The Axon Protective Effects of Syringic Acid on Ischemia/Reperfusion Injury in a Rat Sciatic Nerve Model

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ABSTRACT

AIM: In the relevant literature, there is no experimental study that investigated the axon protective effects of syringic acid- a polyphenol compound- with an anti-oxidant capacity on ischemia/reperfusion injury.

MATERIAL and METHODS: The rats were randomly divided into four groups: Control group (no medication or surgical procedure), Sham group, Syringic acid group, and Methyprednisolone (MP) Group. Ischemia was achieved by abdominal aorta clamping and all animals were sacrificed 24 hours after ischemia. Harvested sciatic nerve segments were investigated histopathologically and for tissue biochemistry.

RESULTS: Ischemic fiber degeneration scores were found significantly lower in syringic acid and MP groups than sham group. Additionally, apoptosis-related cysteine peptidase caspase-3 immunostaining scores were lower in syringic and MP groups. Biochemically, superoxide dismutase and nuclear respiratory factor 1 values were significantly higher in syringic acid group compared to those of control and sham groups while malondialdehyde levels were significantly lower in the syringic acid group.

CONCLUSION: Syringic acid reduces oxidative stress and axonal degeneration in rat sciatic nerve after ischemia/reperfusion injury. Therefore, syringic acid may play a role in the treatment of peripheral nerve injuries due to ischemia/reperfusion.

KEYWORDS: Axon, Sciatic nerve, Syringic acid, Methylprednisolone, Reperfusion, Malondialdehyde, Nuclear respiratory factor 1, Superoxide dismutase

INTRODUCTION

Ischemia is a reversible or irreversible condition due to hypoperfusion of systemic arterial blood to tissues and organs. Ischemia disrupts cellular oxidative phosphorylation process and this in turn causes adenosine triphosphate (ATP)-dependent ionic pump failure and hyperinflux of calcium, sodium, and water into cells (3, 8). Meanwhile, adenine nucleotide breakdown is accelerated resulting in intracellular accumulation of hypoxantine which is the precursor of reactive oxygen derivatives (ROD) (4). Additionally, ischemia increases the synthesis of some proinflammatory gene products (leucocyte adhesion molecule, cytokines, etc.) and bioactive compounds (endothelin-1, thromboxane A2, etc.) in endothelial cells while it suppresses the expression and synthesis of some cytoprotective genes and their enzyme products including nitric oxide (NO) and prostacyclins (5). At this critical stage, reperfusion- the restoration of blood flow following ischemia- must be accomplished in order to prevent irreversible tissue/organ damage. It has been proven that the temporary occlusion of aorta may cause reperfusion injury in the sciatic nerves (17).
We investigated the potential axon protective effects of syringic acid (SA)-a polyphenol compound, a derivative of benzoic acid- on sciatic nerve ischemia and reperfusion. We compared its axon-protective effects to those of methylprednisolone (MP) which was recently shown to be effective in axon protection in sciatic nerve injuries (27).

In this study, the potential beneficial effects of SA on rat sciatic nerves after ischemia/reperfusion were evaluated histopathologically and biochemically by measuring malondialdehyde (MDA), superoxide dismutase (SOD), nuclear respiratory factor-1 (NRF-1).

## MATERIAL and METHODS

### Animals

Prior to the start of the experiments, permission was obtained from Canakkale Onsekiz Mart University Animal Experiments Ethics Committee.

Twenty-four male Sprague–Dawley rats each weighing 300±25 grams and 8 to 12 weeks old were used in the experiment. All rats were fed with standard pellet rat food (Bil-Yem Ltd, Ankara-Turkey) and water ad libitum. An automatic photoperiod with white fluorescent light was used to create an environment with 12 hours light/12 hour darkness. The temperature was set to 21±2 °C and humidity was set to 55-60%. The methods used for animal experiments were organized in accordance with the protocols of the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### Experimental Design

Rats were randomly divided into four equal groups (consisting of six rats each):

- **Group 1**: Control group (n=6, no medication or surgical procedure)
- **Group 2**: Sham group (n=6, Single dose 1 ml 10% ethanol was administered intraperitoneally at 5th minutes following aorta occlusion. Rats were sacrificed at 24th hours after ischemia)
- **Group 3**: Syringic Acid Group-SA (n=6, Single dose 10 mg/kg, SA (Syringic Asid Sigma-Aldrich Interlab, Turkey) was administered intraperitoneally at 5th minutes following aorta occlusion. Rats were sacrificed at 24th hours after ischemia)
- **Group 4**: Methylprednisolone Group-MP (n=6, Single dose 30 mg/kg MP (Prednol, Mustafa Nevzat, Turkey) was administered intraperitoneally immediately following spinal cord ischemia (SCI).

