. The result strongly suggests that D-carnosine treatment significantly reduced the SCI-induced spinal cord tissues alteration and improve the motor function. As previously argued, the action of carnosinases only on L-carnosine would explain the different behaviour between L and D-carnosine in SCI-operated mice.

The purpose of our study was to highlight our current knowledge on the interaction between post-traumatic immune reactivity and the development of complications. A better understanding of these mechanisms might facilitate the translation from preventive and therapeutic strategies into clinical practice. In order to overcome the degradation of L-carnosine *in vivo*, the derivatization of the natural dipeptide as been also proposed as a promising strategy. New carnosine derivatives have been synthesized and characterized, showing chelating and antioxidant properties similar to those of the parent dipeptides [23]. In addition, these new compounds survive to attack by carnosinases. A better understanding of the mechanism of action of L-carnosine derivatives may represent further refinement of our therapeutic approaches, overcoming limitations associated to possible toxicity effects by D-carnosine.

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## References

[1] Ambrozaitis KV, Kontautas E, Spakauskas B, Vaitkaitis D. Pathophysiology of acute spinal cord injury. Medicina (Kaunas Lithuania) 2006;42:255–61.
[2] Calabrese V, Cornelius C, Maiolino L, Luca M, Chiaramonte R, Toscano MA, et al. Oxidative stress, redox homeostasis and cellular stress response in Meniere's disease: role of vitagenes. Neurochem Res 2010;35(12):2208–17.
[3] Carlson GD, Gorden C. Current developments in spinal cord injury research. Spine J 2002;2:116–28.
[4] Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev 2001;53:135–59.
[5] Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev 2007;8:766–75.
[6] Calabrese EJ, Mattson MP, Calabrese V. Resveratrol commonly displays hormesis: occurrence and biomedical significance. Hum Exp Toxicol 2010;29(12): 980–1015.
[7] Calabrese V, Cornelius C, Dinkova-Kostova AT, Calabrese EJ, Mattson MP. Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders. Antioxid Redox Signal 2010;13(11):1763–811.
[8] Genovese T, Mazzon E, Di Paola R, Crisafulli C, Muia C, Bramanti P, et al. Increased oxidative-related mechanisms in the spinal cord injury in old rats. Neurosci Lett 2006;393:141–6.
[9] Perluigi M, Di Domenico F, Giorgi A, Schinina ME, Coccia R, Cini C, et al. Redox proteomics in aging rat brain: involvement of mitochondrial reduced glutathione status and mitochondrial protein oxidation in the aging process. J Neurosci Res 2010;88(16):3498–507.
[10] Kang JH. Protective effects of carnosine and homocarnosine on ferritin and hydrogen peroxide-mediated DNA damage. BMB Rep 2010;43(10):683–7.
[11] Quinn PJ, Boldyrev AA, Formazuyk VE. Carnosine: its properties, functions and potential therapeutic applications. Mol Aspects Med 1992;13:379–444.
[12] Hipkiss AR. Carnosine and its possible roles in nutrition and health. Adv Food Nutr Res 2009;57:87–154.
[13] Corona C, Frazzini V, Silvestri E, Lattanzio R, La Sorda R, Piantelli M, et al. Effects of dietary supplementation of carnosine on mitochondrial dysfunction, amyloid pathology, and cognitive deficits in 3xTg-AD mice. PloS One 2011;6(3):e17971.
[14] Shen Y, He P, Fan YY, Zhang JX, Yan HJ, Hu WW, et al. Carnosine protects against permanent cerebral ischemia in histidine decarboxylase knockout mice by reducing glutamate excitotoxicity. Free Radic Biol Med 2010;48(5):727–35.
[15] Calabrese V, Colombrita C, Guagliano E, Sapienza M, Ravagna A, Cardile V, et al. Protective effect of carnosine during nitrosative stress in astroglial cell cultures. Neurochem Res 2005;30:797–807.
[16] Fontana M, Pinnen F, Lucente G, Pecci L. Prevention of peroxynitrite-dependent damage by carnosine and related sulphonamido pseudodipeptides. Cell Mol Life Sci 2002;59:546–51.
[17] Nicoletti VG, Santoro AM, Grasso G, Vagliasindi LI, Giuffrida ML, Cuppari C, et al. Carnosine interaction with nitric oxide and astroglial cell protection. J Neurosci Res 2007;85:2239–45.
[18] Calabrese V, Cornelius C, Stella AM, Calabrese EJ. Cellular stress responses, mitostress and carnitine insufficiencies as critical determinants in aging and neurodegenerative disorders: role of hormesis and vitagenes. Neurochem Res 2010;35(12):1880–915.
