



Effect of Carvedilol on Secondary Damage in Experimental Spinal Cord Injury in Rats

Ratlarda Deneyisel Omurilik Travmasında Karvedilol'ün İkincil Hasara Etkisi

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ABSTRACT

AIM: Previous studies have shown that carvedilol has a neuroprotective effect in animal models of brain ischemia and brain oxidative damage in vitro. This study was performed to investigate the effect of carvedilol on the secondary damage in experimental spinal cord injury (SCI).

MATERIAL and METHODS: Twenty-four Wistar albino rats were divided into three groups. Group 1 underwent laminectomy alone. Group 2 underwent laminectomy followed by SCI and received carvedilol. Group 3 underwent laminectomy followed by SCI and received no medication. The neurological functions were assessed by Tarlov's motor scale at the first and 24th hours. Oxidative stress status was assessed by MDA, SOD, MPO, GSH activities. A TUNEL-based apoptosis kit was used for evaluating apoptosis in the spinal cord samples and hematoxylin- and eosin-stained specimens were used for light microscopic examination.

RESULTS: Carvedilol reduced apoptosis and regulated oxidant and antioxidant status by increasing SOD and GSH levels and reducing MPO and MDA levels in the spinal tissue homogenate. Neurological examination of rats revealed statistically significant improvement 24 hours after the trauma.

CONCLUSION: Carvedilol has a statistically significant therapeutic effect, especially on functional recovery, and we found that carvedilol reduced secondary damage by inhibiting apoptosis and regulating the oxidant and antioxidant status.

KEYWORDS: Carvedilol, Spinal trauma, Apoptosis, Secondary damage

ÖZ

AMAÇ: Beyinde oksidatif hasar ve iskemi ile ilgili daha önce yapılan in vitro hayvan modeli çalışmalarında karvedilolün nöron koruyucu etkilerinin olduğu gösterildi. Çalışmada karvedilolün deneyisel spinal travma modelinde ikincil hasar üzerine olan etkileri araştırıldı.

YÖNTEM ve GEREÇLER: 24 adet Wistar albino tipi sıçan üç gruba ayrıldı. Spinal kord yaralanması ağırlık düşürme modeli ile gerçekleştirildi. Birinci gruba sadece laminektomi uygulandı. İkinci gruba laminektomi yapılarak spinal kord yaralanması oluşturuldu ve karvedilol verildi. Üçüncü gruba laminektomi yapıp spinal kord yaralanması oluşturuldu ve herhangi bir medikasyon uygulanmadı. Tüm gruplarda deneklerin birinci ve 24. saatlerde Tarlov motor skorlaması ile nörolojik muayeneleri kaydedildi. Oksidatif stres ölçümü için MDA (malonildialdehit), SOD (süperoksit dismutaz), MPO (myeloperoksidaz) ve GSH (glutasyon) aktiviteleri kullanıldı. Apoptozis düzeyini belirlemek için TUNEL kiti ve ışık mikroskobu bulguları için hematoksilen-eozin boyası kullanıldı.

BULGULAR: Karvedilol apoptozis düzeyini azalttı ve SOD ile GSH düzeyini artırıp MPO ve MDA düzeylerini azaltarak oksidasyon ve antioksidasyon olaylarını düzenledi.

SONUÇ: Karvedilol özellikle fonksiyonel iyileşme üzerine istatistiksel olarak olumlu etki oluşturdu ve apoptozisi azaltıcı ve oksidasyon-antioksidasyon olaylarını olumlu yönde düzenleyici etki gösterdi.

ANAHTAR SÖZCÜKLER: Karvedilol, Spinal travma, Apoptozis, İkincil hasar

INTRODUCTION

Spinal cord injury (SCI) is the most destructive neuropathologic condition with neurological deficits and secondary complications (15). SCI occurs in two stages as primary and secondary injury. Mechanical destruction of nerve axons by the primary injury causes the induction of some biochemical and cellular cascades. Secondary injury starts in the first

few minutes and continues for several weeks following the injury (7). Oxidative stress, inflammation, increased release of excitatory amino acids, lipid peroxidation, ion dysregulation and apoptosis are responsible for the secondary damage (8). Free radicals damage various cellular components, including proteins, lipids, and deoxyribonucleic acid (DNA). The central nervous system is highly vulnerable to free radical