### Dosage

The dosage was determined as 10 mg/kg body weight based on preliminary studies with various doses (50, 75, 100 mg) to reveal the biological effects of SA.

### Reagents

Syringic acid (≥98% HPLC) was obtained from Sigma-Aldrich (St Louis, MO, USA). MP was obtained from Mustafa Nevzat Drug Industry Inc. (Istanbul, Turkey). The drugs were dispersed with isotonic NaCl 0.9%. The protein concentrations were indicated by the Bradford method using Bradford reagent (Catalogue No. B6916-1KT) (Sigma Aldrich, St Louis, MO, USA). SOD Assay Kit (Catalogue No. 19160) was obtained from Sigma-Aldrich (St Louis, MO, USA). Lipid peroxidation (MDA) assay kit (Cat. No. MAK085) was obtained from Sigma-Aldrich (St.Louis, MO, USA). Rat Nuclear Respiratory Factor 1 ELISA Kit (Cat. No. CK-E90555) was obtained from Hangzhou Eastbiopharm Co. Ltd. (Hangzhou, China). We preferred ethanol in sham group instead of serum physiologic since syringic acid could be solved in ethanol.

### Surgical Procedure

Rats were administered premedication; intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg). Anesthesia was continued with ketamine injections at intervals without intubation or mechanical ventilation. Surgical approach was supine position. After the operating field was prepared in a sterile fashion, laparotomy was performed with a standard midline incision. After retracting the intestines laterally, the retroperitoneum was opened and the abdominal aorta reached. Aorta occlusion was induced by cross-clamping the aorta with mini aneurysm clip between just below the left renal artery and just proximal to the aortic bifurcation. Loss of aortic pulse was confirmed by palpation. The duration of ischemia was set at 45 minutes and later the cross clamps were removed and distal reperfusion was observed visually. At the end of the procedure, the abdominal wall was closed with 5/0 prolene sutures. Animals in control group underwent a surgical procedure similar to the other groups but the aorta was not clamped. Animals were fed a standard diet and water ad libitum in their cages after surgery. At 24th hour all animals were anesthetized with pentobarbital (20 mg/kg) and sacrificed. Sciatic nerves were removed full length via the gluteal approach in all animals. Half of the specimen taken for histopathological investigation and it was fixed in formalin for 7 days. The other half was stored in a freezer at -80 °C for biochemical estimations.

### Histopathological Evaluation

The sciatic nerve segments were fixed in 10% neutral formalin and then each sample cut into two pieces. One piece was used for transverse and the other is for longitudinal section. After processed histologically, tissue samples were embedded in parafin and 5 μm in thickness sections were taken. Tissue samples were stained with hematoxylin-eosin (H-E) (Sigma) and Modified Gomori trichrome solution (Bio Optica, Milano, Italy) (according to the manufacturer’s protocol) in order to evaluate the neuronal damage and myelination status. Sciatic nerves were graded for ischemic fiber degeneration (IFD) using previously described method. The fibers were evaluated for IFD according to axonal changes such as swollen or shrunken axons and light or dark appearance. Myelin changes were examined in terms of breakdown or collapse. The sections were graded from 0 to 4 for IFD based on the percentage of IFD as follows: ≤ 2%, 3–25%, 26–50%, 51–75%, and >75%, respectively (Table I). Each section of sciatic nerve was examined under light microscope.
The expressions of caspase-3 was investigated by immunohistochemistry. To summarize the protocol; following dewaxing, washing and rehydration of the slides, microwave heating in citrate buffer was used for antigen retrieval. Endogenous peroxidase was blocked by 3% hydrogen peroxidase in methanol. Then, sections were incubated with caspase-3 primary antibody (dilution 1: 100)(AB3623, Millipore, California) at 4°C overnight. The next day, Horseradish peroxidase (HRP) secondary antibody kit (Anti-polyvalent HRP, Lab Vision Corp, Fremont, CA) was applied as a secondary antibody according to the manufacturer’s protocol. 3-amino-9-ethylcarbazole (AEC) kit (Lab Vision Corp, Fremont, CA) was used for chromogen and then the sections were counterstained with Mayer's hematoxylin and mounted with water based mounting medium. For evaluating the percentage of caspase-3 positive cells, fibers were scored using previously described method from 0 to 4 as follows: <5%, 6–15%, 16–25%, 26–35% and >35%, respectively. All the sections were observed under a microscope (Eclipse E-600 Nikon, Japan). Image Analysis Software (NIS Elements Nikon, Japan) was used for assessing the samples.

