Neuroprotective Effects of Dexpanthenol on Rabbit Spinal Cord Ischemia/Reperfusion Injury Model

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■ OBJECTIVE: **Dexpanthenol (DXP) reportedly protects tissues against oxidative damage in various inflammation models. This study aimed to evaluate its effects on oxidative stress, inflammation, apoptosis, and neurological recovery in an experimental rabbit spinal cord ischemia/reperfusion injury (SCIRI) model.**

■ METHODS: **Rabbits were randomized into 5 groups of 8 animals each: group 1 (control), group 2 (ischemia), group 3 (vehicle), group 4 (methylprednisolone, 30 mg/kg), and group 5 (DXP, 500 mg/kg). The control group underwent laparotomy only, whereas other groups were subjected to spinal cord ischemia by aortic occlusion (just caudal to the 2 renal arteries) for 20 min. After 24 h, a modified Tarlov scale was employed to record neurological examination results. Malondialdehyde and caspase-3 levels and catalase and myeloperoxidase activities were analyzed in tissue and serum samples. Xanthine oxidase activity was measured in the serum. Histopathological and ultrastructural evaluations were also performed in the spinal cord.**

■ RESULTS: **After SCIRI, serum and tissue malondialdehyde and caspase-3 levels and myeloperoxidase and serum xanthine oxidase activities were increased ($P < 0.05$–0.001). However, serum and tissue catalase activity decreased significantly ($P < 0.001$). DXP treatment was associated with lower malondialdehyde and caspase-3 levels and reduced myeloperoxidase and xanthine oxidase activities but increased catalase activity ($P < 0.05$–0.001). Furthermore, DXP was associated with better histopathological, ultrastructural, and neurological outcome scores.**

■ CONCLUSIONS: **This study was the first to evaluate antioxidant, anti-inflammatory, antiapoptotic, and neuroprotective effects of DXP on SCIRI. Further experimental and clinical investigations are warranted to confirm that DXP can be administered to treat SCIRI.**

INTRODUCTION

The spine aids in transferring body weight to the lower extremities, thereby protecting body posture and improving balance. In addition, it protects the spinal cord, which transmits neural messages from the brain to the peripheral nervous system and vice versa.[1-3]

Spinal cord injury may develop because of trauma, tumor, infection, neurodegenerative diseases, and thoracoabdominal

Key words
- Antiapoptotic
- Anti-inflammatory
- Antioxidant
- Dexpanthenol
- Ischemia/reperfusion
- Neuroprotection
- Spinal cord

Abbreviations and Acronyms
CAT: Catalase
DXP: Dexpanthenol
ELISA: Enzyme-Linked immunosorbent assay
IR: Ischemia/reperfusion
MDA: Malondialdehyde
MP: Methylprednisolone
MPO: Myeloperoxidase
PA: Pantothenic acid
SCIRI: Spinal cord ischemia/reperfusion injury
TBA: Thiobarbituricacid
XO: Xanthine oxidase

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aortic surgery. Spinal cord injury may disturb motor, sensory, and autonomic functions temporarily or permanently. Injury basically occurs through 2 main mechanisms. Primary injury develops after ischemia occurs, with loss of blood supply in the spinal cord, and this injury is irreversible. Following primary injury, cellular mechanisms are activated and a reversible secondary injury process begins.[4,5]

Reperfusion is the return of blood flow that has stopped or slowed down during ischemia to normal. While blood flow is regulated by reperfusion, tissue damage develops because of oxygenation, biochemical and molecular changes that occur during ischemia, and the formation of free oxygen radicals. This is called ischemia/reperfusion (IR) injury.[6]

In the United States, descending aortic aneurysms in the thoracoabdominal region are the 12th leading cause of death. Approximately 43,000–47,000 patients die each year owing to aneurysms originating from the aorta and its branches. In Europe, approximately 110,000–125,000 patients die every year owing to aortic aneurysms. When thoracic endovascular aneurysm repair is performed for the descending thoracic aorta, the risk of developing spinal cord ischemia is 4%–7%. In open surgery, this risk is 2%–28%.[7,8]

The spinal cord is extremely susceptible to ischemia because of its limited glycogen stores and limited anaerobic metabolic capacity. When blood flow to the tissue decreases, cellular energy stores are rapidly depleted, activating ischemic cascades that cause cellular death.[9] Reperfusion following ischemia results in more cellular death, leading to oxidative stress, increased oxidant mechanisms, inflammation, and increased apoptotic activity.[9-14] Spinal cord injury can also develop in patients undergoing thoracoabdominal surgery.[15] Various pharmacological drugs, cerebrospinal fluid drainage, hypothermia, and shunting have been attempted to prevent spinal cord ischemia/reperfusion injury (SCIRI).[9-14,16-19]

Dexpanthenol (DXP), a stable alcohol analog of pantothenic acid (PA, vitamin B5), has been tested in many IR models and is reportedly effective in treating IR injury owing to its antioxidant, anti-inflammatory, and antiapoptotic properties. DXP is commonly used in clinical practice safely for years. This study investigated the effects of DXP in reducing reperfusion injury and oxidative stress employing various markers of oxidative stress and apoptosis in tissues and serum. In addition, histopathological differences, the number and density of myelinated axons, and ultrastructural changes were measured and analyzed.

MATERIALS AND METHODS

Experimental Groups

Animals were cared for and treated in accordance with the guidelines of the European Communities Council Directive, September 22, 2010 (2010/63/EU). Each aspect of this study was assessed and endorsed by the ethical board of trustees of the Saki Yenilli Laboratory Animals Facility Committee of Animal Ethics (dated 10/03/2019). Thirty-two adult male New Zealand white rabbits weighing 2800–3750 g were arbitrarily separated into the following 5 groups: group 1, control group (n = 8); group 2, ischemia group (n = 8); group 3, vehicle group (n = 8); group 4, methylprednisolone [MP] group (n = 8), and group 5, DXP group (n = 8). Group 1 rabbits underwent a laminectomy, and non-ischemic spinal cord samples were obtained immediately after surgery. This group received no treatment. Group 2 rabbits were subjected to transient global spinal cord ischemia and underwent a laminectomy, following which spinal cord samples were obtained at 24 h post ischemia. Group 3 rabbits were subjected to transient global spinal cord ischemia. The same volume of saline (2 cc 0.9% NaCl) was intraperitoneally infused instantly after the occlusion clamp was removed. The animals then underwent laminectomy and spinal cord samples were obtained 24 h post ischemia. Group 4 rabbits were treated the same as those in group 2 but also received a single intraperitoneal dose of 30 mg/kg MP (Prednol; Mustafa Nevzat, Istanbul, Turkey) after the occlusion clamp was removed. This MP dose was selected based on previous studies.[9-11,13,20,21] Group 5 rabbits were treated the same as those in group 2 but were administered intravenous injection of 500 mg/kg DXP (Bepanthen vial; Roche, Berlin, Germany) following the occlusion period, when the clamps were removed and restoration of blood flow was visually observed. This DXP dose was selected based on previous studies.[22,23]

