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The protective effect of low-dose methotrexate on ischemia–reperfusion injury of the rabbit spinal cord

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ABSTRACT

Methotrexate was developed as a cytostatic agent, but at low doses, it has shown potent anti-inflammatory activity. Previous studies have demonstrated that the anti-inflammatory effects of methotrexate are primarily mediated by the release of adenosine. In this study, we hypothesized that low-dose methotrexate has protective effects in spinal cord ischemia–reperfusion injury. Rabbits were randomized into the following four groups of eight animals each: group 1 (control), group 2 (ischemia), group 3 (methylprednisolone) and group 4 (methotrexate). In the control group only a laparotomy was performed. In all the other groups, the spinal cord ischemia model was created by the occlusion of the aorta just caudal to the renal artery. Neurological evaluation was performed with the Tarlov scoring system. Levels of myeloperoxidase, malondialdehyde and catalase were analyzed, as were the activities of xanthine oxidase and caspase-3. Histopathological and ultrastructural evaluations were also performed. After ischemia–reperfusion injury, increases were found in the serum and tissue myeloperoxidase levels, tissue malondialdehyde levels, xanthine oxidase activity and caspase-3 activity. In contrast, both serum and tissue catalase levels were decreased. After the administration of a low-dose of methotrexate, decreases were observed in the serum and tissue myeloperoxidase levels, tissue malondialdehyde levels, xanthine oxidase activity and caspase-3 activity. In contrast, both the serum and tissue catalase levels were increased. Furthermore, low-dose methotrexate treatment showed improved results concerning the histopathological scores, the ultrastructural score and the Tarlov scores. Our results revealed that low-dose methotrexate exhibits meaningful neuroprotective activity following ischemia–reperfusion injury of the spinal cord.

1. Introduction

Spinal cord ischemia/reperfusion (I/R) injury is a serious complication of thoracoabdominal surgery and may result in paraplegia in up to 40% of patients (Crawford et al., 1986). The exact mechanisms of spinal cord I/R injury are not fully understood. Ischemic injury, which is aggravated by reperfusion, results in neuronal damage (Yilmaz et al., 2012). Although the exact mechanisms underlying spinal cord I/R injury remain uncertain, inflammation is known to play an important role (Lu et al., 2007; Matsumoto et al., 2003). Activation of neutrophils and oxidative stress lead to the production of reactive oxygen species (ROS), causing inflammation, lipid peroxidation and protein and DNA damage (Agee et al., 1991; Ueno et al., 1994). Blocking several of the inflammatory cascades has been shown to prevent injury in experimental spinal cord injury (Fansa et al., 2009; Hirose et al., 2004; Naidu et al., 2003).

The most common pharmacologic approach to the prevention of spinal cord injury from I/R is the use of steroids for their anti-inflammatory effects (Cassada et al., 2001a). Steroids, especially methylprednisolone (MP), are reported to reduce paraplegia and apoptosis in animal studies (Kanellopoulos et al., 1997). Most of the previous studies support the use of anti-inflammatory drugs to attenuate spinal cord I/R injury (Cassada et al., 2001a; Kanellopoulos et al., 1997).

Methotrexate (MTX) is a potent inhibitor of the enzyme dihydrofolate reductase and this inhibition blocks the de novo

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synthesis of purines and pyrimidines. Initially, MTX was developed as a cytostatic agent, and based on these properties, it has been used, at high doses to treat oncological diseases (Johnston et al., 2005; Wessels et al., 2008). However, MTX is also used at low-doses as a potent anti-inflammatory agent (Cronstein et al., 1991, 1993). The anti-inflammatory activity of MTX is primarily mediated by the release of adenosine (Asanuma et al., 2004; Cassada et al., 2001a; Cronstein et al., 1991, 1993; Genestier et al., 1998). In fact, previous studies have concluded that adenosine protects the spinal cord from I/R injury through mediation of anti-inflammatory and anti-apoptotic pathways (Cassada et al., 2001a, 2001b; Reece et al., 2004). Methotrexate has also been reported to limit infarct size and has shown a potent cardioprotective effect against I/R injury of the heart (Asanuma et al., 2004). There are no previous studies examining the neuroprotective effects of MTX in spinal cord I/R injury. Based on these results, the purpose of this study was to evaluate whether MTX administration could protect the spinal cord from I/R injury in rabbits. We also compared MTX with MP, which has been widely used for spinal cord injury (Diaz-Ruiz et al., 2000; Kanellopoulos et al., 1997).

2. Materials and methods

2.1. Experimental groups

Animal care and all experiments were conducted following the European Communities Council Directive of November 24, 1986 (86/609/EEC) concerning the protection of animals for experimental use. All experimental procedures used in this investigation were reviewed and approved by the ethical committee of the Ministry of Health Ankara Education and Research Hospital Committee of Animal Ethics. Thirty-two adult male New Zealand white rabbits, weighing 2.800–3.550 g, were randomly divided into the following four groups of eight rabbits each:

Group 1: Control group ($n=8$): laparotomy only. Rabbits underwent laminectomy, and non-ischemic spinal cord samples were obtained immediately after the surgery. No treatment was given to this group.
Group 2: Ischemia group ($n=8$); Rabbits underwent transient global spinal cord ischemia. The same volume of saline (0.9% NaCl) was injected intravenously immediately after the occlusion clamp was removed. The animals then underwent laminectomy, and spinal cord samples were removed 24 h post-ischemia.
Group 3: Methylprednisolone (MP) group ($n=8$); Treated similar to group 2, but the rabbits received a single intravenous 30 mg/kg dose of MP (Prednol, Mustafa Nevzat, Turkey) immediately after the occlusion clamp was removed. This dosage of the MP was selected based on earlier studies (Sanli et al., 2012; Yilmaz et al., 2012).
Group 4: Methotrexate (MTX) group ($n=8$); Treated similar to group 2, but the rabbits received a single intravenous 0.5 mg/kg dose of MTX (Metoart, Koçak Farma, Istanbul, Turkey) immediately after the occlusion clamp was removed. This dosage of MTX was selected based on past studies (Sanli et al., 2012).