### Statistical Analysis

All experimental data were analyzed by SPSS for Windows 18 statistical analysis software. The Kruskal-Wallis test was used to analyze the differences between the groups and Mann-Whitney U test was performed for pairwise comparisons. P<0.05 value was considered to be statistically significant.

### Biochemical Investigations of Sciatic Nerve Tissues

After macroscopic analysis, rat tissues were kept at -80°C. For biochemical investigation, MDA, NRF1 levels and SOD activities from each supernatant were measured in duplicate with highly sensitive enzyme linked immunosorbent assay (ELISA) spectrophotometry, respectively. The protein concentrations were indicated by the Bradford method using Bradford reagent (Sigma-Aldrich, Bradford reagent-B6916-1KT, USA). All the data was defined as the mean ± standard deviation (SD) results based on per mg of protein.

### Table I: Ischemic Fiber Degeneration (IFD) Scores of Groups According to Mitsui et al. (17)

<table>
<thead>
<tr>
<th>Rats</th>
<th>Control</th>
<th>Ischemia</th>
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<th>MP</th>
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<td>2.38</td>
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### Caspase-3 Immunohistochemistry Protocol

Caspase-3 Immunohistochemistry Protocol

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### Nuclear Respiratory Factor-1 (NRF1)

Rat NRF1 ELISA kit (Hangzhou Eastbiopharm CO., LTD., China) was used to assay NRF1 on the basis of the Biotin double antibody sandwich technology. Absorbances of each well were measured under 450 nm wavelength. The results were expressed as ng/ml NRF1 per milligram protein (ng.ml⁻¹ mg protein⁻¹).

### Superoxide Dismutase (SOD)

Tissue SOD activity was measured with SOD assay kit (Sigma-Aldrich-19160-St.Louis, MO, USA) using highly sensitive ELISA spectrophotometry. The IC50 (50% inhibition activity of SOD) values was determined by this colometric method under 450 nm. The results were expressed as U/ml SOD per milligram protein (U.ml⁻¹.mg protein⁻¹).

### Lipid Peroxidation (MDA)

Lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532 nm) product, proportional to the MDA present. Lipid peroxidation (MDA) assay kit (Sigma-Aldrich-MAK085- St.Louis, MO, USA) was used for determining MDA levels. The results were expressed as nmole/ml MDA per milligram protein (nmole.ml⁻¹.mg protein⁻¹).

### Statistical Analysis

Results were subjected to one-way analysis of variance (ANOVA) using SPSS 21.0 software (SPSS Inc., USA). Differences among the groups were obtained using Tukey’s test option. Statistical significance was accepted as p < 0.05. All data was expressed as mean ± standard deviation (SD) in each group.

### RESULTS

### Histopathological Results

#### Ischemic Fiber Degeneration: For histopathological examination of rat sciatic nerves, H-E and gomori trichrome staining were performed. A nerve fiber consists of an axon and a myelin sheath formed by Schwann cells. Myelin does not stain well in a typical H-E preparation because of its high lipid content.
content that is lost in the staining process. Neurokeratin content can be seen in this area, a sparse proteinaceous network that is the remnants of the myelin sheath following removal of fatty material. H-E staining demonstrates centrally located nerve fiber, which is acidophilic, and surrounding pale-staining myelin sheath. After stained with Gomori trichrome solution, endoneurium was stained as green and neurokeratin of myelin was in red and nucleus of Schwann was in blue colour. In longitudinal sections, the myelin sheath was observed in elongated columns. In transverse sections, centrally placed axons, ensheathing Schwann cells together with endoneurium were observed.