damage because of its high lipid content (12). Free radicals and inflammation lead to apoptosis and are responsible for neuronal and glial cell damage, as well as destruction of the cell membrane (8). Carvedilol, which is used for hypertension and congestive heart failure, is an adrenoceptor antagonist. It also behaves as a neuroprotective agent. (9,17). This effect has been attributed to the free radical scavenging of carvedilol (10). Carvedilol has also been shown to possess neuroprotective effects in several models of transient focal stroke (11). In vitro studies showed that carvedilol inhibited superoxide anion release from activated neutrophils and lipid peroxidation. In addition, carvedilol protected cultured endothelial cells against oxygen-radical-induced cell injury (18,19). However, the effects of carvedilol have not yet been reported in traumatic SCI. In the present study, we aimed to investigate the effects of carvedilol on oxidative damage, neuron cell apoptosis and neurological dysfunction in a rat model of traumatic SCI.

MATERIAL and METHODS

Animals

Twenty-four Wistar albino rats weighing 250-300 g were housed in an air-conditioned room with 12-h light and dark cycles and relative humidity was kept constant. They were given free access to food and water. All experimental protocols were approved by the Necmettin Erbakan University Experimental Medicine Research and Application Center Experimental Animal Ethics Committee. Rats were divided into three groups of eight rats. Group 1 underwent laminectomy alone. Group 2 underwent laminectomy followed by SCI and received carvedilol. Group 3 underwent laminectomy followed by SCI and received no medication

Induction of Spinal Cord Injury

The animals were anesthetized by an intraperitoneal injection of 10 mg/kg xylazine (Rompun, Bayer Turk Kimya Sanayi Limited Sirketi, İstanbul, Turkey) and 50 mg/kg ketamine hydrochloride (Ketalar, Pfizer İlaçları Limited Sirketi, İstanbul, Turkey). Rats were positioned in the prone position. The surgical procedure was performed under sterile conditions.

Three level laminectomy was performed after the T6-10 midline skin incision and paravertebral muscle dissection. The dura was left intact. After visualization of the spinal cord, SCI was produced by using the weight drop technique. The animals were subjected to a 50 g/cm impact to the surface of the spinal cord. The weight was a stainless steel rod with a rounded surface (3 mm in diameter and weighing 10 g), which was dropped through a 5-cm-long guide tube positioned perpendicular to the center of the spinal cord. After SCI, the incision was closed.

Neurological Examination

The neurological examination scores were assessed using Tarlov's scoring system (14). The animals were followed up for 24 h after the surgery and neurological assessment was carried out at the first and twenty-fourth hours. Neurological scores were assessed as follows: 0; Paraplegic, 1; Perceptible

movement, 2; Some functional movement, 3; Hind limb movement and can support body weight, 4; Normal function.

Measurement of Tissue Myeloperoxidase (MPO) Activity

Activity of the neutrophil-specific MPO was measured by slight modifications. The spinal cord segments were homogenized with 50 mmol/L potassium phosphate buffer (pH: 6) on ice and the homogenates were centrifuged at 13.000 g for 20 minutes. The supernatant MPO activity was determined in 0.6 mL of 0.1 M phosphate buffer (pH 6.0) containing o-dianesidine dihydrochloride (0.167 mg/mL) and hydrogen peroxide (0.0005%). One unit of MPO activity was defined using MPO as a standard. The absorbance at 460 nm was monitored with spectrophotometer and calculated using a standard curve prepared for purified MPO. Specific activity is given as units per g of protein.

Measurement of Tissue Glutathione (GSH) Levels

The glutathione (reduced and oxidized form) content of tissues was determined using the assay kits from the Cayman Chemical Company (Ann Arbor, MI, USA) and the method of Elman (4). Glutathione peroxidase (GSH) is reacted with 5,5-dithiobis-2-nitrobenzoic acid resulting in the formation of a product which has absorbance at 410 nm. Results were expressed as micromole per gram tissue (mmol/g).

Superoxide Dismutase (SOD) Assay

SOD activity was determined as described by Sun et al. (13). This method depends on the inhibition of nitroblue tetrazolium (NBT) reduction by xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD activity was expressed as the amount of enzyme that causes 50% inhibition of the rate of NBT reduction. SOD activity was designated as units for mg/protein of spinal tissue.

