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# Effects of Metoprolol on Experimental Spinal Cord Ischemia-Reperfusion Injury in Rats

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**ABSTRACT**

**Aim:** The aim of this study was to investigate the neuroprotective effect of metoprolol and its efficacy in reducing lipid peroxidation levels in the spinal cord ischemia-reperfusion model in rats.

**Material and Methods:** Twenty (20) Sprague-Dawley female rats weighing between 220 gr and 280 gr were randomly divided into 3 groups. Only laparotomy was performed in the control group, and the aorta abdominalis was revealed. In the groups other than the control group, clip compression was applied to the aorta abdominalis for 45 minutes. The ischemia group was not given any medication. Metoprolol was administered intraperitoneally at 0.5 mg/kg to the metoprolol group. Motor examination was made according to Tarlov scale at the 1st and 24th hours and then, spinal cords of all rat models were removed. Spinal cord tissue samples were collected for histopathological examination and for determining malondialdehyde (MDA) level. All rats were sacrificed by draining blood after their motor examinations.

**Results:** According to motor examination findings at the 1st and 24th hours, metoprolol resulted in a statistically significant improvement in recovery ($p$=0.045). Histopathological examinations revealed that metoprolol contributed to neurological recovery by reducing neuronal necrosis. MDA levels, which is an indicator of lipid peroxidation, were significantly lower in the metoprolol group when compared to the ischemia group ($p$=0.001).

**Conclusion:** Metoprolol was found to be significantly effective in reducing and/or preventing spinal cord ischemia-reperfusion injury.

**Keywords:** Ischemia-reperfusion injury; metoprolol; spinal cord.

## Sıçanlarda Metoprololün Deneysel Omurilik İskemisi/Reperfüzyon Hasarı Üzerine Etkileri

**ÖZ**

**Amaç:** Bu çalışmanın amacı, metoprololün nöroprotektif etkisini ve sıçanlarda omurilik iskemisi/reperfüzyon modelinde lipid peroksidasyon düzeylerini azaltmadaki etkinliğini araştırmaktır.

**Gereç ve Yöntemler:** 220 gr ve 280 gr ağırlığında yirmi (20) Sprague-Dawley dişi sıçan rastgele 3 gruba ayrıldı. Kontrol grubunda sadece laparotomi yapıldı ve aort abdominalis ortaya konuldu. Kontrol grubu dışındaki gruplarda, aort abdominalise 45 dakika boyunca klips kompresyon uygulandı. İskemi grubuna herhangi bir ilaç verilmedi. Metoprolol grubuna intraperitonal olarak 0,5 mg/kg metoprolol uygulandı. Motor muayene 1. ve 24. saatlerde Tarlov ölçeğine göre yapıldı ve daha sonra tüm sıçanların omurilikleri çıkarıldı. Histopatolojik inceleme ve malondialdehit (MDA) seviyesini belirlemek için omurilik doku örnekleri toplandı. Tüm sıçanlar motor muayenelerinden sonra kansızlaştırma yoluyla sakrifiye edildi.

**Bulgular:** 1. ve 24. saatteki motor muayene bulgularına göre, metoprolol istatistiksel olarak anlamlı bir iyileşme sağladı (*p=0,045*). Histopatolojik incelemeler, metoprololün nöronal nekrozu azaltarak nörolojik iyileşmeye katkıda bulunduğunu ortaya koydu. Lipid peroksidasyonunun bir göstergesi olan MDA düzeyleri, metoprolol grubunda iskemi grubuna göre anlamlı olarak daha düşüktü (*p=0,001*).

**Sonuç:** Metoprololün omurilik iskemi/reperfüzyon hasarını azaltmada ve/veya önlemede önemli ölçüde etkili olduğu ortaya konulmuştur.

