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Ghrelin Alleviates Spinal Cord Injury in Rats Via Its Anti-inflammatory Effects

Ghrelin Anti-inflamatuar Etkileri Yoluyla Sıçanlarda Spinal Kord Hasarını Azaltır

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ABSTRACT

AIM: Spinal cord injury (SCI) leads to an inflammatory response that generates substantial secondary damage within the tissue besides the primary damage. Ghrelin, 28 amino-acid peptide, has been shown to modulate the release of proinflammatory cytokines and exert anti-inflammatory effects. The aim of the current study was to investigate the anti-inflammatory effects of ghrelin, in a rat model of SCI.

MATERIAL and METHODS: Wistar albino rats were divided as control, SCI, and ghrelin-treated (10 μg/kg/day, ip) SCI groups. In order to induce SCI, a standard weight-drop method that induced a moderately severe injury (100 g/cm force) at T10, was used. Injured animals were given either ghrelin or saline 15 min post-injury.

RESULTS: In plasma samples, neuron-specific enolase (NSE) and S-100β protein levels were evaluated. Spinal cord samples were taken for histological examination or determination of myeloperoxidase (MPO) activity and DNA fragmentation. SCI caused significant increases in plasma NSE and S-100β levels and tissue MPO activity and DNA damage. On the other hand, ghrelin treatment improved histological findings as well as biochemical parameters while it failed to improve the impairment of the neurological functions due to SCI.

CONCLUSION: The present study suggests that ghrelin could reduce SCI-induced oxidative stress and exert anti-inflammatory effects in the spinal cord following trauma.

KEYWORDS: Ghrelin, Spinal cord injury, Neuroprotective, Rat

ÖZ

AMAÇ: Spinal kord hasarı (SKH) primer hasarın yanısıra doku içinde oluşan ikincil hasara yanıt olarak inflamasyona yol açar. Ghrelin 28 amino asit içeren bir peptiddir ve proinflamatuvar sitokinlerin salıverilmesini module ettiği ve antiinflamatuvar etkili olduğu gösterilmiştir. Bu çalışmanın amacı SKH modeli oluşturulan sıçanlarda ghrelinin etkisininin araştırılmasıdır.

YÖNTEM ve GEREÇLER: Wistar albino sıçanlar kontrol, SKH ve SKH + ghrelin (10 μg/kg/day, ip) olarak üç gruba bölünmüştür. SKH oluşturmak için T10 düzeyinde orta şiddette yaralanma yapan (100 g/cm kuvvet) ağırlık düşürme yöntemi kullanıldı. Hasar sonrası ilk 15 dakikada ghrelin veya fizyolojik serum uygulandı. Birinci hafta sonunda motor işlev değerlendirilerek dekapitasyon yapıldı. Kanda nöron-spesifik enolaz (NSE) ve S-100β protein düzeyleri ölçüldü. Omurilik dokusunda ise histolojik ve biyokimyasal incelemeler (miyeloperoksidaz aktivitesi (MPO) ve DNA fragmantasyonu) bakıldı.

BULGULAR: SKH grubunda kanda NSE, S-100β düzeylerinde ve dokuda MPO aktivitesi, DNA hasarında anlamlı bir artış görüldü. Diğer yandan ghrelin tedavisi SKH'ya bağlı olarak gelişen biyokimyasal ve histolojik değişiklerde düzelme sağlarken, motor işlev skorunda değişiklik yapmadı.

SONUÇ: Bu çalışma ghrelinin omurilikte travmayla indüklenen inflamasyonu ve oksidan hasarı azalttığını göstermektedir.

ANAHTAR SÖZCÜKLER: Ghrelin, Spinal kord hasarı, Nöroprotektif, Sıçan

INTRODUCTION

Traumatic spinal cord injury (SCI) primarily produces vascular damage to arterioles, capillaries and venules, limiting the blood flow to cord tissue causing a necrotic lesion (50), while reperfusion of the tissues in the first few days following SCI results in the development of a secondary damage (20). Although the development of secondary damage is not completely understood, cellular apoptosis, increased release of excitatory amino acids, enhanced generation of

reactive oxygen species and excessive cytokine release with subsequent lipid peroxidation are thought to constitute the major pathway of secondary injury in SCI (3, 5, 6, 35, 49). Traumatic injury to the spinal cord leads to a strong inflammatory response with the recruitment of peripherally derived inflammatory cells including macrophages, which initiate the activation and regulation of specific signaling molecules, among which cytokines play a prominent role (2). Thus, the final outcome of SCI is correlated with the extent of the initial physical damage as well as the following secondary events leading to the death of neurons and glia (20).