In the control rats, myelinated area appeared in ordered structure showing neither axonal shrunken nor its swollen (Figures 1A, 2A). Ischemic fiber degeneration (IFD) was graded based on the previous study of Mitsui et al. (17). There was some ischemic fiber degeneration in ischemia group (Figures 1B, 2B). Axonal swelling and shrinkage were observed. The average myelin loss and disorganisation were significantly more in this group in comparison to the controls (p<0.05) and this deterioration was partly restored by syringic acid and MP group. Treatment with syringic acid significantly improved morphology of axon-myelination and it decreased ischemic fiber degeneration (Table I, Figures 1C, 2C) (p<0.05). MP also significantly reduced ischemic fiber degeneration (Figures 1D, 2D) (p<0.05). However, restoration of the nerve fibers was more prominent by treatment with MP group compared to the syringic acid group.

Figure 1: Control group showing normal morphology (A1-A2). Ischemia group showing axonal degeneration (arrows) (B1) swollen nerve fiber with myelin loss (arrowhead) and shrunken and darky axons (thick arrows) (B2). Syringic acid group showing less axonal and myelin degeneration than ischemia group (C1-C2). MP group showing less axonal and myelin degeneration than syringic acid group (D1-D2). (Photomicrographs of longitudinal (A1,B1,C1,D1) and transverse (A2,B2,C2,D2) sections from the sciatic nerves (H-E, X400, scale bar=50 μm).
**Caspase-3 Expression of Sciatic Nerve Fibers:** Caspase-3, an effector caspase, is activated in both intrinsic and the extrinsic pathway of apoptosis. Caspase-3 protein expression were observed by immunohistochemistry and graded based on the scoring system of Schmeichel et al. (22). Stained sections were examined under x40 magnification. Figures show the transvers sections from the nerve fibers under x100 magnification with immersion oil. Caspase-3 staining were observed in both axonal and myelinated region. The caspase expression of the control group was negative (Figure 3A). The percentage of positive nerve fibers for caspase-3 in ischemic nerves was significantly higher than in controls (Table II, Figure 3B, p<0.05). Less positive staining was found in the syringic acid (Figure 3C) and MP (Figure 3D) groups. There was no significant difference in caspase-3 expression between syringic acid and MP groups (p>0.05).

In summary, peripheral nerves subjected to ischemia reperfusion injury underwent IFD with a breakdown in the axon and myelin sheath and apoptosis was increased. Treatment with syringic acid and MP reduced IFD and excess apoptosis.

**Biochemical Results**

The mean and standard deviation values of NRF1, SOD and MDA in each group were given in Table III and Figure 4A-C. NRF1 levels of ischemia/reperfusion group were found to be lower compared to the other groups and this was statistically significant.

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**Figure 2:** Control group showing normal morphology (A1-A2). Ischemia group showing axonal swelling and greatly high both axonal and myelin degeneration (B1-B2). Syringic acid group showing less fiber degeneration (C1-C2). MP group showing less fiber degeneration than syringic acid group (D1-D2). (Photomicrographs of longitudinal (A1,B1,C1,D1) and transverse sections (A2,B2,C2,D2) from the rat sciatic nerves (Gomori’ Trichrome, X400, scale bar = 50 μm).
Table II: The Scores of Caspase-3 Positive Cells According to Schmeichel et al. (22)

<table>
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<tr>
<th>Rats</th>
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Mean value: 0 1.88 1.13 0.88

Table III: The Activity of Superoxide Dismutase (SOD), Levels of Malondialdehyde (MDA) and Nuclear Respiratory Factor-1 (NRF1) of Rat Sciatic Nerve Tissues

