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Original Investigation

Analysis of Serum Pro-Inflammatory Cytokine Levels after Rat Spinal Cord Ischemia/Reperfusion Injury and Correlation with Tissue Damage

Rat Spinal Kord İskemi/Reperfüzyon Hasarı Sonrası Proinflamatuvar Sitokin Düzeyleri ve Doku Hasarı ile İlişkisi

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ABSTRACT

AIM: A rat model of spinal cord ischemia/reperfusion was conducted and the serum cytokine levels and histopathological changes were assessed.

MATERIAL and METHODS: Twenty-four male Sprague-Dawley rats were assigned into four experimental groups. Group-A (the sham operated rats) and group-B (the spinal ischemia/reperfusion group) were sacrificed at 24 hours postoperatively while group-C (the sham operated rats) and group-D (the spinal ischemia/reperfusion group) were sacrificed at 48 hours. Histopathological changes in the spinal cords and serum cytokine levels were analysed.

RESULTS: All three proinflammatory cytokine levels reached significantly higher levels compared to the sham operated groups in both the 24-hour and 48-hour spinal cord ischemia/reperfusion groups.

CONCLUSION: Inflammation is a plausible pathway in spinal cord ishemia/reperfusion injury. However clinical treatment of the damage does not currently include anti-inflammatory therapy. The results of our study supported the hypothesis that inflammatory responses could play a possible role in the ischemia/reperfusion injury of the spinal cord. Characterization of the role of inflammation in the etiopathogenesis of ischemia/reperfusion injury to the spinal cord is important to facilitate the development of novel therapeutic approaches for prevention and/or treatment of this severe condition.

KEYWORDS: Inflammation, Ischemia, Reperfusion, Rat, Spinal cord, Cytokines

ÖZ

AMAÇ: Rat spinal kord iskemi/reperfüzyon modeli oluşturuldu ve serum sitokin düzeyleri ve histopatolojik değişiklikler değerlendirildi.

YÖNTEM ve GEREÇ: Yirmidört Sprague-Dawley rat dört deney grubuna ayrıldı. Grup A sham opereli ve Grup B spinal iskemi/reperfüzyon grubu ratlar postoperatif 24. saatte sakrifiye edildi. Grup C sham opereli ve Grup D spinal iskemi/reperfüzyon grubu ratlar postoperatif 48. saattte sakrifiye edildi. Spinal korddaki histopatolojik değişiklikler ve TNF-α, IL-1ß ve IL-6 için serum sitokin düzeyleri incelendi.

BULGULAR: Hem 24. saat, hem de 48. saat spinal kord iskemi/reperfüzyon gruplarında her üç proinflamatuvar sitokin düzeyleri sham opereli gruplarla karşılaştırıldığında belirgin olarak daha yüksek düzeylere eriştiler.

SONUÇ: Spinal kord iskemi/reperfüzyon hasarında iflamasyon olası bir durumdur. Ancak hasarın klinik tedavisi günümüzde antiinflamatuvar tedaviyi içermemektedir. Bizim çalışmamızın sonuçları spinal kord iskemi/reperfüzyon hasarında inflamatuvar cevapların olası rol oynayabileceği hipotezini desteklemektedir. Spinal kordda iskemi/reperfüzyon hasarının etyopatogenezinde inflamasyonun rolünün tanımlanması bu ciddi durumun önlenmesi ve tedavisi için gelecek terapötik yaklaşımların gelişmesini kolaylaştırmada önemlidir.

ANAHTAR SÖZCÜKLER: İnflamasyon, İskemi, Reperfüzyon, Rat, Spinal kord, Sitokinler

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INTRODUCTION

Ischemic spinal cord injury is a serious complication of thoracoabdominal aortic surgery and can cause paraplegia in up to 40% of the patients (40). This complication is thought to be a consequence of temporary or permanent ischemia of the spinal cord caused by disruption the blood circulation during aortic cross-clamping (9). Inflammatory processes, together with the free oxygen radicals and the activation of phospholipase-A2, have been accused in the etiopathogenesis of the ischemia/reperfusion injury of the spinal cord (7,19,31).