Many pharmacological agents have been used targeting one or more mechanisms of the secondary injury and have been reported to be neuroprotective in a variety of animal models, but none has yet proved to be effective in ameliorating the effects of acute SCI in humans (32, 40, 45). Ghrelin, identified as an endogenous ligand for the GH secretagogue receptor (GHS-R), is a novel 28-amino-acid peptide that is principally released from X/A-like cells in the oxyntic mucosa of the stomach (12, 31). Apart from its effect on energy balance (39), ghrelin has numerous biological actions, such as regulation of cardiovascular actions (47), modulation of cell proliferation and survival (4), inhibition of inflammation (15, 27, 46) and regulation of the immune functions (34). In an animal model of ischemia, ghrelin was shown to reduce infarct volume and inhibit apoptosis in the hypothalamic neurons (9). Miao et al (37) have demonstrated that decreased GHS-R-1a expression in the cerebral cortical neurons of rats with ischemia/reperfusion injury was reversed by ghrelin administration. It was suggested that redox injury and apoptotic mechanisms could be inhibited by ghrelin in cortical neurons subject to injury (37). Recently it was shown that ghrelin also inhibited apoptotic cell death of spinal cord neurons and oligodendrocytes after moderate SCI, while release of mitochondrial cytochrome c and activation of caspase-3 were also significantly inhibited (33). In the light of the aforementioned studies, in the current study we aimed to determine possible anti-inflammatory effects of ghrelin on secondary neuronal damage following SCI using neurological, biochemical and histopathological approaches.

MATERIAL and METHODS

Animals

Wistar albino rats (250-300 g) supplied by the Marmara University (MU) Animal Center (DEHAMER) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature (22±2°C) and relative humidity (65-70%) were kept constant. All experimental protocols were approved by the MU Animal Care and Use Committee.

Rats were randomly divided into three groups with 8 rats in each: 1) control group that underwent sham surgery and received intraperitoneal (ip) saline; 2) SCI group that underwent surgery for SCI induction and injected with ip saline; 3) SCI-induced and ghrelin (10 µg/kg/day, ip) administered group. Induction of SCI Anesthetized (ip ketamine and chlorpromazine; 100 mg/kg and 1 mg/kg, respectively) rats were positioned on a thermistor-controlled heating pad in a prone position and a rectal probe was inserted. Under sterile conditions, following T5-12 midline skin incision and paravertebral muscle dissection, spinous processes and laminar arcs of T7-10 were removed, while dura was left intact. Using modified weight-drop model for SCI (1), the rats were subjected to a 100 g/cm (10 g weight from 10 cm height) force to the dorsal surface of their spinal cord via a stainless steel rod (3 mm diameter tip) that was rounded at the surface. The rod was dropped vertically through a 10 cm guide tube that was positioned perpendicular to the center of the spinal cord. Following the suturing of the incision, the rats were placed in a warming chamber and their body temperatures were maintained at approximately 37 °C until they were completely awake.

A week after SCI induction, neurological examinations were performed in all 3 groups. Following the examination, the rats were decapitated to obtain plasma and spinal cord tissue samples (the epicenter to caudal parts of the injury) for the biochemical and histological analysis. Plasma levels of neuron-spesific enolase (NSE), and soluble protein-100β (S-100β) were assayed respectively as indices of neuron and astrocyte injury using ELISA kits for rats (USCN Life Science & Technology Company, Missouri, TX, USA).

Neurological Examination

The neurological examination scores were assessed according to motor function score of Gale et al. (18). All behavioral tests were conducted by a 'blinded' investigator. The sequence of testing animals by a given task was randomized for the animals.

Measurement of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity in tissues was measured by a procedure similar to that described by Hillegas et al. (25). Spinal cord tissue samples were homogenized in 50 mM potassium phosphate buffer with a pH of 6.0, and centrifuged at 41,400 g for 10 min. The pellets were then suspended in 50 mM PB containing 0.5 % hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw-cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H2O2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance, measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

DNA fragmentation assay

Samples from spinal cord tissues were homogenized in 9x volumes of a lysis buffer (5 mM Tris–HCl, 20 mM ethylene diamine tetra-acetic acid (EDTA) and 0.5% (v/v) t-octylphenoxypolyethoxyethanol (Triton-X 100); pH 8.0). Two separate samples of 1 ml each were taken from the sample and centrifuged at 25 000 g for 30 min to separate the

intact chromatin in the pellet from the fragmented DNA in the supernatant. The supernatant was taken out to be saved and the pellet was re-suspended in 1 ml Tris-EDTA buffer (pH 8,0) (10 mM:1 mM). Both the supernatant and the re-suspended pellet were then assayed for DNA content determination by the diphenylamine reaction described by Burton (7).

