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Effect of heparin on neuroprotection against spinal cord ischemia and reperfusion in rats

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Abstract. – BACKGROUND: Paraplegia due to ischemia/reperfusion (I/R) injury of the spinal cord is a devastating and undesired complication of thoraco-abdominal aortic surgery. Unidentified clots cause a variety of thromboembolic events and deteriorate the severity of ischemia. We investigated the effect of the degree of anticoagulation on spinal cord I/R injury and whether heparin is protective against I/R injury beside its anticoagulant properties.

MATERIALS AND METHODS: Twenty-eight rats were randomly assigned to four groups (n=7 per group) as G1 (no aortic occlusion and heparin administration), G2 (45 min aortic occlusion; no heparin administration), G3 (45 min aortic occlusion; 400 IU/kg heparin to keep activated clotting time (ACT) level around 200 sec), and G4 (45 min aortic occlusion; 800 IU/kg heparin to keep ACT level around 600 sec). After neurologic evaluation at the 48th hour of reperfusion, lumbar spinal cords were removed for histopathologic evaluation and immunohistochemical staining for HSP70 (heat shock protein 70), interleukin-6 and myeloperoxidase (MPO).

RESULTS: The Motor Deficit Index (MDI) scores were lowest in G1 group ($p < 0.05$) and the MDI scores of G3 and G4 were significantly lower than G2 group ($p < 0.05$). The neuronal degeneration in G3 was significantly lower than the other groups, respectively ($p = 0.03$). Histopathological evaluation showed no significant intergroup differences in terms of the degree of edema and inflammatory response. There was no statistically significant difference found among the groups in terms of HSP70 staining, IL-6 staining or the degree of MPO staining.

CONCLUSIONS: Protection of spinal cord from I/R injury requires a multimodal management. We should not miss out the importance of adequate anticoagulation in thoraco-abdominal surgical procedures. Furthermore, the recently discovered anti-inflammatory property of glycosaminoglycans, including heparin, deserves to be investigated.

Key Words:
Heparin, Ischemia and Reperfusion injury, Spinal cord.

Introduction

Paraplegia due to ischemia/reperfusion injury of the spinal cord is a devastating and undesired complication of thoracoabdominal aortic surgery, ranging in prevalence from 2.9% to 38%[1,2]. Temporary or permanent ischemia of the spinal cord is inevitable during the operation and caused by interruption of the blood supply during aortic cross-clamping[3]. Several strategies have been implemented to increase spinal cord perfusion and modulate the immune system[4]. These strategies seem to be partly helpful in reducing the risk of spinal cord deficits. However, even after implementing these different approaches, paraplegia remains a persistent complication.

Oxidative stress due to the reperfusion of aorta and other tissues results in the overexpression of inflammatory mediators, cytokines and initiates a catastrophic chain reaction. The inflammatory response activates cellular and enzymatic components that interact with the activated clotting system. Unidentified macroscopic or microscopic clots cause a variety of thromboembolic events, deteriorate the severity of ischemia and augment the inflammatory response[5,6].

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IL-6 is a pleiotropic cytokine engaged in the differentiation of B lymphocytes. It is generally considered as a non-specific marker of inflammation, that is released in response to infection, burns, trauma, neoplasia[7].

Heat shock proteins (HSPs) are cellular stress proteins which have been shown to have an important role for the survival of cells under stress conditions[8]. Zhang et al[9] pointed out that, HSP70 could respond to a wide variety of stress conditions such as ischemia, and inflammation. It can prevent the irreversible denaturation of proteins[10]. Elevated expression of HSP70 can prevent cell death processes[11]. It has been showed that overexpression of HSP70 attenuate the release of inflammatory factors and interferes with the process of apoptotic cell death[12-14].

Myeloperoxidase (MPO) is one of the distinct indicators for the tissue infiltration of neutrophilic granulocytes. MPO activity increases in response to the I/R injury[15].

Heparin continues to dominate anticoagulation therapy for thoracoabdominal aortic graft replacement and other cardiovascular surgical procedures since it is rapidly acting, easily reversible, cheap, widely available and well tolerated. Its activation of antithrombin III results in inhibition of both thrombin and factor Xa, thereby, preventing clotting[16]. Discerning the degree of anticoagulation to attenuate platelet and thrombin activation but provide sufficient clotting to prevent excessive bleeding is difficult. It is made more difficult because the technology to accurately assess the degree of anticoagulation is not clinically available or developed currently.

