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The efficacy of adalimumab on experimentally induced spinal cord ischemia-reperfusion injury

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ABSTRACT
Objective: Paraplegia is a dangerous complication of thoracoabdominal aortic surgery. Various studies have been conducted on the prevention of this complication and some spinal cord protection methods have been proposed. However, there is not any modality that prevent the development of paraplegia certainly. In the I / R period, primary injury triggers secondary injury due to increased inflammation, apoptosis and free radical formation. In this study, we evaluated that the neuroprotective effect of adalimumab in spinal cord ischemia-reperfusion injury.

Materials and Methods: In total, 24 adult New Zealand rabbits were divided into three groups: Group 1, control; Group 2, ischemia-reperfusion by infrarenal aortic clamping; Group 3, adalimumab treated followed by ischemia. Tissue and plasma tumor necrosis factor alpha, interleukin 6, interleukin 10, thiobarbituric acid reactive substance, total oxidant status and total antioxidant status levels were analyzed as a marker of inflammation and oxidation. Histopathological evaluation of the tissues was performed, and apoptosis was evaluated by TUNNEL method.

Results: I/R injury significantly increases plasma and spinal cord tissue at TNF alpha, TOS, TBARS, IL6 levels and reduces plasma and spinal cord tissue to TAS and IL10 levels. Adalimumab treatment significantly reduces plasma and spinal cord tissue to TNF alpha, TOS, TBARS, IL6 and increases plasma and tissue to TAS and IL10 levels.

Conclusion: Adalimumab treatment significantly reduces the spinal cord neuronal damage score and the number of apoptotic cells. This paper aims to demonstrate the important neuroprotective effects of adalimumab on rabbit spinal cord I/R injury.

1. INTRODUCTION
Spinal cord reperfusion injury is described as cell death of neurons although improvement of blood supply of spinal cord after ischemia. It usually occurs because of oxygen free radical-induced lipid peroxida-
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2. Materials and methods

This research was carried out in the Experimental Medicine Application and Research Center of Necmettin Erbakan University. The experimental protocol was assessed and confirmed by the Ethics Review Committee of Necmettin Erbakan University. The animals were kept at a room temperature (18–21 °C) and fed on a standard diet. A 12-h light-dark cycle (08:00–20:00 hours light/20:01–07:59 hours dark) was preserved. The animals were able to get as much food and water as they wanted.

2.1 Groups

Twenty-four adult New Zealand rabbits were randomly separated into three groups: Group 1, control group (n=8); Group 2, ischemia-reperfusion (I/R) group and group 3 (n=8), I/R injury + adalimumab (40 mg/kg, ip, single dose) treatment group.

All rabbits were anesthetized by intramuscular (i.m.) injection of ketamine (50 mg/kg) (Ketalar, Parke-Davis, Eczacıbaşı, Istanbul, Turkey) and xylazine (10 mg/kg) (Rompun, Bayer, Istanbul, Turkey) and permitted to breathe during the procedure. An intravenous catheter was placed in the auricular vein of the animals and preoperative cefazolin 10 mg/kg (Cefamezin, Eczacıbaşı, Istanbul, Turkey) was given as a single dose. As maintenance, 0.9% NaCl (20 ml/h) was given throughout the experiment.

All rabbits underwent laparotomy in supine position. Aortic cross-clamp was not applied to group 1. In group 2 and 3 the abdominal aorta was detected and dissected carefully from the beginning of the left renal artery by transperitoneal approach. Five minutes before occlusion, 100 IU/kg heparin was given intravenously. The aorta was then cross-clamped using an aneurysm clip with a closing force of 70 grams (Yasargil FE 721, Aesculap). The clipping site was just below the origin of the left renal artery. Pulsation of the femoral artery disappeared after occlusion. The aneurysm clip was removed 30 minutes later, and aortic pulsation was restored. Neither aortic nor caval hemorrhage were observed during surgery. Before closure, rabbits of group 3 received single dose intraperitoneal 40 mg/kg adalimumab treatment. After the closure of laparotomy all rabbits awoke and returned to their cages. The animals were followed neurologically, and motor inefficiency and recovery rates were recorded. Seventy-two hours later, all rabbits were re-anesthetized by intramuscular (i.m.) injection of ketamine (50 mg/kg) (Ketalar, Parke-Davis, Eczacıbaşı, Istanbul, Turkey) and xylazine (10 mg/kg)
Yasar Karatas, Mehmet Fatih Erdi, Bulent Kaya et al. (Rompun, Bayer, Istanbul, Turkey). Blood samples were taken from auricular veins for biochemical examination. For histopathological examination spinal cord samples were taken from lumbar spinal cord segments between L4-L6 by laminectomies and the rabbits were sacrificed.

