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Mildronate Has Ameliorative Effects on the Experimental Ischemia/Reperfusion Injury Model in the Rabbit Spinal Cord

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■ BACKGROUND: **Mildronate is a useful anti-ischemic agent and has antiinflammatory, antioxidant, and neuroprotective activities. The aim of this study is to investigate the potential neuroprotective effects of mildronate in the experimental rabbit spinal cord ischemia/reperfusion injury (SCIRI) model.**

■ METHODS: **Rabbits were randomized into 5 groups of 8 animals as groups 1 (control), 2 (ischemia), 3 (vehicle), 4 (30 mg/kg methylprednisolone [MP]), and 5 (100 mg/kg mildronate). The control group underwent only laparotomy. The other groups have the spinal cord ischemia model by a 20-minute aortic occlusion just caudal to the renal artery. The malondialdehyde and catalase levels and caspase-3, myeloperoxidase, and xanthine oxidase activities were investigated. Neurologic, histopathologic, and ultrastructural evaluations were also performed.**

■ RESULTS: **The serum and tissue myeloperoxidase, malondialdehyde, and caspase-3 values of the ischemia and vehicle groups were statistically significantly higher than those of the MP and mildronate groups ($P < 0.001$). Serum and tissue catalase values of the ischemia and vehicle groups were statistically significantly lower than those of the control, MP, and mildronate groups ($P < 0.001$). The histopathologic evaluation showed a statistically significantly lower score in the mildronate and MP groups than in the ischemia and vehicle groups ($P < 0.001$). The modified Tarlov scores of the ischemia and vehicle groups were statistically significantly lower than those of the control, MP, and mildronate groups ($P < 0.001$).**

■ CONCLUSIONS: **This study presented the antiinflammatory, antioxidant, antiapoptotic, and neuroprotective effects of mildronate on SCIRI. Future studies will elucidate its possible use in clinical settings in SCIRI.**

INTRODUCTION

Spinal cord ischemia/reperfusion injury (SCIRI) is an unwanted event that may be seen during thoracoabdominal surgery.[1] After thoracoabdominal surgery, paraplegia is a devastating complication that affects the patient's life. Paraplegic patient care costs thousands of dollars annually.[2]

The mechanisms of SCIRI are multifactorial.[3] SCIRI has 2 stages in primary and secondary injury. The primary injury involves necrotic cell death caused by acute ischemia.[4] The secondary injury starts minutes after the primary injury. The process involves hypoxia, lipid peroxidation, inflammation, oxidative stress, and apoptosis, which could cause neurologic

Key words
- Antiapoptotic
- Antiinflammatory
- Antioxidant
- Ischemia/reperfusion
- Mildronate
- Neuroprotective

Abbreviations and Acronyms
CAT: Catalase
ELISA: Enzyme-linked immunosorbent assay
MDA: Malondialdehyde
MP: Methylprednisolone
MPO: Myeloperoxidase
ROS: Reactive oxygen species
SCI: Spinal cord ischemia
SCIRI: Spinal cord ischemia/reperfusion injury
XO: Xanthine oxidase

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Citation: World Neurosurg. (2023) 173:e717-e726.
https://doi.org/10.1016/j.wneu.2023.02.139

*Journal homepage: **www.journals.elsevier.com/world-neurosurgery***

*Available online: **www.sciencedirect.com***

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damage.[5-7] Several animal studies have successfully prevented SCIR-related damage; however, further clinical evidence is needed.[6-10] Therefore, conducting more investigation for effective drugs is necessary to protect the spinal cord against SCIRI.

Mildronate is an anti-ischemic agent that reversibly inhibits the carnitine biosynthesis, therefore decreasing fatty acid oxidation and limiting cytotoxic product production in ischemic conditions.[11,12] In addition, it limits the aggregation of toxic acylcarnitines in ischemic tissues. Mildronate has been shown to be effective in several neuroprotection studies, including cerebral ischemia, traumatic brain injury, and encephalopathy models.[13-15] Mildronate decreases neuroinflammation, increases neuronal regeneration, and prevents apoptosis.[13,14] The present study investigated the neuroprotective activity of mildronate in a SCIRI model in rabbits.

METHODS

Experimental Groups

The animals were taken care of and treated following the European Communities Council Directive, September 22, 2010 (2010/63/EU) for experimental use. Ethical approval was obtained from Saki Yenilli Laboratory Animals Facility Committee of Animal Ethics (dated March 10, 2019). Thirty-two adult male New Zealand white rabbits that weighed 2800–3750 g were randomly divided into 5 groups, with 8 rabbits in each group.

The groups were as follows:

Group 1: Control group ($n = 8$); laparotomy only wherein the rabbits underwent laminectomy without aortic cross-clamping. Nonischemic spinal cord samples were immediately obtained after surgery, without treatment.

Group 2: Ischemia group ($n = 8$); 20 minutes after transient global spinal cord ischemia (SCI), 2 mL of 0.9% NaCl was intraperitoneally applied. The spinal cord samples were evacuated 24 hours after ischemia after laminectomy.

Group 3: Vehicle group ($n = 8$); the rabbits underwent transient SCI; 2 mL 0.9% NaCl was intraperitoneally applied.

Group 4: Methylprednisolone (MP) group ($n = 8$), the rabbits were treated like those in group 2 but received a single intraperitoneal dose of 30 mg/kg of MP (Prednol, Mustafa Nevzat, Turkey) after the occlusion clamp evacuation. This MP dose was chosen based on the literature.[5-8,16,17]

Group 5: Mildronate group ($n = 8$); wherein the rabbits were given preoperative intraperitoneal injections of 100 mg/kg of mildronate (JSC Grindeks, Riga, Lithuania). This mildronate dose was chosen based on the literature.[13,14]

Anesthesia and Surgical Procedures

The rabbits were taken care of at standard room conditions. An intramuscular dose of 70 mg/kg of ketamine (Ketalar [Parke Davis Eczacıbaşı, Turkey]) and 5 mg/kg of xylazine (Rompun, Bayer, Turkey) were applied for anesthesia. Body temperatures were maintained at 37°C. An aortic cross-clamping technique was used for SCIRI.[5,6,8,18,19] The 20-minute ischemia and 24-hour reperfusion durations caused adequate injury[20] and resulted in paraplegia. Spinal cord samples between the L2-L5 segments were used for biochemical, histopathologic, and ultrastructural examination. Blood was obtained from the left ventricle for the biochemical investigations.

Serum and Tissue Caspase-3 Concentration

The serum and tissue caspase-3 concentration was measured using enzyme-linked immunosorbent assay (ELISA) (ELISA kit [Cusabio, Hubei, China]). ELISA was performed following the manufacturer's guidelines. The detailed protocol was published previously.[8] The results were expressed in nanograms per milliliter.

Serum and Tissue Myeloperoxidase Analyses

Serum and tissue myeloperoxidase (MPO) activity was measured by competitive inhibition ELISA (Cusabio), following the manufacturer's instructions. The detailed protocol was published previously.[8] The results were presented in nanograms per milliliter.

