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The Effects of Rifampicin on Experimental Cerebral Ischemia/Reperfusion Injury in Rats

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Abstract

Background: Ischemic brain damage can be explained by the emergence of acute focal or global neurological findings caused by vascular occlusions or hemorrhages. Even in non-fatal cases, stroke is an important pathologic condition with a severe impact on the quality of life, and patients require considerable assistance in the daily lives.
Objectives: The purpose of this study was to investigate the effect of rifampicin on malondialdehyde (MDA) levels and neurological examination of the hippocampal region in rats with transient cerebral ischemia.
Methods: This experimental study has been performed in a university-affiliated animal lab, Trabzon, Turkey, in 2016. Thirty-eight Sprague Dawley rats weighing 220 - 280 g were used. In this two-vessel occlusion and hypotension ischemia-reperfusion model, the bilateral carotid arteries were temporarily clipped (30 minutes), and blood was withdrawn up to 3 mL of intracardiac volume before the induction of hypotension. After 30 minutes, the clips were removed, and a reperfusion medium was created. One group of 12 rats received intraperitoneal injections of 30 mg/kg of rifampicin every day, and after a 30-minute bilateral carotid artery clipping and hypotension (10 mL/kg). Another group of 12 rats underwent a 30-minute bilateral carotid artery clipping and hypotension (10 mL/kg). The third group consisting of 7 rats underwent skin laceration only. The final group of 7 rats received anesthesia for only 15 minutes. Neurological examinations were performed at the end of days 1, 4, 7, and 10 in all groups. At the end of the 10th day, the animals were euthanized, and their brain tissues were removed. The hippocampi were removed from the brains for biochemical analysis and stored at -76°C in a deep freeze. Ischemic changes in the brain were assessed biochemically by measuring MDA levels in both blood and brain tissue.
Results: There was no statistically significant difference between the groups in terms of the mean tissue MDA levels (P = 0.112), but a significant difference was determined in the mean serum MDA values (P = 0.033). Serum MDA values significantly differed between the Group 1 and Group 2 (P = 0.030), but not between Group 1 and Group 3 (P = 0.58). Serum MDA values were also significantly different between Group 2 and Group 3 (P = 0.019), and between Group 2 and Group 4 (P = 0.035).
Conclusions: Rifampicin could exhibit a neuroprotective effect on cerebral ischemia-reperfusion injury.

Keywords: Brain Ischemia, Carotid Arteries, Hippocampus, Ischemia/Reperfusion Injury, Ischemic Attack, Malondialdehyde, Neuroprotective Agents, Rats, Rifampin, Transient

1. Background

Ischemic brain damage can be explained by the emergence of acute or ictal, focal, or global neurological findings stemming from vascular occlusions or hemorrhages resulting from the diseases of vascular structures involved in the central nervous system's blood supply (1). In this regard, 80% - 85% of stroke cases are ischemic, and 10% - 15% are hemorrhage-related (2, 3). Approximately 700,000 new stroke cases occur annually in the USA, and 20% of these patients die within the same year (4, 5). Even in non-fatal cases, stroke is an important pathologic condition with a severe impact on the quality of life, and patients with stroke require considerable assistance in their daily lives (6, 7).

A commonly used method for studying the pathophys-

iology of focal cerebral ischemia involves clipping the bilateral common carotid arteries to reduce or stop the cerebral flow in rats (8). We applied this model to our study due to the need for minimally invasive procedures, the ease of operation, and the need for craniectomy to control intracranial pressure.

2. Objectives

Many substances have been studied for their protective effects against acute cerebral ischemia in the experimental setting (9-14). Rifampicin is one of these substances that has been investigated in the context of neuroprotective effects against cerebral ischemia. Rifampicin is an antibiotic from the macrocyclic group used as an anti-tuberculosis agent. The principal effect of rifampicin is the elimination of free radicals. The radical-destroying effects of rifampicin have been reported in many studies. Glucocorticoid receptor activation has also been shown along with its neuroprotective effect (15-17).

