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Amantadine's Neuroprotective Effects in Rabbit Spinal Cord Ischemia/Reperfusion Model

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ABSTRACT

AIM: To examine the effects of amantadine, a drug with neuroprotective and anti-inflammatory activities on oxidative stress, tissue necrosis, apoptosis, and neurological recovery in an experimental rabbit spinal cord ischemia-reperfusion injury (SCIRI) model.

MATERIAL and METHODS: A total of 32 rabbits were randomized into five groups: control, ischemia, vehicle, methylprednisolone (MP), and amantadine (AMT) (n=8/each). At 24th-hour neurological examination was performed, spinal cord tissues were collected, and biochemical and histopathological examinations were performed.

RESULTS: When ischemia and vehicle groups were compared with control group, significant increase was seen in serum and tissue caspase-3, malondialdehyde (MDA), and myeloperoxidase (MPO) levels ($p<0.001$); significant decrease was seen in serum and tissue catalase (CAT) levels ($p<0.001$); and significant increase was seen in serum xanthine oxidase (XO) levels ($p<0.001$). When the ischemia group and the MP and AMT groups were compared, low serum and tissue caspase-3 levels ($p<0.001$), high serum and tissue CAT levels ($p<0.001$), significantly low serum XO levels ($p<0.001$), low serum and tissue MDA levels ($p<0.05$) and tissue MPO levels ($p<0.001$) were found. Both AMT and MP groups showed decreased histopathological score and higher number of normal neurons ($p<0.001$) compared to ischemia group. Both AMT and MP showed better modified Tarlov scores compared to the ischemia group ($p<0.001$).

CONCLUSION: Our study found that AMT had antioxidant, anti-inflammatory, anti-apoptotic, and neuroprotective effects on SCIRI. We used biochemical, microscopic, and ultrastructural approaches to demonstrate these effects. AMT might be a candidate medication for SCIRI prophylaxis and treatment.

KEYWORDS: Amantadine, Anti-inflammatory, Antiapoptotic, Antioxidant, Spinal cord ischemia and reperfusion injury

INTRODUCTION

Spinal cord ischemia-reperfusion injury (SCIRI) is seen after trauma, shock, or surgery that involves temporary vessel occlusion (1). Surgical treatment of thoracoabdominal aortic aneurysm can result in SCIRI at a reported incidence of between 1% and 32% (45). Ischemia-reperfusion (IR) injury of the spinal cord can cause debilitating injuries, including immediate or delayed paraplegia (23). The pathophysiology of IR injury involves lipid peroxidation caused by free oxygen radicals, intracellular calcium excess, leukocyte activation, an inflammatory response and neuronal apoptosis (66).

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Numerous interventional techniques, such as hypothermia, surgical decompression, cerebrospinal fluid drainage to provide reperfusion, antiedema treatment, and many pharmacological agents have been studied experimentally to treat and protect the spinal cord from ischemic injury but complications persist (5,28,34,63). In some patients, neurological deterioration was observed even after spinal cord decompression; this is probably explained by damage from free radicals seen in prolonged exposure of the spinal cord to ischemia despite reperfusion. Following this injury, the patient may experience sensory, motor and/or autonomic impairment due to the neuronal dysfunction of the affected cord region (66). The interactions of local metabolites and oxidative stress cause increased vascular permeability at the membrane level and an increase in apoptotic activity and cell death may occur (67).

Amantadine (AMT, L-Adamantanamine) has cerebral anti-inflammatory effect and acts by upregulating selective neurological dopaminergic pathways, besides its antagonist effect at N-methyl-D-aspartate receptors (NMDAR) (39). The mechanism of action is thought involve increased number of dopaminergic receptors, blocking dopamine re-uptake from the synaptic gap and increasing dopamine release from vesicles (31). AMT is currently used in Parkinson's disease due to its neuroprotective properties resulting from its NMDAR antagonistic activity and dopaminergic effects, and it is thought to have positive effects on neuronal survival (7,58). AMT is currently also used for Huntington's chorea (24), Parkinson's disease related dyskinesia and impulse control (14,47), recovery from traumatic brain injury (TBI) or stroke (25), and neuropathic pain (13,48).

AMT is a medication shown in numerous animal and human trials to have neuroprotective and anti-inflammatory properties, though no prior research has been done on its impact on SCIRI. Our research focused on AMT's antiapoptotic, antioxidant and anti-inflammatory activity in the rabbit SCIRI model and discussed its potential neuroprotective characteristics.

MATERIAL and METHODS

Experimental Groups

This research was carried out in the Saki Yenilli Laboratory Animals Facility (This research was carried out in the Saki Yenilli Laboratory Animals Facility (2019/01/02/03). The animals used during the research were cared for according to the animal protection guidelines the experimental studies published by the European Communities Council Directive on 24.11.1986. Research was performed after the approval of the Local Experimental Animals Ethics Committee (10/03/2019). A total of 40 male New Zealand white rabbits (2900 - 3200 grams) were used in this experiment. The rabbits were cared for in standard room settings (at a temperature of 18-21° C, within a 12-hour dark/light balance, with standard chow).

The groups were as follows:

Control group (n=8): only laparotomy performed.

Ischemia group (n=8): Aortic cross-clamping was performed for 20 minutes to achieve temporary global spinal cord ischemia (SCI).

Vehicle group (n=8): same SCI as in ischemia group. After ischemia, 2 ml 0.9% NaCl was injected intraperitoneally.

Methylprednisolone (MP) group (n=8): same procedures as ischemia group. After ischemia, 30 mg/kg of MP (Prednol, Mustafa Nevzat, Turkiye) was applied once intraperitoneally. MP dosage was selected based on the literature (27,28,34,35).