Anesthesia and Surgical Procedures

The rabbits were fed ad libitum standard chow and water at an ideal room temperature (18°C–21°C) and were kept under a 12-h light/12-h dark cycle. They were anesthetized with an intramuscular dose of 70 mg/kg ketamine (Ketalar; Parke Davis Eczacıbaşı, İstanbul, Turkey) and 5 mg/kg xylazine (Rompun; Bayer, İstanbul, Turkey), which they were permitted to spontaneously inhale. Body temperatures were measured using a rectal thermometer (digital fever thermometer; Becton Dickinson, Franklin Lakes, NJ, USA) and were maintained at 37°C with a warming cushion. The rabbits were placed in a supine position while undergoing surgery. After sterilization, a 10-cm midline incision was made and the abdominal aorta was approached through a transperitoneal route. A 150 U/kg dose of Nevparin (Mustafa Nevzat, İstanbul, Turkey) was intravenously administered as an anticoagulant 5 min before clamping. Approximately 1 cm beneath the renal artery, the aorta was clipped under a surgical microscope using an aneurysm clip with a closing force of 70 g (Yasargil, FE721; Aesculap, Tuttlingen, Germany). The cross-clamping time was 20 min. At the end of the occlusion period, the clamps were removed and restoration of blood flow was visually observed. The rabbit aortic cross-clamping technique used in this study is an established and valuable one,[11,13,20,24] with the 20-min ischemia period providing satisfactory damage to blood flow. The rabbits were permitted free access to food and water 2 h after surgery. Credé's maneuver was performed at least twice a day for rabbits with a neurogenic bladder. All rabbits were sacrificed 24 h after surgery by the infusion of high-dose pentobarbital (200 mg/kg; Nembutal; Oak Pharmaceuticals, Lake Forest, IL, USA). Spinal cord fragments at the L2–L5 level were precisely uprooted by laminectomy and used for biochemical, histopathological, and ultrastructural investigations. Blood (10 cc) was obtained from the left ventricle for biochemical examination and was centrifuged at 1000 × g for 5 min; the upper clear supernatant was used for analysis. All serum and tissue test samples were stored at −80°C until further analysis. On the day of analysis, the tissue was homogenized in physiological saline (1/5 wt/vol) using a

homogenizer (B. Braun Melsungen AG 853202; Melsungen, Germany) and centrifuged at 1780 × g for 20 min. The protein level of the clear supernatant was estimated using the Lowry's method and adjusted to equal concentrations before analyses. Serum samples obtained from the upper clear supernatant of the centrifuged blood samples were used for biochemical examinations.

Serum and Tissue Caspase-3 Levels

Serum and tissue caspase-3 levels were measured using the enzyme-linked immunosorbent assay (ELISA; ELISA kit; Cusabio, Hubei, China). ELISA was performed as per the manufacturer's instructions. This technique includes a quantitative sandwich protein immunoassay. Antibodies specific for caspase-3 were precoated onto a microplate. Standards and samples were pipetted into wells, allowing precoated antibodies to bind the caspase-3 that was present in the samples and standards, if any. After removing unbound substances, a biotin-conjugated antibody specific for caspase-3 was added to the wells. After washing, avidin-conjugated horseradish peroxidase was added to the wells and any unbound substances were removed by 3 washes with a washing buffer before the avidin-protein reagent was added to the wells. The intensity of the color that developed was proportional to the concentration of caspase-3 bound in the initial step. When the color development stopped, the color intensity was measured at 450 nm. Caspase-3 levels were ascertained by comparing the solutions. The results are expressed in nanograms per milliliter.

Serum and Tissue Myeloperoxidase Analyses

Serum and tissue myeloperoxidase (MPO) activity was measured with a competitive inhibition ELISA (Cusabio, Hubei, China) according to the manufacturer's instructions. The provided microtiter plate was precovered with an antibody specific for MPO. Standards or samples were added to appropriate microtiter plate wells with biotin-conjugated MPO, and a competitive inhibition reaction was started between MPO (from standards or samples) and biotin-conjugated MPO with the precoated antibodies specific for MPO. With greater amounts of MPO, lower amounts of antibodies are bound by the biotin-conjugated MPO. After washing, avidin-conjugated horseradish peroxidase was added to the wells. The substrate solution was subsequently added and the color was allowed to develop, which indicated the amount of MPO in the sample. When the color development stopped, the color intensity was measured at 450 nm. MPO activities were determined by comparing the absorbance values of the samples with those of the standard MPO solutions. The results are expressed in nanograms per milliliter.

Serum and Tissue Malondialdehyde Analyses

Serum and tissue malondialdehyde (MDA) levels were determined using thiobarbituric acid (TBA). Briefly, specimens were blended with 2 volumes of cold saline liquid containing 0.001% butylated hydroxytoluene and 0.07% sodium dodecyl sulfate. Next, 1 mL of each of the samples was added to 500 μL of TBA with 0.01 μL of NH_2SO_4 (0.67% TBA in half-acidic corrosive) to precipitate the proteins. The specimens were then warmed in boiling water for 1 h, cooled, and mixed with an equivalent volume (2 mL) of n-butanol. The mixture was centrifuged at 1780 × g for 10 min at room temperature. The absorbance of the organic layer was read at 535 nm in a 1-mL cell (Molecular Devices Corporation, Sunnyvale, CA, USA). MDA levels were determined by comparing the absorbance values of the samples with those of the standard MDA solutions. The results are expressed in nanomoles.

Serum and Tissue Catalase Analyses

Serum and tissue catalase (CAT) activities were measured using the rate of absorbance decrease in H_2O_2 at 240 nm.[25] For calculating CAT levels, extinction coefficients of H_2O_2 (40.98 L mol^{-1} cm^{-1} at 240 nm) were used. The results are expressed in International Units per milliliter.

Serum Xanthine Oxidase Analyses

Serum xanthine oxidase (XO) activity was measured with the technique of Prajda and Weber,[73] in which activity is measured by determining the amount of uric acid formed from xanthine. Using this technique, serum samples (100 μL) were incubated for 30 min at 37°C in 3 mL of phosphate buffer (pH 7.5, 50 nM) containing xanthine (4 mM). The reaction was halted by adding 0.1 mL of 100% (wt/vol) trichloroacetic acid, and the mixture was then centrifuged at 1780 × g for 20 min. Uric acid levels were determined in the supernatant by measuring absorbance at 292 nm compared to a blank, and the results are presented in milli-international units per milliliter. A calibration curve was constructed using 10–50 mU/mL concentrations of standard XO solutions (Sigma X-1875; Sigma Aldrich, St. Louis, MO, USA). One unit of activity was defined as 1 μmol of uric acid that formed per minute at 37°C and pH 7.5.

