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Protective Evidence of Clarithromycin after Ischemic Cerebral Injury; an Experimental Study

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Abstract

Objective: Acute ischemic stroke is caused by a reduction in cerebral blood flow, leading to brain ischemic and subsequent cell death. The therapeutic options available for this condition are limited. The inflammatory response associated with the ischemic injury may influence the outcomes of ischemic stroke. Clarithromycin is a widely used antibiotic in medical practice. This study aimed to investigate the effect of clarithromycin on brain ischemic injury.

Methods: In this study, 38 Sprague Dawley female rats were used and divided into four main groups: the pure control group, the ischemia group, the sham/control group, and the ischemic+claritromycin group. A temporary clip was placed in the bilateral carotid arteries of rats for 45 minutes. One group administered a dose of clarithromycin, and the tissue and blood samples of all four groups underwent biochemical evaluation. The results were subjected to statistical analysis.

Results: The administration of clarithromycin to animals resulted in a reduction in malondialdehyde levels in brain tissues within the study group. Furthermore, Bederson's motor scores were observed to be higher in the clarithromycin-treated group in comparison to the ischemia group (p=0.092).

Conclusion: A potential correlation exists between post-stroke infections and prognosis, suggesting that prophylactic antibiotic treatment may be beneficial. This study indicates that clarithromycin exerts a neuroprotective effect on cerebral ischemic injury following a stroke.

Keyword: Cerebral injury, clarithromycin, ischemic, neuroprotection

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INTRODUCTION

It has been extensively studied that hypoxia-ischemic can cause insufficient blood flow to the brain's tissues, which can cause severe organ damage and neuronal cell apoptosis. Ischemic stroke occurs due to the reduction of cerebral blood flow. Cerebral ischemic/hypoxic damage is a serious medical disorder that can arise from several different illnesses. It is one of the leading causes of worldwide disability and death. Ischemic cerebral injury results from a complicated web of molecular and metabolic processes, but its pathophysiology is still unclear. Knowing the mechanisms underlying white matter damage following a stroke is essential. Neuroprotection may stop the death of neurons. In the last decades, understanding of the pathophysiological events in cerebrovascular diseases has improved (1), but the fatality rate from ischemic stroke is still high. It is therefore imperative to develop novel therapy approaches that can improve functional recovery and lower morbidity (2). The goal of ischemic stroke therapy is to restore perfusion to the brain (3). Investigating appropriate therapeutic approaches for ischemic brain damage is an important issue. Researchers have been trying to find the best therapeutic agent for ischemic injury following stroke. Neuroinflammation is a key aspect of stroke. The inflammation in the ischemic injury may affect determining the outcomes of ischemic stroke, because it may cause a neurological deficit or there may be a link between the occurrence of post-stroke infections and outcomes. It is believed that neuroinflammation, which is a secondary neurological impairment, is the primary cause of cerebral ischemic injury. Increasing levels of cytokines in the central nervous system and systemic circulation mediate inflammatory responses to acute ischemic injury. Antibiotics may have a role in the management of stroke. They have been often used in medical practice. Macrolide antibiotics like clarithromycin have both antibacterial and anti-inflammatory properties, and this antibiotic may have a role in stroke-related ischemic/reperfusion injury. Using antibiotics as a preventative measure may be helpful. Antibiotic drugs may be a reasonable choice in this situation. Macrolides represent the preferred class of antibiotics, and as such, they are of significant clinical interest due to their applicability to human medicine. Currently, major changes in medical practice have been observed, however, despite the increased use of technology, our understanding of the histopathological mechanisms of the effect of clarithromycin on human stroke is still limited. When an ischemic stroke occurs, proinflammatory chemokines to activate leukocytes are released by brain tissues, and inflammation occurs. Antibiotics following a stroke may be a useful choice. Clarithromycin can reduce inflammatory processes in the brain and may have a neuroprotective effect (4).