Serum and Tissue Malondialdehyde Analyses

Serum and tissue malondialdehyde (MDA) levels were determined using thiobarbituric acid. The detailed protocol was published previously.[8] The MDA levels were expressed in nanometers.

Serum and Tissue Catalase Analyses

Serum and tissue catalase (CAT) levels were determined by measuring the rate of absorbance decrease of hydrogen peroxide (H_2O_2) at 240 nm.[21] The results were presented in international units per milliliter.

Serum Xanthine Oxidase Analyses

Serum xanthine oxidase (XO) activity was measured using the technique of Prajda and Weber,[22] wherein the activity was measured by determining the amount of formed uric acid from xanthine. The detailed protocol was published previously.[8]

Histopathologic Evaluation

The obtained spinal cord samples at 24 hours after injury were prepared for histologic evaluation. The detailed protocol was published previously.[8]

Transmission Electron Microscopic Tissue Preparation and Examination Techniques

Methods for this measurement were described previously.[8] A total of 100 large myelinated axons, 100 medium myelinated axons, and 100 small myelinated axons were assessed per sample, scored from 0 to 3, and counted. The scoring was performed on 5 samples of every group. Data were then represented as mean values, as shown by Kaptanoglu et al.[23]

Neurologic Evaluation

The neurologic statuses of the rabbits were scored blindly 24 hours after surgery by evaluating the hind limb neurologic function using the modified Tarlov scoring system.[5,6,17]

Statistical Analysis

All experiments were randomly conducted by blinded investigators. Data were analyzed using GraphPad Prism 8.0

Table 1. Biochemical Results in the Experimental Groups

Variables	Control	Ischemia	Vehicle	MP	Mildronate	*P* Value
Serum caspase-3 (ng/mL)	215.3 ± 31.3*,†	421.5 ± 55.62*,‡,§	403.7 ± 54.81†	205.5 ± 42.2‡,‖	188.0 ± 32.72§,‖	<0.001
Tissue caspase-3 (ng/mL)	172.5 ± 53.98*,¶	642.8 ± 153.0*,‡,§	626.6 ± 116.8†	141.3 ± 75.44‡,‖	115.2 ± 38.30§,‖	<0.001
Serum CAT (IU/mL)	156.1 ± 41.97*,†	40.47 ± 11.90*,‡,§	54.88 ± 11.23†	112.1 ± 22.62‡,‖	111.5 ± 12.89§,‖	<0.001
Tissue CAT (IU/mL)	114.5 ± 1.79*,†	27.47 ± 10.80*,‡,§	25.79 ± 10.92§	111.5 ± 12.82‡,‖	114.9 ± 9.56§,‖	<0.001
Serum MDA (nmol/g tissue)	2.57 ± 0.51*,†	6.41 ± 1.11*,‡,§	6.55 ± 1.05†	2.51 ± 0.57‡,‖	1.82 ± 0.65§,‖	<0.001
Tissue MDA (nmol/g tissue)	3.73 ± 1.23*,†	10.66 ± 2.88*,§,#	10.98 ± 3.40†	6.39 ± 1.20‖,#	5.80 ± 1.28§,‖	<0.001
Serum MPO (ng/mL)	2.39 ± 0.46*,†	5.49 ± 2.34*,††	5.65 ± 1.35†	3.42 ± 0.88#,**	3.45 ± 0.55‖,††	<0.001
Tissue MPO (ng/mL)	3.02 ± 0.78¶,‡‡	5.09 ± 0.96‡,¶	5.51 ± 1.46‡‡	2.47 ± 1.38‡,‖	2.28 ± 1.04§,‖	<0.001
Serum xanthine oxidase (mIU/mL)	10.13 ± 9.20*,†	61.25 ± 12.75*,‡,§	57.50 ± 12.27†	6.00 ± 5.78‡,††	4.62 ± 4.71§,‖	<0.001

MP, methylprednisolone; CAT, catalase; MDA, malondialdehyde; MPO, myeloperoxidase.
*Control versus ischemia ($P < 0.001$).
†Control versus vehicle ($P < 0.001$).
‡Ischemia versus MP ($P < 0.001$).
§Ischemia versus mildronate ($P < 0.001$).
‖MP versus mildronate (not significant).
¶Control versus ischemia ($P < 0.01$).
#Ischemia versus MP ($P < 0.01$).
**Ischemia versus MP ($P < 0.05$).
††Ischemia versus mildronate ($P < 0.05$).
‡‡Control versus vehicle ($P < 0.01$).

statistical software (GraphPad Software Inc., La Jolla, California, USA). Before the analysis, test assumptions were checked. Normality was checked by inspecting the symmetry and the unimodality of histograms. The 1-way analysis of variance with post hoc Tukey multiple comparison test was used for the comparison of the multiple independent groups (comparisons between all groups). The data were expressed as means ± standard error of the mean. P values <0.05 were regarded as significant.

RESULTS

Serum and Tissue Caspase-3 Analyses

SCIRI increased caspase-3 concentrations in the damaged tissue, and there was a significant difference among the control and ischemia groups in their mean serum and tissue caspase-3 concentrations ($P < 0.001$). When MP or mildronate groups were compared with the ischemia group, a significantly decreased serum and tissue caspase-3 concentration was obtained ($P < 0.001$ for both). However, no significant difference was obtained among the MP and mildronate groups, suggesting that treatment with either mildronate or MP prevents apoptosis after SCIRI (Table 1).

Serum and Tissue MPO Analyses

The mean serum and tissue MPO activities were increased in the ischemia groups compared with the control group and the differences were statistically significant ($P < 0.001$, for serum; $P < 0.01$, for tissue MPO). The SCIRI caused increased serum and tissue MPO activities; however, compared with the ischemia group, treatment with either MP ($P < 0.05$ for serum MPO; $P < 0.001$ for tissue MPO) or mildronate ($P < 0.05$ for serum MPO; $P < 0.001$ for tissue MPO) significantly decreased the MPO activities. No significant differences were found in the serum and tissue MPO activities between the MP and mildronate groups (Table 1). Increased activities of the MPO, a marker of neutrophil migration to damaged tissue, were decreased in mildronate and MP groups, exerting the antiinflammatory activity of both drugs.

Serum and Tissue MDA Analyses

The control and ischemia groups showed significant difference in their mean serum and tissue MDA levels ($P < 0.001$, for both), showing that serum and tissue MDA levels increased by SCIRI. The comparison of the ischemia group with either the MP ($P < 0.001$ for serum; $P < 0.01$ for tissue MDA) or mildronate groups showed that the treatment significantly decreased the MDA levels ($P < 0.001$ for serum and tissue MDA). No statistically significant difference was found between the MP and mildronate groups (Table 1). Thus, both mildronate and MP prevented lipid peroxidation in SCIRI.