In light of the above, we aimed to investigate the neuroprotective effects of rifampicin on ischemia and reperfusion injury by generating experimental focal brain ischemia in rats. To the best of our knowledge, this is the first experimental study to show the neuroprotective effect of rifampicin against global cerebral ischemia.

3. Methods

All experimental protocols received approval from the Karadeniz Technical University Experimental Animal Ethics Committee (approval number: 2016/12, date: 29 March 2016), Trabzon, Turkey. All data were extracted from this center, a referral public university medical faculty.

3.1. Test Animals

Thirty-eight female Sprague Dawley rats weighing 220 - 280 g were used. The rats were obtained from the Karadeniz Technical University Experimental Animal Laboratory. This study was performed at the animal laboratory of Karadeniz Technical University, located in Trabzon, Turkey, on June 2016. Rats' general health was monitored under standard conditions. The animals were kept under constant laboratory conditions of 18°C to 21°C with a 12-hour light-dark cycle and continuous access to food and tap water. The animals were housed in separate cages with no pre-study limit on access to water or feed restriction. Each rat was marked according to the group to which it was assigned.

3.1.1. The rats were divided into four main groups:

3.1.1.1. Group 1 (Ischemia and Rifampicin)

This group of 12 rats received the intraperitoneal administration of 30 mg/kg rifampicin every day followed by 30-minute bilateral carotid artery clipping and hypotension.

3.1.1.2. Group 2 (Ischemia)

This group of 12 rats was subjected to a 30-minute bilateral carotid artery clipping and hypotension.

3.1.1.3. Group 3 (Sham)

This group of seven rats was subjected to skin laceration.

3.1.1.4. Group 4 (Pure Control Group = Anesthesia Group)

Seven rats were anesthetized for 15 minutes only.

Neurological examinations were performed at the end of days 1, 4, 7, and 10 in all 4 groups. At the end of the 10th day, the animals were euthanized, and their brain tissues were removed.

Rats in the ischemia and rifampicin group (Group 1) were fasted for 24 hours prior to the surgery, and access to water only was permitted during this period of time. For the induction of anesthesia, 10 mg/kg xylazine hydrochloride (Rompun®; Bayer Healthcare) was administered intraperitoneally, together with 30 mg/kg ketamine hydrochloride (Ketalar®; Pfizer). The anesthetized rats were placed in a supine position, and the area to be treated was shaved and rubbed with 10% povidone-iodine solution (Batticon®, Adeka). A midline skin incision was made, and a retractor was attached. Bilateral paratracheal areas were dissected with blunt dissection to expose the common carotid artery. Once the N. vagus was dissected from the bilateral common carotid arteries, a Yaşargil aneurysm clip was attached to each common carotid artery (Figure 1). Yaşargil aneurysm clips were then attached to the bilateral common carotid arteries for 30 minutes. During this time, approximately 3 mL (10 mL/kg) of intracardiac blood was collected, and hypotension was induced. Arterial flow was inspected once the clips were removed at the end of 30 minutes, and the layers were sutured appropriately. Intermittent neurological examinations were then performed (at 1, 4, 7, and 10 days). Rifampicin was prepared at a dose of 30 mg/kg for ten days and injected intraperitoneally. After 10 days, 4 mL of intracardiac blood was collected from the remaining ten rats, which were then euthanized, and their brains were removed. The hippocampus was separated from the brain tissue for biochemical analysis and stored at -76°C in a deep freezer.

The rats in the ischemic group were subjected only to bilateral common carotid artery clipping for 30 minutes

Figure 1. Clipping of the bilateral A. carotis communis using Yaşargil aneurysm clips is shown

without rifampicin injection. During this time, 3 mL (10 mL/kg) blood was collected, and hypotension was induced. The arterial flow was inspected after the removal of the clip at the end of 30 minutes. The presence of the arterial flow was confirmed, and the folds were closed appropriately. Neurological examinations were performed on the postoperative days 1, 4, 7, and 10. Four cubic centimeters of intracardiac blood were collected at the end of the 10th day, and the animals were euthanized. The hippocampus was then removed from the brain tissue for biochemical analysis and stored at -76°C in a deep freezer.