Many treatments for ischemic cerebral strokes are vessel and blood-based, but brain-based therapies should be evolved. Many stroke studies have focused on antiplatelet drugs such as aspirin, clopidogrel, dipyridamole, ticlopidine as well as warfarin. There are a limited number of studies focused on the effect of clarithromycin. Brambrink et al. showed that group of macrolides (Erythromycin, clarithromycin, azithromycin, spiramycin etc) increases hypoxia tolerance (5). In this study, the neuroprotective effect of clarithromycin on focal brain ischemic and reperfusion injury was investigated.

METHODS

The study was conducted in the Experimental Research Center Laboratory of Karadeniz Technical University, Faculty of Medicine. All experimental protocols were approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University (with the decision of the Animal Experiments Local Ethics Committee of Karadeniz Technical University numbered 2016/55). The study was conducted under the ethical guidelines outlined in the 1964 Declaration of Helsinki and any subsequent changes, as well as any other relevant ethical guidelines. The biochemical analyses were carried out in the Research Laboratory of the Department of Biochemistry. In the study, 38 Sprague Dawley female rats, each weighing between 220 and 280 gr, were used. The general health of the rats was examined before the study and they were monitored under standard conditions and in individual cages without water and feed restrictions, and each rat was marked with the appropriate method according to the group in which it was included. The rats were divided into four groups.

Description of groups

Group 1 (Ischemic+Claritromycin)

Clarithromycin 100 mg/kg/day was intraperitoneally given to this group consisting of 12 rats every day after clipping of the bilateral carotid arteries for 30 min + hypotension induction (10 ml/kg). They underwent a neurological examination at the end of postoperative day 1, day 4, day 7, and day 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

Group 2 (Ischemic)

Clipping of the bilateral carotid arteries for 30 min + hypotension induction (10ml/kg) was performed in this group consisting of 12 rats. They underwent a neurological examination at the end of postoperative day 1, day 4, day 7, and day 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

Group 3 (Sham/Control)

The rats in this group were only anaesthetised, and neurological examinations were performed on day 1, day 4, day 7 and at the end of day 10.

At the conclusion of the 10th day, the rats were euthanised and the brain tissue was extracted.

Group 4 (Pure Control Group "Only Anesthesia and Drug")

Following the administration of anaesthesia, a single skin incision was made in this group of seven rats, and clarithromycin was administered intraperitoneally for a period of 10 days. They underwent a neurological examination at the end of day 1, day 4, day 7, and day 10. At the end of day 10, they were sacrificed, and the brain tissue was removed.

In this study, the experimental procedure of study Arslan et al (6), was used. The rats were individually numbered and placed in cages where they could easily access food and water. The anaesthetic agent was administered intraperitoneally at a dose of 10 mg/kg xylazine hydrochloride (Rompun®; Bayer Healthcare) and 30 mg/kg ketamine hydrochloride (Ketalar®; Pfizer). In the supine position, the surgical site of rats was shaved and stained with 10% povidone-iodine solution (Batticon®; Adeka). A midline skin incision was done, and a retractor was placed. After cervical midline blunt dissection, the common carotid arteries were found, and the arteriae carotid communis were dissected from the N. vagus. Then this artery was clipped by a Yasargil aneurysm clip and waited for 30 minutes. After 30 minutes, 3ccs intracardiac blood was taken and hypotension was induced. After removing the clip at the end of 30 minutes, the arterial flow was checked, the clamps were removed, and the layers were properly sutured. A neurological examination of the rats was performed at intervals (on days 1, 4, 7, and 10). The starting solution (Clarithromycin (Klacid® IV 500mg injectable vial) was prepared by adding 10ml sterile saline to a 500mg vial and administered intraperitoneally at the doses indicated. Clarithromycin 100 mg/kg/day was injected intraperitoneally for 10 days. Ten days later, 4 ccs of intracardiac blood were taken from the surviving rats and the rats were sacrificed and The hippocampus, c. striatum, corpus callosum and thalamus parts of the brain were dissected and stored in deep-freezer at -76^{o}C for histological and biochemical analysis. The rats in the SHAM group underwent a neurological examination at the end of day 1, day 4, day 7, and day 10. At the end of day 10, 4 ccs of intracardiac blood were taken into citrated tubes. Biochemical changes were evaluated by measuring the levels of malonaldehyde (MDA) in both blood and tissue.