Tumor necrosis factor-∞ (TNF-α interleukin-1ß (IL-1ß) and interleukin-6 (IL-6) are the key proinflammatory cytokines and play important functions in inflammatory injury to the central nervous system (CNS). Beside their direct damage to the cell membranes, free oxygen radicals activate the accumulation of neutrophils and stimulate various types of cells to produce TNF-α and IL-1ß (12). These cytokines further contribute to the production of other cytokines and expression of endothelial leukocyte adhesion leading to endothelial cell damage and spinal cord ischemia (16,22,30,32).

Although, the role of inflammatory cascade in the etiopathogenesis of traumatic spinal cord injury has been investigated by a significant number of authors, little is known about the responsibility of inflammation in the initiation and maintenance of spinal cord ischemia/reperfusion injury. In the present study, the authors investigated the serum proinflammatory cytokine levels (TNF-α, IL-1ß and IL-6) after ischemia/reperfusion injury to the spinal cord in a rat model.

METHODS and MATERIALS:

Twenty-four male Sprague-Dawley rats weighing between 300 and 400 grams were randomly assigned into four experimental groups; group-A (n=6) was the sham operated rats and sacrificed at 24 hours postoperatively, group-B (n=6) was the spinal ischemia/reperfusion group and sacrificed at 24 hours, group-C (n=6) was the sham operated rats and sacrificed at 48 hours and, group-D (n=6) was the spinal ischemia/reperfusion group and sacrificed at 48-hours. Approval was obtained from the Local Ethics Committee prior to the commencement of the study.

Animal model of rat spinal ischemia/reperfusion:

The detailed surgical method for transient lower thoracolumbar spinal cord ischemia was described elsewhere (15). Briefly, the animals were anesthetized with intraperitoneal (i.p.) injections of ketamine 40 mg/kg (Ketalar, Parke Davis, Eczacibasi, Istanbul) and xylazine 5 mg/kg (Rompun, Bayer, Istanbul). During the surgery, body temperature was monitored using a rectal probe and maintained at 35.5°C to 37.5°C with a heat lamp. A 1.5 cm midline neck skin incision was made and the left carotid artery, jugular vein and vagus nerve were found with the help of a Zeiss operating microscope. Next, a 1.8 F Magellan single lumen balloon catheter was inserted into the left carotid artery and advanced approximately 3.5 cm proximally until it reached the aortic arc and it was inflated with 0.1 cc air. The catheter was then withdrawn slightly until it reached the origin of the subclavian artery and inflated again with 0.2 cc air (total 0.3 cc air) to occlude aortic blood flow. Intravenous heparin (5 U/100 grams) was administered just before the aortic occlusion to prevent coagulation. The left femoral artery was also explored and cannulated with a 23 F polyethylene catheter simultaneously with the carotid artery exploration and arterial blood (0.5 cc/100 grams) was drawn into an injector after aortic occlusion in order to produce systemic hypovolemia and prevent collateral blood flow to the spinal cord.

Aortic occlusion of the blood flow lasted 10 minutes and the balloon was then deflated. Next, heparin was neutralized by protamine (1 mg/100 U heparin) and the left carotid and femoral arteries were ligated. After skin closure, the animals were returned to their cages and the body temperature was kept at 35.5°C to 37.5°C until they were fully recovered.

In sham-operated animals, the exact same surgical procedure was applied without the inflation of the aortic balloon catheter. All animals were sacrificed by a lethal dose of pentobarbital (120 mg/kg) and decapitated. Blood samples were taken for the measurement of the cytokine levels just before the sacrifice and the lower thoracolumbar segments of the spinal cord were harvested for histopathological examinations.

Histopathological examination of the spinal cord:

Spinal cords were fixed in phosphate-buffered 10% formaldehyde for 72 hours. Transverse sections 3 μm thick were prepared after paraffin embedding. These sections were stained with hematoxylin and eosin and assessed by a pathologist for the presence of hemorrhage, congestion, edema, damaged neurons and tissue integrity. The total number of damaged neurons in the gray matter region was counted using five serial sections of each animal. An Olympus U-DO3 light microscope (Tokyo, Japan) was used for histopathological examinations.