The rats in the sham group were fasted for 24 hours prior to the surgery, and access to water only was permitted during this period of time. To establish anesthesia, we administered 10 mg/kg xylazine hydrochloride (Rompun®; Bayer Healthcare) and 30 mg/kg ketamine hydrochloride (Ketalar®; Pfizer) intraperitoneally. With the anesthetized rats in a supine position, the area to be treated was shaved and rubbed with 10% povidone-iodine solution (Batticon®). A midline skin incision was made and then sutured. Neurological examinations were performed at the end of days 1, 4, 7, and 10. Then, after ten days, 4 mL of intracardiac blood was collected, and the animals were euthanized. The hippocampus was removed from the brain tissue for biochemical analysis and stored at -76°C in a deep freezer.

To induce anesthesia in the pure control group, we administered 10 mg/kg of xylazine hydrochloride (Rompun®; Bayer Healthcare) and 30 mg/kg of ketamine hydrochloride (Ketalar®; Pfizer) intraperitoneally. Neurological examinations were performed at the end of days 1, 4, 7, and 10. After 10 days, 4 mL of intracardiac blood was collected, and the animals were euthanized. The hippocampus was removed from the brain for biochemical analysis and stored at -76°C in a deep freezer.

Ischemic changes in the brain were assessed biochemically by measuring the malondialdehyde (MDA) levels in both blood and brain tissue. A biochemist who was blind to the groups completed these measurements.

3.2. Determination of MDA Levels

3.2.1. Determination of MDA Levels in Tissue Samples

MDA was measured by modifying the method that Uchiyama and Mihara described (18). This method relies on the measurement of the absorbance at 532 nm of a colored molecule that MDA forms with thiobarbituric acid (TBA) in an acidic medium.

Preparation of Samples: Samples approximately 50 mg in size were prepared from each of the tissue specimens. These tissues were then homogenized in a homogenizer (Jane and Kunkel, Germany) at 9500 rpm (4 × 10 seconds, 40°C) in 2 mL phosphate-buffered saline (PBS). Next, the homogenates were centrifuged at 1500 g for ten minutes. The resulting supernatants were diluted 1:10 with PBS, and the MDA levels were measured.

3.2.1.1. Tissue MDA Measurement

1- A total of 500 μL homogenate was prepared by adding 3 mL 1% phosphoric acid.

2- The mixture was combined with 1 mL 0.672% TBA and stirred for 60 minutes in boiling water.

3- The tubes were allowed to cool and were then centrifuged at 1500 g for ten minutes.

4- After centrifugation, 200 μL supernatant was added to 96-well plates, and absorbance was read at 532 nm in a microplate reader spectrophotometer (Versamax, Molecular Devices, California, USA). The standard absorbance results were plotted against concentrations, and an MDA standard chart was drawn. With this chart, the amount of tissue MDA was calculated as nmol MDA/g wet tissue (Figure 2).

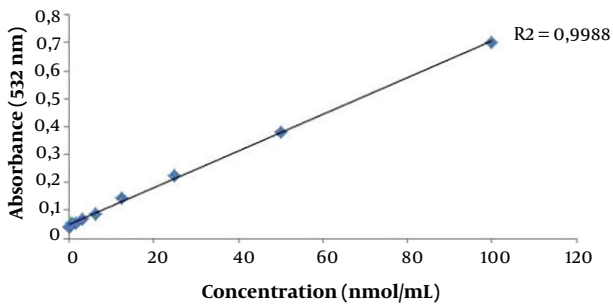

Figure 2. Standard chart used for tissue MDA measurement is indicated

3.2.1.2. Determination of MDA Levels in Serum Samples

Serum samples were maintained at -80°C until biochemical measurements were performed. The amount of MDA in the serum samples was determined using the thiobarbituric acid reactive substance (TBARS) method (19). The red color of the reaction product between the lipid peroxidation product (MDA) and TBA was measured using spectrophotometry. To separate the water-soluble substances that react with TBA and elicit the same color, we precipitated the serum lipids with the phosphotungstic acid/sulfuric acid system together with the protein.