Histopathological Evaluation

Spinal cord samples obtained at 24 h post injury were prepared for histological evaluation. Each cord section was submerged in 4% paraformaldehyde with 0.1 mol/l phosphate buffer and then stored at 4°C. The samples were subsequently embedded in paraffin, cut into 5-μm thick sections, and stained with hematoxylin and eosin. The samples were inspected with light microscopy by a neuropathologist who was blinded to the study plan. Five distinct fields of spinal cord gray matter were assessed using a 40× objective. A semiquantitative scoring system, with scores ranging 0–3, was used to evaluate histopathological changes in the spinal cord tissues of all samples. Six distinct parameters (i.e., hemorrhage, congestion, necrosis, edema, neuronal loss, and inflammation) were histopathologically evaluated and scored as follows: 0 = negligible, 1 = mild, 2 = moderate, and 3 = common. The histopathology score for each spinal cord sample was determined by averaging the scores of the 6 parameters.[12] Furthermore, a point-by-point appraisal of the level of ischemic neuronal injury was performed in a similar manner. In this investigation, the quantity of typical motor neurons in the anterior horn of the spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis) was determined. For every rabbit, 3 areas were assessed using a 40× objective. An average normal motor neuron count for the areas from each rabbit was then obtained. Neurons that contained Nissl substances, loose chromatin, and prominent nucleoli were considered normal viable neurons.[26]

Transmission Electron Microscopic Tissue Preparation and Examination Techniques

The tissue samples were placed in 2.5% glutaraldehyde for 24 h for primary fixation. The samples were then washed with Sorenson's phosphate buffer solution (pH: 7.4) and postfixed in 1% osmium tetroxide for 1 h. After postfixation, they were washed with the same buffer. Following this procedure, the tissue samples were dehydrated in increasing concentrations of alcohol. After dehydration, the tissues were washed with propylene oxide and embedded in epoxy resin embedding media. The semithin and ultrathin sections of the obtained tissue blocks were cut with an ultramicrotome (LKB Nova, Elkin, NC, USA). These semithin sections, which were 2-μm thick, were stained with methylene blue and examined with light microscopy (Nikon, Tokyo, Japan). Following this procedure, the tissue blocks were trimmed into ultrathin sections, which were about 60-nm thick, using the same ultramicrotome. These ultrathin sections were stained with uranyl acetate and lead citrate and then examined under a Jeol JEM 1200 EX (Japan) transmission electron microscope. Electron micrographs of the specimens were obtained using the same transmission electron microscope at 5000× magnification. A total of 100 large, 100 medium, and 100 small myelinated axons were assessed per sample, and they were scored within 0–3 as follows: 0 = normal ultrastructure of myelinated axons, 1 = separation in myelin configuration, 2 = interruption in myelin configuration, and 3 = honeycomb appearance of the myelin configuration and then counted. The scoring was performed for 5 samples from each group. Data were then presented as mean values as reported by Kaptanoglu et al.[27]

Neurological Evaluation

The neurological status of each rabbit was scored at 24 h after surgery by evaluating hindlimb neurological function according to the modified Tarlov scoring system.[10,13] A score of 0–5 was allocated to each rabbit as follows: 0 = no voluntary hindlimb movement; 1 = perceptible joint movement; 2 = active movement but unable to sit without help; 3 = able to sit but unable to hop; 4 = weak hop; and 5 = complete recovery of hindlimb function. A medical doctor blinded to the experimental groups performed the neurological evaluation.

Statistical Analysis

All experiments were randomized and conducted by blinded investigators. Data were analyzed using GraphPad Prism 8.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA). Test assumptions were checked prior to the analyses. Normality was checked by inspecting the symmetry and unimodality of the data histograms. To compare multiple independent groups, a one-way analysis of variance with post hoc Tukey's multiple comparison test was employed (comparisons among all groups). The data were expressed as the mean ± standard error of the mean. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

Serum and Tissue Caspase-3 Analyses

A significant difference was observed between the control and ischemia groups in their mean serum and tissue caspase-3 levels ($P < 0.001$), with SCIRI clearly increasing caspase-3 levels in the injured tissues. When either the MP or DXP group was compared with the ischemia group, a significant decrease in serum and tissue caspase-3 levels was observed ($P < 0.001$ for both). Moreover, no significant difference was noted between the MP and DXP groups, suggesting that treatment with either DXP or MP inhibits apoptosis after SCIRI (Table 1).

Serum and Tissue MPO Analyses

Significant differences were observed between the control and ischemia groups in the mean serum and tissue MPO activities ($P < 0.01$, $P < 0.05$ respectively). Although SCIRI increased serum and tissue MPO activities compared with the ischemia group, treatment with either MP ($P < 0.01$) or DXP ($P < 0.001$) significantly decreased tissue MPO activities but did not change serum MPO activities (Table 1). Thus, the activity of MPO, a marker of neutrophil migration to injured tissues, decreased with DXP and MP treatments, indicating the anti-inflammatory actions of these drugs.

Serum and Tissue MDA Analyses

A significant difference was found between the control and ischemia groups in the mean serum and tissue MDA levels ($P < 0.001$, for both), indicating that serum and tissue MDA levels were increased after SCIRI. Comparison of the ischemia group with either the MP ($P < 0.001$ for serum; $P < 0.01$ for tissue MDA) or DXP group revealed that treatment significantly decreased MDA levels ($P < 0.01$ for serum and $P < 0.05$ for tissue MDA). When the MP and DXP groups were compared, no statistically significant difference was found (Table 1). Thus, both DXP and MP appeared to prevent lipid peroxidation after SCIRI.

Serum and Tissue CAT Analyses

A significant difference was observed between the control and ischemia groups in the mean serum and tissue CAT levels ($P < 0.001$, for both), indicating that tissue CAT levels were decreased after SCIRI. Compared with the ischemia group, serum and tissue CAT levels were significantly increased in the MP and DXP groups ($P < 0.001$, for both), and there were no significant differences between the MP and DXP groups (Table 1). CAT levels decreased owing to oxidative stress after SCIRI, and DXP and MP exerted an antioxidant effect by increasing CAT levels.

Serum XO Analyses

Serum XO activity was associated with a significant increase in the ischemia group compared with the control group ($P < 0.001$). Compared with the ischemia group, serum XO activity was significantly decreased in the MP and DXP groups ($P < 0.001$ for both); again, however, there was no significant difference between the MP and DXP groups. After SCIRI, increased activities of the inflammatory marker XO appeared to be reduced by the secondary anti-inflammatory actions of DXP and MP (Table 1).

Histopathological Evaluations

Light microscopy results from the spinal cord samples of the control group were normal (Figure 1A). In the ischemia and vehicle experimental groups, diffuse hemorrhaging and congestion were observed in the gray matter at 24 h after SCIRI,