Histological analysis

Tissues were investigated at light microscopic level by an experienced histologist who was unaware of the treatment conditions. The paraffin-embedded spinal cord samples were cut (5 µm thick) transversally and then stained with Hematoxylin & Eosin (H&E) to examine the general morphology of white and gray matter in all groups with an Olympus BX51(Tokyo, Japan) photomicroscope. Luxol fast blue (LFB) stain which is specific for myelin sheath (41) is used to investigate myelin damage. Bright blue LFB staining indicates myelinization, whereas pale blue stained regions show myelin damage or loss.

Statistical Analysis

Statistical analysis was done using a GraphPad Prism 3.0 (GraphPad Software, San Diego; CA; USA). All data are expressed as means ± S.E.M. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The neurological examination scores were evaluated by Mann-Whitney U test. Values of $p<0.05$ were considered as significant.

RESULTS

The average neurological examination scores recorded one week after the SCI were significantly higher in the both saline- and ghrelin-treated SCI groups when compared with those of the sham-operated control group ($p<0.001$; Figure 1A), but the scores of the ghrelin-treated SCI group were not different than those of the saline-treated SCI group. On the other hand, plasma levels of S-100β protein and NSE, which were significantly elevated one week after SCI induction in the saline-treated group ($p<0.001$; Figure 1B, 1C), were suppressed in the ghrelin-treated SCI group ($p<0.05$).

MPO activity in the spinal cord tissue was significantly elevated in the saline-treated SCI group, demonstrating enhanced infiltration of neutrophils to the inflamed cord tissue ($p<0.001$; Figure 2A). Although MPO activity in the ghrelin-treated SCI group was not reversed back to control levels, the reduction was significant with respect to saline-treated SCI group ($p<0.05$). Similarly, in the spinal cord tissue samples of the saline-treated SCI group, DNA fragmentation indicating apoptosis was significantly increased as compared to the control group ($p<0.001$; Figure 4). However, SCI-induced increase in apoptosis was significantly suppressed by ghrelin treatment ($p<0.05$).

In histological sections stained with H&E, severe degeneration of the white matter and moderate degeneration of gray matter were observed in the saline-treated SCI group (Figure 3B), while neuropil architecture was apparently disrupted compared