3.2.2. Serum MDA Measurement

1- In brief, 150 μL serum, 1200 μL sulfuric acid, and 150 μL phosphotungstic acid were added to test tubes. These were thoroughly mixed and then allowed to stand at room temperature for 5 minutes.

2- The supernatants were removed after the mixtures were centrifuged at 1500 g for ten minutes.

3- The remaining precipitate fractions were vortexed, after which 2 mL distilled water was added, and the precipitates were re-dissolved.

4- Next, 500 μL TBA was added to the tubes and incubated at 100°C for one hour.

5- Following the incubation, the tubes were centrifuged at 1500 g for ten minutes.

6- Subsequently, 200 μL supernatant was collected and loaded into 96-well plates. The absorbance was read at 532 nm in a microplate reader spectrophotometer (Versamax, Molecular Devices, California, USA). The standard absorbance results were plotted against the concentration to prepare an MDA standard chart. With this chart, the amount of serum MDA was calculated as nmol/mL (Figure 3).

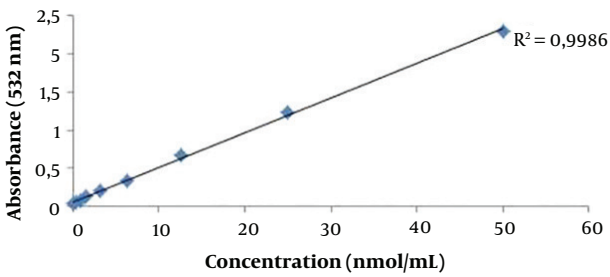

Figure 3. Standard chart used for serum MDA measurement is indicated

3.3. Neurological Examination

Motor examination scoring was performed using the Bederson's scoring system, as Bederson et al. described (20) (Table 1). Twenty-four hours following the global ischemia and recovery from anesthesia, the rats were placed on a flat surface and lifted by their tails. Following a successful ischemic occlusion, rats exhibited specific features, such as twisting and the retraction of the forepaw. Neurological deficits were determined using Bederson's scoring system (20, 21) as follows:

0 = No observable deficit

1 = Forelimb flexion

2 = Decreased resistance to lateral push

3 = Unidirectional circling

Table 1. Bederson's Scores Based on the Neurological Examination Scale

Grade	Status	Score
0	No neurological deficit	5
1	Flexion on front legs	4
2	Reduced resistance to lateral pushing motion without rotational motion	3
3	Addition of rotational motion in addition to Grade 2	2
4	Exitus	1

Motor examination scoring was repeated on days 4, 7, and 10 for each group. Motor examination scores were then calculated on days 1, 4, 7, and 10 and are shown in Table 2.

Table 2. Comparison of the Mean Serum MDA Values Among the Groups

Compared Groups		P Values[a]
Ischemia	Ischemia + Rifampicin	0.030
Ischemia + Rifampicin	Sham	0.588
Sham	Pure Control	0.435
Ischemia	Sham	0.019
Ischemia	Pure Control	0.035
Sham	Pure Control	1.000

[a] $P < 0.05$ was considered significant.

3.4. Statistical Analysis

A statistical software package was used for data analysis. Descriptive statistics are given as numbers and percentages for categorical variables, mean plus standard deviation, and minimum and maximum values for numerical variables. Next, the Shapiro-Wilk test was used to assess the normality of distribution. Analysis of variance (ANOVA) was used for comparisons between groups for normally distributed parameters, and the Kruskal-Wallis test was used for non-parametric tests. If the Kruskal-Wallis

test were significant, the Bonferroni-corrected Mann-Whitney U test was used for binary comparisons. P values less than 0.05 were considered statistically significant.