Amantadine (AMT) group (n=8): same surgery as in ischemia group. Intravenous injections of 135 mg/kg of AMT (PK Merz vial, Merz Pharmaceuticals GmbH, Frankfurt, Germany) were applied just after SCI. AMT dosage was selected based on the literature (54).

Laminectomies were performed 24 hours post-initial surgery in control group as well as 24 hours after reperfusion in ischemia and AMT and MP groups. Spinal cord L2-L5 segments were obtained. Samples were processed for biochemical, light and electron microscopic examinations. Blood samples were collected for biochemical analysis.

Surgery

Ketamine (70 mg/kg, Ketalar, Pfizer, Kirklareli, Turkiye) and xylazine (5 mg/kg, Rompun, Bayer, Turkiye) were administered intramuscularly. Spontaneous breathing and body temperatures of 37 °C were maintained. Aortic cross-clamping for SCIRI was employed at the level of left renal artery which corresponds to the level of proximal lumbar segments (27,28,35,38,68). The paraplegia that followed from the 20-min ischemia and 24-h reperfusion period was sufficient to produce lasting injury (68). After 120th minute of the intervention, the subjects were fed. Rabbits were monitored for neurogenic bladder and rabbits with neurogenic bladder were treated with the Crede maneuver at least twice a day. All subjects were sacrificed with 200 mg/kg pentobarbital (Nembutal, Oak Pharmaceuticals, Lake Forest, IL, USA) at 24 hours postoperatively.

Caspase-3 Levels

Enzyme-linked immunosorbent assay (ELISA) was conducted to investigate the serum and tissue caspase-3 levels (ELISA kit; Cusabio, Hubei, China). The instructions from the manufacturer were followed. Detailed procedure was published (27). The output was given in ng/mL.

Myeloperoxidase (MPO) Activity

Competitive inhibition ELISA was used to detect serum and tissue MPO activity (Cusabio, Hubei, China) according to manufacturer's instructions. Detailed procedure was published (28). The output was given in ng/mL.

Malondialdehyde (MDA) Levels

Thiobarbituric acid was used to detect serum and tissue MDA levels. Detailed procedure was published (27). The output was given in nmol/ml.

Catalase (CAT) Levels

Serum and tissue CAT levels were detected by measuring the rate at which hydrogen peroxide's absorbance decreased at 240 nm (2). The output was given in IU/mL.

Xanthine Oxidase (XO) Activity

The amount of formed uric acid from xanthine was used to detect serum XO activity (46). Detailed procedure was published (27). The output was given in mIU/ml.

Light Microscopic Investigation

Neuronal injury in anterior horn neurons of spinal cord was evaluated and level of injury was scored according to the cell size, nuclear hyperchromasia and presense of nucleolus. A score of 0 indicated no degeneration, 1 indicated mild degeneration (in a few neurons), 2 indicated moderate degeneration (degeneration in almost half of the neurons), 3 indicated severe degeneration (extensive neuronal degeneration or total loss). The complete process was made accessible from previous papers (27).

Electron Microscopic Investigation

For each spinal cord tissue, 100 all sized myelinated axons were graded from 0 to 3. The scoring was applied on 5 samples of all groups (33).

Investigation of Normal Number of Neurons in Different Groups

Motor neurons in the anterior horn of the spinal cord were evaluated in 3 different areas at 40x magnification. The average number of neurons in each group was evaluated for each spinal cord preparation taking the arithmetic average of these 3 different areas. In this calculation, cells with Nissl bodies in their cytoplasm, scattered chromatin structure and nucleolus were defined as healthy neurons (59).

Neurological Examination

The hindlimb neurological status of the rabbits was evalauted with the modified Tarlov scoring system 24 hours following surgery (64). A score from 0 to 5 was assigned to each animal, where 0 indicated no movement and 5 indicated complete recovery.

Statistical Analysis

Data-blind researchers carried out analysis with statistical programme GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Detailed procedure was published previously (27).

RESULTS

Caspase-3 Levels

The ischemia and vehicle groups' serum and tissue caspase-3 levels were significantly higher than those of the control group ($p<0.001$) (Table I, Figure 1).

There was no significant difference in serum and tissue caspase-3 levels between the ischemia and the vehicle group ($p>0.05$).

Table I: Biochemical Results in the Experimental Groups

Variable	Control	Ischemia	Vehicle	MP	AMT	p-value
Serum Caspase-3 (ng/ml)	215.3 ± 31.3 a,c	421.5 ± 55.62 a, f	403.7 ± 54.81 c, i, k	205.5 ± 42.2 f, i	165.2 ± 69.3 h, k	**<0.001**
Tissue Caspase -3 (ng/ml)	172.5 ± 53.98 a,c	642.8 ± 153.0 a, f	626.6 ± 116.8 c, i, k	141.3 ± 75.44 f, i	84.78 ± 74.55 h, k	**<0.001**
Serum MPO (ng/ml)	2.39 ± 0.46 b, m	5.49 ± 2.34 b	5.65 ± 1.35 m	3.80 ± 1.37	3.70 ± 1.51	**<0.001**
Tissue MPO (ng/ml)	3.02 ± 0.78 b, c	5.09 ± 0.96 b, f, h	5.51 ± 1.46 c, i, k	2.47 ± 1.38 f, i	1.42 ± 0.94 h, k	**<0.001**
Serum MDA (nmol/g tissue)	2.57 ± 0.51 a, c, e	6.41 ± 1.11 a, f	6.55 ± 1.05 c, i, l	2.51 ± 0.57 f, i	4.49 ± 1.73 h, l	**<0.001**
Tissue MDA (nmol/g tissue)	3.73 ± 1.23 a, c	10.66 ± 2.88 a, g	10.98 ± 3.40 c, j, k	6.39 ± 1.20 g, j	5.85 ± 1.75 h, k	**<0.001**
Serum CAT (IU/ml)	156.1 ± 41.97 a, c, d	40.47 ± 11.90 a, f	54.88 ± 11.23 c, i, l	112.1 ± 22.62 d, f, i	98.37 ± 15.87 h, l	**<0.001**
Tissue CAT (IU/ml)	114.5 ± 1.79 a, c	27.47 ± 10.80 a, f	25.79 ± 10.92 c, i, k	111.5 ± 12.82 f, i	99.72 ± 15.41 h, k	**<0.001**
Serum XO (mIU/ml)	10.13 ± 9.20 a, c	61.25 ± 12.75 a, f, h	57.50 ± 12.27 c, i, k	6.00 ± 5.78 f, i	7.62 ± 4.50 h, k	**<0.001**