For both MP and MTX, saline (0.9% NaCl) was used to dissolve the drugs.

2.2. Anesthesia and surgical procedures

The animals were kept at an optimal (18–21 °C) room temperature, fed a standard diet and kept under a 12-h light–dark cycle. Free access to food and water was provided. The animals were anesthetized by intramuscular administration of 70 mg/kg ketamine (Ketalar, Parke Davis Eczacıbaşı, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Turkey) and allowed to breathe spontaneously. Body temperatures were measured using an anal thermometer (Digital Fever thermometer, Becton Dickinson, NJ, USA) and maintained at 37 °C with a heating pad. Animals were placed in the supine position for the surgery. After sterile preparation, a 10-cm midline incision was made, and the abdominal aorta was exposed through a transperitoneal approach. Heparin (150 U/kg) was administered intravenously 5 min before clamping for anticoagulation. Approximately 1 cm below the renal artery, the aorta was clamped using an aneurysm clip with 70 g of closing force (Yasargil, FE721, Aesculap, Germany) under a surgical microscope. The cross clamp time was 20 min. At the end of the occlusion period, the clips were removed and restoration of blood flow was visually verified. The drugs were administered immediately after the clamp was removed. The rabbit aortic cross-clamping method, which was used in this study, is a useful method for these procedures. The 20 min ischemia period was chosen to achieve adequate injury (Zivin and DeGirolami, 1980). The rabbits were allowed free access to food and water 2 h after surgery. Crede's maneuver was performed on animals with a neurogenic bladder at least two times a day. The animals were sacrificed 24 h after the operation by injection of pentobarbital (200 mg/kg). Spinal cord segments between L2 and L5 were carefully removed by laminectomy and used for the biochemical, histopathological and ultrastructural analyses. Blood (10 cm^3) was taken from the left ventricle for biochemical analysis. The blood samples were centrifuged at 1000*g* for 5 min, and the upper clear supernatants were removed for analysis. All serum and tissue samples were stored at −80 °C until analyzed. On the day of the analysis, the tissues were homogenized in physiologic saline solution and centrifuged at 1780*g* for 20 min. The serum samples obtained as the upper clear supernatants of the centrifuged blood were used for the biochemical analyses.

2.3. Serum and tissue myeloperoxidase (MPO) analysis

MPO activity was measured using an ELISA kit (Cusabio, Hubei, China). The ELISA procedures were performed according to the manufacturer's instructions. This assay employs the competitive inhibition enzyme immunoassay technique. The microtiter plate provided in this kit was pre-coated with an antibody specific to MPO. Standards or samples were added to the appropriate microtiter plate wells with Biotin-conjugated MPO. A competitive inhibition reaction was initiated between the MPO (from the standards or the samples) and the Biotin-conjugated MPO with the pre-coated antibody specific for MPO. With greater amounts of MPO in the samples, lower amounts of antibodies are bound by the Biotin-conjugated MPO. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. The substrate solution was then added, and the color developed to indicate the amount of MPO in the sample. When color development stopped, the intensity of the color was measured at 450 nm. The MPO concentrations were calculated by comparing the absorbance values of the samples with those of standard MPO solutions. The results are expressed in ng/ml.

2.4. Tissue malondialdehyde (MDA) analyses

Tissue MDA levels were determined using a method based on reaction with thiobarbituric acid (TBA). Briefly, the samples were mixed with two volumes of cold saline solution containing 0.001% butylated hydroxytoluene (BHT) and 0.07% sodium dodecyl sulfate (SDS). Then, 1 ml of the samples was added to 500 μl of 0.01 μl NH_2SO_4 and 500 μl of the thiobarbituric acid reagent (0.67% thiobarbituric acid in 50% acetic acid) to precipitate protein. Then,

the samples were heated in boiling water for 60 min. After cooling, an equal volume (2 ml) of *n*-butanol was added to each test tube and mixed. 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). The malondialdehyde concentrations were calculated by comparing the absorbance values of the samples with those of standard MDA solutions. The MDA concentrations are expressed in nmoles per gram of wet weight tissue.

2.5. Serum and tissue catalase (CAT) analyses

Catalase activity was determined by measuring the absorbance decrease of hydrogen peroxide (H_2O_2) at 240 nm (Aebi, 1984). In the activity calculations, an extinction coefficient of H_2O_2 was used for CAT. The results are expressed in IU/ml.

2.6. Serum xanthine oxidase (XO) analyses

Serum XO activity was measured by the method of Prajda and Weber (1975), where activity is measured by the determination of the amount of uric acid formed from xanthine. Serum samples (100 μl) were incubated for 30 min at 37 °C in 3 ml of the phosphate buffer (pH 7.5, 50 nM) containing xanthine (4 mM). The reaction was stopped by the addition of 0.1 ml 100% (w/v) TCA, and the mixture was then centrifuged at 1780*g* for 20 min. Uric acid was determined in the supernatant by measuring the absorbance at 292 nm against a blank and expressed as mIU/ml. A calibration curve was constructed using 10–50 mU/ml concentrations of standard XO solutions (Sigma X-1875, Sigma-Aldrich, St. Louis, MO). One unit of activity was defined as 1 μmol of uric acid formed per minute at 37 °C and pH 7.5.