Cytokine assays:

Blood samples from each rat were centrifuged at 1500 rpm for 15 minutes and the serum was kept at -86°C until it was analyzed. The serum concentrations of TNF-α, IL-1ß and IL-6 were determined using enzyme-linked immunosorbent assay kits (Biosource International Inc., California, USA; TNF-α: KRC3011, IL-1ß: KRC0011 and IL-6: KRC0061) according to the manufacturer's specifications. Results were expressed as picograms per milliliter (pg/ml) of serum.

Statistical analyses were performed by using the paired-samples T-test on the SPSS 13.0 computer program (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±SD. A probability value less then 0.05 was considered significant ($p<0.05$).

RESULTS

Histopathological observations:

No significant damage to the spinal cord was observed either in the 24-hour or 48-hour sham operated groups (group-A and group-C). There was no spinal cord hemorrhage, congestion or damaged neurons in the sham-operated groups (Figure 1A,C). On the other hand, spinal cord edema was observed in all rats in groups of ischemia that showed prominent cellular edema.

In the 24-hour ischemia/reperfusion group (group-B), spinal cord hemorrhage was observed in 50% of the rats (n=3/6) and congestion was observed in 66.6% of the rats (n=4/6) (Figure 1B). The rate of damaged neurons with pale karyolitic nuclei was less then 50% in this group.

In the 48-hour ischemia/reperfusion group (group-D), significant spinal cord hemorrhage and congestion was observed in 83.3% of the rats (n=5/6) (Figure 1D). The rate of neurons with pale karyolitic nuclei was higher then 50% in this group.

Inflammatory cell infiltration with a predominance of monocytes in the spinal cord sections was observed both in group-B and group-D rats (Figure 2A,B).

Cytokine levels:

TNF-∞ levels in the 24-hour sham operated group (group-A) and 24-hour ischemia/reperfusion group (group-B) were 48.30±11.11 pg/ml and 138.62±78.58 pg/ml respectively. The level of TNF-α in group-B was significantly higher as compared to group-A ($p<0.05$). TNF-α levels in the 48-hour sham operated group (group-C) and 48-hour ischemia/reperfusion group (group-D) were 68.65±25.15 pg/ml and 129.16±51.27 pg/ml respectively. The level of

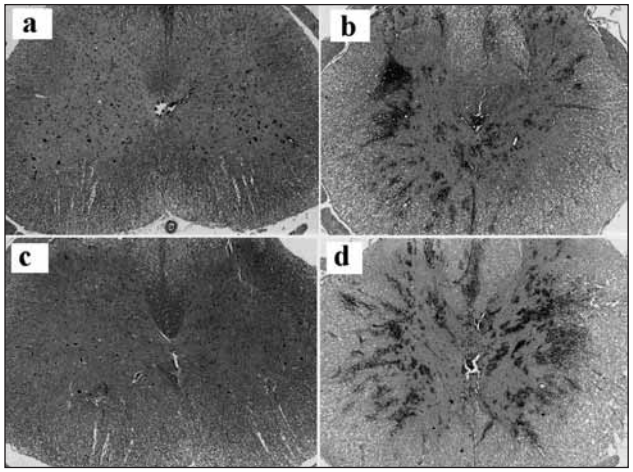

Figure 1: *Photomicrographs of the rat spinal cords. No spinal cord hemorrhage, congestion or damaged neuron was observed in the 24-hour sham-operated group (**A**) and 48-hour sham-operated group (**C**). Spinal cord hemorrhage and congestion was observed in the 24-hour ischemia/reperfusion group (**B**) and 48-hour ischemia/reperfusion group (**D**) (hematoxylin-and-eosin x40).*

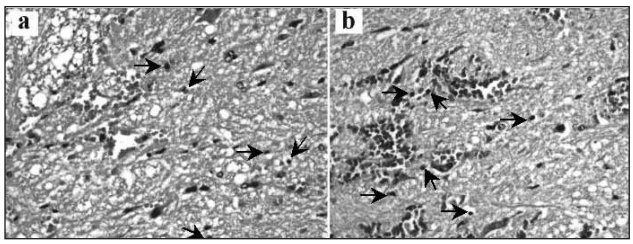

Figure 2: *Inflammatory cell infiltration (black arrows) in the spinal cord sections was observed both in the 24-hour ischemia/reperfusion (**A**) and 48-hour ischemia/reperfusion (**B**) groups (hematoxylin-and-eosin x400).*

TNF- α in group-D was significantly higher compared to group-C ($p<0.05$) (Figure 3). There were no significant changes for TNF- α levels between the 24-hour ischemia/ reperfusion group (group-B) and 48-hour ischemia/reperfusion group (group-D) ($p>0.05$).