[19] Teufel M, Saudek V, Ledig JP, Bernhardt A, Boularand S, Carreau A, et al. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. J Biol Chem 2003;278:6521–31.
[20] Bellia F, Calabrese V, Guarino F, Cavallaro M, Cornelius C, De Pinto V, et al. Carnosinase levels in aging brain: redox state induction and cellular stress response. Antioxid Redox Signal 2009;11:2759–75.
[21] Boldyrev A, Bulygina E, Leinsoo T, Petrushanko I, Tsubone S, Abe H. Protection of neuronal cells against reactive oxygen species by carnosine and related compounds. Comp Biochem Physiol 2004;137:81–8.
[22] Lanza V, Bellia F, D'Agata R, Grasso G, Rizzarelli E, Vecchio G. New glycoside derivatives of carnosine and analogs resistant to carnosinase hydrolysis: synthesis and characterization of their copper(II) complexes. J Inorg Biochem 2011;105(2):181–8.
[23] Bellia F, Amorini AM, La Mendola D, Vecchio G, Tavazzi B, Giardina B, et al. New glycosidic derivatives of histidine-containing dipeptides with antioxidant properties and resistant to carnosinase activity. Eur J Med Chem 2008;43: 373–80.
[24] Bertinaria M, Rolando B, Giorgis M, Montanaro G, Guglielmo S, Buonsanti MF, et al. Synthesis, physicochemical characterization, and biological activities of new carnosine derivatives stable in human serum as potential neuroprotective agents. J Med Chem 2011;54(2):611–21.
[25] Aldini G, Canevotti R, Negrisoli G. Compositions containing D-carnosine. Patent WO 2005/120543 2005.
[26] Sirin BH, Ortac R, Cerrahoglu M, Saribulbul O, Baltalarli A, Celebisoy N, et al. Ischaemic preconditioning reduces spinal cord injury in transient ischaemia. Acta Cardiol 2002;57:279–85.
[27] Mullane K. Neutrophil-platelet interactions and post-ischemic myocardial injury. Prog Clin Biol Res 1989;301:39–51.
[28] Bethea JR, Castro M, Keane RW, Lee TT, Dietrich WD, Yezierski RP. Traumatic spinal cord injury induces nuclear factor-kappaB activation. J Neurosci 1998; 18:3251–60.
[29] Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 2006;23:635–59.
[30] Hobart LJ, Seibel I, Yeargans GS, Seidler NW. Anti-crosslinking properties of carnosine: significance of histidine. Life Sci 2004;75:1379–89.
[31] Chez MG, Buchanan CP, Aimonovitch MC, Becker M, Schaefer K, Black C, et al. Double-blind, placebo-controlled study of L-carnosine supplementation in children with autistic spectrum disorders. J Child Neurol 2002;17:833–7.
[32] Stvolinsky SL, Kukley ML, Dobrota D, Matejovicova M, Tkac I, Boldyrev AA. Carnosine: an endogenous neuroprotector in the ischemic brain. Cell Mol Neurobiol 1999;19:45–56.
[33] Kang JH, Kim KS. Enhanced oligomerization of the alpha-synuclein mutant by the Cu,Zn-superoxide dismutase and hydrogen peroxide system. Mol Cells 2003;15:87–93.
[34] Thiel R, Fowkes SW. Can cognitive deterioration associated with Down syndrome be reduced? Med Hypotheses 2005;64:524–32.
[35] Petroff OA, Hyder F, Rothman DL, Mattson RH. Homocarnosine and seizure control in juvenile myoclonic epilepsy and complex partial seizures. Neurology 2001;56:709–15.
[36] Ferrari CK. Functional foods, herbs and nutraceuticals: towards biochemical mechanisms of healthy aging. Biogerontology 2004;5:275–89.
[37] Pubill D, Verdaguer E, Sureda FX, Camins A, Pallas M, Camarasa J, et al. Carnosine prevents methamphetamine-induced gliosis but not dopamine terminal loss in rats. Eur J Pharmacol 2002;448:165–8.
[38] Gallant S, Kukley M, Stvolinsky S, Bulygina E, Boldyrev A. Effect of carnosine on rats under experimental brain ischemia. Tohoku J Exp Med 2000;191:85–99.
[39] Stover SL, Fine PR. The epidemiology and economics of spinal cord injury. Paraplegia 1987;25:225–8.
[40] Beattie MS, Hermann GE, Rogers RC, Bresnahan JC. Cell death in models of spinal cord injury. Prog Brain Res 2002;137:37–47.