Measurement of Tissue Malondialdehyde (MDA) Levels

MDA levels of the samples were measured by Draper and Hadley's method (3) based on thiobarbituric acid (TBA) reactivity. MDA, an end-product of fatty acid peroxidation, reacts with TBA to form a coloured complex that has maximum absorbance at 532 nm. MDA activity was designated as unit for micromole per gram tissue (mmol/g).

Histopathological Analysis

Spinal cord samples were fixed in 10% paraformaldehyde and prepared with the Autotechnicon and then embedded in paraffin. Slices (5 mm) were obtained with a microtome and stained with hematoxylin and eosin. Hematoxylin and eosin-stained specimens were examined under a Nikon Eclipse E400 light microscope (Nikon Instruments Inc, Melville, NY, USA). For detecting apoptosis in samples, a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling)-based apoptosis kit (Fragel DNA fragmentation kit, Calbiochem, Darmstadt, Germany) was used. In this method, 4-micron thick sections cut from paraffin blocks were first deparaffinized and rehydrated. They were permeabilized with proteinase K and endogenous peroxidase inactivated by 10% H₂O₂. For DNA

labeling, Tdt labeling reaction mix and Tdt enzyme mixture were used. For detection of the reaction, DAB solution in H_2O_2 /urea mixture was used. Methyl green (3%) was used for counter staining. Samples were then dehydrated and mounted with xylene. Terminal deoxynucleotidyl transferase dUTP nick end labeling-positive brown-stained cells were accepted as apoptotic in concordance with the positive control supplied by the manufacturer.

Statistical Analysis

For statistical evaluation, we used the software package SPSS 15.0. Statistical comparisons between groups were tested with the Kruskal–Wallis test, and the Mann–Whitney U test was used for dual comparison. $p < 0.05$ was considered statistically significant. The results are given as the mean $\pm$ standard deviation of the mean (SD).

RESULTS

Biochemical Assessment

Figure 1 shows the mean levels of MPO in all groups. MPO activity, which is accepted as a determinant of neutrophil infiltration, was lower in the spinal cord tissues of rats that received carvedilol than in the trauma group. MPO levels were 1.67 ± 0.84 in the laminectomy alone group, 2.55 ± 0.70 in the carvedilol treatment group and 4.36 ± 3.27 in the trauma group. Carvedilol decreased MPO levels but this was not statistically significant ($p > 0.05$).

Figure 2 shows the mean levels of MDA in all groups. MDA levels, an indicator of lipid peroxidation, were higher in the trauma group and lower in the carvedilol group. MDA levels were 2.49 ± 1.58 in the laminectomy alone group, 2.57 ± 1.85 in the carvedilol treatment group and 2.95 ± 1.94 in the trauma group. Carvedilol decreased MDA levels but this was not statistically significant ($p > 0.05$).

Figure 3A and 3B shows the mean levels of GSH and SOD in all groups. GSH and SOD levels, which are the important antioxidant enzymes, were higher in the carvedilol treatment group than the trauma group. Comparisons between the carvedilol treatment group and trauma group showed no statistically difference ($p > 0.05$).

GSH levels were 1249.25 ± 227.88 in the laminectomy alone group, 1330.23 ± 491.97 in the carvedilol treatment group and 1190.43 ± 255.77 in the trauma group.

SOD levels were 845.46 ± 362.46 in the laminectomy alone group, 883.77 ± 332.15 in the carvedilol treatment group and 522.88 ± 389.86 in the trauma group.

Neurological Assessment

Figure 4 shows the mean levels of neurological examination in all groups. For each group, neurological examinations were performed at the first and 24th hours. The functional recovery was better in the carvedilol treatment group than the trauma group. Neurological examination showed recovery especially in the 24th hour and this was statistically significant ($p < 0.05$).

Histopathological Assessment

Photomicrographs show the degenerated glial cells with the yellow arrows and degenerated neurons with black arrows (hematoxylin eosin staining) in Figure 5: A) Laminectomy group, B) Trauma group, C) Carvedilol treatment group. Photomicrographs show apoptotic cells with black arrows (TUNEL staining) in Figure 5: D) Laminectomy group, E) Trauma group, F) Carvedilol treatment group.

Table I shows the histopathological analysis in all groups. The highest apoptosis values were in the trauma group. Carvedilol reduced apoptosis in the white and gray matter but there was no statistically significant difference between the trauma and carvedilol treatment groups ($p > 0.05$).