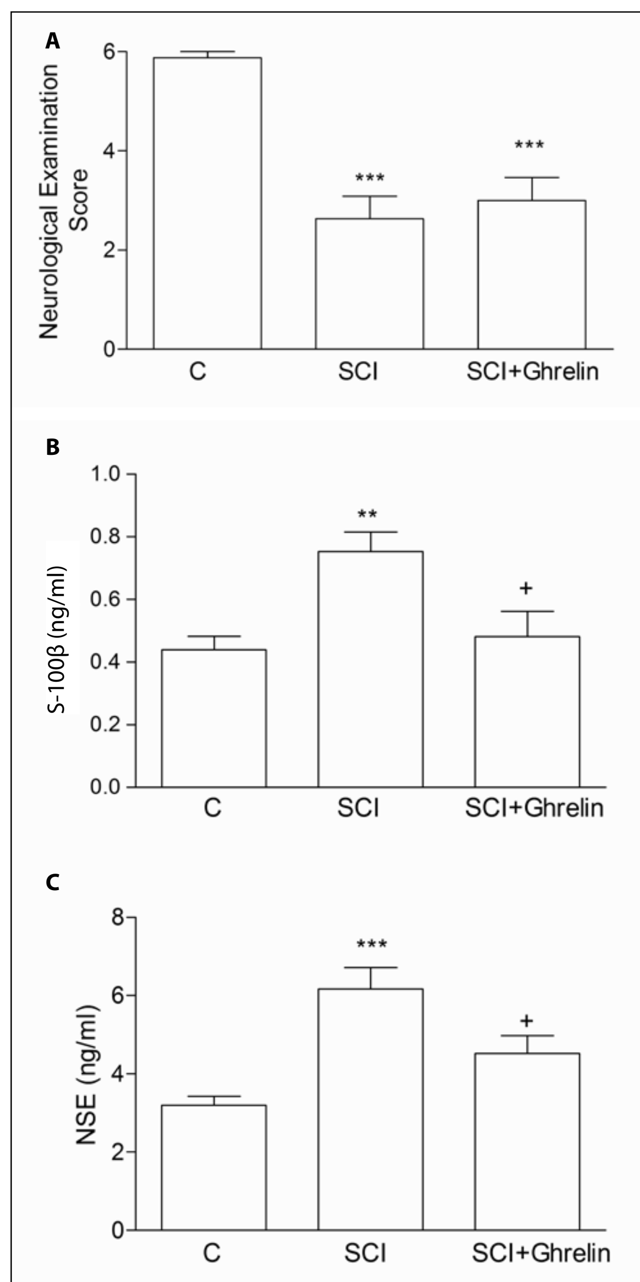

Figure 1: A) Gale's motor function scores, **B)** plasma S-100β and, **C)** plasma neuron specific enolase (NSE) levels of the rats in the control (C), saline-treated spinal cord injury (SCI) and ghrelin-treated SCI (SCI + Ghrelin) groups. Each group consists of 8 rats. Values are represented as mean ± SEM. **P<0.01, ***P<0.001 compared to control group; +P<0.05 compared to saline-treated SCI group.

to control group (Figure 3A). Prominent vacuolization and decreased staining intensity for LFB were observed when compared to control rats that had no evidence of myelin damage. The ghrelin-treated SCI group revealed near-normal morphological pattern in the H&E stained sections of both gray and white matter with preserved neuropil architecture (Figure 3C). Staining intensity for LFB was similar to that of

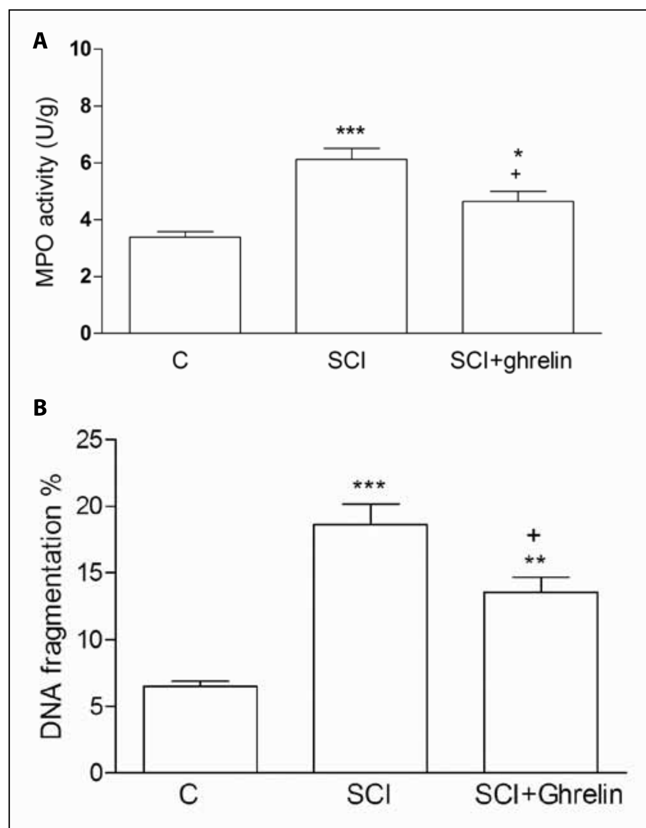

Figure 2: A) Myeloperoxidase (MPO) activity and **B)** DNA fragmentation (%) in the spinal cord tissues of the rats in the control, saline-treated spinal cord injury (SCI) and ghrelin-treated SCI (SCI + Ghrelin) groups. Each group consists of 8 rats. Values are represented as mean ± SEM. *p<0.05, **P<0.01, *** p<0.001 versus control group; +p<0.05, vs saline-treated SCI group.

the control group with less vacuole formation as compared to saline-treated SCI group.

DISCUSSION

It has been well established that free radical damage is an important pathological consequence of acute neural injury, while the generation of free radicals leads to subsequent lipid peroxidation and inflammation, which are thought to constitute a major pathway of secondary injury in SCI. Our data clearly demonstrates that treatment with ghrelin as a neuroprotective agent significantly inhibited SCI-induced neutrophil infiltration, lipid peroxidation and DNA damage in the spinal cord tissue, while the increased plasma S100-β and NSE levels were reduced back to control levels. However the findings revealed that ghrelin treatment failed to improve neurological deterioration observed following SCI.