a: *Control vs Ischemia (p<0.001),* ***b:*** *Control vs Ischemia (p<0.01),* ***c:*** *Control vs Vehicle (p<0.001),* ***d:*** *Control vs MP (p<0.01),* ***e:*** *Control vs AMT (p<0.05)* ***f:*** *Ischemia vs MP (p<0.001),* ***g:*** *Ischemia vs MP (p<0.01),* ***h:*** *Ischemia vs AMT (p<0.001),* ***i:*** *Vehicle vs MP (p<0.001),* ***j:*** *Vehicle vs MP (p<0.01),* ***k:*** *Vehicle vs AMT (p<0.001),* ***l:*** *Vehicle vs AMT (p<0.01),* ***m:*** *Control vs Vehicle (p<0.01),* ***AMT:*** *Amantadine,* ***CAT:*** *Catalase,* ***MDA:*** *Malondialdehyde,* ***MP:*** *Methylprednisolone,* ***MPO:*** *Myeloperoxidase,* ***XO:*** *Xanthine oxidase.*

A significant reduction in serum and tissue caspase-3 levels was seen in the MP and AMT groups compared to the ischemia group when the ischemia group was compared with the MP and AMT groups ($p<0.001$).

There was no significant difference in the serum and tissue caspase-3 levels between the MP and AMT groups ($p>0.05$).

The data shows that ischemia increases serum and tissue caspase-3 levels. The decrease in serum and tissue caspase-3 levels following AMT and MP administration reveals the antioxidant and antiapoptotic effects of these agents.

MPO Activity

Examination of the control, ischemia, and vehicle groups showed significantly higher serum and tissue MPO activity in the ischemia and vehicle groups than the control group ($p<0.001$) (Table I, Figure 1).

Serum and tissue MPO activity did not differ significantly between the ischemia and the vehicle group ($p>0.05$).

Tissue MPO activity in the MP and AMT groups were significantly lower than in the ischemia group ($p<0.001$). Serum MPO activity showed no significant difference among groups ($p>0.05$).

Serum and tissue MPO activity between the MP and AMT groups did not differ significantly ($p>0.05$).

The data shows that ischemia increased serum and tissue MPO activity, indicating inflammatory response. Administration of AMT and MP was followed by decreased serum and tissue MPO activity, revealing the anti-inflammatory effects of these agents.

MDA Levels

Serum and tissue MDA levels in the ischemia and vehicle groups were significantly higher than those of the control group ($p<0.001$) (Table I, Figure 1).

Serum MDA levels in the AMT group were significantly higher than those of the control group ($p<0.05$).

Comparing the ischemia group with the MP and AMT groups showed the ischemia group's serum and tissue MDA levels were significantly greater than those of the MP and AMT groups ($p<0.001$).

The vehicle group's serum and tissue MDA levels were significantly higher than those of the MP and AMT groups ($p<0.01$).

There was no significant difference between the vehicle group and the ischemia group in terms of serum and tissue MDA levels ($p>0.05$).

The significant statistical data indicates that both MP and AMT inhibit rising serum and tissue MDA levels after SCIRI, showing that MP and AMT inhibit lipid peroxidation.

CAT Levels

Serum and tissue CAT levels in the ischemia and vehicle groups were significantly lower than in the control group ($p<0.001$) (Table I, Figure 1).

Serum and tissue CAT levels did not differ statistically between the ischemia and the vehicle groups ($p>0.05$).

The MP group's serum and tissue CAT levels were significantly greater than those of the vehicle group ($p<0.001$).

The MP and AMT group's serum and tissue CAT levels were significantly greater than those of the ischemia group ($p<0.001$).

There was no significant difference in serum and tissue CAT levels between the MP and AMT groups ($p>0.05$).

Serum and tissue CAT levels dropped due to oxidative stress following SCIRI. The data showed that MP and AMT treatment exerted antioxidant effects, increasing serum and tissue CAT levels.

XO Activity

Serum XO levels in the control group were significantly lower than those in the ischemia and vehicle groups ($p<0.001$) (Table I, Figure 1).

Serum XO levels between the vehicle and ischemia groups did not differ significantly ($p>0.05$).

The MP and AMT group's serum XO levels were significantly lower than the ischemia group's levels ($p<0.001$).

The serum XO levels in the AMT group were discovered to be significantly lower than those in the vehicle group.

There was no significant difference in serum XO levels between the MP and AMT groups ($p>0.05$).

Increased serum XO levels in the ischemia and vehicle groups is a marker of oxidative damage. Decreased serum XO levels after MP and AMT administration indicates that MP and AMT have antioxidant activity after SCIRI.