2.7. Tissue caspase-3 activities

Caspase-3 activity was measured using an ELISA kit (Cusabio, Hubei, China). The ELISA procedures were performed according to the manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. Antibodies specific for caspase-3 had been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any caspase-3 present was bound by the immobilized antibody. After removal of any unbound substances, a biotin-conjugated antibody specific for caspase-3 was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. Any unbound substances had been removed by the three washes with washing buffer. Following the washing procedure, the avidin-enzyme reagent was added to the wells. The color develops in proportion to the amount of caspase-3 bound in the initial step. When the color development stopped, the intensity of the color was measured at 450 nm. Caspase-3 concentrations were calculated by comparing the absorbance values of the samples with those of standard caspase-3 solutions. The results are expressed in ng/ml.

2.8. Histopathological procedures

The cord specimens obtained at 24 h post-injury were prepared for histological study. Each cord segment was immersed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer and stored at 4 °C. The specimens were then embedded in paraffin, cut into 5 μm thick sections and stained with hematoxylin–eosin (H&E). The specimens were examined under a light microscope by a neuropathologist, who was blinded to the study design. Five different fields of the gray matter of the spinal cord were evaluated using a 40× objective.

A semi-quantitative scoring system, ranging between 0 and 3, was used for grading the histopathological changes in the spinal cord tissues of all samples. Six different parameters (hemorrhage, congestion, necrosis, edema, neuronal loss and inflammation) were assessed histopathologically and were scored as follows: 0=absent, 1=mild, 2=moderate, and 3=common. The pathological score for each spinal cord was calculated by averaging the scores of these six parameters.

In addition, a more detailed assessment of the degree of ischemic neuronal injury was also performed. For this analysis, the number of normal 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 counted. For each animal, Section 3 were evaluated using a 40× objective. An average normal motor neuron count for the sections from each animal was then determined. Neurons that contained Nissl substances in the cytoplasm, loose chromatin and prominent nucleoli were considered normal viable neurons (Umehara et al., 2010).

2.9. Ultrastructural examinations

Tissue samples were cleared of blood using a scalpel and the meninges were carefully removed. The tissue samples were then fixed in 2.5% glutaraldehyde for 24 h, followed by washing in phosphate buffer (pH: 7.4). They were next post-fixed in 1% osmium tetroxide in phosphate buffer (pH: 7.4) for 2 h and dehydrated with increasing concentrations of alcohol. Then, the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections approximately 2 μm thick and ultra-thin sections approximately 60 nm thick were cut with a glass knife on a LKB-Nova ultramicrotome (LKB-Produkter AB, Bromma, Sweden). The semi-thin sections were stained with methylene blue and examined with a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscope. Following this examination, the tissue blocks were trimmed, and ultra-thin sections were made using the same ultramicrotome; these sections were stained with uranyl acetate and lead citrate. After staining, all of the ultra-thin sections were examined with a Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan) transmission electron microscope. The electron micrographs were taken by the same transmission electron microscope at 5000× magnification. A total of 100 large-sized myelinated axons, 100 medium-sized myelinated axons and 100 small-sized myelinated axons were evaluated for every sample, scored from 0 to 3, and counted, and the data are presented as the mean values, as described by Kaptanoglu et al. (2002).

The scoring system was as follows:

0. Ultrastructurally normal myelinated axon
1. Separation in myelin configuration
2. Interruption in myelin configuration
3. Honeycomb appearance in myelin configuration.

2.10. Neurologic evaluations

The neurologic statuses of the animals were scored 24 h after the procedure by assessing hind-limb neurologic function using the modified Tarlov Scoring System (Yilmaz et al., 2012). A score of 0 to 5 was assigned to each animal as follows: 0=no voluntary hind-limb movement, 1=movement of joints perceptible, 2=active movement but unable to sit without assistance, 3=able to sit but unable to hop, 4=weak hop and 5=complete recovery of hind-limb function. A medical doctor blinded to the experimental groups performed the neurologic evaluations.

2.11. Statistical analyses

Data analysis was performed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). Whether the continuous variables were normally distributed was determined by the Shapiro Wilk test. The Levene test was used for the evaluation of the homogeneity of the variances. Where applicable, the data are shown as the means ± standard deviation or the medians (IQR). The mean differences among groups were analyzed using One-Way ANOVA. Otherwise, the Kruskal Wallis test was applied for comparison of the median values. When the P value from the One-Way ANOVA or the Kruskal Wallis test was statistically significant, post hoc Tukey HSD or Conover's non-parametric multiple comparison tests were used to evaluate the differences between the various groups. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Serum and tissue myeloperoxidase (MPO) analysis

Statistically significant differences were observed between the control and the ischemia groups with regard to the mean serum and tissue MPO levels ($P<0.001$ for both). However, these data showed that I/R injury clearly elevated both the serum and tissue MPO levels. Treatment with MTX led to a statistically significant decrease in the MPO levels both in serum and tissue when compared to ischemia group ($P<0.001$). As in the MTX group, the MP and the ischemia groups showed a statistically significant difference in their MPO levels ($P<0.001$). There were no statistically significant differences between the MP and the MTX groups for both the serum and tissue MPO levels ($P=0.873$ and $P=0.358$, respectively).

3.2. Tissue malondialdehyde (MDA) analyses

When the mean tissue MDA levels were compared between the control and the ischemia group, statistically significant differences were observed ($P<0.001$). Furthermore, these data showed that after I/R injury, the tissue MDA levels were increased. When we compared the ischemia and the MTX groups, there was a statistically significant difference between these groups ($P<0.001$). As in the MTX group, a comparison between the MP and the ischemia groups revealed a statistically significant difference in the MDA levels ($P<0.001$). When the MTX and the MP groups were compared, no statistical significance was found ($P=0.711$). These data showed that both MP and MTX prevented an increase in the MDA levels.