Serum and Tissue CAT Analyses

There was a statistically significant difference in the mean serum and tissue CAT levels among the control and ischemia groups ($P < 0.001$ for both), showing that serum and tissue CAT levels were decreased after SCIRI. Serum and tissue CAT levels were statistically significantly increased in the MP and mildronate groups compared with the ischemia group ($P < 0.001$ for both). There was no significant difference among the MP and mildronate groups (Table 1). Oxidative stress that was seen after SCIRI caused

decreased CAT levels, and mildronate and MP showed an antioxidant activity by increasing the CAT levels.

Serum XO Analyses

Compared with the control group, the serum XO activity was associated with a significant increase in the ischemia group ($P < 0.001$), whereas a significant decrease was observed in the MP and mildronate groups compared with the ischemia group ($P < 0.001$ for both). However, no significant difference was found among the MP and mildronate groups. After the SCIRI, the increased XO levels were reduced by the antiinflammatory effects of mildronate and MP (Table 1).

Histopathologic Evaluation

Normal morphology in the spinal cord samples were observed in the control group (Figure 1A). Diffuse hemorrhage and congestion were observed in the gray matter, and marked necrosis and diffuse edema were obvious in the white and gray matter in the ischemia and vehicle groups. Polymorphonuclear leukocytes, lymphocytes, and plasma cells invasion were prominent in the damaged areas. Cytoplasmic eosinophilia, loss of cytoplasmic elements, and neuronal pyknosis were observed in the ischemia groups (Figure 1B and C). In the MP and mildronate groups, spinal cord tissue was protected from ischemia and reperfusion injury (Figure 1D and E). The ischemia group showed significantly higher histopathology scores than did the control group ($P < 0.001$; Figure 2). The histopathology scores were significantly lower in the MP and mildronate groups than in the ischemia group ($P < 0.001$ for both; Figure 2). No significant difference was observed between the MP and mildronate groups (Figure 2).

The number of normal motor neurons in the anterior spinal cord was significantly lower in the ischemia group than in the control group ($P < 0.001$; Figure 3). In the MP and mildronate groups, the number of normal motor neurons in the anterior spinal cord was significantly higher than in the ischemia group ($P < 0.001$ for both; Figure 3). No significant difference was observed between the MP and mildronate groups. Either mildronate and MP seemed to prevent SCIRI histopathologically (Figure 1D and E).

Ultrastructural Evaluation

The ultrastructure of the tissue samples was evaluated with transmission electron microscopic examination. There were no ultrastructural pathologic changes in the gray and white matters of the spinal cord in the control group (Figure 4A). In only a few of the large-sized myelinated axons, mild separations were observed in a small part of the myelin sheath. This situation may be related to delayed tissue fixation. In the ischemia and vehicle groups, severe ultrastructural pathologic changes were detected both in the gray and in the white matter of the spinal cord samples (Figure 4B and C). In the gray matter, vacuoles inside the cytoplasm of the neurons were prominent. Besides, perineuronal

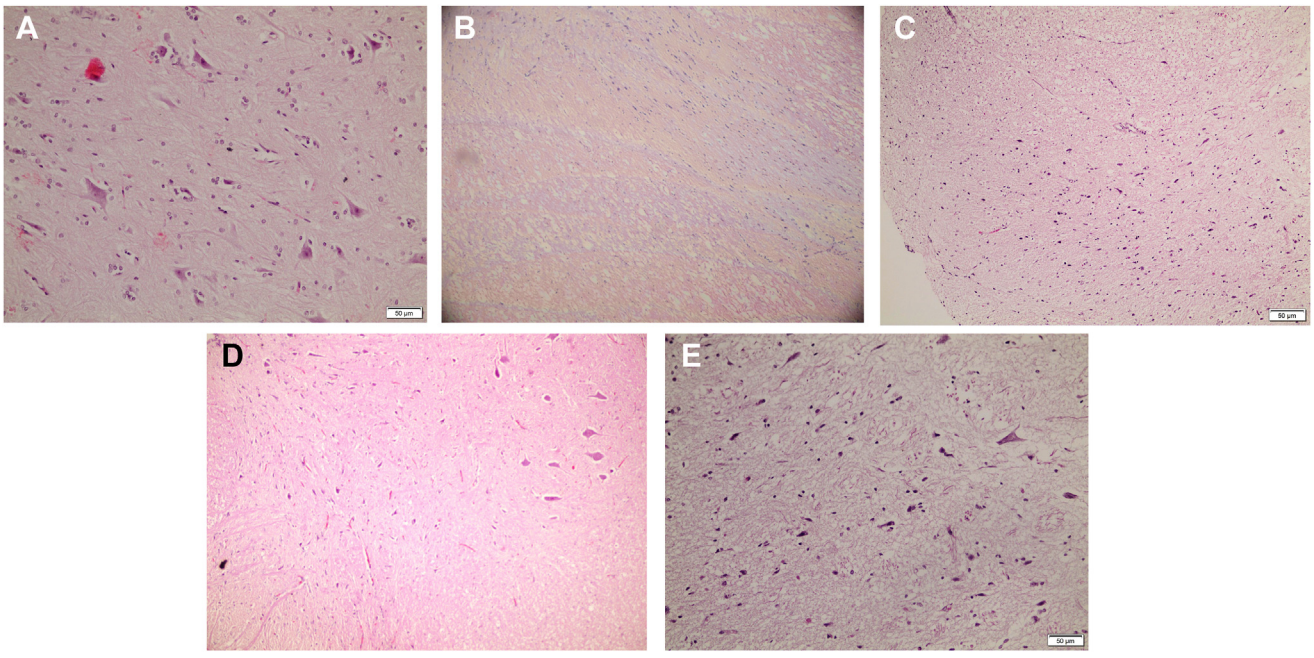

Figure 1. Photomicrographs of 5-μm spinal cord tissue sections from each study group. Images are shown with hematoxylin-eosin staining under a 10× objective. (**A**) Control group, showing normal spinal cord parenchyma and normal neurons (**B**, **C**) Ischemia and vehicle groups, showing degenerated neurons on the edematous surface. (**D**) Methylprednisolone group, showing less degenerated neurons and the normal neurons. (**E**) Mildronate group, showing less degenerated neurons and more normal neurons. Spinal cord tissue was protected from injury by mildronate pretreatment.

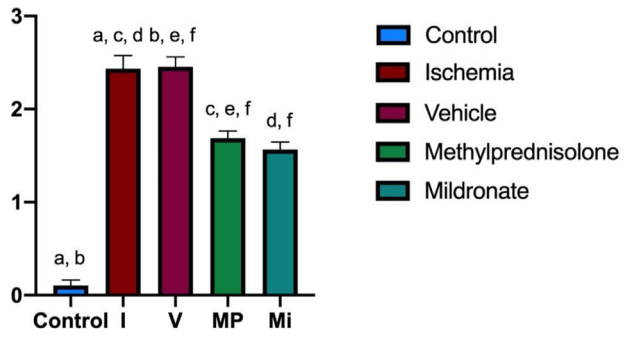

Figure 2. Distribution of histopathology score among groups. a, control versus ischemia (I) ($P < 0.001$); b, control versus vehicle (V) ($P < 0.001$); c, ischemia versus methylprednisolone (Mp) ($P < 0.001$); d, ischemia versus mildronate (Mi) ($P < 0.001$); e, vehicle versus methylprednisolone ($P < 0.001$); f, vehicle versus mildronate ($P < 0.001$).