One-way ANOVA was identified as the hypothesis test, which was appropriate for this study due to the continuous numerical variables being employed. The effect sizes obtained from the previous literature were evaluated in the calculation of the requisite sample size, and the large effect size that Cohen described (22), f = 0.57, was used in the power analysis. Accordingly, a minimum sample size of 40 rats with 10 rats in each group was planned to provide 80% test power at a 95% confidence interval and at an effect size of f = 0.57 for one-way ANOVA (partial $\eta^2 \cong 0.25$; in other words, under the assumption that at least 25% of the total variation could be explained with the variable in question). However, considering the probability of a higher number of deaths in rats in Groups 1 and 2, the numbers in these groups were kept high. Therefore, the total sample size was 38, and the actual power was determined to be 0.8386755.

4. Results

The mean tissue MDA value of the rats in the study (n: 35) was 399.3 ± 127.3, whereas the mean serum MDA value was 0.44 ± 0.23. In the ischemia and reperfusion group, the mean tissue MDA value was 370.8 ± 125.6, and the mean serum MDA value was 0.41 ± 0.20. In the ischemia group, the mean tissue MDA value was 471.0 ± 114.6, and the mean serum MDA value was 0.6 ± 0.22. While in the skin laceration group, the mean tissue MDA value was 350.6 ± 100.6, and the mean serum MDA value was 0.32 ± 0.20. In the anesthesia group, the mean tissue MDA was 358.8 ± 140.8, and the mean serum MDA was 0.37 ± 0.21. No statistically significant difference was determined between the groups in terms of the mean tissue MDA values (P = 0.112). However, the mean serum MDA values differed significantly (P = 0.033). The comparison of the mean serum MDA values is shown in Table 2.

Serum MDA values were significantly different between the ischemia and rifampicin group, and the ischemia group (P = 0.030). No significant difference was observed in the serum MDA values between the ischemia and rifampicin group, and the skin laceration group (P = 0.58). In addition, no significant difference was determined in the serum MDA levels between the ischemia and rifampicin group, and the anesthesia group (P = 0.43). The serum MDA values significantly differed between the ischemic and skin laceration groups (P = 0.019), as well as between the ischemic and anesthesia groups (P = 0.035). Whereas, no significant difference was observed between the skin laceration and anesthesia groups (P = 1.00).

Moreover, significant differences were observed in the Bederson motor scores on all days in all groups (Table 3 and Figure 4). However, no significant differences were determined between the groups in the two-way analysis.

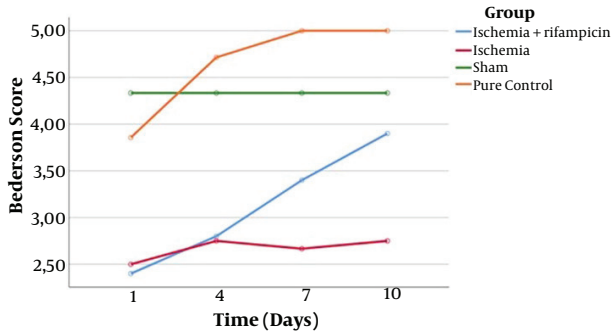

Figure 4. Plot graph of the groups and the times corresponding to their Bederson's scores are shown

5. Discussion

Bilateral common carotid artery ligation and middle cerebral artery ligation have frequently been used in previous experimental models to investigate the pathophysiology of cerebral ischemia-reperfusion injury and to develop therapeutic options. Hypoxic environments were created in this context, and the response of the resulting ischemic tissue was investigated. These previous studies evaluated the immunohistochemical responses to the ischemia-reperfusion of trigger factors, such as cellular response, neurotransmitter release, ion gradient disorders, and cytokines (23, 24).

During the stroke, brain tissue damage occurs as a result of numerous cascade interactions, including acidotoxicity, excitotoxicity, ionic imbalance, oxidative and nitrate stress, infarction, and apoptosis (25, 26). Numerous agents, including glutamate receptor antagonists, membrane stabilizers, calcium channel blockers, free radical scavengers, anti-inflammatories, antiemetics, and antiaggregant agents, are used to prevent the damage that occurs during ischemia-reperfusion and to ameliorate it subsequently (26, 27).