2.2 Biochemical Analysis
Venous blood samples were collected by centrifugation at 4° C and 1,000 g for 10 minutes to remove plasma. Plasma samples were kept at -80 ° C until the parameters were studied. Spinal cord tissue samples were provided in pH 7.4 50 mM phosphate buffer and kept at -80 ° C until they were analyzed. The thawed tissue samples were weighed and homogenized in ice using a mechanical homogenizer and an ultrasonic homogenizer in a 10fold (w / v) cold phosphate buffer (50 mM, pH: 7.4). The supernatants were separated by centrifuging the homogenates for 10 min at 4 ° C and 10,000 g. Pierce bicinechonic acid-BCA (Thermo Scientific, Illinois, USA) was used to measure spectrophotometrically plasma and spinal cord tissue total oxidant (TOS) and antioxidant status (TAS) (Rel Assay Diagnostics, Gaziantep, Turkey), thiobarbituric acid reactive substances (TBARS) (Oxford Biomedical Research, Missouri, USA) and tissue protein levels. Plasma and spinal cord tissue IL-6, IL-10 and TNF alpha levels were examined by using ELISA antigens that were intrinsic to rabbits (Elabscience Biotechnology Co., Wuhan, China).

2.3 Histopathological Studies
Spinal cord samples were stabilized by 10% formaldehyde for two days and then embedded in paraffin blocks. After dehydration, coronal sections of the spinal cord segment were severed at a thickness of 4 μm and stained with hematoxylin and eosin (HE) in order to examine the structural changes. Gray matter was checked in five different areas in each section. Depending on the degree of inflammation, hemorrhage, axonal swelling, congestion, neuronal degeneration and vacuolization of the spinal cord, the light microscopic findings were graded on a scale ranging from 0 to 3, corresponding to “no change”, “mild”, “moderate” and “severe” changes, respectively. The histopathological score was calculated for each spinal cord sample [12].

Apoptotic cells were labeled using an ApopTag In Situ Apoptosis Detection Kit (Millipore). DNA fragments in spinal cord regions were altered by the action of terminal deoxynucleotidyl transferase. The manufacturer’s instructions were followed during procedures. In each section five dark visual fields were randomly chosen, and the TUNEL-positive neurons and the total number of neurons in the selective visual fields was counted. TUNEL-positive index (the TUNEL-positive to whole neurons ratio) was computed. Eight sections from each group were used for measurement, and five high-powered visuals were indiscriminately picked from every section to carry out measurement of the TUNEL-positive indices [13].

2.4 Statistical analysis
Data were analyzed using SPSS (version 24.0, SPSS Inc.) and expressed as mean ± SD. Comparisons were made by the Kruskal-Wallis test. Differences among the groups were evaluated by the Mann-Whitney U test. A p < 0.05 was considered statistically significant. Histopathological score and TUNEL positive cell count were contrasted using a one-way analysis of variance (ANOVA) with TUKEY test.