Histopathological Evaluation

The histopathology scores of the ischemia and vehicle groups were significantly higher than the control group ($p<0.001$) (Figure 2).

There was no significant difference in the histopathology scores between the ischemia and vehicle groups ($p>0.05$).

The histopathology scores of the MP and AMT group were significantly lower than those of the ischemia group ($p<0.001$).

When the histopathological scores of the MP and AMT groups were evaluated, no significant difference was discovered ($p>0.05$).

The data indicates that MP and AMT show neuroprotective activity with low occurrence of histopathology changes after SCIRI.

Evaluation of Normal Number of Neurons in Different Groups

The ischemia and vehicle groups showed considerably worsened tissue structure and integrity and significantly decreased number of neurons ($p<0.001$) (Figure 3).

There was no significant difference in the normal number of neurons between the ischemia and the vehicle group.

In terms of the preservation of the typical number of neurons, the number of neurons was significantly preserved in the MP and AMT groups as opposed to the ischemia group ($p<0.001$).

There was no significant difference in the typical number of neurons between the MP and AMT groups.

The data indicates that MP and AMT show neuroprotective effects in maintaining the normal number of neurons after SCIRI.

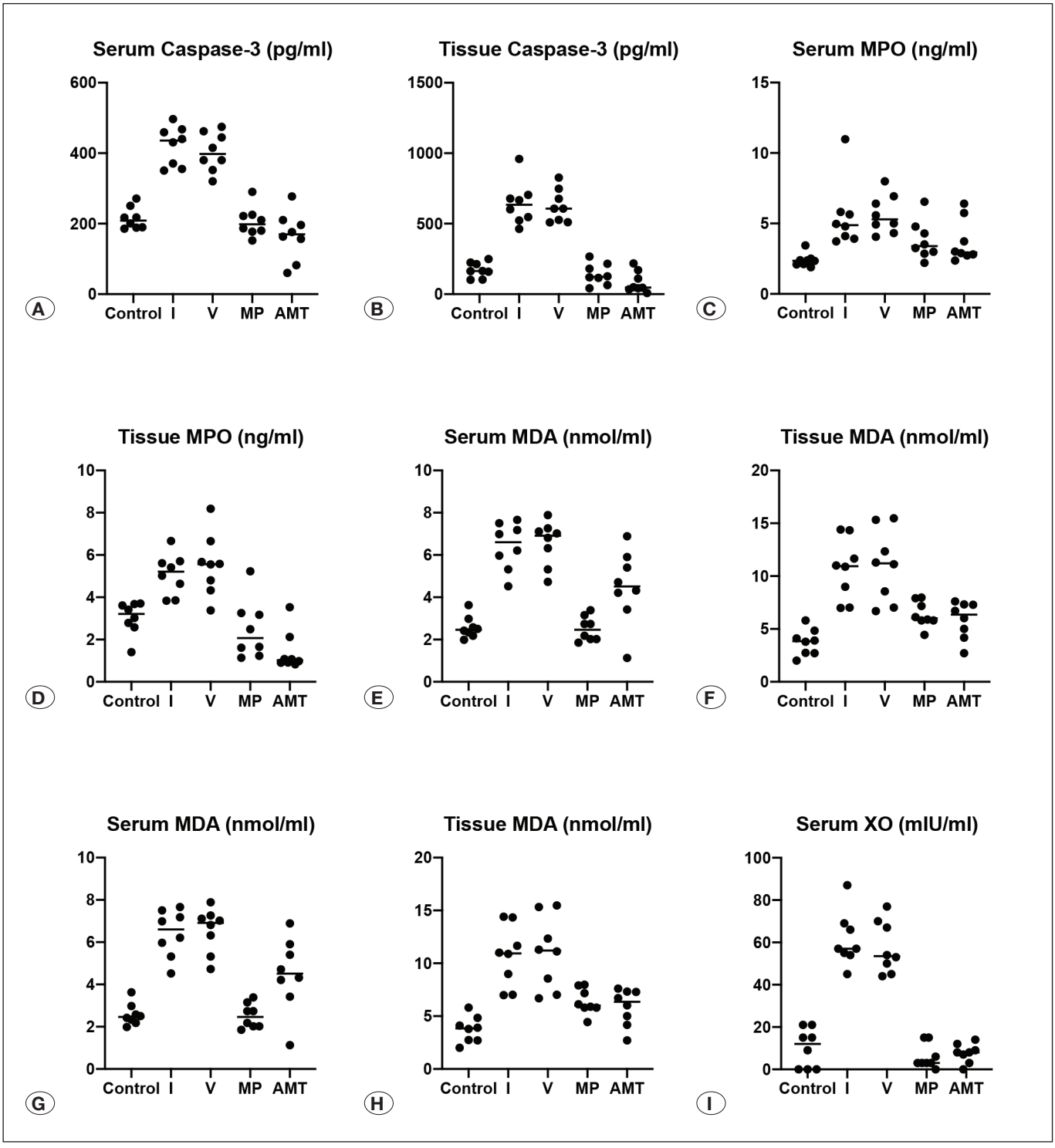

Figure 1: Biochemical results in the experimental groups.

