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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,