Malondialdeyde Determination

In MDA measurement, the absorbance at 532 nm of the color of the component that the MDA produces with thiobarbituric acid (TBA) in an acidic state is measured. The preparation of solutions used in tissue and serum MDA measurements was made. Figure 1 shows the MDA standard chart. Using this chart, the tissue MDA level was calculated as nmol MDA/gram

wet tissue. The standard absorbance values are seen in Figure 2.

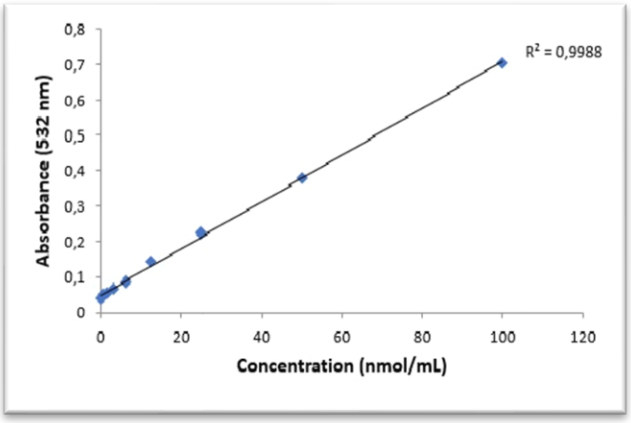

Figure 1: Shows the MDA standard chart.

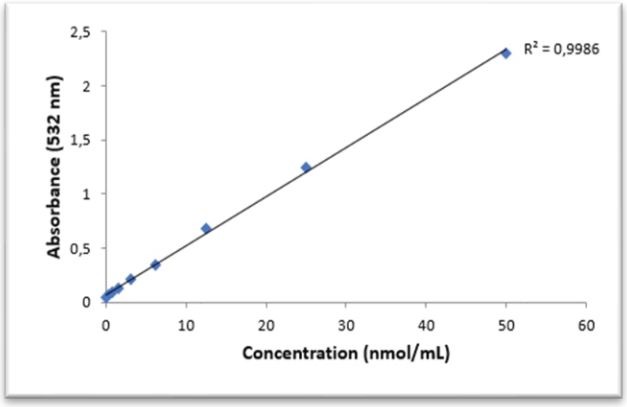

Figure 2: The standard absorbance values are seen.

Preparation of Solutions Used in Tissue MDA Measurement

1) Tissue homogenization buffer (0.01 M Phosphate Buffer Solution (PBS), pH: 7.4) Ten PBS tablets (Medicano, Uppsala, Sweden) were dissolved in a beaker containing approximately 900 mL of distilled water, then the pH of the solution was adjusted to 7.4 at a pH meter (Hanna Instrument, USA). The pH of the adjusted solution was brought to a final volume of 1 L.

2) 1% H_3POI_4 solution 2.94 mL of 85% H_3PO_4 (Sigma, St. Louis, MO, USA) was added to the distilled water and the volume was adjusted to 250 mL with the distilled water.

3) TBA solution: 0.67 g TBA (Sigma, St. was weighed, 50 mL of distilled water and 50 mL of acetic acid (Sigma, St. Louis, MO, USA) was added and dissolved by mixing with a magnetic bar.

4) Standard solutions: 82.5 µL of 1,1,3,3-tetrametoxypropan (Sigma, St. Louis, MO, USA) was added to 50 ml of 0.1 M HCL (Sigma, St. Louis, MO, USA) solution. The solution was incubated at 50°C for 1 hour. Thus, by diluting the main stock solution prepared at certain ratios, the standard solutions of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.195 nmol/mL along with blind were prepared.