3.3. Serum and tissue catalase (CAT) analyses

When the mean serum and tissue CAT levels of the control group were compared with the ischemia group, there were statistically significant differences observed ($P<0.001$ for both); these data showed that after I/R, both serum and tissue CAT levels were decreased. Treatment with MTX was associated with statistically significant increases in the CAT levels in both serum and tissue ($P<0.001$ and $P=0.017$, respectively). As in the MTX group, MP was also associated with statistically significant increases in the CAT levels in serum and tissue ($P<0.001$ for both). There were no differences between the MP and the MTX groups in the serum and the tissue CAT levels ($P=0.516$ and $P=0.493$, respectively).

3.4. Serum xanthine oxidase (XO) analyses

Serum XO activity was linked to a statistically significant increase in the ischemia group compared with the control group ($P<0.001$). In the MTX group, serum XO activity was significantly decreased when compared to the ischemia group ($P<0.001$). Similar to the MTX group, the XO activity of the MP group showed a statistically significant decrease compared to the ischemia group ($P=0.003$). There was no difference between the MP and the MTX groups ($P=0.088$).

3.5. Tissue caspase-3 analyses

There was a statistically significant difference between the control and the ischemia groups in the means of their caspase-3 activity ($P<0.001$). These data showed that I/R injury clearly elevated caspase-3 activity in the damaged tissue. When the MTX group was compared with the ischemia group, there was a statistically significant decrease in caspase-3 levels ($P<0.001$). Similar to the MTX group, the MP group also showed a statistically significant decrease in caspase-3 levels ($P=0.004$). In addition, the MTX group showed a statistically significant decrease in caspase-3 levels when compared to the MP group ($P=0.002$).

All of the biochemical results are summarized in Table 1.

3.6. Histopathological procedures

Light microscopic examinations of the spinal cord samples from the control group were normal (Fig. 1a). In the ischemia group (Fig. 1b), diffuse hemorrhage and congestion in the gray matter were

Table 1
Biochemical alterations among the groups.

Variables	Control ($n=8$)	Ischemia ($n=8$)	MP ($n=8$)	MTX ($n=8$)	*P*-value
Serum MPO (ng/ml)	1.33 ± 0.32[a,b,c]	4.19 ± 0.71[a,d,e]	2.47 ± 0.33[b,e]	2.29 ± 0.43[c,d]	< 0.001
Tissue MPO (ng/ml)	2.38 ± 0.61[a]	4.98 ± 0.45[a,d,e]	3.14 ± 0.94[e]	2.46 ± 1.08[d]	< 0.001
Tissue MDA (nmol/ml)	2.39 ± 0.98[a]	5.21 ± 0.94[a,d,e]	3.18 ± 0.86[e]	2.73 ± 0.51[d]	< 0.001
Serum CAT (IU/ml)	168.36 (77.99)[a,b,c]	53.55 (34.00)[a,d,e]	117.12 (29.28)[b,e]	125.42 (53.09)[c,d]	< 0.001
Tissue CAT (IU/ml)	161.43 ± 34.71[a]	70.95 ± 22.10[a,d,e]	144.94 ± 34.23[e]	122.04 ± 35.33[d]	< 0.001
Serum XO (mIU/ml)	0.05 (0.07)[a,b,c]	0.36 (0.15)[a,d,e]	0.12 (0.03)[b,e]	0.14 (0.06)[c,d]	< 0.001
Tissue caspase-3 (ng/ml)	0.48 (0.28)[a,b]	2.19 (0.64)[a,d,e]	1.25 (0.75)[b,e,f]	0.40 (0.66)[d,f]	< 0.001

MP=methylprednisolone, MTX=methotrexate, MPO=myeloperoxidase, MDA=malondialdehyde, CAT=catalase, XO=xanthine oxidase. The data are shown as the means ± standard deviation or the medians (IQR).

[a] Control vs Ischemia ($P<0.001$).
[b] Control vs MP ($P<0.05$).
[c] Control vs MTX ($P<0.05$).
[d] Ischemia vs MTX ($P<0.05$).
[e] Ischemia vs MP ($P<0.01$).
[f] MP vs MTX ($P<0.05$).

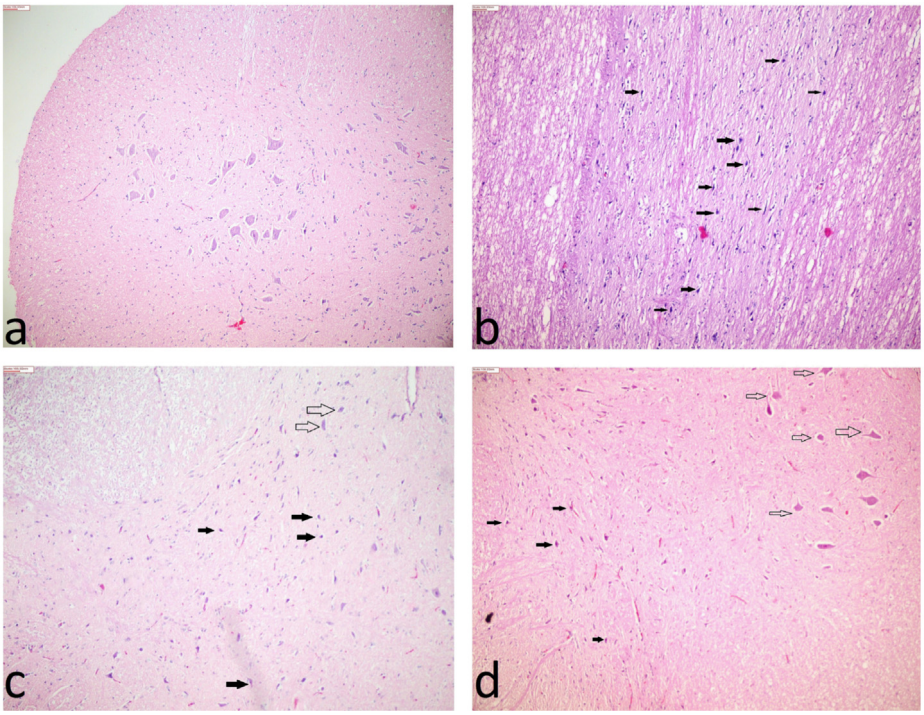

Fig. 1. Photomicrographs of 5 μm spinal cord tissue sections from the different treatment groups (H&E, × 10obj.). (a) Control group ($n=8$), showing normal spinal cord parenchyma. (b) Ischemia group ($n=8$), showing degenerated neurons (filled arrows) in the edematous surface. (c) Methylprednisolone group ($n=8$), showing less degenerated neurons (filled arrows); note the normal neurons (hollow arrows). (d) Methotrexate group ($n=8$), showing less degenerated neurons (filled arrows), and more normal neurons (hollow arrows). The spinal cord tissues were protected from injury.