Table 1. Biochemical Results in the Experimental Groups

Variable	Control	Ischemia	Vehicle	MP	DXP	*P* value
Serum caspase-3 (ng/ml)	215.3 ± 31.3*§	421.5 ± 55.62*	403.7 ± 54.81§	205.5 ± 42.2¶	170.9 ± 69.6**	<0.001
Tissue caspase -3 (ng/ml)	172.5 ± 53.98*§	642.8 ± 153.0*	626.6 ± 116.8§	141.3 ± 75.44¶	85.42 ± 76.22**	<0.001
Serum CAT (IU/ml)	156.1 ± 41.97*§	40.47 ± 11.90*	54.88 ± 11.23§	112.1 ± 22.62¶	109.5 ± 17.93**	<0.001
Tissue CAT (IU/ml)	114.5 ± 1.79*§	27.47 ± 10.80*	25.79 ± 10.92§	111.5 ± 12.82¶	103.9 ± 18.55**	<0.001
Serum MDA (nmol/g tissue)	2.57 ± 0.51*§	6.41 ± 1.11*	6.55 ± 1.05§	2.51 ± 0.57¶	4.50 ± 1.48††	<0.001
Tissue MDA (nmol/g tissue)	3.73 ± 1.23*§	10.66 ± 2.88*	10.98 ± 3.40§	6.39 ± 1.20#	6.68 ± 1.84‡‡	<0.001
Serum MPO (ng/ml)	2.39 ± 0.46†§	5.49 ± 2.34†	5.65 ± 1.35§	3.80 ± 1.37	3.77 ± 1.30	<0.001
Tissue MPO (ng/ml)	3.02 ± 0.78‡‖	5.09 ± 0.96‡	5.51 ± 1.46‖	2.47 ± 1.38#	1.48 ± 1.32**	<0.001
Serum XO (mIU/ml)	10.13 ± 9.20*§	61.25 ± 12.75*	57.50 ± 12.27§	6.00 ± 5.78¶	9.50 ± 6.02**	<0.001

CAT, catalase; DXP, dexpanthenol; MDA, malondialdehyde; MP, methylprednisolone; MPO, myeloperoxidase; XO, xanthine oxidase.
*Control versus ischemia ($P < 0.001$).
†Control versus ischemia ($P < 0.01$).
‡Control versus ischemia ($P < 0.05$).
§Control versus vehicle ($P < 0.001$).
‖Control versus vehicle ($P < 0.01$).
¶Ischemia versus MP ($P < 0.001$).
#Ischemia versus MP ($P < 0.01$).
**Ischemia versus DXP ($P < 0.001$).
††Ischemia versus DXP ($P < 0.01$).
‡‡Ischemia versus DXP ($P < 0.05$).

and marked necrosis and diffuse edema were observed in the white and gray matter. In the injured areas, invading polymorphonuclear leukocytes, lymphocytes, and plasma cells were observed. Neuronal pyknosis, loss of cytoplasmic elements, and cytoplasmic eosinophilia were also observed in the ischemia group (Figure 1B and C). In the MP and DXP groups, the spinal cord tissue was protected from IR damage (Figure 1D and E). When the histopathology scores were analyzed, the ischemia and vehicle experimental groups demonstrated significantly higher scores than the control group ($P < 0.001$ for both, Figure 2). The histopathology scores were significantly lower in the MP and DXP groups than in the ischemia group ($P < 0.001$ and $P < 0.01$ respectively, Figure 2). Furthermore, no significant difference was observed between the MP and DXP groups (Figure 2).

In the ischemia and vehicle experimental groups, the number of normal motor neurons in the anterior spinal cord was significantly lower than that in the control group ($P < 0.001$, Figure 3). In the MP and DXP groups, the number of normal motor neurons in the anterior spinal cord was significantly higher than that in the ischemia group ($P < 0.001$ for both, Figure 3). No significant difference was observed between the MP and DXP groups. Histopathologically, both DXP and MP appeared to prevent SCIRI (Figure 1D and E).

Ultrastructural Evaluation

In the transmission electron microscopy results for the tissue samples of the control group, ultrastructural pathological changes were not observed in the gray matter and white matter of the spinal cord (Figure 4A). The neurons in the gray matter were ultrastructurally normal in appearance, and the intracellular organelles, nuclei, membranes, and perineuronal tissues did not show any pathological changes. In this group, all of the small- and medium-sized myelinated axons had a normal ultrastructure. However, mild separations were observed in a small part of the myelin sheath in only a few of the large-sized myelinated axons. This may be related to delayed fixation of the tissue.

The transmission electron microscopic results for the tissue samples of the ischemia and vehicle groups showed severe ultrastructural pathological changes both in the gray matter and white matter of the spinal cord samples (Figure 4B and C). In the ultrastructural examination of the gray matter, vacuoles were found inside the cytoplasm of the neurons. In addition, perineuronal edema was present. In all of the neurons, the ultrastructures of the nuclei and cell membranes were normal. In the examination of the white matter, ultrastructural pathological changes were observed in the myelinated axons. In most of the small-sized, medium-sized, and large-sized myelinated axons, separations in the myelin configuration were observed. Furthermore, in some of the large-sized and medium-sized myelinated axons, interruptions were observed in the myelin configurations. The severity of the ultrastructural pathological changes was the highest in the large-sized myelinated axons and the lowest in the small-sized myelinated axons. Additionally, no interruption in the myelin configuration was observed in the small-sized myelinated axons.

The transmission electron microscopy results from the tissue samples of the MP group showed severe ultrastructural