**Anahtar Kelimeler:** İskemi-reperfüzyon hasarı; metoprolol; omurilik.

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## INTRODUCTION

Spinal cord injuries can be caused by traffic accidents, sports activities, occupational accidents and other reasons, and they are associated with high mortality rates as well as serious socio-economic problems for surviving patients (1-3). Prevention and treatment of common spinal cord injuries are highly important. In acute spinal cord injury, neurological damage is caused by two basic mechanisms. The first one is primary (mechanical) injury, which results in the death of cells that become necrotic due to the primary trauma caused by tissue damage. Secondary trauma is the damage that starts and progresses after a certain period of time as a result of various cascades occurring after the primary injury (2).

In recent years, experimental studies on pharmacological applications for preventing secondary damage have become more common. Owing to their neuroprotective properties, the effects of many agents such as mexiletine, nebivolol, carvedilol, methylprednisolone and melatonin in preventing and/or reducing secondary damage have been investigated in experimental spinal cord ischemia-reperfusion injury models (4-8). Metoprolol is a cardioselective β1-adrenergic blocker (9). Recent studies have shown that by indirectly affecting the renin-angiotensin system, which can help in reducing the effects of heart attack, metoprolol reduces the symptoms of drug users, dilates peripheral blood vessels, significantly reduces water and sodium retention (10), reduces myocardial damage caused by an excess of myocardial calcium ion, and reduces blood pressure and cardiac output by stabilizing heart rate (11). β-blockers can effectively stop over-activation of the noradrenergic nervous system, improve cardiac function in patients with severe heart failure, and block ventricular remodeling (12). When the effects of metoprolol on myocardial ischemia and reperfusion injury were examined, it was determined that it reduced ischemia damage by inhibiting lipid peroxidation and decreased myocardial energy demand by reducing heart rate (13). Although metoprolol is used to regulate cardiac autonomic regulation in individuals with spinal cord injury, the long-term effects of metoprolol on spinal cord injury have not been investigated in these individuals (14). While the therapeutic effects of metoprolol have been studied in the experimental traumatic spinal cord injury model (15), to the best of our knowledge, its effects on the spinal cord ischemia-reperfusion model have not been included in the literature.

In this study, the effects of systemically administered metoprolol were investigated by using motor function, biochemical and histopathological methods in the rat spinal cord ischemia reperfusion model, and its effects as a therapeutic agent were examined.

## MATERIAL AND METHODS

This study was carried out in the Experimental Research Center of the Karadeniz Technical University (KTU) Faculty of Medicine (Trabzon, Turkey) with the approval of KTU Animal Experiments Local Ethics Committee (Date of Approval: 08 December 2016, Protocol number: 53488718-665).

### Experimental Groups

Twenty (20) healthy Sprague Dawley female rats (250 ± 30 g), obtained from the Karadeniz Technical University Experimental Animal Laboratory were used in the study. During the experiment, the rats were fed with standard animal feed and water, and kept in separate cages at 20-22°C temperature, and under a 12 hours' day/night (light/dark) cycle. The experimental animals were randomly divided into three groups of seven. Two rats died due to side effects of the surgical procedure in metoprolol group and were excluded from the experiment.

Group 1 (n=6): control group; laparotomy was performed, abdominal aorta was revealed, and ischemia was not performed.

Group 2 (n=7): ischemia group; laparotomy was performed, and abdominal aorta was clipped.

Group 3 (n=5): ischemia and metoprolol group; laparotomy was performed, abdominal aorta was clipped, and metoprolol was administered following these procedures.

All rats were sacrificed by draining blood after their motor examinations, and their T8-T12 spinal cords were then removed. Labeled samples belonging to the subjects were divided into two, and stored in 1.5 mL Eppendorf tubes at -80 ºC for biochemical analyses to be performed at the KTU Faculty of Medicine's Department of Biochemistry, and in a 10% formaldehyde solution for histopathological evaluations at the KTU Faculty of Medicine's Department of Pathology.