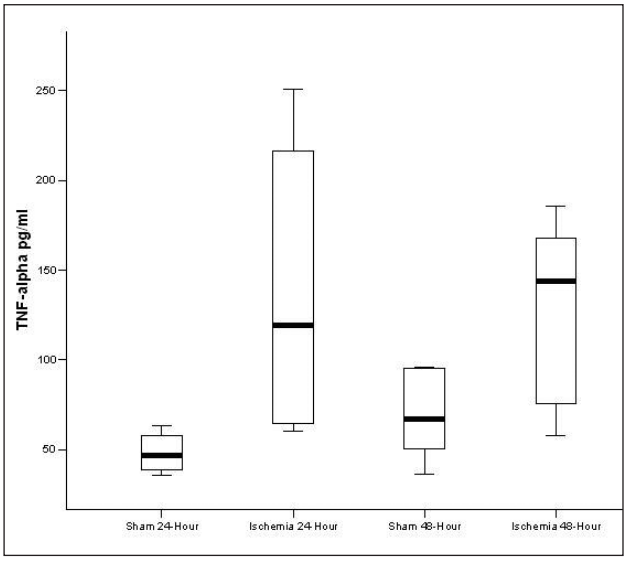

Figure 3: *Boxplot of the TNF-alpha levels of each group animals.*

IL-1ß levels in the 24-hour sham operated group (group-A) and 24-hour ischemia/reperfusion group (group-B) were 22.21±8.64 pg/ml and 58.01±26.46 pg/ml respectively. The level of IL-1ß in group-B was significantly higher compared to group-A ($p<0.05$). IL-1ß levels in the 48-hour sham operated group (group-C) and 48-hour ischemia/reperfusion group (group-D) were 31.45±16.43 pg/ml and 71.65±15.90 pg/ml respectively. The level of IL-1ß in group-D was significantly higher than group-C ($p<0.05$) (Figure 4). There were no significant changes for IL-1ß levels between 24-hour ischemia/reperfusion group (group-B) and 48-hour ischemia/reperfusion group (group-D) ($p>0.05$).

IL-6 levels in the 24-hour sham operated group (group-A) and 24-hour ischemia/reperfusion group (group-B) were 48.21±19.79 pg/ml and 372.50±134.62 pg/ml respectively. The level of IL-6 in group-B was significantly higher compared to group-A ($p<0.05$). IL-6 levels in the 48-hour sham operated group (group-C) and 48-hour ischemia/reperfusion group (group-D) were 216.51±74.48 pg/ml and 847.20±350.28 pg/ml respectively. The level of IL-6 in group-D was significantly higher compared to group-C ($p<0.05$) (Figure 5). In addition, the level of IL-6 in group-D was significantly higher than group-B ($p<0.05$).