Preparation of Samples: Approximately 50 mg slices were cut from all of the tissues. These tissues were then homogenized in 2 mL of PBS at 9500 rpm (4x10s, 40 °C) with a homogenizer (Jane and Kunkel, Germany). The homogenates were centrifuged at 4000 rpm for 10 minutes. After the supernatants obtained after centrifugation were diluted at a ratio of 1:10 with PBS, MDA values were measured.

Measurement of Tissue MDA

1. 3 mL 1% H_3POI_4 was added to 500 µL of homogenate and a mixture was prepared.

2. 1 mL of 0.672% TBA was added to the mixture and mixed, then incubated in boiling water for 60 minutes.

3. At the end of the period, the tubes were placed at room temperature to cool down and centrifuged at 4000 rpm for 10 minutes at room temperature.

4. After the centrifugation, 200 µL was taken from the supernatant part and filled into 96-well plates, absorbances were read in a microplate reader spectrophotometer (Versamax, Molecular Devices, California, USA) at 532 nm wavelength. The standard absorbance results obtained were plotted against concentration and the MDA standard chart was created. (Figüre 4) Using this chart, tissue MDA level was calculated as nmol MDA/gram wet tissue.

Determination of malondialdehyde in plasma

The rat serum samples were kept at -80°C until biochemical measurements. The amount of malondialdehyde in the serum samples was calculated with the TBARS (Tiobarbituric Acid Reactive Substance) method developed by Yagi (90). The red color of the reaction between the lipid peroxidation product (MDA) and thiobarbituric acid (TBA) was measured spectrophotometrically. In order to separate the water-soluble components that react with thiobarbituric acid and give the same color, serum lipids were separated together with the protein in the phosphotungstic acid/sulfuric acid assembly.

Preparation of Solutions Used for Measurement of Tissue MDA

1) 0.084 N Sulfuric Acid (H_2SOA_4)

577 µL of 97% H_2SOA_4 (Sigma, St. Louis, MO, USA) was taken and the volume brought to 250 mL with deionized water.

2) 10% Phosphotungstic Acid ($H_3(W_3O_{10}).4H_2O$)

5.55 g of phosphotungstic acid (Sigma, St. (Louis, MO, USA) was dissolved in 50 mL of deionized water.

3)Thiobarbituric acid (TBA) solution

0.67 g of TBA (Sigma, St. was weighed, 50 mL of distilled water and 50 mL of acetic acid (Sigma, St. Louis, MO, USA) was added and dissolved by mixing with a magnetic bar.

4) Standard solutions

82.5 µL of 1,1,3,3-tetrametoxypropan (Sigma, St. Louis, MO, USA) was added to 50 ml of 0.1 M HCL (Sigma, St. Louis, MO, USA) solution. The solution was incubated at 50°C for 1 hour. By diluting the main stock solution prepared at certain ratios, the standard solutions of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 and 0.195 nmol/mL and blind were prepared.

Measurement of Serum MDA

1. 150 µL of serum, 1200 µL of H_2SOA_4 and 150 µL of phosphotungstic acid were added to test tubes, and the tubes were kept at room temperature for 5 minutes after thoroughly mixed.

2. The mixtures were centrifuged at 1500 g for 10 minutes and the supernatants were discarded.

3. 2 mL of distilled water was added to the remaining precipitate and vortexed until redissolved.

4. 500 µL of TBA was added to the tubes and they were incubated at 100°C for 1 hour.

5. After the incubation, the tubes were centrifuged at 1000 g for 10 minutes.

6. Taking 200 µL from the transparent area above, it was filled in 96-well plates and absorbances were read in a microplate reader spectrophotometer (Versamax, Molecular Devices, California, USA) at 532 nm wavelength. The resulting standard absorbance values were plotted against concentration and the MDA standard chart was created (Figüre 5). Using this chart, serum MDA amount was calculated as nmol/mL.

Motor Examination

The motor scores of the rats in each group were recorded on the 1st, 4th, 7th, and 10th days according to the Bederson scale.