### Inducing Spinal Cord Ischemia Reperfusion

The rats were fasted for 12 hours before the procedure and only fed with water. Anesthesia was achieved by intraperitoneal administration of 10 mg/kg Xylazine as a pre-anesthetic agent (Rompin, Bayer Turkish Chemical Industry Limited Company, Istanbul, Turkey), followed by intraperitoneal administration of 50 mg/kg of ketamine HCl (Ketalar, Pfizer Pharmaceutical Company Limited, Istanbul, Turkey). The subjects were then taken to the operation table and the surgical area was stained with povidone iodine.

Laparotomies were performed with a midline incision on the anterior abdominal wall of rats. Abdominal organs were deviated to the right to reach the abdominal aorta. No additional procedure was performed on the rats in the control group. In the ischemia group, the aorta was compressed with a flat, transient, *Yaşargil* aneurysm clip for 45 minutes, after which the clip was removed. Compression was applied to the aorta of rats in the metoprolol group for 45 minutes with the *Yaşargil* aneurysm clip, after which the clip was removed, and the rats were intraperitoneally administered with 0.5 mg/kg metoprolol immediately before the peritoneal closure. All closures were performed according to the layers method.

### Motor Examination

Motor examination was performed with the naked eye using Tarlov motor scale (16) before the surgery and following the surgery at the 1$^{st}$ and 24$^{th}$ hours. During this observation, motor responses were scored between 0 to 4 as follows: 0=fully plegic; 1=minimal movement in the joints; 2=moves hind legs well, but cannot stand up; 3=can stand, but cannot walk normally; 4=can walk normally.

**Determination of MDA Level in Tissues**

Malondialdehyde (MDA) levels of the tissues were determined with Uchiyama and Mihara's method (17). This method measures the absorbance value at 532 nm of the color of the molecule formed by the reaction of thiobarbituric acid (TBA) in an acidic environment with MDA. Tetraethoxypropane was used as a standard. The MDA standard graph was generated by plotting the measured standard absorbance values against the concentration, and the amount of MDA (nmol MDA/g wet tissue) was calculated based on this graph (Figure 1). All the samples were assayed at the same time.

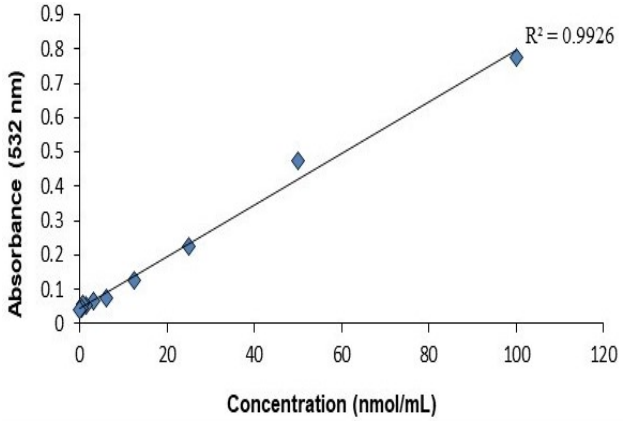


**Figure 1.** MDA standard curve used for TBA method, prepared in the same condition with the sample.

**Histopathologic Examination**

Histopathological examinations were performed with an Olympus BX51 light microscope, and the evaluations were documented at 40 magnification (x400) with microphotographs. Samples of approximately 5 mm thickness taken from the spinal cord pieces were applied with alcohol, xylol and formal solutions, and then embedded in paraffin blocks. Following this, tissue samples were cut at 5 µm thickness using a microtome. Deparaffinization was performed three times at 60ºC in an incubator by using the xylol pretreatment method. Samples were rehydrated with alcohol, and then washed with water and stained with hematoxylin-eosin (HE). The prepared samples were histopathologically examined under light microscopy, and findings of edema, axonal degeneration, myelin damage and inflammatory response were noted.