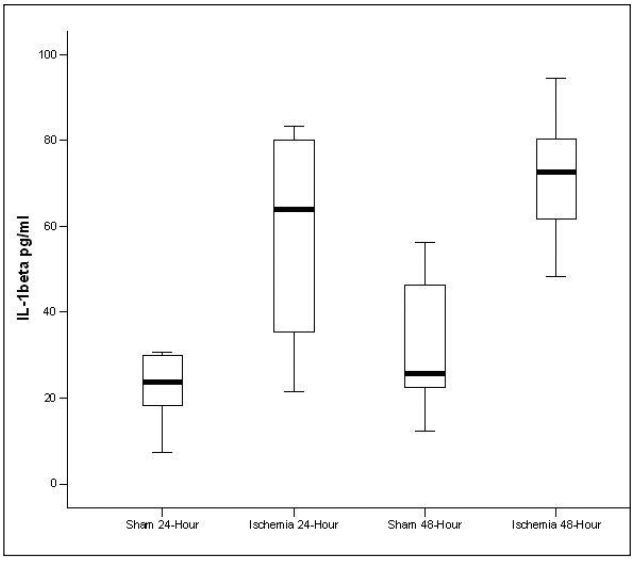

Figure 4: *Boxplot of the Interleukin-1beta levels of each group animals.*

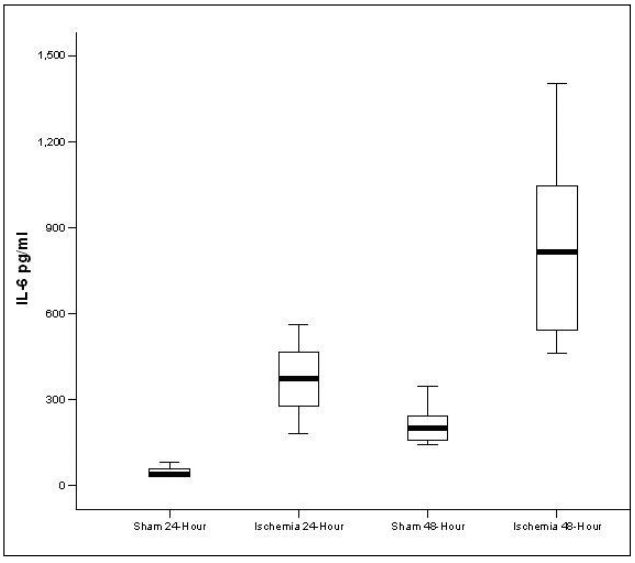

Figure 5: *Boxplot of the Interleukin-6 levels of each group animals.*

DISCUSSION

Paraplegia is a serious complication of thoracoabdominal aortic surgery and thought to be the result of temporary or permanent ischemia of the spinal cord caused by disruption the blood circulation. Ischemia/reperfusion injury occurs when blood flow to an organ is restored after such an ischemic period. Inflammatory processes, together

with the free oxygen radicals and the activation of phospholipase-A2, have been accused in the aetiology of this type of injury (3,4,14). Generation of free oxygen radicals and the activation of phospholipase-A2 trigger the accumulation of neutrophils in the ischemic tissue and stimulate monocytes to produce various types of cytokines (21,27).

Cytokines are a group of proteins produced during the activation and effector phases of the immune response. Proinflammatory cytokines such as TNF-α, IL-1ß and IL-6 are the key inflammatory mediators in several kinds of central nervous system diseases and play an important role in the inflammatory response to traumatic CNS injury (11,13,33). Elevated expression of TNF-α, IL-1ß and IL-6 have been demonstrated in animal models of traumatic SCI (2,11,36). TNF-α and IL-1ß are the early mediators of inflammation and share the same signaling molecules as NF-κB and AP-1 transcription factor (23). Thus both cytokines have similar biological effects.

Several cell types have been shown to synthesize proinflammatory cytokines. TNF-α, for instance, is produced by circulating monocytes and microglia (2,29). TNF-α contributes to neutrophil-induced endothelial cell damage by activation of neutrophils and increasing the expression of endothelial leukocyte adhesion molecules (16). Activated neutrophils damage the endothelium by releasing mediators such as neutrophil elastase and free oxygen radicals. As a result, endothelial cell damage leads to ischemic spinal cord injury. Additionally, TNF-α has toxic effects on oligodendrocytes, promotes apoptosis in neurons and induces IL-6 production (20,25,28,36). However, TNF-α is a pleiotropic cytokine and has been shown to have regenerative effects on axons and neurotrophic effects by inducing nerve growth factor in astrocytes (26,39).

IL-1ß is another key cytokine in the inflammatory cascade that plays a role in neuronal necrosis, apoptosis, leukocyte infiltration and stimulates IL-6 production (1). Similar to TNF-α, IL-1ß has also neuroprotective effects such as enhancing leukemia inhibitory factor synthesis in astrocytes. Both TNF-α and IL-1ß control the production of IL-6, which is synthesized by mononuclear phagocytes, T cells and endothelial cells. IL-6 stimulates the growth of mature B cells and is responsible for the respiratory burst of neutrophils and release of free radicals (5,21,35).