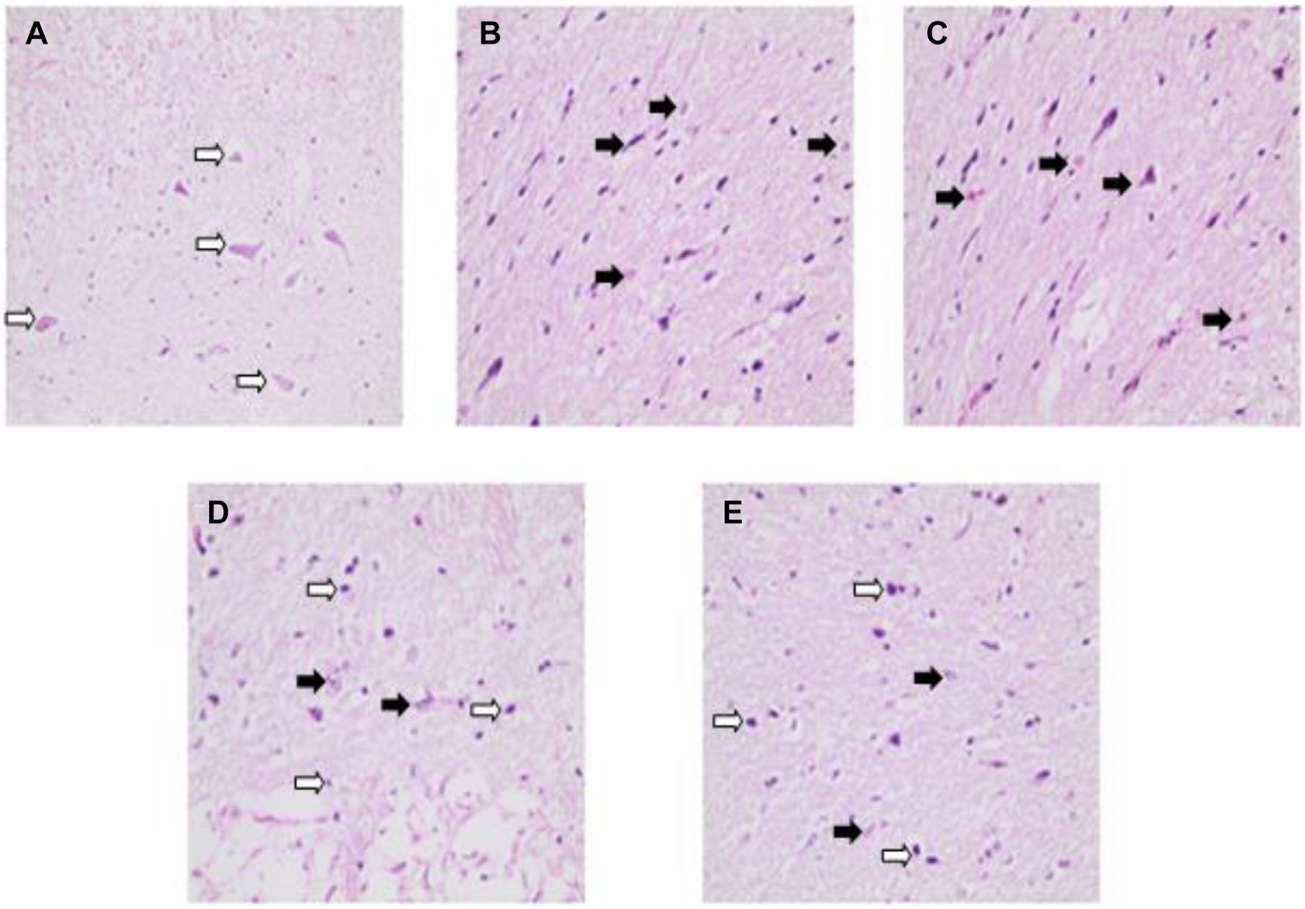

Figure 1. Photomicrographs of 5-μm spinal cord tissue sections from each study group. Images are shown for hematoxylin–eosin staining under a 40× objective. (**A**) Control group showing normal spinal cord parenchyma. Normal neurons are indicated with *arrows*. (**B**–**C**) Ischemia and vehicle experimental groups showing hyperchromatic degenerated neurons (*filled arrows*) on an edematous surface. (**D**) Methylprednisolone group showing less degenerated neurons (*filled arrows*); note the normal neurons (*hollow arrows*). (**E**) Dexpanthenol group showing less degenerated neurons (*filled arrows*) and more normal neurons (*hollow arrows*). Spinal cord tissue was protected from injury by dexpanthenol treatment.

pathological changes both in the gray matter and white matter of the spinal cord samples (Figure 4D). In the ultrastructural examination of the gray matter, vacuoles were observed inside the cytoplasm of the neurons. Additionally, perineuronal edema was present in these groups. The nuclei of the neurons and the cell membranes were ultrastructurally normal. In the examination of the white matter, ultrastructural pathological changes were observed in the myelinated axons. In small-sized, medium-sized, and large-sized myelinated axons, separations in the myelin configuration were observed in most of the myelinated axons. In addition, in some large-sized and medium-sized myelinated axons, interruptions were observed in the myelin configurations. The severity of the ultrastructural pathological changes was the highest in the large-sized myelinated axons and the lowest in the small-sized myelinated axons. Further, no interruption in the myelin configuration was observed in the small-sized myelinated axons.

In the transmission electron microscopic examination of the gray matter in the tissue samples of the DXP group, nuclei and membranes of the neurons were found to be damaged ultrastructurally (Figure 4E). In most medium-sized and large-sized myelinated axons, separations were observed in the myelin configuration.

The ischemia group had more prominent disturbances in the myelinated axons of all sizes compared to the control group ($P < 0.001$ for all). Compared with the ischemia control group, MP protected myelinated axons of all sizes from interruption ($P < 0.001$ for all). Significant differences were observed between the MP and DXP treatments in protecting the myelinated axons of all sizes and DXP did not show any protective efficacy in SCIRI at an ultrastructural level (Table 2).

Neurological Evaluation

The mean Tarlov scores in the ischemia and vehicle experimental groups were significantly lower than that in the control group

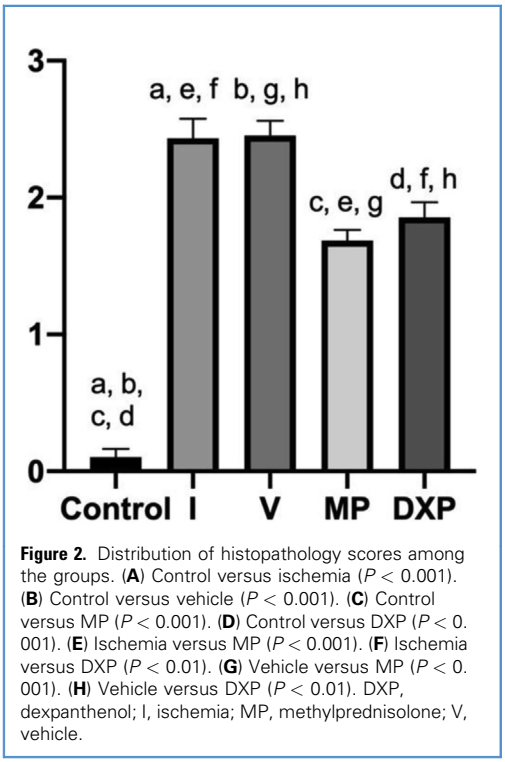

Figure 2. Distribution of histopathology scores among the groups. (**A**) Control versus ischemia ($P < 0.001$). (**B**) Control versus vehicle ($P < 0.001$). (**C**) Control versus MP ($P < 0.001$). (**D**) Control versus DXP ($P < 0.001$). (**E**) Ischemia versus MP ($P < 0.001$). (**F**) Ischemia versus DXP ($P < 0.01$). (**G**) Vehicle versus MP ($P < 0.001$). (**H**) Vehicle versus DXP ($P < 0.01$). DXP, dexpanthenol; I, ischemia; MP, methylprednisolone; V, vehicle.

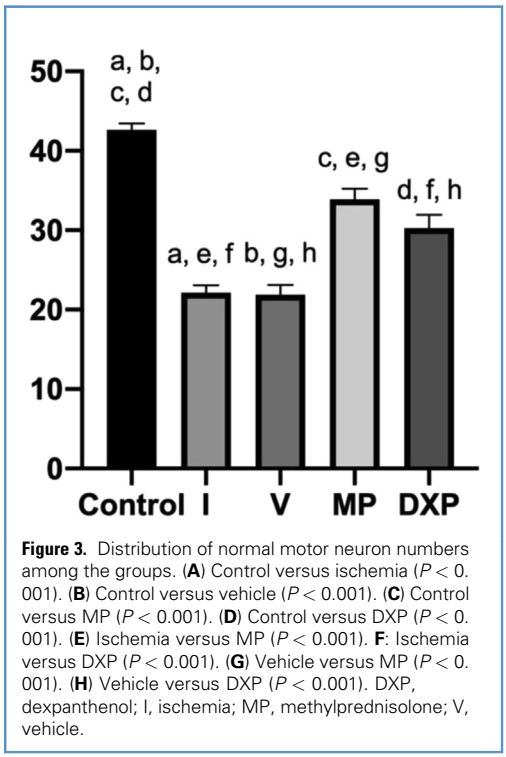

Figure 3. Distribution of normal motor neuron numbers among the groups. (**A**) Control versus ischemia ($P < 0.001$). (**B**) Control versus vehicle ($P < 0.001$). (**C**) Control versus MP ($P < 0.001$). (**D**) Control versus DXP ($P < 0.001$). (**E**) Ischemia versus MP ($P < 0.001$). **F**: Ischemia versus DXP ($P < 0.001$). (**G**) Vehicle versus MP ($P < 0.001$). (**H**) Vehicle versus DXP ($P < 0.001$). DXP, dexpanthenol; I, ischemia; MP, methylprednisolone; V, vehicle.