The highest number of neurons and glial cells were in the laminectomy alone group. The neuron and glial cell counts

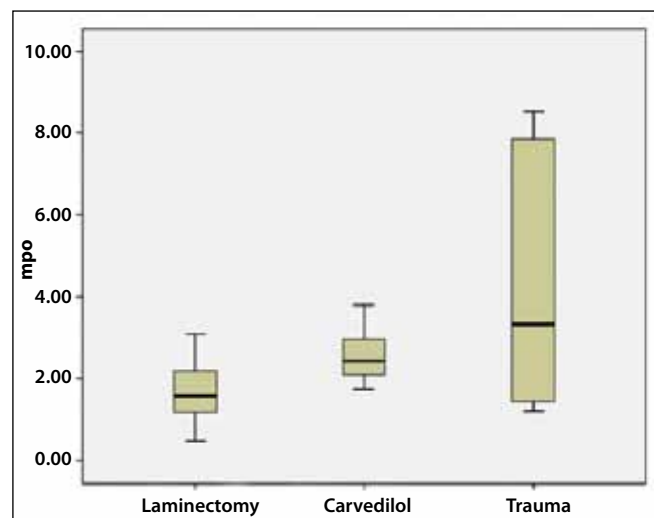


Figure 1: Graphic showing the mean values of MPO levels as u/g protein.

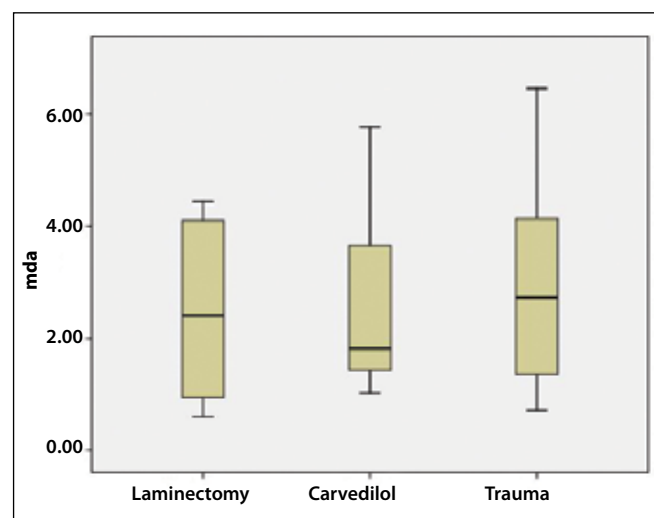


Figure 2: Graphic showing the mean values of MDA levels as micromol/g protein.

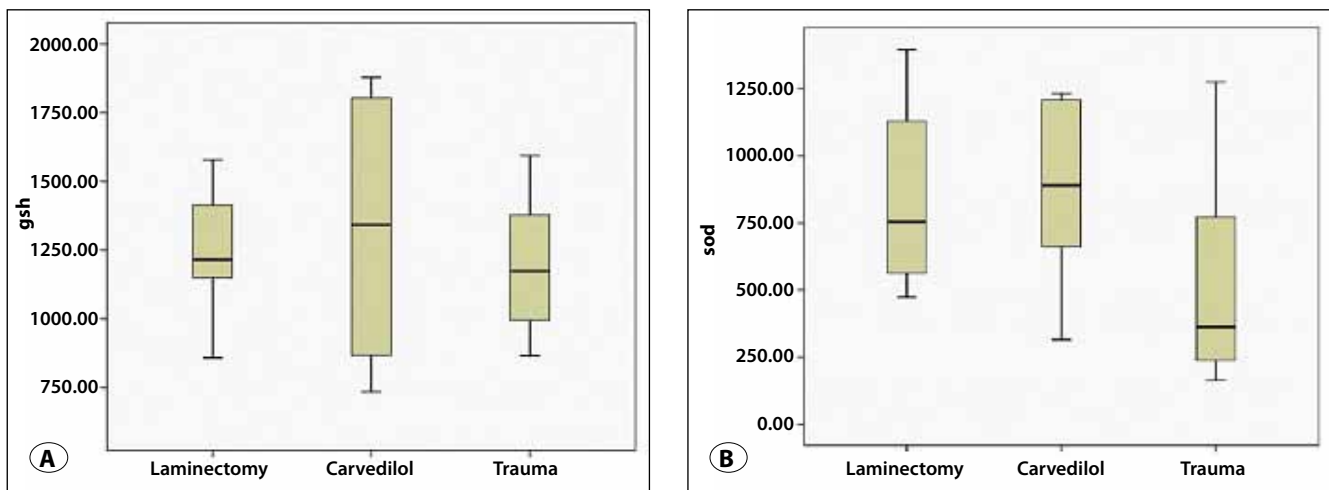


Figure 3A, B: Graphics showing the mean values of SOD as u/g protein and GSH levels as micromol/g protein.