Bederson motor assessment

Grade 0: no neurological deficit 5 points,
Grade 1: Stretching the front legs 4 points,
Grade 2: Reduced resistance to lateral thrust without rolling movement 3 points,
Grade 3: addition of rotational movement in addition to grade 2, 2 points
Grade 4: Exitus receives 1 point.(7).

Statistical Analyses

Using the IBM SPSS software package, version 22, all statistical analyses were conducted. The Mann-Whitney U test was used to determine statistical differences and significance levels in all measurements, and results with a p-value of 0.05 or lower were regarded as significant. Multiple comparisons were made using Duncan's Multiple Range Test.

RESULTS

It was found that the brain tissue MDA level of the group 2 (ischemia) was significantly increased compared to the brain MDA levels of the group 3 (control) ($p<0.05$). The administration of clarithromycin significantly decreased the level of MDA when the group 2 (ischemia) and the group 1 (clarithromycin) were compared ($p<0.05$) (Figure 3). It was observed that the MDA level of the group 2 was significantly increased compared to the serum MDA level of the group 3 ($p<0.05$). Again, this result showed that significant ischemia was achieved. When the group 1 and group 2 values were compared, there was a significant decrease in the serum MDA level ($p <0.05$). (Figure 4)

No statistically significant difference in Bederson motor scores was found between the group 1 and groups 2 ($p>0.05$). (Table 1). A graphic abstract summarizes the result of the present study (Figure 5).

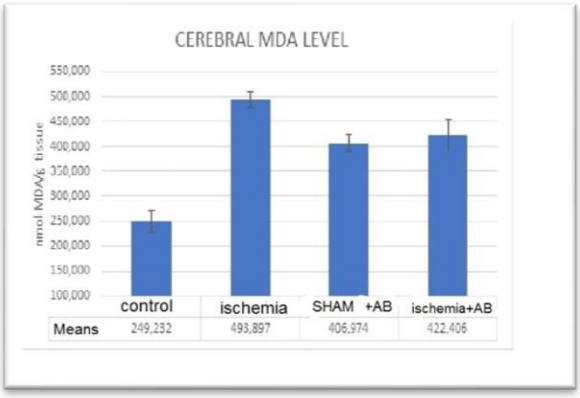

Figure 3. Group brain tissue MDA levels

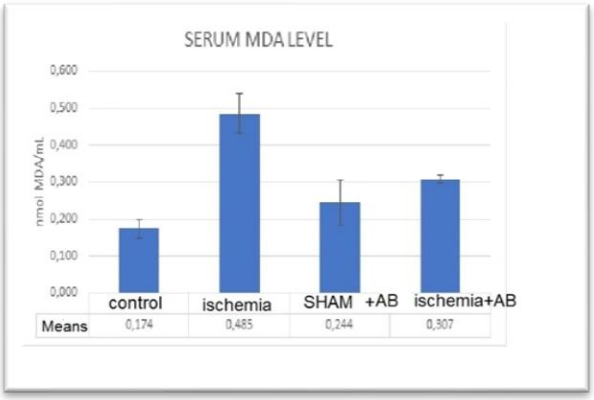

Figure 4. Serum MDA levels of the groups

Table 1: Means and minimum and maximum values of the Bederson scores of the groups on days 1, 4, 7 and 10.

Group	Day 1		Day 4		Day 7		Day 10	
	Mean	**Min-Max.**	**Mean.**	**Min-Max.**	**Mean**	**Min-Max.**	**Mean**	**Min-Max.**
Ischemia + Clarithromycin	2.25	1-5	2.75	1-5	2.83	1-5	3.25	1-5
Ischemia	2.16	2-5	2.25	2-5	2.33	1-5	2.41	1-5
Sham	4.85	4-5	5	5-5	5	5-5	5	5-5
Pure Control	4.14	3-5	4.71	4-5	4.85	4-5	5	5-5