[41] Tator CH, Fehlings MG. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 1991;75:15–26.
[42] Tator CH. Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathol (Zurich Switzerland) 1995;5:407–13.
[43] Dusart I, Schwab ME. Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. Eur J Neurosci 1994;6:712–24.
[44] de Castro Jr R, Hughes MG, Xu GY, Clifton C, Calingasan NY, Gelman BB, et al. Evidence that infiltrating neutrophils do not release reactive oxygen species in the site of spinal cord injury. Exp Neurol 2004;190:414–24.
[45] Klusman I, Schwab ME. Effects of pro-inflammatory cytokines in experimental spinal cord injury. Brain Res 1997;762:173–84.
[46] Genovese T, Esposito E, Mazzon E, Crisafulli C, Paterniti I, Di Paola R, et al. PPAR-alpha modulate the anti-inflammatory effect of glucocorticoids in the secondary damage in experimental spinal cord trauma. Pharmacol Res 2009;59:338–50.
[47] Brewer KL, Bethea JR, Yezierski RP. Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury. Exp Neurol 1999;159:484–93.
[48] Murphy S, Simmons ML, Agullo L, Garcia A, Feinstein DL, Galea E, et al. Synthesis of nitric oxide in CNS glial cells. Trends Neurosci 1993;16:323–8.
[49] Hamada Y, Ikata T, Katoh S, Tsuchiya K, Niwa M, Tsutsumishita Y, et al. Roles of nitric oxide in compression injury of rat spinal cord. Free Radic Biol Med 1996;20:1–9.
[50] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620–4.
[51] Xu J, Kim GM, Chen S, Yan P, Ahmed SH, Ku G, et al. iNOS and nitrotyrosine expression after spinal cord injury. J Neurotrauma 2001;18:523–32.
[52] Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. Chem Res Toxicol 1996;9:836–44.
[53] Endoh M, Maiese K, Wagner J. Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia. Brain Res 1994;651:92–100.
[54] Merrill JE, Ignarro LJ, Sherman MP, Melinek J, Lane TE. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. J Immunol 1993; 151:2132–41.
[55] Skaper SD. Poly(ADP-ribosyl)ation enzyme-1 as a target for neuroprotection in acute central nervous system injury. Curr Drug Targets 2003;2:279–91.
[56] LaPlaca MC, Zhang J, Raghupathi R, Li JH, Smith F, Bareyre FM, et al. Pharmacologic inhibition of poly(ADP-ribose) polymerase is neuroprotective following traumatic brain injury in rats. J Neurotrauma 2001;18:369–76.
[57] Whalen MJ, Clark RS, Dixon CE, Robichaud P, Marion DW, Vagni V, et al. Reduction of cognitive and motor deficits after traumatic brain injury in mice deficient in poly(ADP-ribose) polymerase. J Cereb Blood Flow Metab 1999;19: 835–42.
[58] Szabo C. Pathophysiological aspects of cellular pyridine nucleotide metabolism: focus on the vascular endothelium [Review]. Acta Physiol Hung 2003;90:175–93.

[59] Burkle A. Physiology and pathophysiology of poly(ADP-ribosyl)ation. Bioessays 2001;23:795–806.
[60] Chiarugi A. Poly(ADP-ribose) polymerase: killer or conspirator? The 'suicide hypothesis' revisited. Trends Pharmacol Sci 2002;23:122–9.
[61] Lacza Z, Horvath EM, Komjati K, Hortobagyi T, Szabo C, Busija DW. PARP inhibition improves the effectiveness of neural stem cell transplantation in experimental brain trauma. Int J Mol Med 2003;12:153–9.
[62] Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI, et al. Induction of apoptosis by the Bcl-2 homologue Bak. Nature 1995;374: 733–6.
[63] Bar-Peled O, Knudson M, Korsmeyer SJ, Rothstein JD. Motor neuron degeneration is attenuated in bax-deficient neurons in vitro. J Neurosci Res 1999;55:542–56.
[64] Nesic-Taylor O, Cittelly D, Ye Z, Xu GY, Unabia G, Lee JC, et al. Exogenous Bcl-xL fusion protein spares neurons after spinal cord injury. J Neurosci Res 2005;79:628–37.
[65] Ackery A, Robins S, Fehlings MG. Inhibition of Fas-mediated apoptosis through administration of soluble Fas receptor improves functional outcome and reduces posttraumatic axonal degeneration after acute spinal cord injury. J Neurotrauma 2006;23:604–16.