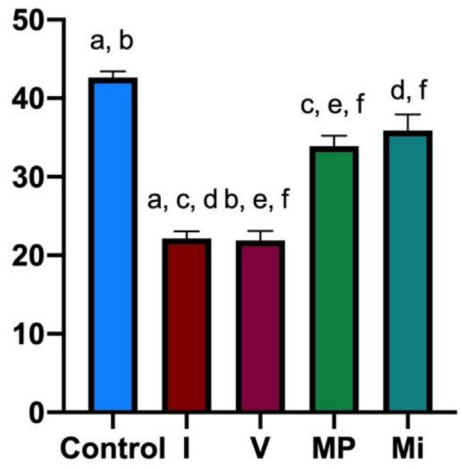

Figure 3. Distribution of normal motor neuron numbers among groups. a, control versus ischemia (I) ($P < 0.001$); b, control versus vehicle (V) ($P < 0.001$); c, ischemia versus methylprednisolone (Mp) ($P < 0.001$); d, ischemia versus mildronate (Mi) ($P < 0.001$); e, vehicle versus methylprednisolone ($P < 0.001$); f, vehicle versus mildronate ($P < 0.001$).

edema was present in these groups. The nuclei of the neurons and cell membranes were ultrastructurally normal. In the white matter, ultrastructural pathologic changes in the myelinated axons were observed. Separations in myelin configuration were seen in most of the small, medium-sized, and large myelinated axons. Interruptions were shown in the myelin configurations in some of the large and medium-sized myelinated axons. The severity of the ultrastructural pathologic changes was higher in large myelinated axons and was lowest in small myelinated axons. There was no interruption in myelin configuration in small myelinated axons. In the MP group, severe ultrastructural pathologic changes were observed both in the gray and in the white matter of the spinal cord samples (Figure 4D). In the mildronate group, ultrastructurally normal nuclei of neurons and membranes of neurons were observed (Figure 4E). Vacuoles were detected inside the cytoplasm of neurons. Perineuronal tissues did not show any ultrastructural pathologic changes. In the mildronate group, the white matter showed ultrastructurally normal small myelinated axons. Separations in myelin configuration were detected in some of these axons. The medium-sized and large myelinated axons showed separations in myelin configuration. None of the myelinated axons showed interruption in myelin configuration in the mildronate group.

The ischemia and vehicle groups had more prominent disturbances in small myelinated axons compared with the control group ($P < 0.001$). In the ischemia group, compared with the MP and mildronate groups, the small myelinated axons were protected from interruption ($P < 0.001$ for both). The MP group was more effective than the mildronate group in protecting the small myelinated axons ($P < 0.001$). In the control group, compared with the ischemia group, the medium-sized myelinated axons were damaged ($P < 0.001$). There were significant differences among the ischemia group and the MP and mildronate groups, with both treatments preserving the medium-sized axons from SCIRI ($P < 0.001$ for both). Besides, large myelinated axons were more damaged in the ischemia group than in the control group ($P < 0.001$). Significant differences were found between the ischemia group and the MP and mildronate groups, with both treatments preserving the medium-sized axons from SCIRI ($P < 0.001$ for both). No difference was observed in the MP and mildronate groups in terms of protecting the medium-sized and large myelinated axons (Table 2).

Neurologic Evaluation

In the ischemia and vehicle groups, the mean Tarlov score was significantly lower than in the control group ($P < 0.001$ for both). The mean Tarlov scores in the MP and mildronate groups were significantly higher than the ischemia group ($P < 0.001$ for both). No significant difference was found in the Tarlov scores between the MP and mildronate groups (Figure 5).

DISCUSSION

Nervous tissue, including the spinal cord, has restricted anaerobic capacity, making it vulnerable to ischemia.[8] The stored cellular energy is promptly consumed after ischemia, triggering cellular death cascades. There is paradoxic tissue response in ischemic tissues after reperfusion.[24] Accelerated apoptotic cascades, increased inflammatory response, and formation of free radicals are seen after abrupt and excessive presence of oxygen after ischemia.[5,6,17,25] There are 2 main mechanisms responsible for SCI.[8] Primary injury is irreversible and occurs when the oxygen is deprived because of the spinal cord blood flow interruption. Secondary biochemical activities are caused by primary injury[26,27] and include apoptosis, inflammation, lipid peroxidation, accumulation of reactive oxygen species (ROS), and increase in excitatory neurotransmitter.[5,6,17,25]

Ischemic injuries of the spinal cord, which lead to irreversible lesions, are seen in acute mechanical traumas secondary to vascular and tissue injuries.[4] In addition, etiopathologic