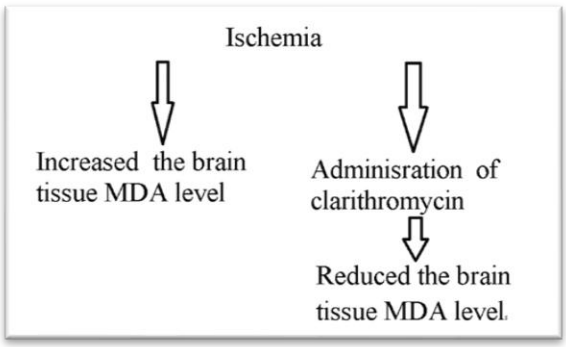

Figure 5: The results of the study are summarised below

DISCUSSION

This study indicates that clarithromycin has a neuroprotective effect on cerebral ischemic injury following a stroke.

Interpretation: In medical practice, having a thorough understanding of pathophysiology is crucial (8). An improved understanding of the pathophysiology of cerebrovascular disorders has been observed in the last decades (9). A continuous supply of oxygen is necessary for the proper functioning of brain cells (10). During a stroke, the blood flow decreases below the critical threshold in the cerebral tissue and

causes ischemic degenerative changes in the cerebral tissues. Ischemic neuronal damage after stroke remains a common cause of neurological disability and death. Reductions in infarct volumes can be associated with better neurological outcomes. Oxygen supplementation has been suggested to reduce secondary injury by minimizing damage to cerebral tissue in stroke because ischemic injury results in cell loss. There is a balance between cell death and cell proliferation in the nervous system. Restoring blood supply may lead to reperfusion injury, this reperfusion can paradoxically contribute to more brain damage. The intact blood-brain barrier (BBB) is required for maintaining the microenvironmental homeostasis of the CNS. BBB disruption following ischemia may lead to brain injury. Though contemporary healthcare has made significant advancements (11), post-stroke systemic inflammation can increase morbidity and mortality in stroke patients. Macrolide antibiotics have potent anti-inflammatory activities, likely by reducing pro-inflammatory cytokine production. During ischemia, structural and metabolic changes begin in the brain. Hypoxic-ischemic insult causes oxidative stress and the neuroinflammatory signaling cascade starts. Inflammation and oxidative stress have been linked to cerebral injury after stroke. This injury can be related to the activation of metalloproteases. Metalloprotease leads to the destruction of BBB. Systemic inflammation is known to change the permeability of the BBB (12), and BBB disruption following stroke promotes inflammation. Cerebral ischemia may lead to an inflammatory response that is believed to contribute to cell death. The fever sometimes increases following cerebral ischemia. The impairment and recovery of neurological functioning are impacted by post-stroke neuroinflammation. The occurrence of secondary neurologic deterioration in cerebral ischemia or cerebral tissue hypoxia may be linked to a pronounced increase in the inflammatory parameters inflammation, and apoptosis. Neutrophils have important functions in post-stroke pathogenesis. The anti-inflammatory actions of macrolide antibiotics are well-known. Inhibition of neuronal apoptosis by the inflammatory effect of clarithromycin may be a promising treatment strategy to improve neurological deficits following stroke. These changes can overly stimulate N-methyl-d-aspartate (NMDA) receptors, causing calcium overload and triggering apoptotic cell death in neurons. In our study, a significantly decreased level of MDA after the administration of clarithromycin may be explained by this mechanism. The blood-brain barrier is necessary for the normal function of the brain (10). Some antibiotics cannot pass between the blood-brain and blood-CSF barriers unless there is an infective process, such as meningitis (13).

Clarithromycin administration can prevent BBB disruption following ischemic stroke. In this study, the benefit of clarithromycin was shown in ischemic cerebral injury after stroke. Based on this property, we measured tissue and serum. In this study, the only outcome measures are malondialdehyde (MDA) levels in brain tissue and a crude assessment of behavioral outcome on the Bederson scale (7). MDA levels in this study found that the brain tissue MDA level of the ischemia group was significantly increased compared to the brain MDA levels of the control (sham) group ($p<0.05$), but the administration of clarithromycin significantly decreased the level of MDA in comparing the ischemia group and the clarithromycin group ($p<0.05$). It is an interesting part of this study, that functional recovery was not seen with no significant change in Bederson motor scores between groups ($p>0.05$). No statistically significant difference in Bederson motor scores was observed between the clarithromycin and ischemia groups ($p>0.05$).