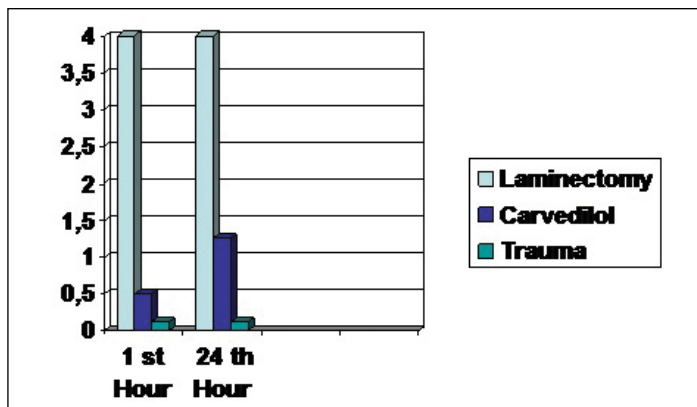


Figure 4: Graphics showing the mean levels of neurological examination in all groups.

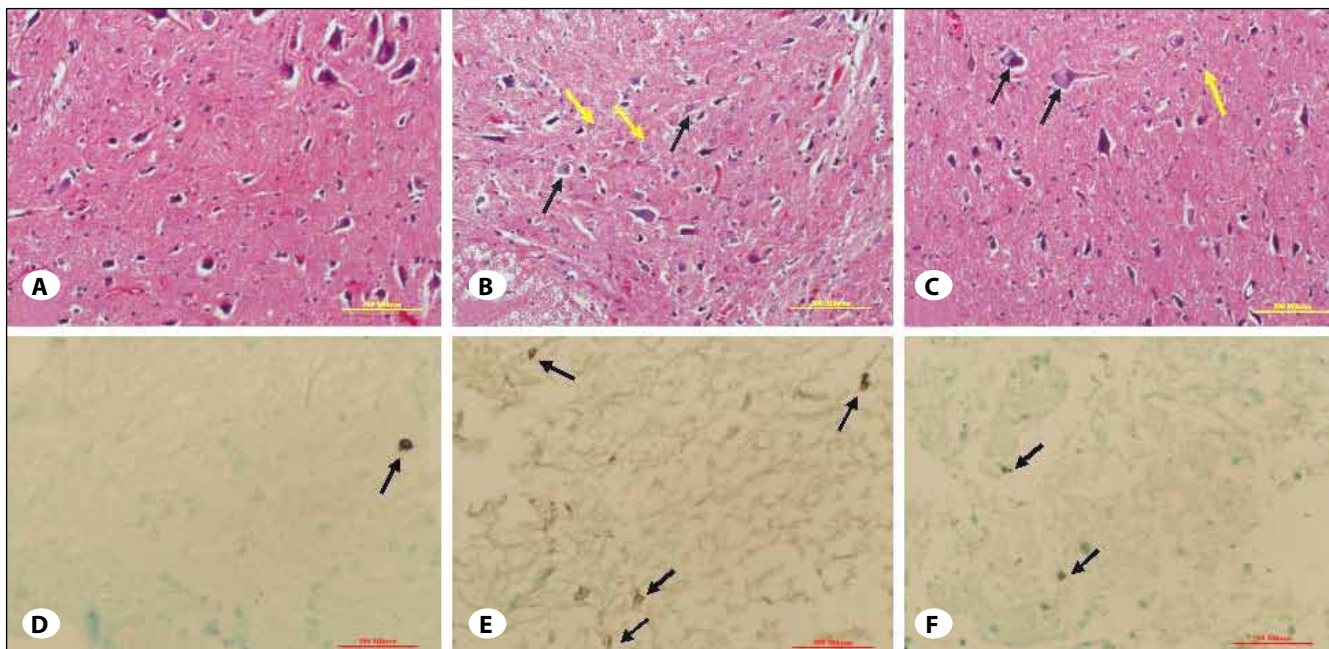


Figure 5: Photomicrographs showing degenerated glial cells with the yellow arrows and degenerated neurons with the black arrows (hematoxylin-eosin staining). **A)** Laminectomy group, **B)** Trauma group, **C)** Carvedilol treatment group. Photomicrographs showing apoptotic cells with the black arrows (TUNEL staining). **D)** Laminectomy group, **E)** Trauma group, **F)** Carvedilol treatment group.