observed 24 h after the I/R injury. There was marked necrosis and widespread edema in both the white and gray matter. In the damaged areas, there were infiltrating polymorphonuclear leukocytes, lymphocytes, and plasma cells observed. Neuronal pyknosis, a loss of cytoplasmic features and cytoplasmic eosinophilia was also observed in the ischemia group. In the MTX group as well as the MP group, the cord tissues were protected from I/R injury (Fig. 1c and d).

When the pathological scores were compared, the ischemia group showed statistically higher scores than the control group ($P<0.001$). In the MTX group, the pathological score was significantly lower than that for the ischemia group ($P=0.001$). In the MP group, the pathology score was significantly lower than the ischemia group ($P=0.003$). The difference between the MP and the MTX groups was not statistically significant ($P=0.56$) (Fig. 2a).

In the ischemia group the number of normal motor neurons in the anterior spinal cord was significantly decreased compared with that of the control group ($P<0.001$). In the MTX group, the number of normal motor neurons in the anterior spinal cord was significantly higher when compared with that of the ischemia group ($P<0.001$). Similar to the MTX group, the MP group showed a statistically significant higher number of normal neurons when compared with the ischemia group ($P<0.001$). The comparison between the MTX and the MP groups did not show a statistical difference ($P=0.745$). Histopathologically, both MTX and MP protected the brain from TBI (Fig. 2b).

3.7. Ultrastructural examinations

In the transmission electron microscopic examination of the tissue samples of the control group, no ultrastructural pathological changes were observed in the gray and white matter of the spinal cord. The neurons were ultrastructurally normal in appearance, and the intracellular organelles, nuclei, membranes and perineuronal tissues did not show any pathological changes. However, in a few of the large-sized myelinated axons, mild separations were observed in a small part of the myelin sheath. This may be related to delayed fixation of the tissue. The rest of the large-sized myelinated axons and the whole medium- and small-sized myelinated axons were found to be ultrastructurally normal (Fig. 3a).

The transmission electron microscopic examination of the ischemia group showed separations in the myelin configuration of small-sized, medium-sized and large-sized myelinated axons. When all of the groups were compared, the ischemia group showed the greatest ultrastructural damage in the myelinated axons (Fig. 3b).

In the MP group, separations were observed in the myelin configuration of the medium-sized and large-sized myelinated axons. Additionally, in a few of the small-sized myelinated axons, separations in the myelin configuration were observed (Fig. 3c).

In the MTX group, separations were observed in the myelin configuration of large-sized myelinated axons. All of the small-sized and most of the medium-sized myelinated axons of this group were ultrastructurally normal (Fig. 3d). The ultrastructural appearances of the myelinated axons of the MTX group were better than those of the MP group, which was also observed after the scoring.

Compared to the control group, the ischemia group showed greater disruption in the small-sized myelinated axons ($P=0.008$). When compared to the ischemia group, both MTX and MP protected the small-sized myelinated axons from disruption ($P=0.008$ for both). When the MTX group was compared to the MP group, the MTX group revealed statistically significant improvements in terms of protecting small-sized myelinated axons ($P=0.008$).

In the ischemia group, I/R injured the medium-sized myelinated axons compared to the control group ($P=0.008$). Similarly, there was a significant difference between both the MTX and the MP groups when compared to the ischemia group, in which both MTX and MP treatments protected the medium-sized axons from I/R injury ($P=0.008$ for both). In addition, when the MTX group