The rationale for choosing MDA; why other markers were not selected:

Stroke is one of the leading causes of mortality and morbidity worldwide, for that reason, many studies have been conducted and many experimental models have been used for the pathology. In the early stages of an ischemic episode, oxidative stress and lipid peroxidation are significant factors. One form of secondary brain damage brought on by reperfusion following an ischemic stroke because of vascular obstruction is known as cerebral ischemia-reperfusion injury. Neuronal apoptosis, blood-brain barrier disruption, and malignant brain edema occur secondary to cerebral ischemia-reperfusion injury. There are reasons to concentrate only on experimental studies because the biomechanical, molecular, and cellular effects of diseases can only be investigated in experimental animal studies. The experimental studies may provide better evidence of the effect of clarithromycin in experimental brain ischemic injury than those of human subjects. malondialdehyde (MDA), is a biomarker of oxidative stress. The rationale behind the selection of MDA is attributable to its well-established role as a reliable indicator of oxidative stress (14). Rising MDA levels may be a sign of increased oxidative stress in patients with stroke. In this study, only the MDA level shows an effect of clarithromycin, while this agent does not show a significant improvement in stroke-induced behavioral deficits.

Limitation

Experimental studies have highlighted neuroprotective agents after ischemic strokes and neuroprotective drugs show efficacy in animal studies. Although the idea of neuroprotection has shown promise in experimental research, it cannot be successfully implemented in clinical settings. Although numerous neuroprotective substances have

demonstrated promise as potential therapies for ischemic stroke in both vitro and in vivo models of cerebral ischemia, patient outcomes have been inconsistent. Another limitation is the limited sample size of the study. The sample size may not be adequate. In studies, the calculation of sample size is an important issue (3). If a researcher selects a smaller number of animals, it may lead to missing significant differences even if they exist in the population. A good statistical analysis with more cases is required. If a greater number of animals are selected, then it may lead to unnecessary wastage of resources (3). Table 1 shows the mean values of all groups. Some changes in the SHAM group are also seen. The SHAM operation can sometimes lead to some changes in animals. In this study, the SHAM group also showed some improvement which may raise some concerns. We preferred to use malondialdehyde which may not be the best approach to measure "ischemia/reperfusion injury". Higher levels of MDA were associated with higher moralities; however, the infarct volume, edema volume, and cerebral blood flow (CBF) could have been measured and would be more clinically related. We are aware that mortality and other critical physiological parameters (cerebral blood flow, systemic blood pressure, and body temperature) are important factors in these kinds of studies but were not assessed in the present study.

CONCLUSION

Currently, there have been tremendous efforts made to manage patients with stroke. The disturbances in cerebral circulation are of particular importance after a stroke. This study provides novel insights into the effect of clarithromycin in reducing ischemic damage following a stroke. There may be a link between post-stroke infections and prognosis, making antibiotic treatment as a prophylactic measure advantageous, and this study indicates that clarithromycin has a neuroprotective effect on cerebral ischemic injury following a stroke. Restriction of oxidative stress in ischemic brain injury can be provided with substances such as clarithromycin that were found to be potentially beneficial in the present study. Clarithromycin may be adopted as a neuroprotective agent for the treatment of ischemic brain injury. More studies are needed.

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Ethics Committee Approval: Ethics approval for this study was obtained from the Karadeniz Tecnic University Animal Experimentation Ethics Committee (ethics committee date: 08/12/2016, ethics committee number: 53488718-667).

Peer-review: Externally peer-reviewed

Author Contributions: Concept: Design: Data Collection and Processing: Analysis and

Interpretation: Writing: ARG, MA, EA, AK, UY, MOB, AA.

Conflict of Interest: The author declared no conflict of interest.

Financial Disclosure: The authors declared that this study has not received no financial support.

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