($P < 0.001$, for both). Consistent with the other analyses, the mean Tarlov scores in the MP and DXP groups were significantly higher than in the ischemia group ($P < 0.001$, for both). Clinically, there was also no significant difference in Tarlov scores between the MP and DXP groups (Figure 5).

DISCUSSION

Spinal Cord Ischemia and Reperfusion Injury

IR injury is a common condition that occurs after myocardial and cerebral infarction, major trauma, shock, and surgeries requiring temporary vascular clamping.[28] The central nervous system and spinal cord are extremely susceptible to the effects of ischemia because of low glycogen stores and limited anaerobic metabolism capacity. When blood flow to neuroglial tissue is reduced, cellular energy stores are rapidly depleted and ischemic cascades are activated, which cause cell death.[9] Reperfusion following ischemia causes additional cellular death due to oxidative stress, increased oxidant mechanisms, inflammation, and increased apoptotic activity.[9-14] Spinal cord damage basically occurs as a result of 2 mechanisms. Primary injury is irreversible and occurs when the blood supply to the spinal cord is lost and the oxygen supply thereby reduced. Furthermore, following primary injury, some cellular mechanisms are activated that cause a secondary injury, which is reversible.[4,5]

Paraplegia secondary to SCIRI after surgical interventions to the descending and thoracoabdominal aorta is an unpredictable and catastrophic complication. While hypoperfusion occurring during aortic cross-clamping causes a primary injury, reperfusion occurring after clamp removal activates secondary cellular mechanisms, resulting in additional spinal cord injury. Though the mechanism of primary injury is hypoxia, reactive oxygen species accumulate with reperfusion, lipid peroxidation, and inflammation, and advanced cellular death occurs with apoptosis.[5] Cerebrospinal fluid drainage,[16] hypothermia,[18] and shunting[19] have been attempted to prevent SCIRI. In addition, several pharmacological agents have been studied experimentally to prevent this type of damage.[9-14]

Dexpanthenol

DXP [(2R)-2,4-dihydroxy-N-(3-hydroxypropyl)-3,3-dimethylbutanamide] is a stable alcohol analog of PA (vitamin B5).[21] DXP is converted intracellularly to PA,[29] which is a member of the vitamin B group (vitamin B5) and the basic building block of CoA.[21] PA has been known for its antioxidant, anti-inflammatory, and antiapoptotic effects since a very long time.[20,21] PA and its derivatives have been shown to reduce oxidative stress due to IR damage in many tissues.[30,31] A previous study on monkeys showed a reduction in the negative effects of alcohol on motor activity with PA.[32] In addition, PA not only exerts a serious antioxidant effect by increasing intracellular glutathione levels[33] but also exerts an anti-inflammatory effect by inhibiting the release of MPO in human polymorphonuclear neutrophils.[34] Furthermore, the antiapoptotic properties of PA have been previously demonstrated.[21]

DXP is a widely available and easily accessible drug that has been safely used for a long time for its anti-inflammatory, antioxidant, and fibroblast proliferation–stimulating effects.[35] In

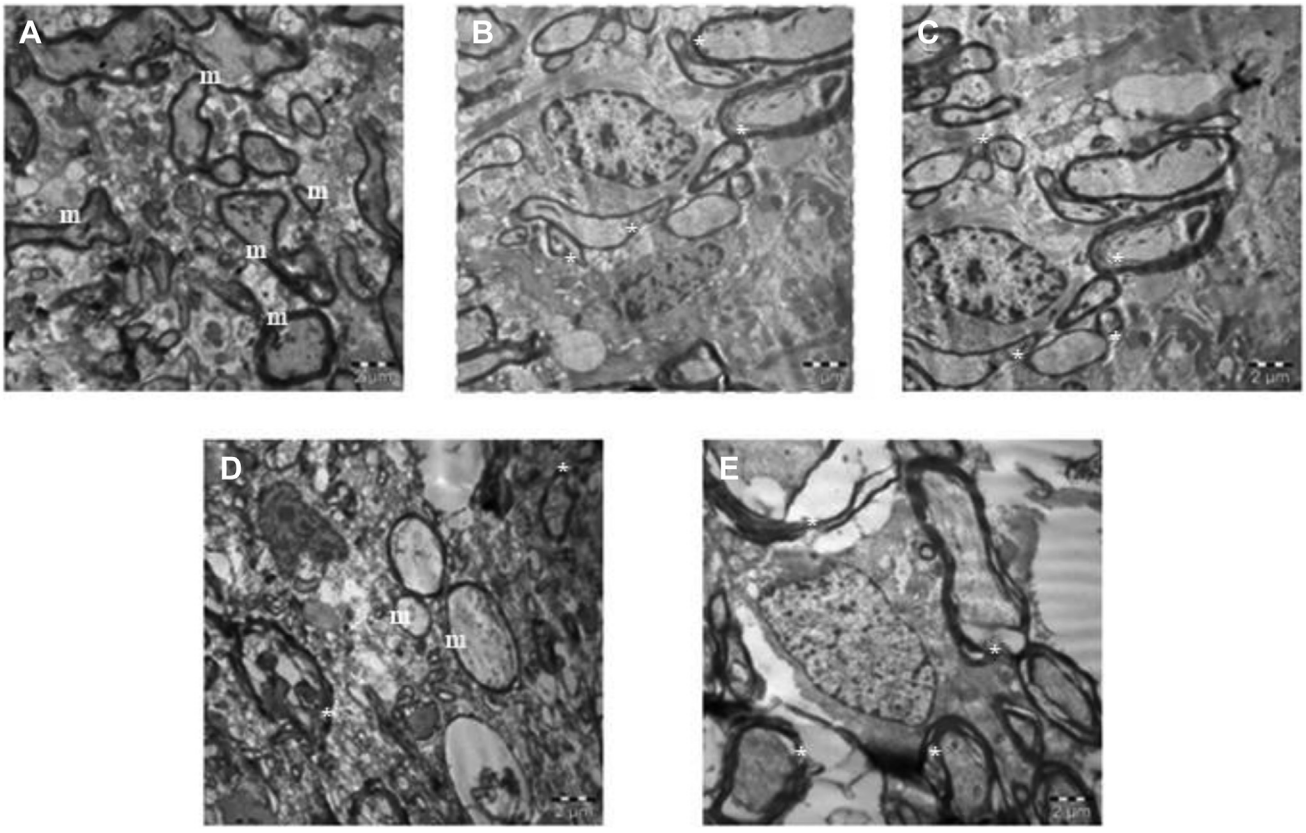

Figure 4. Representative transmission electron micrographs for each group. (**A**) Control group showing ultrastructurally normal myelinated axons (m). (**B, C**) Ischemia and vehicle experimental groups showing small-, medium-, and large-sized axons with separations in the myelin configuration (*).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

previous studies, it has been found to be effective in the experimental treatment of ovarian IR,[36] testicular IR,[37] renal IR,[38,39] mesenteric IR,[35] and liver IR.[40] Its neuroprotective effects have also been demonstrated experimentally.[22,41,42] Studies on sciatic nerve damage[41,42] have shown that DXP has antioxidant and anti-inflammatory effects on neural tissue. In addition, Zakaria et al.[22] treated brain IR injury experimentally with DXP in a middle cerebral artery occlusion model in rats and demonstrated its antioxidant activity in brain tissue.[22]