The wide distribution of ghrelin in various organs, including lymphoid tissues (12, 23) suggests that it has other functions not directly associated with appetite control. Ghrelin has been shown to exhibit anti-inflammatory functions in vitro(8, 14) and in several animal models by enhancing immune

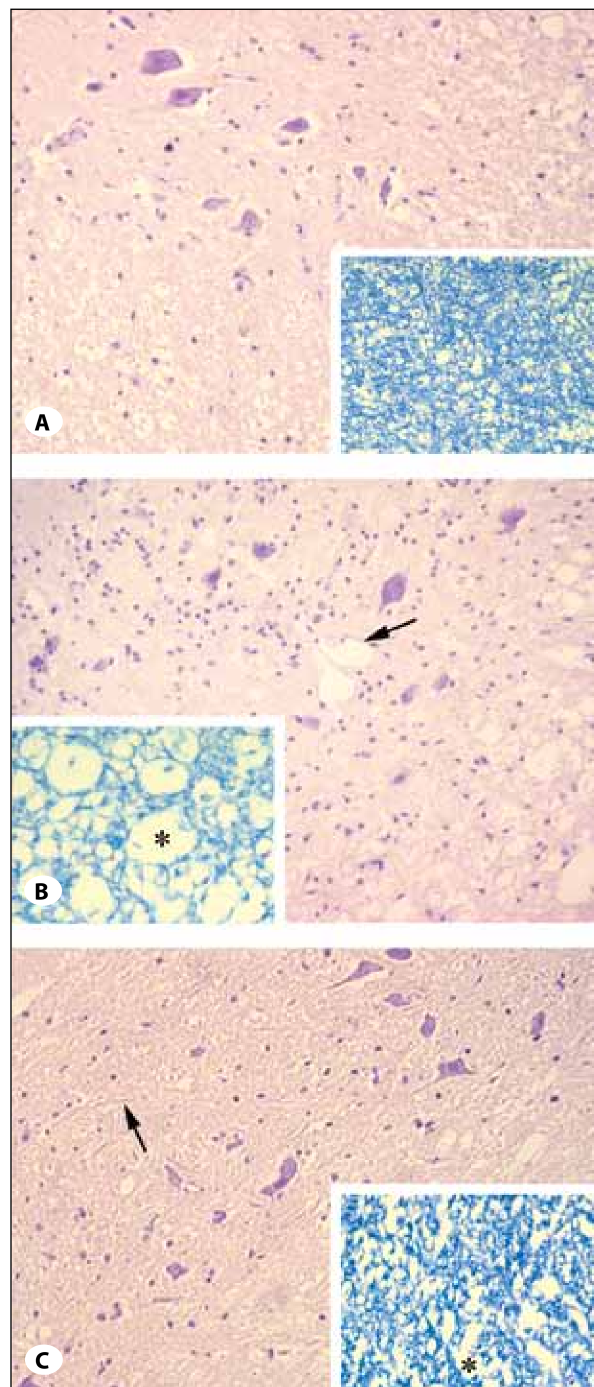

Figure 3: A) The spinal cord of control group: Regular morphology of the gray and white matter; normal Luxol fast blue (LFB) staining intensity is shown in the inset. **B)** Vehicle–treated SCI group: Marked vacuolization of the neuropil in the gray matter was observed (arrow); severe axonal degeneration with vacuole formation ("*" in the inset) was apparent. Note the myelin sheaths with decreased staining intensity compared to the control group in the inset. **C)** Ghrelin-treated SCI group: Reduced damage in the white and gray matter with near-regular neuronal process morphology (arrow); normal staining intensity and less vacuole formation ("*") with LFB is seen in the inset. Hematoxylene and Eosin stain X 400, insets LFB stain X 400, original magnification.