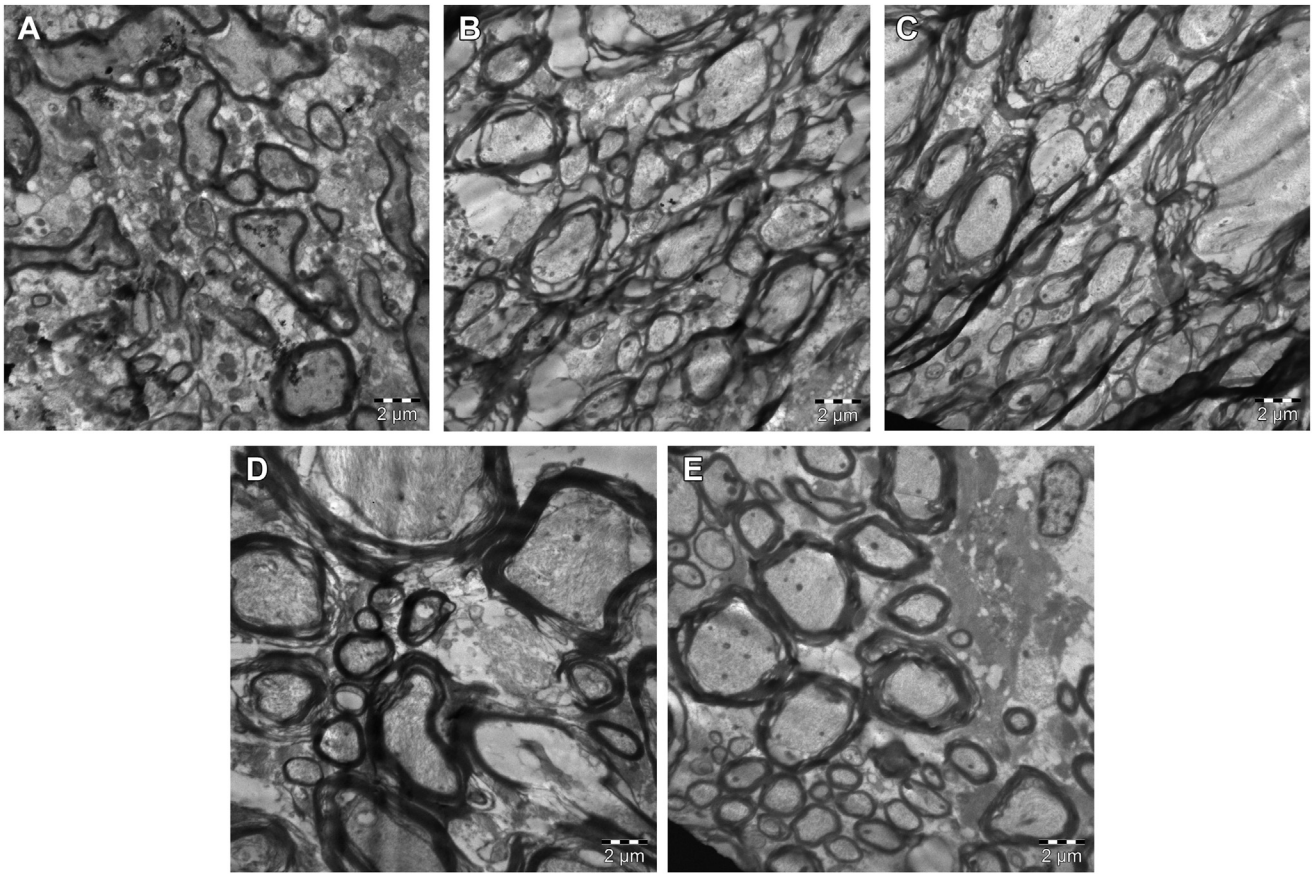

Figure 4. Representative transmission electron micrographs for each group. (**A**) Control group, showing ultrastructurally normal myelinated axons. (**B**, **C**) Ischemia and vehicle groups, showing small, medium-sized, and large axons with myelin configuration separations. (**D**) Methylprednisolone group, showing myelin configuration separations in medium-sized and large myelinated axons less than the ischemia groups. (**E**) Mildronate group, showing small, medium-sized, and large myelinated axons with myelin configuration separations less than the ischemia groups (original amplification = 5000, scale bar = 2 µm, for all).

importance of nontraumatic injuries has emerged, involving those that are caused by spinal ischemia, especially paraplegia, which affects up to 22% of patients having thoracoabdominal aneurysm surgery.[28] During thoracic aortic surgery, the main targets are to support the blood flow to the spinal cord, shorten the cord ischemia duration during the surgery, immediately intervene with SCI, and make the spinal cord less vulnerable to infarction.[29] Plenty of therapeutic approaches have been tried (e.g., drainage of cerebrospinal fluid,[30] shunt use,[31] hypothermia treatment,[32] and experimental pharmacologic drugs[5,6,17,25,33]).

Mildronate was produced in the 1980s as an anti-ischemic agent.[34] In ischemic tissues, the biosynthesis of carnitine and cytotoxic degradation products of fatty acid β-oxidation were inhibited with mildronate.[35] Mildronate decreases the level of carnitine that plays role in the transfer of long-chain fatty acids into the mitochondrial matrix for β-oxidation. Moreover, it controls the ratio of acetyl coenzyme A to coenzyme A in the mitochondria, thus regulating glycolysis and pyruvate dehydrogenase activity.[36,37] Mildronate increases the amount of γ-butyrobetaine and inhibits L-carnitine biosynthesis and fatty acid oxidation. When fatty acid oxidation is inhibited in ischemic tissues, glucose is used as the energy substrate.[38-40] In healthy people, the administration of mildronate decreases plasma carnitine levels.[41] Glucose is the main energy source of the mammalian brain,[42] rather than a fatty acid. Rather than fatty acid metabolism, membrane receptors and secondary messengers of these receptors are the main ways for mildronate to show its activity on the central nervous system.[43] In neuronal cells, it triggers DNA replication, repair, methylation, and RNA polymerase activity.[43] In addition, a previous study[14] investigated the anti-ischemic effect of mildronate in a rabbit subarachnoid hemorrhage—induced vasospasm model and showed that mildronate exerted vasodilatory and neuroprotective effects.

After SCIRI, apoptosis is one of the most important pathways that resulted in cellular death.[5,8] Decreased blood flow causes acute ischemia, which results in adenosine triphosphate depletion and necrosis.[44,45] Mild and major ischemia and reperfusion cause apoptosis[46,47] via caspases, which are cysteine

Table 2. Electron Microscopic Results

Myelinated Axon	Control	Ischemia	Vehicle	MP	Mildronate	*P* Value
Small-sized	0.0 ± 0.0*,†	88.40 ± 1.14*,‡	88.40 ± 1.51†,§	0.0 ± 0.0‡,§	22.60 ± 2.60‖,¶,#	<0.001
Middle-sized	0.0 ± 0.0*,†	109.8 ± 1.92*,‡	109.0 ± 2.23†,§	70.60 ± 2.30‡,§	74.00 ± 1.58‖,¶	<0.001
Large-sized	5.0 ± 1.58*,†	124.2 ± 2.04*,‡	123.0 ± 1.0†,§	89.0 ±1.58‡,§	91.60 ± 1.14‖,¶	<0.001

MP, methylprednisolone.
*Control versus ischemia ($P < 0.001$).
†Control versus vehicle ($P < 0.001$).
‡Ischemia versus MP ($P < 0.001$).
§Vehicle versus MP ($P < 0.001$).
‖Ischemia versus mildronate ($P < 0.001$).
¶Vehicle versus mildronate ($P < 0.001$).
#MP versus mildronate ($P < 0.001$).

protease family enzymes.[48] Caspase-3, one of the main apoptotic enzymes, transforms interleukin into its mature form.[49] Previous SCIRI studies have shown that caspase-3 is a trustworthy marker of apoptosis.[5,6,17,25] Caspase-3 concentration increases after 15 minutes of SCI.[45] Thus, after ischemic events, increased caspase-3 concentration triggers DNA fragmentation.[49] Previously, it was shown that mildronate had antiapoptotic activities,[13] with evidence that it decreased caspase-3 levels at the tissue level. Our study showed that caspase-3 concentrations were increased 24 hours after SCIRI. Mildronate and MP treatments were linked with lower spinal cord caspase-3 concentrations, which preserved the spinal cord from apoptotic damage.