3. Results
3.1 Histopathological evaluation
I/R injury significantly increased the spinal cord neuronal damage score and apoptotic cell count. Adalimumab treatment statistically substantially decreased spinal cord neuronal injury score and apoptotic cell count (p=0). Large motor cells were observed in anterior horn of the spinal cord in the control group (Figure 1A). No changes were observed in the neurons. The most serious injury was seen in ischemia-reperfusion group in spinal cord in HE sections (Figure 1B, C, D). Necrosis, hemorrhage and congestion were noticed in ischemia-reperfusion group. Nissl substances disappeared in necrotic neurons. In addition, neuropil vacuolization and tissue loss were observed in the gray matter (Figure 1E, F, G). Compared with control group, it was noticed that histopathological score rose in ischemia-reperfusion group. Histopathological alterations and score significantly reduced in adalimumab treatment group (Fig 1H). Myelin swelling determined in white matter in ischemia group and the adalimumab group had less myelin swelling compared to the ischemia group. (Figure 2A, B, C). TUNEL positive cells count
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increased in I/R group when compared with control group (Figure 3A, B). Adalimumab treatment decreased TUNEL positive cells count (Figure 3C). Figure 3D showed differences among groups.

Figure 1. Histopathological photomicrographs of spinal cord tissue stained HE: A: Control Group : Neurons in grey matter and White matter B: Necrotic neurons in Ischemia-Reperfusion Group (black arrow), Haemorrhage ve (arrows), *vacuoles. C: chromatolyses in Ischemia-Reperfusion Group (arrow head), *vacuoles. D: pyknosis in Ischemia-Reperfusion Group (arrows), necrotic neurons (arrows). E: Normal neurons in Adalimumab treatment group F: haemorrhage in Adalimumab treatment group (arrow). G: Congestion in Adalimumab treatment group (arrows). H: Histopathological assessment of spinal cord, (* P < 0.05, compared to group 1; &P < 0.05, compared to group 2).
3.2 Biochemical evaluation

I/R injury significantly increased the plasma and spinal cord tissue TNF alpha, TOS, TBARS, IL6 levels and reduced the plasma and spinal cord tissue TAS and IL10 levels. Adalimumab treatment significantly decreased the plasma and spinal cord tissue TNF alpha, TOS, TBARS, IL6 levels and raised plasma and tissue TAS and IL10 levels (Figure 4, Figure 5).
4. DISCUSSION
Neural tissues can be said to be very sensitive to ischemia. I / R damage of the spinal cord during thoracoabdominal vascular surgery can cause serious discomfort such as paraplegia. Primary injury triggers secondary injury with increased inflammation, apoptosis and free radical formation during the I / R period [14]. Secondary injury may result in endothelial dysfunction and increase vascular permeability that promotes migration and activation of immune cells. These activated immune cells infiltrates the related area and secrete some proinflammatory cytokines. Enhanced inflammation causes reactive oxygen species produce and induces lipid peroxidation which causes injury in the ultrastructure of neural cell membranes and hinders their critical functions [15].

The spinal cord is very sensitive to ischemia because it has its own anatomical features. “The infrarenal aortic cross-clamp” method used in this study leads to severe spinal cord injury. It was first described by Liang et al. as an experimental method [16,17]. Rabbits have segmental blood supply in their lumbosacral spinal cord. Thus, rabbit model of spinal cord I/R injury is commonly used. It is clear that there are many causes of paraplegia. Long-term ischemia, interruption of critical intercostal and lumbar arteries, decrease in spinal cord perfusion pressure and postoperative reperfusion injury are some of these reasons [18]. Therefore, this method is thought to be appropriate to imitate the complications of aortic surgery.

High levels of plasma and spinal cord tissue TNF alpha, TOS, TBARS and IL6 in I/R group signifies
increased inflammation and oxidative stress. Neuronal damage score and apoptotic cell count increase after I/R injury. Adalimumab treatment significantly improves biochemical and histopathological adverse impacts of I/R injury.

5. Conclusion
In this study, it was found that adalimumab had significant neuroprotective effects on rabbit spinal cord I/R injury. After I/R injury, high inflammation and oxidative stress were successfully reversed by adalimumab, and the worse effects of biochemical, histopathological and neurological I/R damage were mitigated. Further studies are needed to carry out this treatment in clinical practices.

References