responses and downregulating anti-inflammatory molecules (21, 22). Both in vivo and in vitro studies have shown that ghrelin inhibited neuronal apoptosis. In the hypothalamic neuronal cells ghrelin was shown to inhibit apoptosis during glucose-oxygen deprivation (9), while its receptor agonist, GH-releasing peptide (GHRP)-6, attenuated glutamate-induced apoptosis in the hypothalamus and cerebellum (13). Similarly, ghrelin prevented ischemia- or neurotoxicity-induced cortical neuronal cell death (29, 37, 38). We have previously demonstrated that ghrelin alleviates subarachnoid hemorrhage-induced oxidative brain damage, and exerts neuroprotection by maintaining a balance in oxidant-antioxidant status (15). In support of our findings, recently Lee et al. (33) have reported that systemic administration of ghrelin improved functional recovery after SCI in part by inhibiting apoptotic cell death of neurons and oligodendrocytes. It was further suggested that the neuroprotective effect of ghrelin on SCI injury was mediated through the ghrelin receptor expressed on the spinal cord neurons and oligodendrocytes. Ghrelin inhibits TNF-α and IL-6 expression and nitric oxide production in peritoneal macrophages, human umbilical vein endothelial cells, and in arthritic rats (22, 34). We have also reported that ghrelin possessed a neutrophil-dependent anti-inflammatory effect in the gastric, pulmonary, hepatic and pancreatic tissues of rats induced with different inflammatory models (27, 28, 30, 46). Similarly, administration of a GHS-R agonist in a mouse model of arthritis reduced signs of inflammation and resulted in decreased IL-6 levels (22). These anti-inflammatory effects of the GHS-R agonist were suggested to be mediated by ghrelin receptors directly expressed in T lymphocytes and monocytes, where ghrelin inhibits the expression of pro-inflammatory cytokines (14).

Following the SCI, neutrophils begin to infiltrate the lesion site and accumulate within 3 h and reach a peak within 24 h (16, 26). Due to the accumulation of neutrophils, a number of substances such as ROS, reactive nitrogen radicals, cytokines, chemokines and a variety of enzymes are released to the lesion site. Spinal cord cells exposed to both NO and ROS undergo oxidative stress caused both by ROS and nitrogen reactive species. This severe oxidative stress produces strand breaks in DNA, which triggers energy-consuming DNA repair mechanisms resulting in the depletion of its substrate NAD+ and reduction in the glycolysis rate which finally results in cell death (10). The present findings revealed that a week after the injury of the spinal cord, neutrophil recruitment to the tissue was still high, while ghrelin treatment reduced the accumulation of neutrophils. In accordance with that, lower percentage of apoptotic DNA was observed in the ghrelin-treated SCI rats, suggesting that the inhibitory effect of ghrelin on neutrophil infiltration may be in part responsible for its protective effect against SCI-induced oxidative injury.

Lee et al. (33) have shown that ghrelin reduced the size of the lesion volume with a concomitant reduction in the loss of axons and myelin, while locomotor functional recovery assessed during the 35-day-follow-up period was also significantly improved at the 80 µg/kg dose of ghrelin, but the 10 µg/kg dose was found to be not effective. Ghrelin was administered immediately after injury and then further injected every 6 h for 1 day. In the present study, the neurological examination based on motor function scores was not improved by 10 µg/kg ghrelin treatment. Although the treatment was continued daily for 1 week, it appears that the dose was not sufficient to reverse the neurological scores that included behavioral and motor aspect. On the other hand, ghrelin treatment depressed the elevated serum S100-β and NSE levels, which are early predictors of outcome in head-injured and stroke patients (17, 24, 42) and their elevation reflects increased severity of the primary hypoxic ischemic insult (19). The neuronal marker studied most extensively in cerebral ischemia is NSE, a neuron-specific intracytoplasmic enzyme in the glycolytic pathway, which is released into cerebrospinal fluid (CSF) and blood after cerebral injury (11). Serum and CSF NSE levels were shown to be elevated in rodent models of focal ischemia in proportion to the eventual infarct volume. Moreover, increases in the peripheral levels of NSE have been specifically related to neuronal injury in traumatic brain injury (24), stroke (42), and epileptic seizures (43). Another glycolytic pathway enzyme, S100-β, is released mainly from astrocytes in multiple forms of CNS damage, including ischemic stroke, CNS trauma, and neurodegenerative diseases (44).

Several pathophysiological events have been proposed to contribute to secondary neuronal dysfunction and death, and included events such as ischemia, edema, ionic imbalances, compromised energy metabolism, and biochemical changes as responsible of the resulting neurotoxicity (3, 35). During secondary degenerative response following SCI, apoptosis of the neurons and oligodendrocytes causes progressive degeneration of the spinal cord and spinal cord dysfunction (36, 48). The results confirm that the ghrelin alleviates secondary degenerative response and inhibits apoptosis of the neurons following SCI. Based on the current data, ghrelin was shown to have efficacy in abating neural damage in spinal cord injury and further experimental studies would provide fundamental information for the effective design and execution of clinical trials using ghrelin as a neuroprotective treatment.

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