Experimental studies have involved numerous animal models. The most widely recognized among these experimental models is the two-vessel occlusion and hypotension model in rats. Hypotension at 50 mmHg is induced by transient occlusion and the simultaneous withdrawal of blood from the bilateral main carotid arteries (28, 29). We also applied the bilateral carotid artery clipping and hypotension model in our study to create

Table 3. Mean, Median, Standard Deviation (SD), and Interquartile Ranges (IQRs) of Bederson's Score Values on Days 1, 4, 7, 10

Groups	1st Day				4th Day				7th Day				10th Day			
	Mean	Med.	SD	IQR	Mean	Med.	SD	IQR	Mean	Med.	SD	IQR	Mean	Med.	SD	IQR
Ischemia + Rifampicin	2.4	2	1.26	1.5	2.8	3	1.23	1.5	3.4	4	1.43	1.75	3.9	4.5	1.6	1.75
Ischemia	2.5	2	1	0.75	2.75	2.5	0.96	1	2.67	2	0.98	1	2.75	2.5	0.97	1
Sham	4.33	5	1.63	1	4.33	5	1.63	1	4.33	5	1.63	1	4.33	5	1.63	1
Pure Control	3.86	4	0.69	1	4.71	5	0.49	1	5	5	0	0	5	5	0	0
P Values[a]	0.009				0.003				0.001				0.003			

[a] $P < 0.05$ was considered significant.

ischemia-reperfusion injuries in the brain, together with rifampicin, the neuroprotective effects of which have been demonstrated in previous studies.

The early emergence of excitotoxicity in consecutive cross-linked pathways, which occurs rapidly in ischemia, stems from cellular and other ions entering the cell, cellular edema, the activation of the intracellular kinase and protease enzymes, the excessive production of reactive nitrosative and oxidative products, cell membrane damage, and organelle malfunction. This leads to a slowly developing inflammatory response commencing in the following hours. This, in turn, results in the upregulation of intracellular adhesion molecule-1 (ICAM-1) and endothelium adhesion molecules, which induce P-selectin and chemokines to infiltrate macrophages and neutrophils in the tissue. Increased calcium levels in the cytoplasm inhibit the electron transport chain at the mitochondrial level and enhance the formation of superoxide anion, as well as the nitric oxide synthesis that supports the formation of peroxynitrite (30-32). In addition, endothelin release, leukocyte, and platelet activation, delayed coagulation, and endothelial dysfunction are involved in pathophysiological changes (33, 34). The ischemia results in astrocyte function impairment, an increased glutamate release, and disruption of glutamate uptake in astrocytes (35-38). Mechanisms such as the lipid peroxidation of calcium, the activation of proteolytic enzymes, free radical production, and gene activation result in an increase in irreversible neuronal damage (33, 39).

In addition to these effects, calcium also affects the smooth muscle in damaged veins following the ischemia. This results in contractile vessels and eventually vasospasm. Similarly, it initiates the metabolism of arachidonic acid, which contributes to free radical production, and other products of the cyclooxygenase pathway contribute to the production of free radicals. Phospholipids, glycolipids, glycerides, and sterols in the plasma membrane, transmembrane proteins containing amino acids, which can be oxidized, mannitol, glucose, and deoxy-sugars are susceptible to damage by free radicals. The most important reaction is the initiation of lipid peroxidation, as hydroxyl radical (OH^-) reacts with the membrane lipids. The oxidative degradation of polyunsaturated lipids is known as lipid peroxidation (40-42). This destruction continues as a chain-extending reaction. Lipid peroxidation, that potentiate all free radical sources and potentially mediate the transient metals, such as Fe and Cu in the environment, is a reaction that occurs in the plasma membrane and intracellular organelles. This reaction continues until the newly formed chemical free radicals are depleted. Lipid peroxidation is the most important cause of reperfusion injury. Increased free radicals initiate lipid peroxidation and are seen in myelin through peroxidation, neuronal cell, plasma, organelle membranes, and vascular endothelial cell membranes. The effect of rifampicin on ischemia-reperfusion injury has been shown to impact levels of MDA, a product of lipid peroxidation, in the blood and ischemic tissues. Glucocorticoid receptor activation has also been reported along with the neuroprotective effect of rifampicin (9-11). Similar to the mechanism of the action of glucocorticosteroid in ischemic injury and post-reperfusion injury in focal cerebral ischemia, in vitro studies have shown that rifampicin also acts in a comparable manner by activating glucocorticosteroid receptors.