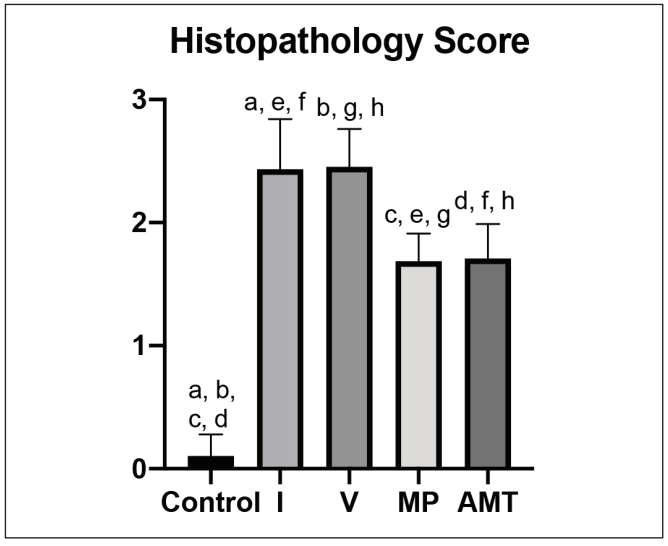

Figure 2: Histopathological score among groups. **AMT:** Amantadine, **I:** Ischemia, **MP:** methylprednisolone, **V:** Vehicle. **a:** Control vs Ischemia (p<0.001), **b:** Control vs Vehicle (p<0.001), **c:** Control vs MP (p<0.001), **d:** Control vs AMT (p<0.001), ***e:*** Ischemia vs MP (p<0.001), **f:** Ischemia vs AMT (p<0.001), **g:** Vehicle vs MP (p<0.001), **h:** Vehicle vs AMT (p<0.001).

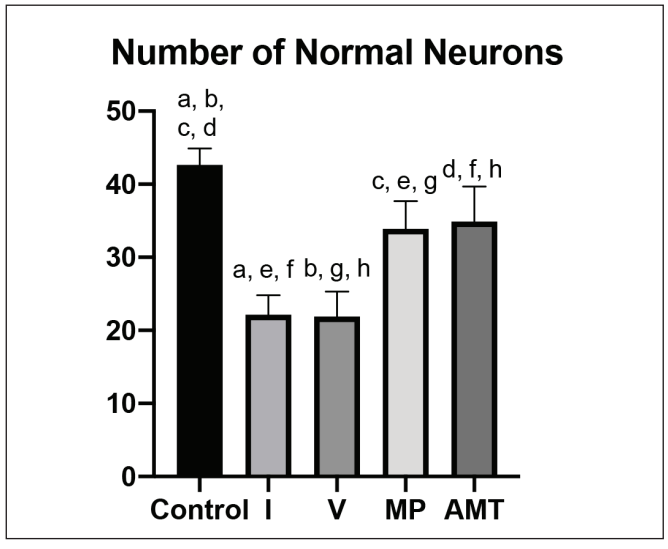

Figure 3: Number of normal motor neurons among groups. **AMT:** Amantadine, **I:** Ischemia, **MP:** methylprednisolone, **V:** Vehicle. **a:** Control vs Ischemia (p<0.001), **b:** Control vs Vehicle (p<0.001), **c:** Control vs MP (p<0.001), **d:** Control vs AMT (p<0.001), **e:** Ischemia vs MP (p<0.001), **f:** Ischemia vs AMT (p<0.001), **g:** Vehicle vs MP (p<0.001), **h:** Vehicle vs AMT (p<0.001).

Ultrastructural Evaluation

The white and grey matter of the spinal cord showed no ultrastructural pathological alterations in the control group. All small- and medium-diameter myelinated axons in this group had normal ultrastructure; there were only a few large-diameter axons that showed modest dissociations in the myelin sheath, likely due to delayed tissue fixation.

When the tissue samples from the ischemia group were examined, substantial ultrastructural alterations were found in the spinal cord samples' grey and white matter. In the cytoplasm of the neurons, vacuoles were seen during the ultrastructural analysis of grey matter. However, perineural edema was seen in these subjects, though the ultrastructure of their neuronal nuclei was proven to be normal. All neurons had ultrastructurally normal cell membranes. Myelinated axons showed ultrastructural pathological alterations during the white matter investigation. Small-diameter myelinated axons showed less severe ultrastructural pathological alterations than large-diameter myelinated axons did.

As in the ischemia group, substantial ultrastructural pathological alterations were found in both the spinal cord grey matter and the white matter of the vehicle group. Vacuoles and perineural edema were found in the neuronal cytoplasm during the ultrastructural analysis of the grey matter. The ultrastructure of the nuclei and cell membranes were normal in all neurons. Myelinated axons showed ultrastructural pathological alterations during the analysis of the white matter. Seperations in myelin configuration have been seen in most of the myelinated axons, including those with small, medium, and large diameters. Ultrastructural degradation was weaker in small-diameter myelinated axons and more severe in large-diameter myelinated axons.

Normal grey matter ultrastructure was observed in the MP group. Small-diameter myelinated axons were ultrastructurally normal despite the MP group's white matter being unusually thin. In medium and large diameter myelinated axons, separations in myelin configuration were also found.

Both the grey matter and the white matter of spinal cord samples showed severe ultrastructural pathological alterations in AMT group. Vacuoles were discovered in the cytoplasm of neurons. Additionally, some groups had perineuronal edema. The ultrastructure of the neurons' nucleus and cell membranes were normal. Ultrastructural pathological abnormalities were discovered in myelinated axons during white matter examination. Most small, medium, and large myelinated axons showed separations in the myelin structure and disruptions in myelin configuration were seen in several large and medium myelinated axons. Small myelinated axons had the least severe ultrastructural pathological abnormalities, while large myelinated axons had the most severe changes. Small myelinated axons showed no disruption in myelin structure.