**Statistical Analysis**

A sample size of 7 animals per group would provide the appropriate power ($1 - \beta = 0.8$) to identify significant differences in MDA (adjusted $\alpha = 0.016$ for two comparisons), taking into account an effect size d = 2.0, a two-sided t-test, and a sample size ratio = 1 (G*Power 3.1.9.2, Kiel University, Kiel, Germany). Statistical analyses were made by using the SPSS 23.0 statistical package software. The Shapiro-Wilk test was used to check whether the data were normally distributed. As all of the MDA data fitted non-normal distribution, the Kruskal Wallis test was used for overall comparison of the groups. Comparisons between groups were performed using the Mann-Whitney U test with the Bonferroni correction. Tarlov motor examination results were compared using the Wilcoxon test, which are non-parametric method. Data were expressed as the median (first quartile-third quartile) for non-normal distribution. The level of statistical significance was accepted as $p<0.05$. For the all Bonferroni-corrected tests, the statistical significance was set at $p < 0.016$.

**RESULTS**

**Evaluation of Motor Examination**

The rats were evaluated with the Tarlov motor scale before the experiment to determine their normal values, and all of the rats were found to have a Tarlov motor score of 4. Motor responses were evaluated again at the 1st and 24th hours after the procedure (Figure 2). At the 1st hour, 60% of the rats in the metoprolol group were moving their hind legs but could not stand up, while 40% in the same group were standing up but could not walk normally. At 24th hour, 20% had complete recovery, while 80% could stand up but walked partially. In the control group, there was no change in the Tarlov motor scores throughout the experiment. According to motor examination findings, the rate of improvement in ischemia group was slower, and complete recovery was not observed in any of the rats at the end of 24 hours.

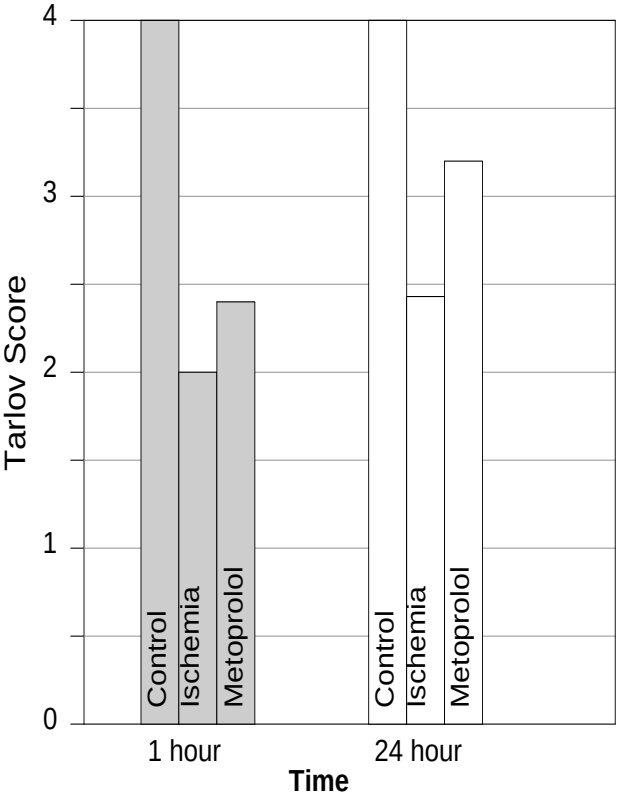


**Figure 2.** The median Tarlov scores of the experimental groups at various time points following ischemia-reperfusion injury.

There was no significant difference in motor examinations between the 1st hour and 24th hours of the control group ($p$=1.000, Table 1). Similar result was statistically obtained for the ischemia group ($p$=0.317, Table 1). On the other hand, there was a significant difference between the motor examination results of the metoprolol group at the 1st hour and 24th hour ($p$=0.045, Table 1).