Although, the precise role of the inflammatory process in the etiology of SCI is not clearly understood, there is growing amount of information implicating a possible responsibility of inflammatory mediators in the pathogenesis spinal cord injury. In a rat model of traumatic SCI, Xu et al. have demonstrated that tissue levels of TNF-α in the spinal cord are significantly increased 24 hours after SCI (37). Similarly, in a mouse model of traumatic SCI, Pineau et al. showed that TNF-α and IL-1ß were produced almost immediately following the spinal cord injury and this production is followed by the expression of IL-6 (24). Recently, Yang et al. revealed increased immunoreactivity of TNF-α, IL-1ß and IL-6 in neurons at both early and late phases of trauma in human spinal cord tissues after injury (38]. Klusman et al. administered a mixture of recombinant murine TNF-α, IL-1ß and IL-6 cocktail to the injured mice spinal cord and showed that early administration of this cocktail increased the recruitment and activation of macrophages and microglial cells in the lesion area, whereas late injection of the cocktail resulted in less tissue loss as compared to Ringer solution injected control group (17). Takoa et al. have demonstrated that intravenous injection of activated protein-C (APC) significantly reduced the number of intramedullary hemorrhages as well as the severity of motor disturbances by inhibiting the production of TNF-α in the rat traumatic spinal cord injury model (29). Additionally, they induced leukocytopenia in rats by injecting nitrogen mustard (NM) and reported that NM-induced leukocytopenia markedly reduced the motor disturbances as well as accumulation of neutrophils in traumatized spinal cord tissue, suggesting that both TNF-α and activated neutrophils might play role in the secondary injury process of trauma induced SCI in rats. There are several other reports supporting the possible role of inflammation in the pathogenesis of ischemia/reperfusion injury to spinal cord. Clark et al. have reported that administration of anti-intercellular adhesion molecule-1 (anti-ICAM-1) reduced spinal cord injury after transient ischemia in a rabbit model (6). Similarly, Giulian et al. showed that early administration of chloroquine and colchicine

decreased the ischemic damage to the spinal cord by inhibiting the functions of mononuclear phagocytes (10).

In the present study, we demonstrated that serum proinflammatory cytokine levels (TNF- α, IL-1ß and IL-6) significantly increased after spinal cord ischemia/reperfusion in rats. These increases of serum cytokine levels were accompanied with tissue damage to the spinal cord in the histopathological specimens. Briefly, in both 24-hour and 48-hour spinal cord ischemia/reperfusion groups, all three proinflammatory cytokine levels reached significantly higher levels as compared to the sham-operated groups. These observations complied with the data revealed by traumatic spinal cord injury studies in the literature. Furthermore, the histopathological examinations of the spinal cords in our study revealed that there was significant hemorrhage, congestion and neuronal loss in both the 24-hour and 48-hour ischemia/reperfusion groups as they were compared to the sham operated groups. These findings were more prominent in the 48-hour ischemia/reperfusion group. In addition, we have observed inflammatory cell infiltration in the gray matters of the spinal cords in the ischemia/reperfusion groups. Consistent with the early reports in the literature, the observations in our study supported the hypothesis that inflammatory responses could play a possible role in the ischemia/reperfusion injury to spinal cord.

The rat model of spinal cord ischemia/reperfusion utilized in the present study simulated thoraco-abdominal surgery leading to transient spinal cord ischemia. Anatomical similarities of the spinal cord vasculature between rats and human have been previously reported (18,34). These similarities allow the close experimental reproduction of spinal cord ischemia/perfusion and the associated neuropathology that occurs after occlusion of the descending thoracic aorta in humans. Ten minutes of aortic occlusion resulted in various levels of histopathological changes in all the ischemia/reperfusion group animals. This time period is comparable to periods of aortic occlusion previously reported as sufficient to cause motor deficits in rats (8,15).

In conclusion, ischemia/reperfusion injury to the spinal cord is a potentially devastating and incompletely understood complication of thoraco-abdominal vascular surgery. Since inflammatory response is a plausible candidate pathway and clinical treatment of spinal cord ischemia/reperfusion injury does not currently include anti-inflammatory therapy, it is particularly important to characterize the role of inflammation in the etipathogenesis of ischemia/reperfusion injury to spinal cord. Such knowledge may facilitate the development of novel therapeutic approaches for prevention and/or treatment of this severe condition.

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