Besides its well-known anticoagulant properties, heparin displays a role in anti-inflammatory activities in asthma and ulcerative colitis[17,18]. Heparin has an ability to bind different cytokines, such as chemokines. Chemokines are a group of small proteins which has an influence on leukocyte migration and activation[19].

In this study, we investigated the effect of the degree of anticoagulation on spinal cord I/R injury and whether heparin is protective against I/R injury beside its anticoagulant properties.

Materials and Methods

Animal Care

This study was approved by the Animal Experiment Committee of Düzce University Graduate School of Medicine. All rats were treated in compliance with the European Convention on Animal Care. 28 male Sprague-Dawley rats weighing 250 to 350 g were housed in cages and maintained on a 12-hour light/dark cycle with free access to food and water. The animals were kept within the same unit at a room temperature between 18°C and 21°C. The animals were followed for 15 days before the procedure. None of the animals had any neurological abnormality before anesthesia and surgery.

Anesthesia and Monitoring

Rats did not receive food or water within 8 hours before anesthesia. Anesthesia was induced with intramuscular administration of ketamine (50 mg/kg) and xylazine (5 mg/kg). Anesthesia was maintained by intermittent delivery of ketamine (25 mg/kg). Animals were allowed to breathe spontaneously without mechanical ventilation and core temperature was maintained between 36.5-37.5°C by means of a heating lamp. The animals received oxygen at 200 mL/minute via a pediatric face mask throughout the procedure. Each operation was performed in the same operating room at ambient temperature. Ensuring adequate depth of anesthesia, a 24G catheter was surgically inserted into the left jugular vein to provide intravenous fluid replacement (0.9% isotonic saline solution). An arterial 24G catheter was inserted into the left carotid artery for monitoring the arterial blood pressure. The core temperature above 36°C was followed with a rectal probe. The animals received prophylactic antibiotics (procaine penicillin, 200,000 units administered intramuscularly twice a day) for 2 days in the immediate postoperative period. Postoperative analgesia was maintained by subcutaneous injection of tramadol. Hemochron Jr signature plus (Keller Medical GmbH, Bad Soden, Germany) was used to monitor activated clotting time (ACT) levels.

Study Groups

Twenty-eight male Sprague-Dawley rats weighing between 250-350 g were enrolled in the study. Intraperitoneal heparin was administered immediately before the procedure to animals in the study groups. The animals were divided into four groups as (Table I):

Group G1 (Vehicle-treated Sham-operation group): The operation was performed in the same fashion, but without aortic occlusion and heparin administration.

Table I. Age distribution of subjects and varicocele presence.

Group	Duration of ischemia (min)	Heparin before laparatomy	n
G1	–	–	7
G2	45	–	7
G3	45	400 IU/kg	7
G4	45	800 IU/kg	7

Group G2 (Vehicle-treated ischemia group): Aorta was cross-clamped for 45 minutes. No heparin administration.

Group G3 (Low-dose heparin treated group): Aorta was cross-clamped for 45 minutes and 400 IU/kg of heparin was administered. ACT was kept around 200 during the procedure.

Group G4 (High-dose heparin treated group): Aorta was cross-clamped for 45 minutes and 800 IU/kg of heparin was administered. ACT was kept around 600 during the procedure.

Surgical Procedure

Animals were placed in supine position. After sterile preparation, a standard midline laparotomy incision was made, and the infrarenal abdominal aorta was exposed via a transperitoneal approach with the abdominal contents reflected to the right. The aorta was isolated from the left renal artery down to the aortic bifurcation. Heparin was not reversed at the end of the procedure in the study groups. The aorta was cross-clamped at 2 sites: just distal to the left renal artery and proximal to the aortic bifurcation. Loss of aortic pulse was confirmed by palpation. The duration of the ischemic insult was 45 minutes. Following removal of the cross-clamp, distal perfusion was observed visually. Upon completion of the procedure, the abdominal wall was closed in a double-layer fashion with 4-0 Prolene suture. After anesthesia was discontinued, the rats were separated from each other to allow optimal recovery from anesthesia.