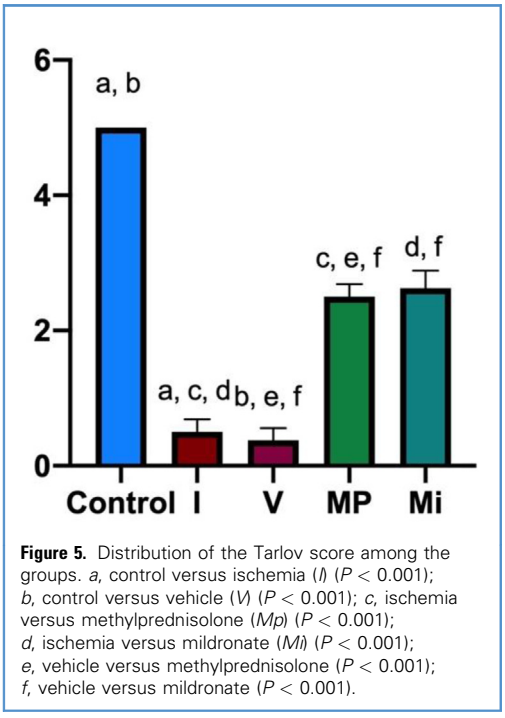

Figure 5. Distribution of the Tarlov score among the groups. *a*, control versus ischemia (*I*) ($P < 0.001$); *b*, control versus vehicle (*V*) ($P < 0.001$); *c*, ischemia versus methylprednisolone (*Mp*) ($P < 0.001$); *d*, ischemia versus mildronate (*Mi*) ($P < 0.001$); *e*, vehicle versus methylprednisolone ($P < 0.001$); *f*, vehicle versus mildronate ($P < 0.001$).

Neuroinflammation is an essential part of cellular death after SCIRI.[50] Mildronate has an antiinflammatory effect on the nervous and other tissues.[13,34,35,43,51] In traumatic brain injury and ischemic stroke models in rats, the neuroinflammation was decreased with the effect of mildronate.[13,51] The inflammatory response after SCIRI play an important role in pathophysiology.[27,52] In the spinal cord, proinflammatory cytokines were released from microglial cells and resulted in inflammatory cells entering the spinal cord tissue.[53] Neutrophils, monocytes, and macrophages are important in the inflammatory response and have a central role in reperfusion injury.[54] It was hypothesized that antiinflammatory substances can prevent SCIRI.[26,27,54] In the injured spinal cord, MPO activity is increased.[55] Our study showed that in the serum and tissue after SCIRI, MPO activity was increased, and mildronate and MP decreased that MPO activity. Increased MPO activity after SCIRI indicated the increased accumulation of neutrophils and their inflammatory activity, whereas treatment with mildronate decreased MPO activity via an antiinflammatory activity. This result was consistent with previous studies that showed the antiinflammatory activity of mildronate.[13]

Neuronal damage from SCIRI involves increased free radical production and lipid peroxidation. Increased free radical production was associated with cellular death.[56] Homeostasis was found between oxygen-free radicals and antioxidative molecules during physiologic cellular oxidative respiratory activities.[47] Cellular membrane destruction is the most essential step in neuronal injury.[57,58] Lipid peroxidation, which is a major factor in secondary SCIRI, occurs at the lipid bilayer.[59] MDA is an indicator of lipid peroxidation and is the waste product of polyunsaturated fatty acid degeneration.[60] MDA levels, showing lipid peroxidation secondary to reperfusion injury, increase after SCIRI.[61] In addition, our study showed that both serum and tissue MDA levels increased in the ischemia group. Mildronate and MP treatments preserved the increased MDA levels associated with SCIRI, which addressed that both treatments could decrease lipid peroxidation within the spinal cord.

ROS production after SCIRI is one of the important parts of secondary injury.[62] An antioxidant enzyme, CAT, has a buffering effect on ROS.[18] Severe molecular damage occurs after excessive

oxidative stress caused by consumed CAT.[5,6,17,25] After traumatic brain injury, mildronate decreases superoxide dismutase levels, which has a similar action mechanism to CAT.[13] Consistent with this finding, serum and tissue CAT levels decreased after the SCIRI in this study, and mildronate and MP treatments increased the CAT levels. This finding proved their high antioxidant properties. XO enzyme is another ROS source that is reliable for assessing the degree of oxidative stress.[24,63] Our study showed increased serum XO activity in the ischemia group, and mildronate and MP dramatically decreased the XO activity. Therefore, this finding suggested that mildronate potentially has an important role in neuroprotection through its antioxidant activity on CAT and XO.

Our study showed that in the ischemia and vehicle groups, prominent hemorrhage, increased edema, and necrosis that resulted from SCIRI were evident. Polymorphonuclear leukocytes, lymphocytes, and plasma cells, which are well-known inflammatory indicators, were seen to migrate to the damaged areas. In the ischemia and vehicle groups, the normal motor neuron number decreased compared with the control group. The histopathologic results in the mildronate and MP groups were better than those in the ischemia and vehicle groups, with the number of motor neurons preserved in both treatment groups. Ultrastructural changes showed the numerous segregations in small, medium-sized, and large myelinated axons. Mildronate or MP treatment preserved the small, medium-sized, and large axons.

Moreover, this study assessed the functional outcomes and evaluated the neurologic functions of the rabbits using the Tarlov scoring system. Some degree of paresis was observed in all rabbits; however, the severity was ameliorated in the mildronate and MP groups. Thus, mildronate and MP improved the functional outcomes besides their ameliorative effects on biochemical and histopathologic changes.

Methylprednisolone is an antiinflammatory and antioxidant agent that has historical importance in spinal cord injury.[4] No randomized controlled trial data have suggested that MP is effective in SCI[64]; however, it is preferred in control groups in animal experiments because of its experimental efficacy.[5,6,17,25] Thus, we compared mildronate with MP as an active control group. Because we do not have an effective pharmacologic alternative in the clinical settings of SCIRI, mildronate gave us hope that this medication could be an effective and affordable treatment alternative.

As in every experimental study, this study also had limitations. The number of animals could have been higher to increase the reliability of the results. In addition, various dosage regimens, with different treatment timing, could have been preferred. This study showed the results at 24 hours after injury, which limits the interpretation of the results in the clinical settings. More comprehensive parameters could have been studied. Also, changes over time with an extended study protocol are therefore needed. Mildronate was applied before SCIRI, which could decrease its daily use, especially in emergencies. Thus, further research is required to clarify the role of mildronate treatment in SCIRI.

CONCLUSIONS

Biochemically, histologically and functionally mildronate provided promising neuroprotection against SCIRI in a rabbit model. Additional experimental and randomized controlled studies are required to identify the promising neuroprotective effects of mildronate in SCIRI.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Dilan Ozaydin: Conceptualization, Methodology, Writing — original draft. **Pınar Kuru Bektaşoğlu:** Conceptualization, Methodology, Formal analysis, Writing — original draft, Visualization. **Durukan Türe:** Methodology. **Hüseyin Bozkurt:** Methodology. **Berrin İmge Ergüder:** Data curation. **Mustafa Fevzi Sargon:** Data curation. **Ata Türker Arıkök:** Data curation. **Hayri Kertmen:** Conceptualization, Methodology, Investigation, Writing — review & editing, Funding acquisition, Resources, Supervision. **Bora Gürer:** Conceptualization, Methodology, Investigation, Data curation, Writing — review & editing, Project administration.

REFERENCES

1. Crawford ES, Crawford JL, Safi HJ, et al. Thoracoabdominal aortic aneurysms: preoperative and intraoperative factors determining immediate and long-term results of operations in 605 patients. *J Vasc Surg*. 1986;3:389-404.