The number of small, medium, and large diameter myelinated axons was significantly higher in the ischemia and vehicle groups compared to the control group (p<0.001) (Table II, Figure 4). There was no significant difference in the quantity of small, medium, and large diameter myelinated axons between the ischemia group and the vehicle group. The number of small, medium, and large diameter myelinated axons in the MP group was significantly higher than the ischemia group (p<0.001), while the number of neurons between the AMT group and the ischemia group did not differ statistically (p>0.05). It was

Table II: Evaluation of Myelinated Axons in Transmission Electron Microscope

Myelinated Axon	Control	Ischemia	Vehicle	MP	AMT	p-value
Small-sized	0.0 ± 0.0 a, b, d	88.40 ± 1.14 a, e	88.40 ± 1.51 b, f	0.0 ± 0.0 g	90.00.0 ± 1.22 d, g	**<0.001**
Medium-sized	0.0 ± 0.0 a, b, c, d	109.8 ± 1.92 a, e	109.0 ± 2.23 b, f	70.60 ± 2.30 c, g	110.0 ± 0.70 d, g	**<0.001**
Large-sized	5.0 ± 1.58 a, b, c, d	124.2 ± 2.04 a, e	123.0 ± 1.0 b, f	89.0 ± 1.58 c, g	125.2 ± 1.30 d, g	**<0.001**

***a:** Control vs Ischemia (p<0.001), **b:** Control vs Vehicle (p<0.001), **c:** Control vs MP (p<0.001), **d:** Control vs AMT (p<0.001), **e:** Ischemia vs MP (p<0.001), **f:** Vehicle vs MP (p<0.001), **g:** MP vs AMT (p<0.001). **AMT:** Amantadine, **MP:** Methylprednisolone.*

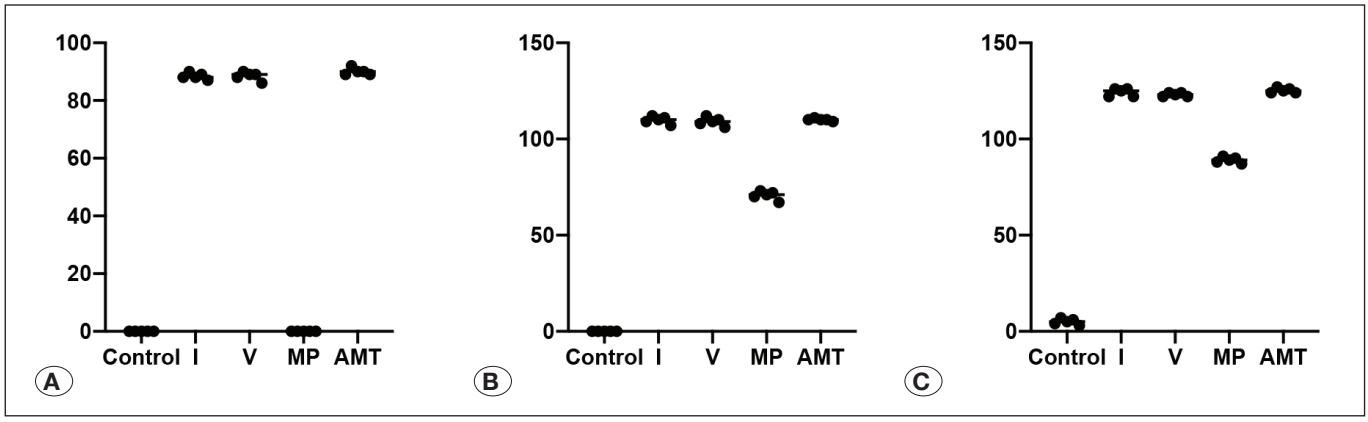

Figure 4: Axonal diameter comparison among groups. **A)** Small-sized myelinated axons, **B)** Medium-sized myelinated axons, **C)** Large-sized myelinated axons.

discovered that the AMT group had significantly more small, medium, and large diameter myelinated axons than the MP group did (p<0.001).

Neurological Evaluation

The Tarlov score of the control group and the ischemia and vehicle group was significantly decreased in the ischemia and the vehicle group compared to control group (p<0.001) (Figure 5).

There was no significant difference in Tarlov score between the ischemia and the vehicle group.

The modified Tarlov score in the MP and AMT groups was significantly higher than in the ischemia group (p<0.001).

There was no significant difference in Tarlov score between the MP and AMT groups.

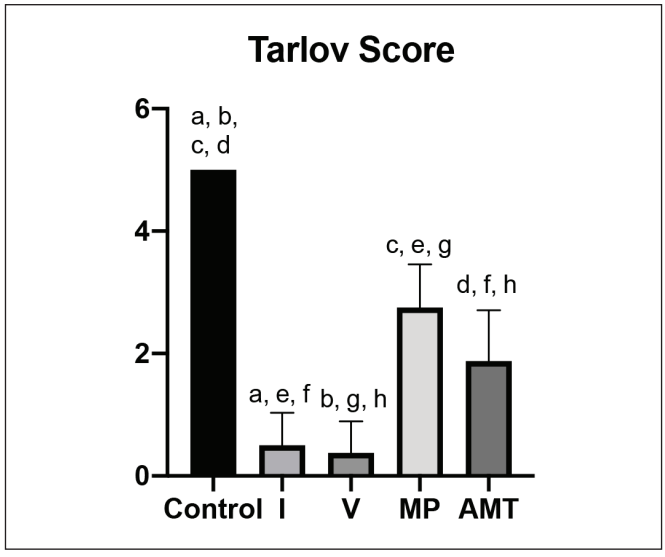

Figure 5: Tarlov score among groups. AMT: Amantadine, **I:** Ischemia, **MP:** methylprednisolone, **V:** Vehicle. **a:** Control vs Ischemia (p<0.001), **b:** Control vs Vehicle (p<0.001), **c:** Control vs MP (p<0.001), **d:** Control vs AMT (p<0.001), **e:** Ischemia vs MP (p<0.001), **f:** Ischemia vs AMT (p<0.001), **g:** Vehicle vs MP (p<0.001), **h:** Vehicle vs AMT (p<0.001).