Table I: Table Showing the Histopathological Analysis In all Groups

	Apoptosis in the white matter	Apoptosis in the gray matter	Number of neuron	Number of degenerated neuron	Number of glial cells	Number of degenerated glial cells
Laminectomy	0.62500±.2	0.6250±0.2	21.8750±.4	1.0000±0.2	21,2500±3.8	0.7500±0.8
Carvedilol	4.5000±0.8	3.8750±0.5	18.75001±0	2.00000±.2	18.25002±.4	2.25000±0.7
Trauma	7.3750±0.8	5.8750±0.4	14.6250±0.8	3.25000±.3	14.6250±2.6	3.7500±1.4

were higher in the carvedilol treatment group than the trauma group but these differences were not statistically significant ($p>0.05$).

The lowest number of degenerated neurons and glial cells were in the laminectomy alone group. Degenerated neuron and glial cell counts were lower in the carvedilol treatment group than the trauma group but these differences were not statistically significant ($p>0.05$).

DISCUSSION

There is no still potent treatment of secondary damage after traumatic spinal cord injury. We know that the most important pathophysiological mechanisms of neuronal degeneration after SCI are free radical-induced oxidative damage, lipid peroxidation and apoptotic cell death (6). The nervous system is quite sensitive to free radical damage because of its high oxygen content and limited antioxidant enzyme activity. Lipids are the biological molecules most sensitive to oxidative damage. The nervous system is therefore very sensitive to lipid peroxidation (2). It has been shown that carvedilol scavenges free radicals and inhibits lipid peroxidation in swine ventricular membranes and rat brain homogenates (16). There is no previous study that has investigated the neuroprotective effect of carvedilol in SCI. In the present study, we investigated the neuroprotective effects of carvedilol after SCI. MDA levels make up the most important indicator for lipid peroxidation (1). In our study, we used MDA levels for to determine the rate of lipid peroxidation. MDA levels were lower in the only laminectomy group than the trauma group. These results showed that carvedilol treatment was effective at reducing lipid peroxidation.

Reactive oxygen species have destructive effects on neuronal cells after SCI. Antioxidant mechanisms play a valuable role in reversing these destructive effects. Reduced glutathione and SOD are endogenous antioxidants that neutralize the negative effects of free radicals. In our study, SOD and GSH levels were higher in the carvedilol treatment group than the trauma group. Our study showed that treatment with carvedilol reduced oxidative damage.

MPO is an enzyme located in azurophilic granules within neutrophils. Elevated MPO levels are seen in injured tissue because of neutrophil infiltration as an indicator of inflammation (5). We found that the highest level of MPO was seen in the trauma group and it was lower in the carvedilol treatment group. Based on these results, we can say that carvedilol may reduce inflammation in SCI.

Reactive oxygen species cause neuronal damage by stimulating lipid peroxidation, DNA damage and neuronal apoptosis. Savitz et al. (2000) has previously shown the antiapoptotic effects of carvedilol in transient focal cerebral ischemia (11). In the present study, we showed increased apoptosis levels in SCI. Carvedilol reduced apoptotic cell counts in the traumatic spinal cord. Light microscopic examination showed neuronal and glial cell degeneration in the white and gray matter. In our study, degenerated neuronal cell and glial cell counts were lower in the carvedilol treatment group than the trauma group. However, we found that the normal glial and neuronal cell counts were higher in the carvedilol treatment group than the trauma group. These light microscopic findings showed that carvedilol reduced apoptosis and neuronal loss (Figure 5A-F).

Behavioral tests are used to determine the functional recovery after SCI. In our study we used Tarlov's motor scale to evaluate the clinical outcome. We found that carvedilol provides improvement after SCI. The functional recovery rate was statistically significant especially after the 24th hour of the SCI in the carvedilol treatment group.

In conclusion, the present study showed that carvedilol has neuroprotective effects after SCI and this is the first study that reveals the neuroprotective effect of carvedilol on neurobehavioral, biochemical and histopathological recovery after SCI. Although this study has some limitations such as the number of rats, we believe that it may guide future studies.

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