2. French DD, Campbell RR, Sabharwal S, Nelson AL, Palacios PA, Gavin-Dreschnack D. Health care costs for patients with chronic spinal cord injury in the Veterans Health Administration. *J Spinal Cord Med*. 2007;30:477-481.

3. Melissano G, Bertoglio L, Mascia D, et al. Spinal cord ischemia is multifactorial: what is the best protocol? *J Cardiovasc Surg*. 2016;57:191-201.

4. Kwon BK, Tetzlaff W, Grauer JN, Beiner J, Vaccaro AR. Pathophysiology and pharmacologic treatment of acute spinal cord injury. *Spine J*. 2004;4:451-464.

5. Gürer B, Kertmen H, Kasim E, et al. Neuroprotective effects of testosterone on ischemia/reperfusion injury of the rabbit spinal cord. *Injury*. 2015;46:240-248.

6. Kertmen H, Gürer B, Yılmaz ER, et al. The protective effect of low-dose methotrexate on ischemia-reperfusion injury of the rabbit spinal cord. *Eur J Pharmacol*. 2013;714:148-156.

7. Kertmen H, Celikoglu E, Ozturk OC, et al. Comparative effects of methylprednisolone and tetracosactide (ACTH1-24) on ischemia/reperfusion injury of the rabbit spinal cord. *Arch Med Sci*. 2018;14:1459-1470.

8. Gürer B, Karakoç A, Bektaşoğlu PK, et al. Comparative effects of vitamin D and methylprednisolone against ischemia/reperfusion injury of rabbit spinal cords. *Eur J Pharmacol*. 2017;813:50-60.

9. Hou J, Li H, Xue C, Ma J. Lidocaine relieves spinal cord ischemia-reperfusion injury via long non-coding RNA MIAT-mediated Notch1 down-regulation. *J Biochem*. 2022;171:411-420.

10. Zhang S, Yan Y, Wang Y, et al. Inhibition of MALT1 alleviates spinal ischemia/reperfusion injury-induced neuroinflammation by modulating glial endoplasmic reticulum stress in rats. *J Inflamm Res*. 2021;14:4329-4345.

11. Dambrova M, Liepinsh E, Kalvinsh I. Mildronate: cardioprotective action through carnitine-lowering effect. *Trends Cardiovasc Med*. 2002;12:275-279.

12. Matsuoka M, Igisu H. Preservation of energy metabolites by carnitine in the mouse brain under ischemia. *Brain Res*. 1992;590:334-336.

13. Demir D, Kuru Bektaşoğlu P, Koyuncuoğlu T, et al. Neuroprotective effects of mildronate in a rat model of traumatic brain injury. *Injury*. 2019;50:1586-1592.

14. Eser MT, Bektasoglu PK, Gurer B, et al. The vasorelaxant and neuroprotective effects of mildronate in A rabbit subarachnoid hemorrhage model. *Turk Neurosurg*. 2020;30:163-170.

15. Dziak LA, Golik VA. Primenenie Mildronata pri lechenii bol'nykh distsirkuliatornoĭéntsefalopatieĭ na fone stenoziruiushchego porazheniia magistral'nykh arteriĭ golovy [Use of mildronate for the treatment of patients with circulatory encephalopathy against a background of stenosis of major arteries of the head]. *Lik Sprava*. 2003:98-101. Russian.

16. Kahveci R, Kahveci FO, Gokce EC, et al. Effects of ganoderma lucidum polysaccharides on different pathways involved in the Development of spinal cord ischemia reperfusion injury: biochemical, histopathologic, and ultrastructural analysis in a rat model. *World Neurosurg*. 2021;150:e287-e297.

17. Yilmaz ER, Kertmen H, Dolgun H, et al. Effects of darbepoetin-α in spinal cord ischemia-reperfusion injury in the rabbit. *Acta Neurochir*. 2012;154:1037-1043 [discussion: 1043-1044].

18. Ilhan A, Yilmaz HR, Armutcu F, Gurel A, Akyol O. The protective effect of nebivolol on ischemia/reperfusion injury in rabbit spinal cord. *Prog Neuro-Psychopharmacol Biol Psychiatry*. 2004;28:1153-1160.

19. Lukácová N, Halát G, Chavko M, Marsala J. Ischemia-reperfusion injury in the spinal cord of rabbits strongly enhances lipid peroxidation and modifies phospholipid profiles. *Neurochem Res*. 1996;21:869-873.

20. Zivin JA, DeGirolami U. Spinal cord infarction: a highly reproducible stroke model. *Stroke*. 1980;11:200-202.

21. Aebi H. [13] catalase in vitro. *Methods Enzymol*. 1984;105:121-126.

22. Prajda N, Weber G. Malignant transformation-linked imbalance: decreased xanthine oxidase activity in hepatomas. *FEBS Lett*. 1975;59:245-249.

23. Kaptanoglu E, Palaoglu S, Surucu HS, Hayran M, Beskonakli E. Ultrastructural scoring of graded acute spinal cord injury in the rat. *J Neurosurg*. 2002;97(1 Suppl):49-56.

24. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: the evolution of a concept. *Redox Biol*. 2015;6:524-551.

25. Gokce EC, Kahveci R, Atanur OM, et al. Neuroprotective effects of Ganoderma lucidum polysaccharides against traumatic spinal cord injury in rats. *Injury*. 2015;46:2146-2155.

26. Cassada DC, Gangemi JJ, Rieger JM, et al. Systemic adenosine A2A agonist ameliorates ischemic reperfusion injury in the rabbit spinal cord. *Ann Thorac Surg*. 2001;72:1245-1250.

27. Fan L, Wang K, Shi Z, Die J, Wang C, Dang X. Tetramethylpyrazine protects spinal cord and reduces inflammation in a rat model of spinal cord ischemia-reperfusion injury. *J Vasc Surg*. 2011;54:192-200.

28. van den Berg ME, Castellote JM, Mahillo-Fernandez I, de Pedro-Cuesta J. Incidence of spinal cord injury worldwide: a systematic review. *Neuroepidemiology*. 2010;210:184-192.

29. Bicknell CD, Riga CV, Wolfe JH. Prevention of paraplegia during thoracoabdominal aortic aneurysm repair. *Eur J Vasc Endovasc Surg*. 2009;37:654-660.

30. Safi HJ, Miller CC 3rd, Huynh TT, et al. Distal aortic perfusion and cerebrospinal fluid drainage for thoracoabdominal and descending thoracic aortic repair: ten years of organ protection. *Ann Surg*. 2003;238:372-380 [discussion: 380-381].

31. Brodbelt AR, Stoodley MA. Post-traumatic syringomyelia: a review. *J Clin Neurosci*. 2003;10:401-408.

32. Dietrich WD, Levi AD, Wang M, Green BA. Hypothermic treatment for acute spinal cord injury. *Neurotherapeutics*. 2011;8:229-239.