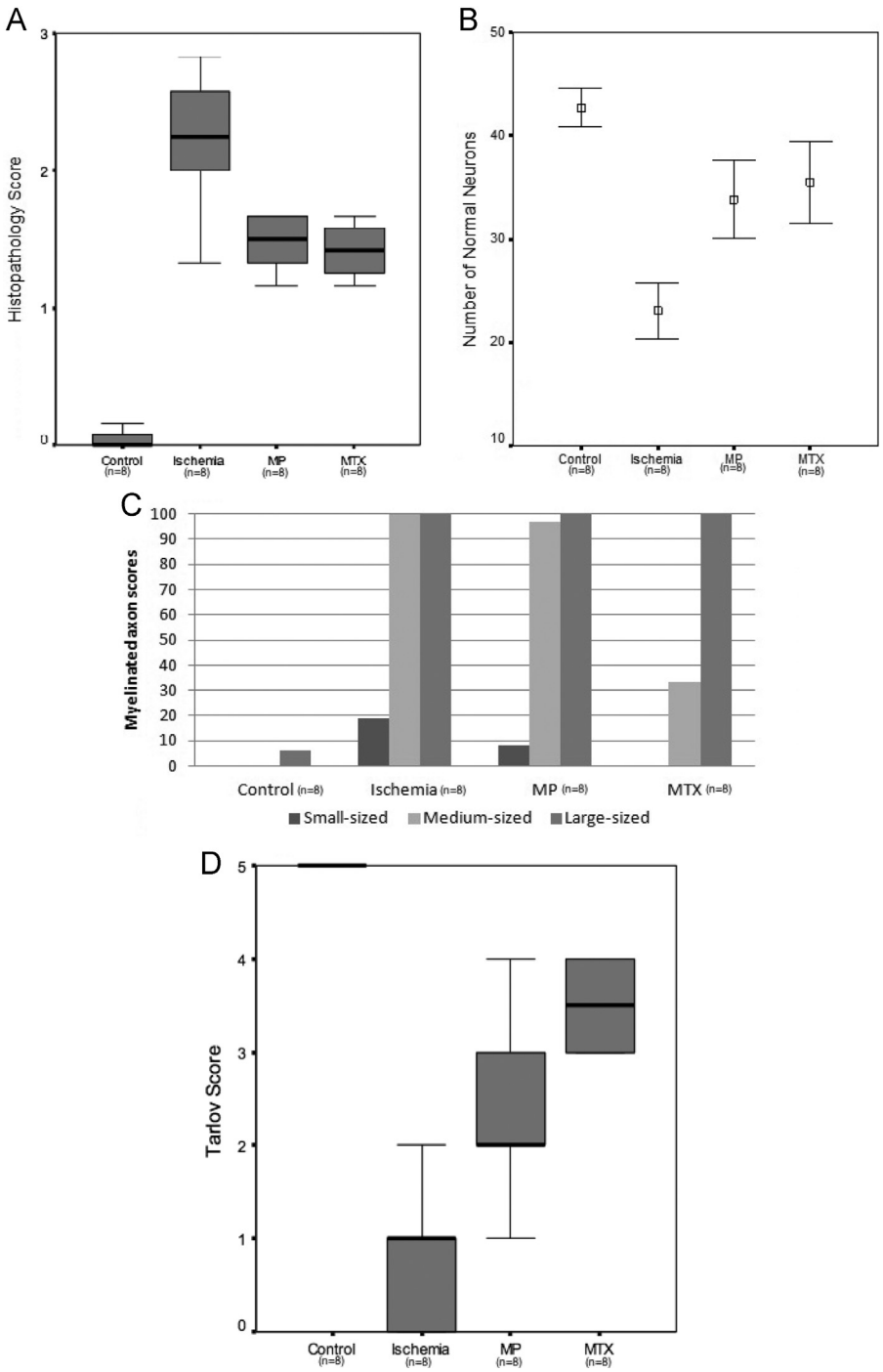

Fig. 2. Graphs showing the histopathological ((a) and (b)), ultrastructural (c) and neurological examination results (d) of the groups ($n=8$ for each group). (a) Comparison of the histopathologic scores among the groups. Five different fields of spinal cord gray matter were evaluated using a 40 × objective. The horizontal lines in the middle of each box indicates the median, while the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. The whiskers above and below the box mark the maximum and minimum levels observed. (b) Comparison of the number of normal neurons in the anterior horn among the groups. The box in the middle of each whisker indicates the arithmetic mean, while the whiskers above and below the box mark the +1 S.D. and −1 S.D. levels, respectively. (c) Bar graph showing the results of the ultrastructural analysis. (d) Comparison of the Tarlov scores among the groups. The horizontal lines in the middle of each box indicates the median, while the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. The whiskers above and below the box mark the maximum and minimum levels observed. MP: methylprednisolone, MTX: methotrexate.

was compared to the MP group, the MTX group revealed statistically significant improvements in terms of protecting medium-sized myelinated axons.

Further, large-sized axons were more damaged after the I/R injury in the ischemia group than the control group. Unfortunately, neither MP nor MTX protected the large-sized axons of the spinal cord from I/R injury (Fig. 2c).

3.8. Neurologic evaluations

The mean Tarlov score of the ischemia group was significantly lower than that of the control group ($P<0.001$). However, the mean Tarlov score of the MTX group was significantly higher than that of the ischemia group ($P<0.001$). The mean Tarlov score of the MP group was also significantly higher than that of the