**Table 1.** Motor functions assessed with Tarlov score at the 1st and 24th hour after surgery.

| Groups | 1st hour | 24nd hour | *p* |
|---|---|---|---|
| Control | 4.0 (4.0-4.0) | 4.0 (4.0-4.0) | 1.000 |
| Ischemia | 2.0 (2.0-3.0) | 2.0 (2.0-3.0) | 0.317 |
| Metoprolol | 2.0 (2.0-3.0)* | 2.0 (2.0-3.5)* | **0.045** |

Values are expressed in median (first quartile-third quartile)
Statistical analysis was performed by means of the Wilcoxon test

**Histopathological Changes**

HE staining was performed on all groups, and ischemia levels was classified into the three scores according to Nazli et al. (18) (Figure 3). Score 1 indicates normal histological appearance, while score 2 shows mild to moderate impact, and score 3 indicates significant impact. Based on this approach, the control group was defined as score 1, and contains motor neurons with normal histological appearance showing Nissl body in the cytoplasm, prominent nucleoli, and fine chromatin (Figure 3a). The ischemia group had a score of 3, and multiple red neurons showing loss of Nissl bodies, intense eosinophilic cytoplasm, nuclear pyknosis and loss of nucleoli were observed (Figure 3b). The metoprolol group contained a small number of red neurons indicating mild to moderate impact, alongside neurons with a normal histological appearance showing Nissl body in the cytoplasm, prominent nucleoli, and fine chromatin, and was defined as Score 2 (Figure 3c).

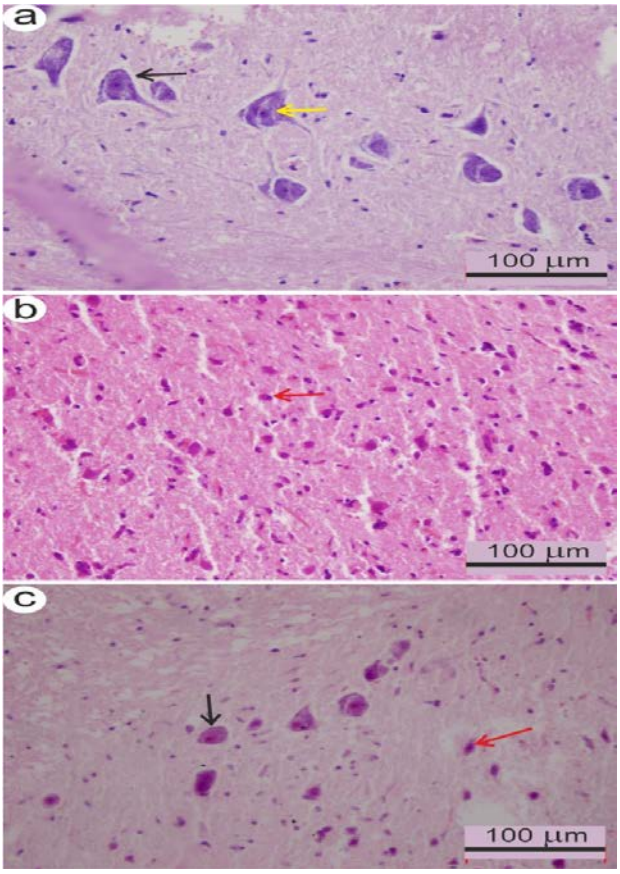


**Figure 3.** Micro-photographs showing the histopathological findings of (a) the control group, containing motor neurons with normal histological appearance showing Nissl bodies in the cytoplasm, prominent nucleoli, and fine chromatin; (b) the ischemia group, composing of multiple red neurons showing loss of Nissl bodies, intense eosinophilic cytoplasm, nuclear pyknosis and loss of nucleoli; c) the metoprolol group, including a few red neurons, alongside motor neurons with normal histological appearance showing Nissl bodies in the cytoplasm, prominent nucleoli, and fine chromatin. (HEx400). Nissl body, yellow arrow; motor neurons, black arrow; red neuron, red arrow.

**Tissue MDA Levels**

MDA levels of the experimental groups are given in Table 2 and Figure 4. Median MDA levels were 695 (581-887) nmol/g wet tissue in the control group, 1323 (1246-1373) nmol/g wet tissue in the ischemia group and 924 (768-1018) nmol/g wet tissue in the metoprolol group. Significant differences were found for MDA levels in post hoc evaluations between the groups performed with a Mann-Whitney U test with Bonferroni correction (Table 2).