33. Sanli AM, Serbes G, Sargon MF, et al. Methotrexate attenuates early neutrophil infiltration and the associated lipid peroxidation in the injured spinal cord but does not induce neurotoxicity in the uninjured spinal cord in rats. *Acta Neurochir*. 2012;154:1045-1054.

34. Pupure J, Isajevs S, Skapare E, et al. Neuroprotective properties of mildronate, a mitochondria-targeted small molecule. *Neurosci Lett*. 2010;470:100-105.

35. Sjakste N, Kleschyov AL, Boucher JL, et al. Endothelium- and nitric oxide-dependent vasorelaxing activities of gamma-butyrobetaine esters: possible link to the antiischemic activities of mildronate. *Eur J Pharmacol*. 2004;495:67-73.

36. Klusa V, Beitnere U, Pupure J, et al. Mildronate and its neuroregulatory mechanisms: targeting the mitochondria, neuroinflammation, and protein expression. *Medicina (Kaunas)*. 2013;49:301-309.

37. Vilskersts R, Liepinsh E, Kuka J, et al. Myocardial infarct size-limiting and anti-arrhythmic effects of mildronate orotate in the rat heart. *Cardiovasc Drugs Ther*. 2009;23:281-288.

38. Shutenko ZhV, Kagan TI, Sjakste NI, Kalvin'sh IIa. Mildronat: mechanisms of action, perspective for pathology correction. *Khim-Pharm Zh*. 1995;29:13-17.

39. Zvejniece L, Svalbe B, Makrecka M, Liepinsh E, Kalvinsh I, Dambrova M. Mildronat exerts acute anticonvulsant and antihypnotic effects. *Behav Pharmacol*. 2010;21:548-555.

40. Dzintare M, Baumane L, Meirena D, Lauberte L, Kalvinsh I, Sjakste N. Involvement of nitric oxide production in the mildronat mechanism of action. *Pharmacol Rev Commun*. 2002;12:163-170.

41. Liepinsh E, Konrade I, Skapare E, et al. Mildronate treatment alters gamma-butyrobetaine and l-carnitine concentrations in healthy volunteers. *J Pharm Pharmacol*. 2011;63:1195-1201.

42. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends Neurosci*. 2013;36:587-597.

43. Sjakste N, Gutcaits A, Kalvinsh I. Mildronat: an antiischemic drug for neurological indications. *CNS Drug Rev*. 2005;11:151-168.

44. Hayashi T, Sakurai M, Abe K, Sadahiro M, Tabayashi K, Itoyama Y. Apoptosis of motor neurons with induction of caspases in the spinal cord after ischemia. *Stroke*. 1998;29:1007-1012 [discussion: 1013].

45. Sakurai M, Nagata T, Abe K, Horinouchi T, Itoyama Y, Tabayashi K. Survival and death-promoting events after transient spinal cord ischemia in rabbits: induction of Akt and caspase 3 in motor neurons. *J Thorac Cardiovasc Surg*. 2003;125:370-377.

46. Hearse DJ, Bolli R. Reperfusion induced injury: manifestations, mechanisms, and clinical relevance. *Cardiovasc Res*. 1992;26:101-108.

47. Lou J, Lenke LG, Ludwig FJ, O'Brien MF. Apoptosis as a mechanism of neuronal cell death following acute experimental spinal cord injury. *Spinal Cord*. 1998;36:683-690.

48. Emery E, Aldana P, Bunge MB, et al. Apoptosis after traumatic human spinal cord injury. *J Neurosurg*. 1998;89:911-920.

49. Li M, Ona VO, Chen M, et al. Functional role and therapeutic implications of neuronal caspase-1 and -3 in a mouse model of traumatic spinal cord injury. *Neuroscience*. 2000;99:333-342.

50. Zhu P, Li JX, Fujino M, Zhuang J, Li XK. Development and treatments of inflammatory cells and cytokines in spinal cord ischemia-reperfusion injury. *Mediators Inflamm*. 2013;2013:701970.

51. Rumaks J, Pupure J, Svirskis S, et al. Search for stroke-protecting agents in endothelin-1-induced ischemic stroke model in rats. *Medicina (Kaunas)*. 2012;48:525-531.

52. Hasturk A, Atalay B, Calisaneller T, Ozdemir O, Oruckaptan H, Altinors N. Analysis of serum pro-inflammatory cytokine levels after rat spinal cord ischemia/reperfusion injury and correlation with tissue damage. *Turk Neurosurg*. 2009;19:353-359.

53. Li BC, Li Y, Chen LF, Chang JY, Duan ZX. Olfactory ensheathing cells can reduce the tissue loss but not the cavity formation in contused spinal cord of rats. *J Neurol Sci*. 2011;303:67-74.

54. Reece TB, Okonkwo DO, Ellman PI, et al. The evolution of ischemic spinal cord injury in function, cytoarchitecture, and inflammation and the effects of adenosine A 2A receptor activation. *J Thorac Cardiovasc Surg*. 2004;128:925-932.

55. Taoka Y, Okajima K, Uchiba M, et al. Role of neutrophils in spinal cord injury in the rat. *Neuroscience*. 1997;79:1177-1182.

56. Bethea JR, Dietrich WD. Targeting the host inflammatory response in traumatic spinal cord injury. *Curr Opin Neurol*. 2002;15:355-360.

57. Demopoulos HB, Flamm ES, Pietronigro DD, Seligman ML. The free radical pathology and the microcirculation in the major central nervous system disorders. *Acta Physiol Scand Suppl*. 1980; 492:91-119.

58. Solaroglu I, Kaptanoglu E, Okutan O, Beskonakli E, Attar A, Kilinc K. Magnesium sulfate treatment decreases caspase-3 activity after experimental spinal cord injury in rats. *Surg Neurol*. 2005;64:S17-S21.

59. Diaz-Ruiz A, Rios C, Duarte I, et al. Lipid peroxidation inhibition in spinal cord injury: cyclosporin-A vs methylprednisolone. *Neuroreport*. 2000;11:1765-1767.

60. Pandey KB, Rizvi SI. Markers of oxidative stress in erythrocytes and plasma during aging in humans. *Oxid Med Cell Longev*. 2010;3:2-12.

61. Christie SD, Comeau B, Myers T, Sadi D, Purdy M, Mendez I. Duration of 1lipid peroxidation after acute spinal cord injury in rats and the effect of methylprednisolone. *Neurosurg Focus*. 2008;25:E5.

62. Chan PH. Role of oxidants in ischemic brain damage. *Stroke*. 1996;27:1124-1129.

63. Hille R, Nishino T. Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. *FASEB J*. 1995;9:995-1003.

64. Cheung V, Hoshide R, Bansal V, Kasper E, Chen CC. Methylprednisolone in the management of spinal cord injuries: Lessons from randomized, controlled trials. *Surg Neurol Int*. 2015;6:142.

Received 29 November 2022; accepted 28 February 2023

Citation: World Neurosurg. (2023) 173:e717-e726.
https://doi.org/10.1016/j.wneu.2023.02.139

Journal homepage: ***www.journals.elsevier.com/world-neurosurgery***

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