As mentioned above, DXP is a drug that has been tested and shown to be effective in many IR models. However, its effects on SCIRI have not been studied to date. In this study, the antioxidant, anti-inflammatory, and antiapoptotic properties of DXP were tested in a rabbit SCIRI model and its possible neuroprotective activity was investigated. In addition, DXP efficiency was compared with MP. MP is an antioxidant and anti-inflammatory agent that has traditionally been used in spinal cord injury.[43] It has lost its place in today's clinical practice and many authors currently question its routine clinical use. However, it remains valuable for use as an effective control in experimental SCIRI and trauma studies.[9-14]

In this study, a rabbit aortic cross-clamping method was used. After a 20-min ischemia period, the animals exposed to perfusion for 24 h were shown to have severe spinal cord injury. This experimental method has been used in previous studies, and it has been a proven and reliable experimental model for SCIRI.[9-14]

Oxidative Stress, SCIRI, and DXP

Tissue hypoxia is the initiator of secondary injuries that occur with reperfusion after spinal cord ischemia. Oxidative damage is one of the leading mechanisms of neuronal death due to excessive production of reactive oxygen radicals during both ischemia and reperfusion processes.[44] The negative effects of reactive oxygen radicals on cellular integrity and functions are well known. Various mechanisms underlie the destructive effects, some of which are lipid peroxidation, degradation of membrane proteins, and DNA damage.[45-47] The antioxidant and free radical-binding activity of DXP has been repeatedly reported in the literature.[22,23,35-37,48-51] The mechanisms underlying the antioxidant activity of DXP, however, remain unclear. When oxygen radicals are formed in the cell, endogenous antioxidant

Table 2. Electron Microscopic Results

Myelinated Axon	Control	Ischemia	Vehicle	MP	DXP	*P* value
Small sized	0.0 ± 0.0*†§	88.40 ± 1.14*‖	88.40 ± 1.51†¶	0.0 ± 0.0‖¶#	90.40 ± 1.81§#	<0.001
Middle sized	0.0 ± 0.0*†‡§	109.8 ± 1.92*‖	109.0 ± 2.23†¶	70.60 ± 2.23‡‖¶#	111.2 ± 1.30§#	<0.001
Large sized	5.0 ± 1.58*†‡§	124.2 ± 2.04*‖	123.0 ± 1.0†¶	89.0 ± 1.58‡‖¶#	125.4 ± 1.67§#	<0.001

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

mechanisms are activated. Superoxide, glutathione, and CAT are the most important of these antioxidant enzymes.[52] Glutathione rapidly eliminates oxygen radicals inside cells. It is also effective in DNA repair, activation of transcription factors, regulation of the cell cycle, organizing enzyme activity, and providing calcium hemostasis.[33,53] Previous studies have shown that DXP has the effect of increasing glutathione levels under its antioxidant activity[22,54] In addition, DXP is converted to PA inside the cell. It increases the levels of PA, coenzyme A, and glutathione.[37] Furthermore, PA prevents collapse of the mitochondrial membrane potential and maintains ATP synthesis at an optimum level. It also increases antioxidant enzyme levels, such as CAT, glutathione peroxidase, and glutathione reductase.[21,54,55]

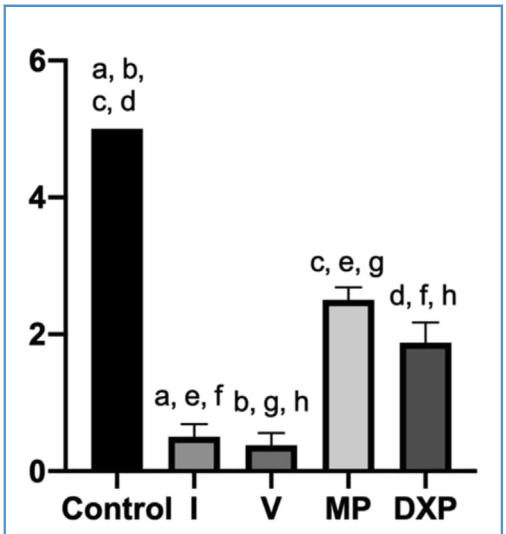

Figure 5. Distribution of Tarlov scores among the groups. (**A**) Control versus ischemia ($P < 0.001$). (**B**) Control versus vehicle ($P < 0.001$). (**C**) Control versus MP ($P < 0.001$). (**D**) Control versus DXP ($P < 0.001$). (**E**) Ischemia versus MP ($P < 0.001$). (**F**) Ischemia versus DXP ($P < 0.001$). (**G**) Vehicle versus MP ($P < 0.001$). (**H**) Vehicle versus DXP ($P < 0.001$). DXP, dexpanthenol; I, ischemia; MP, methylprednisolone; V, vehicle.

CAT is an antioxidant enzyme that also has oxidative stress-reducing activity by binding free oxygen radicals.[56] CAT activity decreases when oxidative stress increases.[57] Previous SCIRI studies have shown that serum and tissue CAT levels decrease as a result of increased oxidative stress.[9-14] In our study, as an indicator of increased oxidative stress, both serum and tissue CAT levels were shown to be decreased in both ischemia and vehicle experimental groups. Following DXP and MP treatments, both serum and tissue CAT levels were elevated in these groups. These data showed that intense oxidative stress due to SCIR injury decreased CAT levels, while DXP treatment increased CAT levels as a result of antioxidant properties. The CAT-enhancing effect of DXP has also been reported in several previous studies, which is in agreement with the results of our study.[22,36,38,50,51,58]

XO is elevated after SCIRI and is an important indicator of oxidative stress.[9-14] In our study, serum XO levels increased after IR injury in both the ischemia and vehicle experimental groups as an indicator of increased oxidative damage. It was observed that serum XO levels were significantly decreased after DXP and MP treatments. It is clear that this decrease in serum XO levels is additional evidence of the antioxidant activity of DXP.