Postoperative Care and Neurological Evaluation

At 1 hour of reperfusion, arterial and venous lines were removed, and all medications were stopped. The animals were returned to their cages following their recovery from anesthesia. The Crede maneuver was used to empty the bladders of the paraplegic animals at least twice daily.

An independent observer, who was blinded to the protocol and group assignments, assessed the motor deficit index (MDI)[20] of the animals after 48 hours of reperfusion. MDI was scored using the assessment of ambulation using the hindlimbs and by the placing/stepping reflex. Assessment of the ambulation of lower extremities was quantified as:

0: normal (symmetric and coordinated ambulation)
1: toes flat beneath the body while walking but ataxia is present
2: knuckle walking
3: unable to knuckle walk but there is some movement in lower extremities
4: no movement of the lower extremities

The placing/stepping reflex was evaluated by dragging the dorsum of the hindpaw along the edge of the surface. This movement causes a response of coordinating lifting and placing. It was graded as:

0: normal
1: weak
2: no stepping response

The MDI score of a rat was the sum of ambulation and placing/stepping reflex score. The maximum deficit was indicated by a score of 6. Animals with MDI < 3 were considered as non-paraplegic, and animals with MDI ≥ 3 were considered as paraplegic.

Sacrifice and Tissue Preparation

Immediately after functional assessment, all animals were killed at 48 hours postoperatively by a lethal cardiac injection of sodium pentobarbital (100 mg/kg). The spinal cords between L1 and S1 were harvested immediately via an anterior approach. Each spinal cord was longitudinally divided into 2 equal parts with a fine scalpel. One of the halves was fixed in 10% neutral buffered formalin solution and embedded in paraffin. The other half was snap-frozen for histopathologic examination. The experimental model was carried out according to the experimental studies in the literature that investigate the spinal cord and visceral organ damage after cross-clamping the aorta[21-23].

Histopathologic Evaluation

The paraffin-embedded spinal cord samples were sectioned into 4-μm-thick transverse sec-

tions, which were then stained with hematoxylin and eosin. The histopathological investigation was carried out by two pathologists blinded to the group assignments. The slides were examined using a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at × 400 magnification to assess the degree of spinal cord injury. The gray matter (motor neurons) and white matter (axonal structure and glial cells) were assessed for ischemic injury. A semi-quantitative scale was devised to assess ischemic features. Cells, which had eosinophilic cytoplasm and lost their nucleus, were considered as injured. Neurons, which had prominent nucleolus with fine chromatins and contained cytoplasmic Nissl bodies were considered as viable cells. Four spinal cord injury parameters were evaluated: neuronal degeneration, axonal vacuole formation, edema and inflammation. At least 10 fields from each spinal cord section were examined for the severity of these changes.

Spinal cord injury was scaled relatively as 0, 1 and 2 for absent, moderate, and severe injury, respectively. Edema and vacuolar congestion was scaled as 0 and 1 for absent and present. Inflammatory response was graded by counting the number of leukocytes that infiltrated in randomly selected fields and scored according to the number of leukocytes as following: '0' for none, '1' for less than 20, '2' for 20 to 50, '3' for more than 50 leukocytes.

Immunohistochemistry Examination

Blood samples from each rat were obtained at the end of 48h immediately before the sacrification. The chest wall was cleansed with cholorohexidine in spirit, and a sterile 10ml syringe was then used to obtain a blood sample by direct cardiac puncture. Blood samples for cytokine assay were collected into heparinized (20 unit/ml blood) sterile tubes and immediately transferred on ice to be centrifuged at 2000 rpm (at 4°C) for 10 minutes. They were stored at –70°C until the time of assay for IL-6, MPO and HSP-70.

Analysis of HSP70: Paraffin sections (4 m thick) were prepared. Tissue sections were deparaffinized and hydrated in xylenes and graded alcohol. The sections were incubated with primary anti-HSP70 (clone BRM.22, dilution 1/80, Biogenex, San Ramon, CA, USA) diluted in buffer. Phosphate buffered saline (PBS) was used as negative control.

Analysis of IL-6: The polyclonal anti-human IL-6 receptor antibody C-20 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was used for the detection of IL-6 receptor. This antibody was diluted 1: 20. IL-6 receptor immunostaining was also performed according to a streptavidin-biotin-peroxidase protocol. The secondary anti-rabbit antibody was diluted 1: 500. Negative controls were performed by omitting the first antibody.