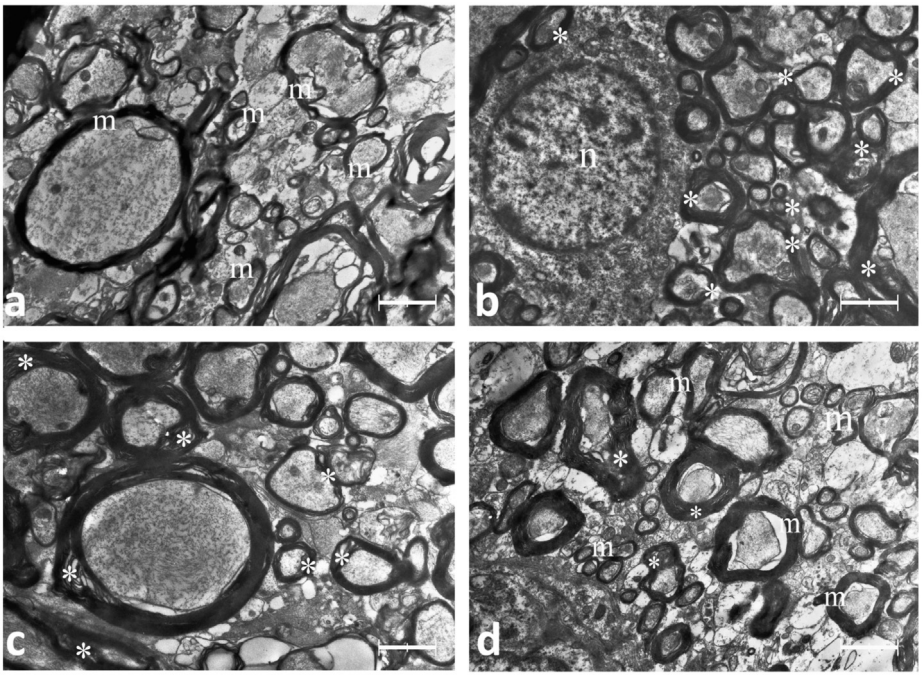

Fig. 3. Transmission electron microscopy of the groups. (a) Electron micrograph from the control group ($n=8$) showing ultrastructurally normal myelinated axons (m) (original magnification × 5000, scale bar is 2 μ long). (b) Electron micrograph from the ischemia group ($n=8$) showing the small and medium-sized axons with separations in myelin configuration (*) (original magnification × 5000, scale bar is 2 μ long). n: nucleus of a neuron. (c) Electron micrograph from the MP group ($n=8$) showing separations in myelin configurations (*) in small-, medium- and large-sized myelinated axons (original magnification × 5000, scale bar is 2 μ long). (d) Electron micrograph from the MTX group ($n=8$) showing medium-sized myelinated axons with mild separation in myelin configuration (*) and ultrastructurally normal small-sized and medium-sized myelinated axons (m) (original magnification × 5000, scale bar is 2 μ long). MP: methylprednisolone, MTX: methotrexate.

ischemia group ($P=0.007$). Additionally, the MTX group showed statistically significant higher Tarlov score compared to those of the MP group ($P=0.035$) (Fig. 2d).

4. Discussion

After surgery involving the descending and thoracoabdominal aorta, spinal cord I/R injury as a post-surgical complication may cause catastrophic consequences, such as paraplegia. The main cause of spinal cord injury is believed to be the result of ischemia due to hypoperfusion during aortic cross clamping. This injury is followed by a secondary injury caused by the reestablishment of blood flow (Dumont et al., 2001). The "primary injury" of spinal cord I/R injury occurs when the loss of nutrients and oxygen activates devastating biochemical cascades. These would include the generation of ROS, apoptosis, necrosis, lipid peroxidation, excessive excitatory amino acid release and inflammation (Hasturk et al., 2009; Reece et al., 2004). After the primary injury, reestablishment of the blood flow, called reperfusion, initiates additional spinal cord damage, which results in a decline of function and the "secondary injury" (Dumont et al., 2001; Lukácová et al., 1996).

Inflammatory processes are one of the most important mechanisms responsible for I/R injury of the spinal cord (Fan et al., 2011; Hasturk et al., 2009). Inflammatory cells, such as neutrophils, macrophages and monocytes play an important role in the inflammatory response of the spinal cord to ischemic injury and cause the expansion of neuronal damage after reperfusion (Reece et al., 2004). The resident glial cells, especially the microglia, also play a role in secreting proinflammatory factors that recruit peripheral immune cells into the spinal cord (Li et al., 2011). By producing a number of proinflammatory mediators, such as cytokines and adhesion molecules, these cells recruit peripheral inflammatory cells into the spinal cord and cause further neuronal death (Fan et al., 2011; Ilhan et al., 2004). The importance of inflammation in spinal cord I/R injury has become better understood and strategies to attenuate I/R injury of the spinal cord have focused on preventing inflammation (Cassada et al., 2001b; Fan et al., 2011; Reece et al., 2004).

Methotrexate, a folate antagonist, is known as a potent anti-inflammatory agent (Mitchell et al., 1969). Despite showing immunosuppressive effects at high doses, MTX has been used in the treatment of inflammatory diseases (Dalmarco et al., 2002; Weinblatt et al., 1985). In several studies, MTX has exerted a wide-range of anti-inflammatory activities that are primarily mediated via the release of adenosine from cells that express ecto-5′-nucleotidase (Cronstein et al., 1991, 1993; Genestier et al., 1998; Wessels et al., 2008).

Adenosine is a purine nucleoside that has four specific receptors: A1, A2A, A2B and A3 (Haskó and Cronstein, 2004). These four receptors differ in their affinity for adenosine, their predominance in different cells and their effects on immunoregulation (Haskó and Cronstein, 2004; Wessels et al., 2008). It is commonly accepted that the anti-inflammatory effects of adenosine are predominantly due to A2A-receptor stimulation (Haskó and Cronstein, 2004). Adenosine A2A-receptor activation has been shown to have protective anti-inflammatory effects against spinal cord I/R injury via suppression of leukocyte recruitment and by reducing cytokine release, including the release of tumor necrosis factor-α and interleukin-1 (Cassada et al., 2001a, 2001b; Reece et al., 2004). However, selective stimulation of the adenosine A2A receptor was shown to be protective against intestinal I/R injury by reducing inflammatory mediators and apoptosis (Di Paola et al., 2010). Paterniti et al. (2011) reported that both adenosine A2A receptor agonism and antagonism protects the spinal cord against traumatic injury. Genovese et al. (2010) demonstrated that treatment with adenosine A2A receptor agonists attenuates traumatic spinal cord injury by preventing apoptotic processes. Concurrently, MTX administration has been shown to increase adenosine concentrations by a factor of 2- to 4-times and activate adenosine

A2A -receptors (Montesinos et al., 2003, 2007). Therefore, we hypothesized that MTX may have a neuroprotective effect in spinal cord I/R injury. A low MTX dose, 0.5 mg/kg, was selected on the basis of past studies (Asanuma et al., 2004; Sanli et al., 2012).

In the present study, spinal cord I/R injury caused a significant increase in MPO activity in both serum and the spinal cord. Myeloperoxidase was shown to be elevated in both serum and tissue; this provides evidence that spinal cord injury is associated with elevated serum levels of MPO. Myeloperoxidase activity is a reliable marker of neutrophil infiltration into injured tissue (Sanli et al., 2012; Taoka et al., 1997). Myeloperoxidase is present in large quantities in the azurophilic granules of neutrophils and contributes to their oxygen-dependent bactericidal activity (Sanli et al., 2012). Myeloperoxidase activity is associated with the number of neutrophils that infiltrate the spinal cord and their activation (Taoka et al., 1997). Our findings showed that both MTX and MP were effective in reducing MPO activity. Thus, the observed decrease in MPO activity in response to low doses of MTX indicates a reduction in the number of neutrophils at the site of injury. This also supports the existence of anti-inflammatory activity for MTX.