Reactive oxygen species (ROS) are one of the main causes of post-ischemic neuron death. The level of MDA in tissue is used as a marker of ROS-related cell death.

In our study, MDA levels in the hippocampus tissue increased significantly due to ischemia compared with the anesthesia and sham groups. Owing to these effects, rifampicin activates glucocorticosteroid receptors and reduces the levels of MDA used to measure ischemia-reperfusion injury. We, therefore, measured the brain tissue and serum MDA levels in this experimental study. The MDA levels in the sham and anesthesia groups were very close to each other, whereas the mean MDA level in the ischemia group increased significantly. In the ischemia and rifampicin group, the MDA levels in the brain tissue and serum decreased compared with the ischemia group. This result was indicative of an increase in tissue MDA levels after the ischemia. No statistically significant difference was

found in terms of tissue MDA values among all groups (P = 0.112). However, serum MDA values significantly differed among all groups (P = 0.033). A significant difference was also observed in serum MDA values between the ischemia and rifampicin group, and the ischemic group (P = 0.030). The serum MDA values were not significantly different between the ischemia and rifampicin group, and the sham group (P = 0.58). They also were not significantly different between the ischemia and rifampicin group, and the anesthesia group (P = 0.43). In addition, no difference was determined between the serum MDA values of the ischemic and skin laceration groups (P = 0.019). However, a significant difference was observed between the ischemic and anesthesia groups (p = 0.035). The serum MDA values did not significantly differ between the sham and anesthesia groups (P = 1.00).

In our study, statistically significant results were also obtained for the groups' Bederson's neurological examination results for motor scores on days 1, 4, 7, and 10. These variations stemmed from differences between the anesthesia group and the ischemia and rifampicin group, the sham group and the ischemia and rifampicin group, the sham and ischemia groups, and the ischemia and anesthesia groups. This is explained by the presence of a sufficient neurological deficit in all groups in which ischemia-reperfusion injury was performed. Motor scores in the rifampicin group approaching those of the sham group were interpreted as a sign of a better neurological outcome than in the ischemia-treated group. Additionally, neurological scoring in the ischemia group and in the ischemia and rifampicin group, as well as the biochemically intergroup serum and the tissue MDA values suggested that rifampicin may be of therapeutic importance in ischemia-reperfusion injury.

5.1. Conclusions

The effects of rifampicin on experimental brain ischemia and reperfusion injury in rats, bilateral carotid artery clipping, and intracardiac blood collection in our study resulted in cerebral ischemia-reperfusion injury. Our analysis revealed varying degrees of neurological damage in the various groups. The efficacy of rifampicin in the prevention of cerebral ischemia-reperfusion injury was statistically significant in terms of the findings determined in the other groups. In conclusion, the healing that occurred with rifampicin used in appropriate doses and durations for preventing cerebral ischemia-reperfusion injury, could stem from its neuroprotective effect.

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Footnotes

Authors' Contribution: Erhan Arslan: performing the experiments and writing the manuscript. Mehmet Selim Gel: analysis of data and designing the study. Selim Demir: analysis of data. Elif Acar Arslan: designing the study and writing the manuscript. Müge Koşucu: writing the manuscript. Süleyman Caner Karahan: analysis of the data and conducting the study.

Conflict of Interests: We declare that we have no conflict of interests.

Ethical Approval: All experimental protocols received approval from the Karadeniz Technical University Experimental Animal Ethics Committee (approval number: 2016/12, date: March 29, 2016), Trabzon, Turkey.

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