Analysis of MPO: The spinal cord MPO activities were evaluated using an anti-MPO kit according to the manufacturer's protocol (Cytostore Inc., Calgary, Alberta, Canada). Briefly, samples on polylysine-coated slides were deparaffinized and rehydrated. Then, the microwave antigen retrieval procedure was performed, and the samples were incubated in a 3% H_2O_2 solution to inhibit endogenous peroxidase. To block nonspecific background staining, the sections were incubated with a blocking solution. Then the sections were incubated with primary anti-MPO antibody, followed by incubation with biotinylated goat anti-mouse antibody. After incubating with the chromogenic substrate 3,3' diaminobenzidine (DAB: Sigma Aldrich, St Louis, MO, USA), the sections were counterstained with HE. The staining of cytoplasmic MPO in the neutrophils was evaluated, and the results were expressed as the percentage of neutrophils cytoplasmically stained positive for MPO. Tissues with no evidence of staining, or only rare scattered positive cells, less than 3%, were recorded as negative. The results were evaluated for intensity and frequency of staining. The intensity of staining was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The frequency was graded from 0 to 4 by the percentage of positive cells as follows: grade 0, < 3%; grade 1, 3-25%; grade 2, 25-50%; grade 3, 50-75%; grade 4, more than 75%. The index score was the product of multiplication of the intensity and frequency grades, which was then classified into a 4 point scale: index score 0 = product of 0, index score 1 = products 1 and 2, index score 2 = products 3 and 4, index score 3 = products 6 through 12.

Statistical Analysis

Statistical analysis and calculations were performed by using SPSS Inc. 15 for Windows (Chicago, IL, USA). Results were expressed as the mean (standard error mean). Kruksal-Wallis

analysis of variance was used to detect differences between groups and statistical comparisons were made using the Mann-Whitney U test. A p value of < 0.05 was considered statistically significant.

Results

There was no significant difference in terms of body temperature, mean arterial pressure, heart rate and body temperature among the groups. One animal was dead in control group due to anesthesia.

Neurological Outcome

Neurological assessment based on the MDI is shown in Figure 1. All rats in the G1 group had a normal neurological status. The ranges of MDI scores were significantly higher in the other groups (spinal cord ischemia-induced rat groups) than the G1 group ($p < 0.05$). The ranges of MDI scores were 2.7 (1-5) and 3.1 (2-6) in G3 and G4 groups, respectively; these values were significantly lower than 5.5 (5-6) in the G2 group ($p < 0.05$).

Histopathologic Evaluation

As depicted in Table II, the neuronal degeneration in G3 was significantly lower than the other groups, respectively ($p = 0.03$).

There was no vacuolar congestion in the spinal cord specimens of G1 group, while vacuolar congestion was evident in the other animals' spinal cords; as showed in Table III.

Histopathological evaluation of the tissue samples taken from the spinal cord showed no significant intergroup differences in terms of the degree of edema ($p = 0.293$). An example of neuronal degeneration and edema that was found in a specimen taken from an animal in G2 is shown in Figure 2.

Evaluation of the spinal cord specimens in terms of inflammatory response showed no significant difference between the groups (Table IV).

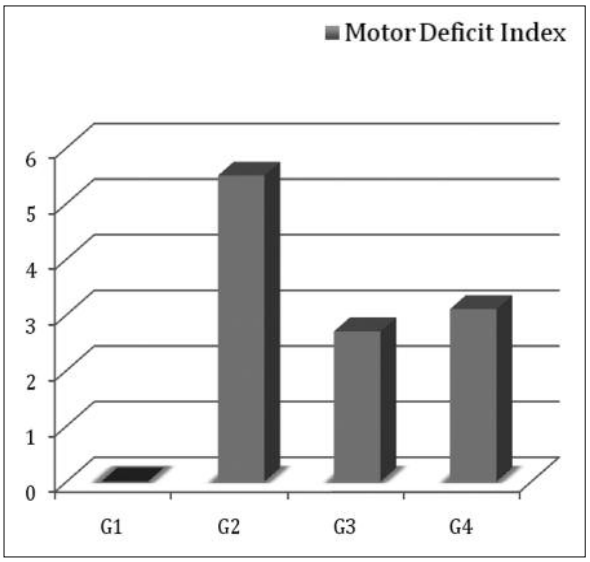

Figure 1. Motor deficit index scores of the animals 48 hours after reperfusion.