<table>
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<tr>
<th>Groups</th>
<th>NRF1 (n mg^-1 ml^-1 mg protein^-1)</th>
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<th>MDA (nmole mg^-1 ml^-1 mg protein^-1)</th>
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<tr>
<td>Control</td>
<td>10.52 ± 0.85 a</td>
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<td>9.94 ± 0.39 c</td>
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<tr>
<td>I/R</td>
<td>2.72 ± 0.62 a</td>
<td>3.18 ± 0.37 e</td>
<td>23.66 ± 2.28 a</td>
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<tr>
<td>I/R + SA</td>
<td>7.85 ± 1.07 e</td>
<td>5.50 ± 0.25 b</td>
<td>13.95 ± 1.99 b</td>
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<tr>
<td>MP</td>
<td>9.27 ± 0.66 b</td>
<td>5.84 ± 0.47 b</td>
<td>11.62 ± 1.48 c</td>
</tr>
</tbody>
</table>

I/R: Ischemia/Reperfusion, SA: Syringic acid, MP: Methylprednisolone 30mg/kg.

Means in the same column by the different letter are significantly different to the One-way ANOVA-Tukey's test (p<0.05). Data was expressed as mean ± SD.

Figure 3: Control group showing no caspase-3 immunopositivity (A). Ischemia group showing increased number of caspase-3 positive fibers (B). Syringic acid group showing less caspase-3 immunopositivity than ischemia group (C). MP group showing less caspase-3 immunopositivity compared to ischemia (D). (Transverse sections of caspase-3 immunohistochemical staining from the sciatic nerves). (caspase-3 antibody, X1000, scale bar = 10 μm. Arrows indicate caspase-3 positive nerve fibers).
There was a significant difference between Syringic acid group and ischemia/reperfusion group (p < 0.05) like MP group. SOD activities of syringic acid group were significantly different compared to the control and ischemia/reperfusion groups (p < 0.05). Syringic acid group was not statistically different compared to MP group (p > 0.05) but highly significant. MDA levels of ischemia/reperfusion group were found to be significantly higher than each group and syringic acid group was statistically significant compared to each other (p < 0.05).

**DISCUSSION**

Although the mechanism for peripheral nerve injury after ischemia and reperfusion is poorly understood, various mechanisms have been proposed (3, 17, 23, 24). Axons are more resistant to ischemia than neurons since they have lower energy requirements (13, 24). Axonal ischemia and reperfusion leads to acute inflammatory changes including the influx of leukocytes at the lesion site in peripheral nerves thus inducing an inflammatory and metabolic cascade leading to cellular edema and disruption of cellular integrity (3, 8, 17).

In this study, we used a polyphenol that can be obtained naturally from plants known as SA. In the induced sciatic nerve ischemia model in rats, SA exerted a histopathologically verified axonoprotective effect on the ischemic sciatic nerve segments. Furthermore, following the treatment with SA, MDA level was decreased and SOD and NRF-1 values increased in biochemical investigations, indicating a significant reduction in the oxidative stress after ischemia/reperfusion. Since there is no definitive evidence regarding the toxicity of SA in human and animals, it is not considered to be toxic by ingestion and inhalation. However, it may cause skin irritation, allergic reactions, nausea and vomiting and exacerbate the ongoing liver and pulmonary diseases.

Surgical approaches to the descending aorta (cross-clamping the aorta) for thoraco-abdominal aortic diseases may result in temporary or permanent ischemia of the peripheral nerves including the sciatic nerve and its branches. Several strategies such as distal aortic perfusion, intrathecal vasodilators, reattachment of intercostal and lumbar vessels, and decreasing cerebrospinal fluid pressure have been used to overcome the hypoperfusion. Other measures including the free radical scavengers and immune system modulation also help to decrease the reperfusion injury (1, 10, 19, 21).

It is reported that polyphenol compounds exert anti-inflammatory and immunomodulatory effects via clearing ROS in oxidative stress and are commonly found naturally in herbs and herbal preparations containing biflavonoids. Although biflavonoids are known to display a variety of biological activities, such as anti-inflammatory activity, inhibition of cytochrome P450 enzymes, and antiviral activity, their neuroprotective roles have not been known (7, 9, 16). Simonyi et al. demonstrated the neuroprotective effects of polyphenols in cerebral ischemia (23). Additionally, Mortonet al. revealed that SA helps in degrading free radicals by inhibiting the oxidation of low density lipoproteins as well as decelerates atherosclerosis by decreasing MDA production (18). Biflavonoids and particu-
larly SA demonstrated a neuroprotective effect against ROS-induced insult (10, 21). Kang et al. reported that biflavonoids neuroprotection might be mediated by a direct blockade of the cell death cascades, but not by their anti-oxidative activity (11). To test this idea further, they examined the neuroprotective effects of biflavonoids against cytotoxic insult induced by staurosporine, which has been known to mediate apoptosis via the caspase-dependent mitochondrial pathway. They also suggested that biflavonoid neuroprotection appeared to be mediated, in part if not all, by direct blockade of the signaling events leading to apoptosis upon cellular stresses. However, in the current literature, an experimental study regarding the axon protective effects of SA on sciatic nerves does not exist.