DISCUSSION

SCI secondary to ischemia after thoracoabominal or cardiovascular surgery may result in catastrophic complications such as paraplegia (23). There are many studies investigating techniques for the preservation and revascularization of the nervous system during intraoperative ischemia. However, the cause of brain and SCIRI is poorly understood and therapeutically unsatisfactory (53).

Due to low glycogen stores and constrained ability to perform anaerobic metabolism, the central nervous system is more vulnerable to ischemia than other tissues. When blood flow declines, cellular energy reserves abruptly fall and ischemia cascades are quickly activated in neuroglial tissue, leading to cell death (27). Two processes contribute to SCI: when the spinal cord's blood supply is cut off and its oxygen supply is lost, the spinal cord suffers primary and irreparable damage; ischemia cellular mechanisms are then triggered, initiating secondary damage, which is reversible (10,22). IR injury results from the restoration of blood perfusion after a prolonged ischemic injury and affects all oxygen-dependent aerobic cells (15). IR injury is a complicated event that involves harmful inflammatory reactions and intracellular damage (6,30). Activation of intracellular signaling cascades and apoptotic pathways may also occur (6,30).

First reported in the early 90's by Gualtieri et al. (26), AMT, an NMDAR antagonist, has neuroprotective, anti-inflammatory effects (42). Many studies over the years have demonstrated the positive effects of AMT on functional recovery after TBI (25,50,51). Excitation produced by NMDAR binding can be harmful in excess. N-methyl-D-aspartate (NMDA) antagonism is anti-excitotoxic (9). Excessive glutamate levels result in neurotoxicity, in part through overactivation of NMDARs, while NMDAR antagonists such as AMT and memantine block NMDA glutamate receptors to suppress the glutamatergic system (29). AMT's NMDA-independent neuroprotective activities are linked to the toxins lipopolysaccharide (LPS) and 1-methyl-4-phenylpyridine (MPP+) (55). AMT has been demonstrated in cell cultures to boost the expression of neurotrophic protective factors, such as Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor and decrease the release of proinflammatory mediators from active microglia (49,62). Low neurotrophin levels seen in diseases of the spinal cord are believed to pose harm (56).

The current study examined AMT's neuroprotective potential in a rabbit SCIRI model. Aortic cross-clamping was applied in rabbits to create the SCIRI model. In the first stage, subjects exposed to ischemia with a 20-minute clamping period were left to reperfuse for 24 hours by removing the clamp, and IR injury was introduced to the spinal cord. This experimental paradigm is dependable and has been successfully applied in numerous SCIRI research (5,19,34). The efficacy of AMT has been compared with MP, which has been used clinically for years due to its anti-inflammatory and antioxidant effects in SCI (21). Today MP, whose effectiveness in the treatment of acute SCI is controversial, continues to be valuable as a strong positive control group in experimental SCIRI research (8). Because of the need for clinically effective treatments for SCI and SCIRI, our study investigated AMT for SCIRI. MP is experimentally effective but clinically controversial; AMT is an alternative.

A crucial processes causing neuronal cell death following IR injury, apoptosis is the most prevalent type of programmed cell death in multicellular animals (18,57). Cell shrinkage and cytoplasmic condensation are the first morphological changes in apoptosis, followed by nuclear membrane breakdown and the creation of apoptotic bodies—all without an inflammatory reaction (3). Studies have demonstrated that apoptosis is reversible and that same characteristics have also been seen in neurons that have sustained postischemic damage (37). The cysteine protease family of enzymes known as caspases control apoptosis. In numerous SCIRI experiments, caspase-3 activity has been employed as an accurate indicator of apoptosis (34,65). Disrupted ionic gradients in the central nervous system lead to ischemia, which depolarizes neurons and releases excitatory neurotransmitters, mainly glutamate (61). Glutamate plays a crucial part in the regulation of apoptosis. Numerous investigations have demonstrated that AMT, an NMDAR antagonist, exerts this antiapoptotic effect (16,43,60). As a marker of SCIRI damage and apoptosis, our work demonstrated that serum and tissue caspase-3 activity were increased in the ischemia and vehicle groups. Serum and tissue caspase-3 levels considerably dropped after AMT and MP treatments. According to these findings, AMT exhibits antiapoptotic effect by greatly lowering the apoptosis that ensues from SCIRI.

After oxygen reduction or restriction, an organ sustains oxidative damage when its blood and oxygen supply are restored, a phenomenon known as IR injury (20). The abnormal accumulation of intracellular calcium and the buildup of reactive oxygen radicals in cells are two crucial mechanical characteristics of oxidative damage following IR injury (40). Reactive oxygen radicals destroy DNA, lipids, and membrane proteins, which compromises the integrity and functionality of cells and ultimately harms neurons (11). Numerous intracellular components, including as mitochondria, Nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, XO, and unbound nitric oxide synthase, are generators of reactive oxygen radicals (4) and capable of triggering programmed cell death or necrosis, inducing or suppressing the expression of many genes and activating cell signaling cascades. Selective inhibition of XO, a reactive oxygen product, may result in a broad spectrum therapeutic for oxidative and reperfusion injury (36,41). In this study we looked at the effects of AMT on SCIRI and found heightened levels of XO levels, which an indication of oxidative stress after IR injury (28,34,65), were increased in blood samples taken from the ischemia and vehicle groups. Serum XO levels dropped significantly following AMT and MP treatments. This drop in serum XO levels can be seen as evidence of AMT's antioxidant effect.