Immunohistochemical Evaluation

There was no statistically significant difference found among the groups in terms of HSP70 staining ($p = 0.511$) (Table V). An example to grade 0 HSP70 staining in a sample taken from an animal in G4 is shown in Figure 3.

IL6 staining results are given in Table VI. There was no significant difference found between the groups ($p = 0.306$). An example to grade 1 IL6 staining in a sample taken from an animal in G3 is shown in Figure 4.

No significant difference was observed between the groups in terms of the degree of MPO staining. An example to grade1 MPO staining in a sample taken from an animal in G2 is shown in Figure 5.

Discussion

Neural tissue is very vulnerable to ischemia. Cross-clamping the aorta during the surgical

Table II. Comparison of neuronal degeneration of the spinal cords.

Group	Neuronal degeneration			p
	0	1	2	
G1	4 (57.1%)	3 (42.9%)	0 (0%)	0.03
G2	1 (14.3%)	2 (28.6%)	4 (57.1%)	
G3	6 (85.7%)	1 (14.3%)	0 (0%)	
G4	4 (57.1%)	2 (28.6%)	1 (14.3%)	

Table III. Comparison of vacuolar congestion in the spinal cord specimens.

Group	Vacuolar congestion		p
	0	1	
G1	7 (100.0%)	0 (0%)	0.001
G2	1 (14.3%)	6 (85.7%)	
G3	1 (14.3%)	6 (85.7%)	
G4	2 (28.6%)	5 (71.4%)	

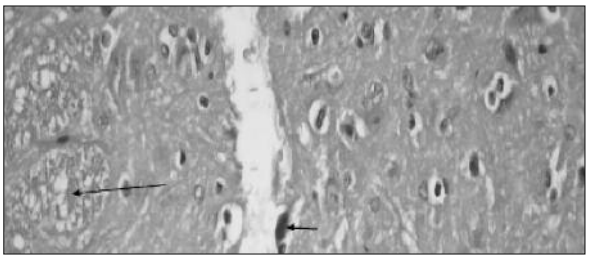

Figure 2. An example of neuronal degeneration and edema that was found in a specimen taken from an animal in G2 (× 200). (*Short arrow*) Degeneration in neurons. (*Long arrow*) Edema.

treatment of descending thoracic and thoracoabdominal aortic disease inevitably results in temporary or permanent ischemia of the spinal cord. Among various factors that has been related with spinal cord ischemia such as distal aortic hypotension after aortic occlusion, duration of ischemia and aneurysm extent, microthrombus formation and thrombosis and/or embolism of intercostal arteries have been one of the most important and avoidable factor[1,4-6,24].

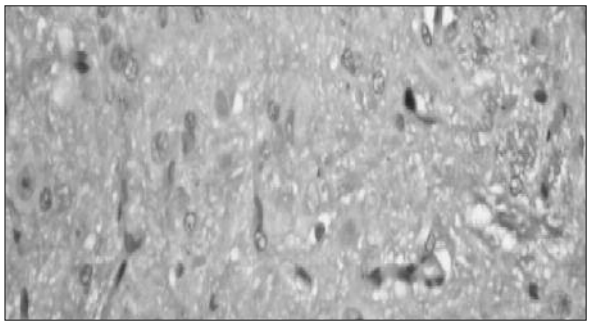

Figure 3. An example to grade 0 HSP70 staining in a sample taken from an animal in G4 (× 200).

Heparin is still the leading choice for anticoagulation in cardiovascular procedures. After injection of heparin, it quickly binds to plasma proteins like platelet factor 4 and fibronectin which reduces its bioavailability[25]. Heparin also binds to macrophages and endothelium that makes its pharmacokinetic properties more complicated. The half-life is affected by the dose and the biologic activity depends on systemic heparin concentration, so the anticoagulant response is not linear.

To provide equilibrium between preventing coagulation and in the mean time preventing excessive bleeding is difficult. This difficulty has increased the importance of the identification of the level of anticoagulation. The most popular test to monitor anticoagulation is ACT, although

Table IV. Comparison of the degree of inflammatory response.