The central nervous system and spinal cord are composed of large amounts of lipid-derived substances and oxidative damage that occurs after IR manifests in the form of lipid peroxidation and cell death.[59] Lipid peroxidation is responsible for cell membrane damage after SCIRI. This mechanism is one of the most fundamental pathophysiological pathways of secondary injury.[60] MDA is an end product of polyunsaturated fatty acids, that is, lipid peroxidation, and is a reliable indicator of peroxidation reactions.[13] Because of cross-linking with membrane lipids, MDA is an indicator of severe tissue damage.[61] In many previous studies, DXP has been shown to inhibit lipid peroxidation and reduce tissue MDA levels.[22,35-38,42,50,62] Similarly, PA has a reduction effect on lipid peroxidation.[53] In SCIRI studies, elevated serum and tissue MDA levels following injury is an important indicator of lipid peroxidation and tissue damage.[9-14] In our study, increased serum and tissue MDA levels were observed in the ischemia and vehicle experimental groups

compared to the control group, which is an indicator of increased lipid peroxidation following IR injury. As a result of the inhibitory effect of DXP and MP treatments on lipid peroxidation, both serum and tissue MDA levels were significantly decreased in these groups.

Inflammation, SCIRI, and DXP

An important component of secondary injury after SCIRI is the inflammatory response.[5] Neutrophils, macrophages, and monocytes are essential mediators of the inflammatory response and are a major component of tissue damage after reperfusion.[63] Microglial cells of the spinal cord secrete pro-inflammatory cytokines, enabling inflammatory cells to migrate to the spinal cord tissue.[64] In addition, these pro-inflammatory cytokines and adhesion molecules are responsible for progressive neuronal damage.[56] Anti-inflammatory agents are believed to be therapeutic in SCIRI and the associated inflammatory response has been shown to be effective.[4,5,63] The anti-inflammatory activity of DXP has been demonstrated in previous studies.[23,49,65] Tumor necrosis factor α and interleukin 1 beta released from human polymorphonuclear neutrophils is inhibited by DXP.[53] MPO activity is a reliable indicator of neutrophil infiltration in injured tissues.[12,20,66] The MPO enzyme is found in large amounts in the azurophilic granules of neutrophils and mediates oxygen-dependent bactericidal activity.[12] MPO activity indicates the extent of neutrophils infiltrating the spinal cord and the extent of activation, that is, the degree of inflammation.[66] In previous SCIRI studies, increased serum and tissue MPO activity levels have also been used as an indicator of an inflammatory response.[9-14] In this study, wherein we investigated the effects of DXP on SCIRI, similarly, both serum and tissue MPO activities were found to be increased in the ischemia and vehicle experimental groups as an indicator of an inflammatory response. DXP and MP treatments reduced tissue MPO activity, which is evidence that both drugs have anti-inflammatory activity. However, this finding was not consistent with serum MPO results.

Apoptosis, SCIRI, and DXP

Apoptosis following SCIRI is one of the most critical mechanisms of neuronal cell death.[67] Reduction in spinal blood flow resulted in adenosine triphosphate depletion and triggers apoptotic cascades. Apoptosis is governed by enzymes known as caspases, which are members of the cysteine protease family. Caspase-3, an interleukin-converting enzyme, is the main driver of an apoptotic cascade.[68] Sakurai et al.[69] showed that caspase-3 immunoreaction is increased when the spinal cord is subjected to 15 min of ischemia.[69] The increase in caspase-3 levels as a result of ischemic events initiates DNA fragmentation and results in apoptotic cell death.[70] Caspase-3 activity has been used as a reliable marker of apoptosis in many SCIRI studies.[9-14] In previous studies, DXP was shown to have similar antiapoptotic properties.[50,71] In this study, serum and tissue caspase-3 levels were increased in the ischemia and vehicle experimental groups as an indicator of SCIRI and apoptosis as shown previously.[72] Serum and tissue caspase-3 levels were significantly decreased following DXP and MP treatments. The results of this study showed DXP significantly reduced apoptosis following SCIRI.

Tissue Damage, SCIRI, and DXP

In the light microscopy analyses in this study, spinal cord sections with completely normal morphology were observed in the control groups. Following IR injury, highly prominent hemorrhaging, congestion, increased edema, necrosis, and inflammation were observed in the spinal cord tissue in the ischemia and vehicle experimental groups. The number of neurons in the normal structures were decreased significantly in both groups. Polymorphonuclear neutrophils, lymphocytes, and plasma cells were increased in the damaged spinal cord regions, which indicated inflammation secondary to ischemia. In the light microscopy examination of the DXP and MP groups, the spinal cord tissue was morphologically preserved. Both DXP- and MP-treated neuron numbers were higher compared to the ischemic tissues. Both drugs were found to be significantly neuroprotective in the spinal cord.

Transmission electron microscopy examination was performed in each group to examine the myelin sheaths of tissues in more detail. Significant dissociation was observed in the small-, medium-, and large-sized myelinated axons in the ischemia and vehicle experimental groups. It was observed that the separation in small-, medium-, and large-sized axons was preserved after MP treatment. No protective effect of DXP treatment on tissue ultrastructure was found.

Neurological Examination, SCIRI and DXP

Neurological examinations of the subjects included in our study were performed using the Tarlov scoring system. All subjects in the ischemia and vehicle experimental groups were paraplegic after 20 min of ischemia and 24 h of reperfusion. Both DXP and MP treatments protected the spinal cord functionally, and the Tarlov scores of these groups were higher than those of the ischemia and vehicle experimental groups.

Limitations

In light of the biochemical, histopathological, and functional findings of this study, DXP was shown to have neuroprotective effects on SCIRI owing to its antioxidant, anti-inflammatory, and antiapoptotic properties. However, as in every study, this study also has some limitations. MP is an antioxidant and anti-inflammatory agent that has traditionally been used in spinal cord injury.[43] It has lost its place in today's clinical practice and many authors currently question its routine clinical use. However, it remains valuable for use as an effective control in experimental SCIRI and trauma studies.[9,10,12-14] In further studies, clinically effective alternative molecules could be replaced as active control. More detailed results could be obtained by increasing the number of subjects in each group. Dose-dependent results can be obtained by changing the dose ranges and posologies of the drugs used. Long-term efficacy could be investigated using longer reperfusion times. By increasing the biochemical parameters in future studies, detailed information on the mechanisms can be obtained.

CONCLUSIONS

As a result of the biochemical, histopathological, and neurological examinations in this study, it has been shown for the first time

that DXP has an antioxidant, anti-inflammatory, and antiapoptotic effect and a significant neuroprotective effect on SCIRI. DXP is an Food and Drug Administration—approved drug that is economical and easily available. DXP has been used to treat bronchitis, cervical erosion, stomatitis, and wound healing for a long time without significant side effects. Therefore, we believe that it can prevent and treat SCIRI in humans as well. We hope that DXP, which we have shown to be neuroprotective in SCIRI, will inspire future human studies.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Ahmet Gülmez: Conceptualization, Methodology, Writing — original draft. **Pınar Kuru Bektaşoğlu:** Conceptualization, Methodology, Formal analysis, Writing — original draft, Visualization. **Çağhan Tönge:** Methodology. **Ahmet Yaprak:** Methodology. **M. Erhan Türkoğlu:** Validation. **Evrim Önder:** Data curation. **Berrin İmge Ergüder:** Data curation. **Mustafa Fevzi Sargon:** Data curation. **Bora Gürer:** Conceptualization, Methodology, Investigation, Data curation, Writing — review & editing, Project administration. **Hayri Kertmen:** Conceptualization, Methodology, Investigation, Writing — review & editing, Funding acquisition, Resources, Supervision.

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