When oxygen interacts with unsaturated lipids, lipid peroxidation occurs, yielding a wide range of oxidation products. MDA is recognised as the most mutagenic product of lipid peroxidation of all the numerous distinct aldehydes that might be produced (46). Numerous SCIRI investigations have utilised MDA, which damages tissue severely by cross-linking with membrane lipids. The increase in serum and tissue MDA after injury is recognised as a key sign of lipid peroxidation and tissue damage (28,34,65). Our investigation demonstrated that after IR injury, serum and tissue MDA levels were significantly higher in the ischemia and vehicle groups than in the control group. After the injection of AMT and MP, we measured significant decreases in both the serum and tissue MDA levels, additional evidence that AMT therapy exerts an antioxidant

effect by lowering lipid peroxidation and secondary SCIRI-induced cell membrane damage.

Following the formation of oxygen radicals in the cell, the most important antioxidant agents are catalase, superoxide dismutase and glutathione (32). Catalase, an enzyme used to identify peroxisomes, catalyses the oxidation of H_2O and a variety of substrates, including ethanol, methanol, phenol and nitrites (44). Catalase efficiently eliminates peroxides produced in peroxisomes and provides crucial protection against their harmful consequences (52). Numerous studies have demonstrated that following IR injury, catalase activity is reduced considerably by oxidative stress (28,34). As a sign of increased oxidative stress in our investigation, serum and tissue CAT levels fell in the ischemia and vehicle groups. Both in the ischemia and vehicle groups, serum and tissue CAT levels were increased following AMT and MP treatments, leading us to conclude that severe oxidative stress after SCIRI lowers CAT levels, but AMT treatment's antioxidative effects raise CAT levels. The increasing effect of AMT on CAT levels has been reported in several previous studies, supporting our results (27,28).

MPO is a crucial inflammatory enzyme and causes both oxidative stress and neuroinflammation in the pathological phase of IR injury. To lessen oxidative harm and neuroinflammation in IR injury, MPO may present a therapeutic target (12). Earlier SCIRI research ihas determined increased blood and tissue MPO activity levels to be signs of oxidative stress and inflammatory response (27,28). We found both serum and tissue MPO activity to be significantly increased in ischemia and vehicle groups and valid markers of inflammatory response and oxidative stress. AMT and MP treatments decreased both serum and tissue MPO activity, and we concluded that both drugs showed anti-inflammatory properties.

Light microscopy analysis of the samples taken from the spinal cord showed that the tissues of the control group have completely normal morphology. Significant hemorrhage, congestion, increased edema, necrosis and inflammation due to IR damage were seen in the spinal cord tissue samples taken from the ischemia and vehicle groups. While a significant increase was observed in the number of degenerated neurons in both groups, the number of neurons with intact morphology was decreased. In tissues with IR injury, polymorphonuclear leukocytes, lymphocytes and monocytes were increased, indicating neuroinflammation. In tissue samples taken from AMT and MP groups, neuronal morphology was better preserved compared to the ischemia and vehicle groups, thanks to the antiapoptotic, antioxidant and anti-inflammatory properties of these agents, and more morphologically normal neurons were observed in the ischemia and vehicle groups. Recently Dogan and Karaca also investigated the ameliorative effects of amantadine on SCI and reported that amantadine exerted anti-inflammatory and neuroprotective activity on rat spinal cord damage (17).

In transmission electron microscopy in the ischemia and vehicle groups, clear separations were observed in small, medium and large sized myelinated axons. While MP treatment caused improvement in small, medium and large size myelin separations, no protective effect was observed in the AMT group.

Neurological examinations were evaluated using the modified Tarlov scoring system. While all subjects were paraplegic following the IR process in the ischemia and vehicle groups, the spinal cord was functionally preserved in the groups given AMT and MP, and biochemical and histopathological improvement was detected after treatment. Modified Tarlov scores were higher in AMT and MP groups compared to ischemia and vehicle groups.

Considering the biochemical, histopathological and neurological examination data from this work, we conclude that AMT has neuroprotective properties given its anti-apoptotic, antioxidant and anti-inflammatory effects on SCIRI. Dose-dependency is a limitation of this study; more detailed results can be obtained by increasing the number of subjects in the groups and by changing the dose intervals and amounts of the agents used. Further, long-term drug efficacy should be studied by prolonging the reperfusion time. Finally, biochemical parameters should be examined in more detail.

CONCLUSION

For the first time in the literature, AMT has been shown to have antiapoptotic, antioxidant and anti-inflammatory effects on SCIRI and a remarkable neuroprotective effect. AMT, which has long been FDA approved for the treatment of many neurological, infectious and psychiatric diseases and whose safety has been shown many times, can also be used to prevent and treat SCIRI. In this we hope that AMT, whose neuroprotective effects we have demonstrated, will be the subject of further research.

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AUTHORSHIP CONTRIBUTION

Study conception and design: CU, PKB, BG, HK
Data collection: CU, PKB, BIE, ATA, IE, BG, HK
Analysis and interpretation of results: PKB, CU
Draft manuscript preparation: CU, PKB
Critical revision of the article: HK, BG
Other (study supervision, fundings, materials, etc...): HK
All authors (CU, PKB, BIE, ATA, IE, BG, HK) reviewed the results and approved the final version of the manuscript.

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