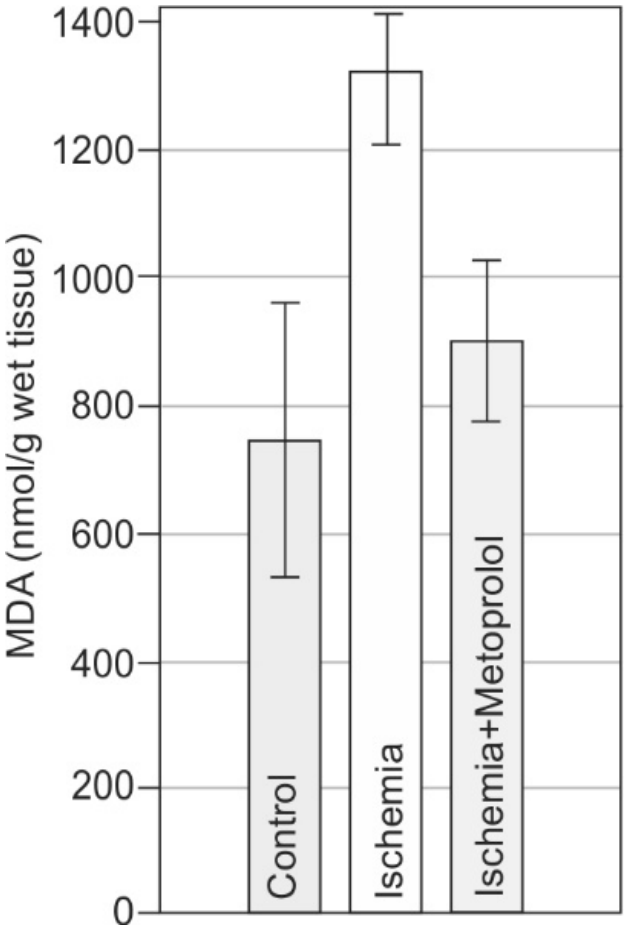


**Figure 4.** Mean (±standard deviation) values of MDA (nmol/g wet tissue) in the experimental groups.

Accordingly, when MDA levels were evaluated, there was a statistically significant difference between the control group and the ischemia group ($p$=0.0001). There was no statistically significant difference between the control group and the metoprolol group in terms of MDA levels ($p$=0.277). There was a statistically significant difference between the MDA levels of the ischemia group and the metoprolol group ($p$=0.001; Table 2).

**Table 2.** MDA (nmol /g wet tissue) levels of the experimental groups.

| Parameter | Control (n=6) | Ischemia (n=7) | Ischemia+ Metoprolol (n=5) | *p* |
|---|---|---|---|---|
| **MDA (nmol/mg wet tissue)** | 695 (581-887) | *1323 (1246-1373)[a] | *924 (768-1018)[b] | 0.002 |

Values are expressed in median (first quartile-third quartile). MDA, malondialdehyde; n, sample size
Comparisons between groups were calculated by means of Kruskal Wallis followed by the Mann-Whitney U test with Bonferroni post hoc correction)
Compared to Control group, [a]: $p$=0.0001
Compared to Ischemia group, [b]:$p$=0.001
*Adjusted significance level $p$<0.016

## DISCUSSION

Spinal cord injury (SCI) is a serious traumatic disease resulting in neurological deficits and motor dysfunction (19-21). According to 2016 data, the incidence of spinal cord injury (SCI) is approximately 54/1,000,000 in the United States (US), corresponding to 17,700 injuries annually (22). In Turkey, there is no current data at a national level about acute spinal cord injuries. According to 1992 data, the incidence of spinal cord injuries is 12.7/1,000,000 (1). Most common causes of spinal cord injury are vehicle accidents and falls, while lesser common causes include violent acts, such as gunshot wounds, and sports activities (1, 19, 22). Incomplete tetraplegia is the most common neurological injury. The incidences of incomplete and complete paraplegia are the same. Complete neurological recovery is achieved in less than 1% of the patients discharged (19).