We found that SA as a biflavonoid appeared to have an axonoprotective effect on the sciatic nerves histopathologically. In addition to these histopathological finding, syringic acid decreased the MDA levels and increased the SOD and NRF-1 activities in biochemical analysis reducing oxidative stress after ischemia. Akdemir et al. reported that aortic clamping resulted in increase in MDA levels in peripheral nerves and edema that leads to axonal injury and myelin damage (2). The occurrence of IFD and edema indicates that long-term ischemia/reperfusion has a devastating impact on the peripheral nerves and that there is a direct link between paraplegia, one of the most common postoperative complications of aorta surgery, and the cross-clamping. Mitsui et al. proposed that the molecular mechanisms of nerve ischemia are similar to those of tissues like heart, gut and brain, but modified in terms of their threshold to ischemic and reperfusion damage (17). Ischemia would result in phospholipase activation and phospholipid breakdown, liberating arachidonic acid with its cascade producing the prostaglandins and leukotrienes. Oxygen free radicals would be generated during ischemia and especially during reperfusion. The formation of lipid hydroperoxides would inhibit prostacyclin synthetase (6, 15, 25). The increased biosynthetic rate of TxA2 coupled with the ischemia-related inhibition of prostacyclin production by endothelial cells would result in vasoconstriction, aggravating the ischemic insult (6). Eventually, this fatal cascade leads to formation of MDA as the end product of lipid peroxidation and at the same time is considered as one of the most sensitive markers of lipid peroxidation. Akdemir et al. (2) reported that aortic clamping resulted in increase in MDA levels in peripheral nerves and edema that leads to axonal injury and myelin damage. Syringic acid may enable neural tissues to become more resistant to lipid peroxidation, thereby activating anti-oxidant enzymes. In our study MDA values were reduced more resistant to lipid peroxidation, thereby activating anti-oxidant enzymes. In our study MDA values were reduced significantly after SA treatment as compared to the untreated control group. Additionally, there was no significant difference in MDA values between the MP group and SA group. Peripheral nerves harbour Schwann cells with potentially enzymatic and non-enzymatic anti-oxidant capacity to protect peripheral nerves from the harmful effects of the free oxygen radicals. SOD activity may reduce (1,4,6,14,20). Similarly, we found that the SOD activity in the SA and MP group was significantly increased in comparison to that of the control group.

During the breakdown of free oxygen radicals, mitochondria are important regulators of the metabolic activity of neuronal cells. Mitochondrial biogenesis is activated in response to cellular oxidative stress via environmental stimuli and other many different signals. NRF-1 is a mitochondrial transcription factor that activates the majority of genes coding subunits of the respiratory complex. Kumari et al. documented the decreased level of NRF-1 at the induced neural ischemia and an in increase during reperfusion period in normal and hyperglycemic rats (12). Likewise, we found that the SA and MP groups exhibited higher NRF-1 values compared to the Sham group. Activation of caspase 3 is known to be important and irreversible in apoptosis caused by ischemia of neuronal cells (3,4,12). Recently, Unsain et al. reported that caspase 3 negative axons are protected from degeneration (26).

**CONCLUSION**

In our study, SA and MP groups displayed fewer caspase 3 immunopositive fibers than Control and Sham groups. We, for the first time, demonstrated the axon protective role of syringic acid in an animal model by histopathological analysis and biochemical means. Lack of neurophysiological evaluation of rats by electromyography was a limitation of our study. In conclusion, syringic acid may become an alternative or a supplementary agent to MP in the treatment of axonal ischemia/reperfusion injury.

**REFERENCES**