Neutrophils are also major sources of ROS in injured tissues. The central nervous system consists primarily of lipids, which are easily damaged by free-radical-induced lipid peroxidation (Yilmaz et al., 2012). Following spinal cord ischemia and during reperfusion, lipid peroxidation occurs in cell membranes. Lipid peroxidation is one of the main pathophysiological mechanisms involved in secondary damage (Diaz-Ruiz et al., 2000). Malondialdehyde is a degeneration product of polyunsaturated fatty acids and serves as a reliable index for determining the extent of peroxidation reactions. Malondialdehyde levels increase after spinal cord I/R, demonstrating the involvement of lipid peroxidation, thus supporting the presence of reperfusion injury (Qian and Liu, 1997; Yilmaz et al., 2012). This study showed a significant increase in MDA levels from I/R injury; however, both MTX and MP protected the spinal cord from lipid peroxidation by lowering the MDA levels.

Reactive oxygen species play a key role in mediating secondary injury insult by reperfusion (Chan, 1996). Neutrophil activation causes the generation of ROS and thus results in a considerable amount of damage to the tissue (Krieglstein and Granger, 2001). The antioxidant enzyme CAT has the capacity to scavenge ROS (Ilhan et al., 2004). Antioxidant enzyme activities are diminished under highly elevated oxidative stress conditions because of molecular damage (Ustün et al., 2001). The present study showed that CAT levels decreased significantly after I/R injury. After administration of either MTX or MP, CAT levels increased significantly, thus showing the effects of antioxidant activity. XO is another important source of ROS (Hille and Nishino, 1995). In the present study, XO levels were found to be elevated after I/R injury. It was also demonstrated that both MTX and MP reduced XO levels significantly. As mentioned above, neutrophils are one of the main sources of ROS, and in low doses, MTX has anti-inflammatory activity; therefore, we attributed the antioxidant effect of low-dose MTX to the anti-inflammatory activity of the drug.

Spinal cord injury involves the apoptotic death of neurons after injury, which can be further exacerbated by inflammation (Schwab and Bartholdi, 1996). Apoptosis is activated by members of the cysteine protease family known as caspases. Caspase-3 is an interleukin converting enzyme and has been suggested to be the principal effector in the mammalian apoptotic and inflammatory pathways (Keane et al., 2001). Past studies have demonstrated that caspase-3 is a reliable marker to reflect apoptotic activity in I/R injury (Yilmaz et al., 2012). In the present study, we demonstrated that caspase-3 activity was increased following I/R injury. Notably, low doses of MTX decreased the caspase-3 activity in both the serum and spinal cord, thus demonstrating its antiapoptotic activity. Furthermore, MTX showed greater antiapoptotic activity than MP. Previous studies have reported a dose- and time-dependant effect of MTX on the inhibition of cell proliferation and the induction of apoptosis (Wessels et al., 2008). In contrast, Cassada et al. (Cassada et al., 2001a) demonstrated that adenosine A2A-agonist had antiapoptotic activity. Although this study did not examine the MTX/adenosine pathways, low doses of MTX may have shown anti-apoptotic activity, most probably, as previously specified MTX actions are mediated by adenosine, which acts on A2A receptors, and not by a direct effect of MTX on A2A receptors.

Histopathologic examination of the spinal cord samples revealed that I/R injury caused diffuse hemorrhage, congestion, marked edema, and necrosis in both white and gray matter. In the damaged portions of the spinal cord, infiltrating polymorphonuclear leukocytes, lymphocytes and plasma cells were observed, indicating an inflammatory response. In the ischemia group, the number of normal motor neurons in the anterior horn of the spinal cord was significantly decreased. Both MTX and MP showed better morphological results and a higher number of the normal motor neurons when compared with the ischemia group.

Nevertheless, evaluation of the neuroprotection using histopathologic techniques did not provide fully adequate results. Because of this, we also evaluated the ultrastructural changes with a transmission electron microscope. The transmission electron microscopic results revealed that I/R caused significant disruption in small, medium and large-sized myelinated axons. Both the MTX and MP treatments protected the small and medium-sized myelinated axons from I/R injury. MTX showed significantly better results than MP. Nevertheless, neither MTX nor MP showed significant protection of large-sized myelinated axons.

The rabbit aortic cross-clamping method, which was used in this study, is a useful method for research; the 20 min ischemia period was chosen to achieve sufficient injury (Zivin and DeGirolami, 1980). In our ischemia group, aortic cross clamping caused paraplegia in all animals. Both MTX and MP treatments, administered after I/R injury, protected the spinal cord and improved neurological function, as determined by the Tarlov Scores. Furthermore, the MTX group showed better neurological function compared to the MP group.

All results from this study suggest that low doses of MTX and MP have beneficial effects on preserving normal spinal cord morphology and ultrastructure by reducing inflammation, lipid peroxidation, oxidative stress and inhibiting apoptosis.

There were some limitations of this study. The number of rabbits in each group and the periods for functional, biochemical and histopathological assessment should be augmented. A more detailed examination of the dose-dependence should be conducted. In addition, the study protocol did not contain a procedure to verify that MTX is showing its protective effects via adenosine pathways. The relationship between MTX and adenosine in the neuroprotective mechanisms should be examined in future studies.

5. Conclusion

In conclusion, biochemical, histopathological, ultrastructural and neurological functional analysis revealed that low doses of MTX exhibit meaningful neuroprotective activity in I/R injury of the spinal cord. Moreover, MTX has been shown to be at least as effective as MP. Further studies based on these findings may be helpful for further evaluating this promising medication for I/R injury of the spinal cord.

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