The literature describes in detail the secondary damage mechanisms that occur after spinal cord injury (2). Secondary damage occurs within minutes after spinal cord injury, and its effects last for weeks or even months (23). In this process, the trauma area expands significantly. Secondary damage is the series of events that occur following the acute phase, such as electrolyte shifts, edema and necrotic cell death, formation of free radicals, delay of calcium influx, immune system-related problems and/or inflammation, and apoptotic cell death (24). Increased intracellular calcium levels, mitochondrial dysfunction, arachidonic acid degradation, and inducible nitric oxide synthase activation (INOS) processes (25-26) are followed by formation of reactive oxygen (ROS) and reactive nitrogen (RNS) species are (27-28). ROS and RNS also cause oxidative and nitrative damage to proteins and nucleic acids, as well as lipid peroxidation (29). There are ongoing studies on pharmacological interventions for the preventing and/or reducing the secondary damage frequently encountered in SCI. As is the case in the present study, these studies generally aim to prevent lipid peroxidation cascades through the removal of lipid peroxyl radicals (4, 13, 30). In this study performed on rats with spinal cord ischemia-reperfusion injury, it was demonstrated with neurological, biochemical and histopathological assessments that metoprolol had a significant neuroprotective effect through the removal of free radicals. Furthermore, although metoprolol is used to regulate cardiac autonomic regulation in individuals with spinal cord injury, the long-term effects of metoprolol on spinal cord injury were not monitored in these individuals (14). While the cardiac effects of metoprolol (11-13) and its neuroprotective effect on spinal cord trauma model (14) have been reported in the literature, this study is the first to investigate the therapeutic effects of metoprolol on the spinal cord ischemia-reperfusion model in rats.

Following the spinal cord injury, the Tarlov motor examination, a behavioral test, was used to demonstrate the functional recovery. According to the results, metoprolol administered after spinal cord ischemia-reperfusion injury resulted in a significant improvement in motor examination findings.

It is thought that the removal of free radicals after SCI through pharmacological applications, which in turn prevents lipid peroxidation, results in the protection of neurons, axons, myelin and intracellular organelles such as mitochondria and nuclei (4, 13). It has been shown that the use of metoprolol in rats with traumatic spinal cord injury reduced tissue myoloperoxidase (MPO) levels. According to histopathological examinations, decreased MPO activity leads to a decrease in traumatic spinal cord injury, and to greater neurological improvement with better neuronal functional outcome in the chronic phase of SCI (14). It has been stated that since metoprolol has a potential histological protective effect, a good clinical outcome can be expected in spinal cord injuries through its use. In our study, histopathological evaluation of tissue samples revealed that while all groups had ischemic damage characterized by pyknotic nucleus, loss of nucleoli, neuronal necrosis, and chromatin clustering, there was a decrease in neuronal necrosis in the metoprolol group compared to the ischemia group. This finding shows that, as described by Gök et al. (15), metoprolol contributes to neurological recovery by reducing neuronal necrosis and decreasing MPO levels, which also reduces neutrophil count.

Lipid peroxidation is determined effectively in an indirect manner by measuring MDA levels (31). Different researchers have reported that beta-blockers prevent the increase in tissue MDA levels; prevent lipid peroxidation biochemically; contribute to tissue healing; and have a neuroprotective effect (5, 6). The present study showed that MDA level, a marker of lipid peroxidation, is effectively reduced by metoprolol administration. Statistically, there was no significant difference between the MDA values of the metoprolol group and the control group and recovery via metoprolol administration was demonstrated by the significant difference between the metoprolol group and the ischemia group.

## CONCLUSION

The improvement of neurological function that was supported by histopathological findings, the speed of recovery, and the contribution to the prevention of lipid peroxidation indicated that, with the administered dose and time used this study, metoprolol had sufficient efficacy in reducing spinal cord ischemia-reperfusion injury.

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