Group	Inflammation				p
	0	1	2	3	
G1	5 (71.4%)	0 (0%)	2 (28.6%)	0 (0%)	0.366
G2	3 (42.9%)	0 (0%)	2 (28.6%)	2 (28.6%)	
G3	4 (57.1%)	0 (0%)	3 (42.9%)	0 (0%)	
G4	5 (71.4%)	0 (0%)	2 (28.6%)	0 (0%)	

Table V. Comparison of the groups in terms of the degree of HSP70 staining.

Group	HSP-70			p
	0	1	2	
G1	6 (85.7%)	1 (14.3%)	0 (0%)	0.511
G2	3 (42.9%)	3 (42.%)	1 (14.3%)	
G3	4 (57.1%)	3 (42.9%)	0 (0%)	
G4	5 (71.4%)	2 (28.6%)	0 (0%)	

Table VI. Comparison of the groups in terms of the degree of IL6 staining.

Group	IL6			p
	0	1	2	
G1	5 (71.4%)	2 (28.6%)	0 (0%)	0.306
G2	2 (28.6%)	4 (57.1%)	1 (14.3%)	
G3	6 (85.7%)	1 (14.3%)	0 (0%)	
G4	4 (57.1%)	3 (42.9%)	0 (0%)	

it's capability to correctly measure the degree of anticoagulation has been controversial[26]. It has been reported that the test results are affected by a lot of factor such as platelet dysfunction, hypothermia, antitrombin level, age, hemodilution, sample size, temperature and venous or arterial blood[26-28].

Although more sensitive tests are available like viscoelastic tests, measurement of activated partial thromboplastin time or heparin concentration, ACT remains the predominant test to manage heparin anticoagulation. Since we know the fact that unidentified macroscopic or microscopic clots cause a variety of thromboembolic events in patients considered adequately anticoagulated, we thought to investigate the effect of ACT levels on spinal cord ischemia in an experimental model.

The pathogenesis of neurologic complications after spinal cord ischemia is also closely associated with reperfusion injury. Actually, ischemia and reperfusion is a chain reaction resulting in free oxygen radical generation, respiratory burst of activated neutrophils that occurs in response to tissue injury and the autooxidation of catecholamines[4]. Free and unstable radicals damage DNA, initiate protein degradation and lipid peroxidation. Ueno et al[29] showed that lipid peroxidation can spread to circumferential neuronal tissue. Lipid peroxidation breaks down the membrane integrity and inactivates the critical membrane bound enzyme systems. The overexpression and release of inflammatory mediators, cytokines, activation of phospholipase A2 and complement system, effects of adhesion molecules, activation of arachidonic acid system make I/R injury very complicated and difficult to understand[4,23,24].

Severin et al[19] reported that heparin, a glycosaminoglycan, has anti-inflammatory properties beside its anti-coagulant effects. This characteristic of heparin has been attributed to binding certain cytokines, like chemokines which mediate inflammation through their control of leukocyte migration and activation. We investigated the levels of IL-6, MPO and HSP-70 to evaluate the inflammatory response to I/R injury and the effect of heparin on it.

We could not find a significant difference between the groups in terms of the degree of inflammatory response, degree of IL6, HSP-70 or MPO staining. But the MDI scores of heparin treated groups were significantly lower and neuronal degeneration was significantly less in heparin treated G3 group.

The half-life of heparin increases to 30 minutes following an intravenous bolus of 25 U/kg, increases to 60 minutes with a bolus of 100 U/kg and increases to 150 minutes following a bolus dose of 400 U/kg[30]. Our clinical routine in ab-

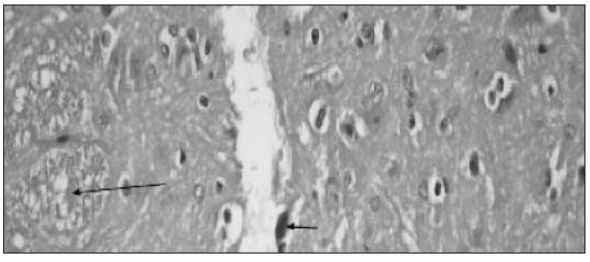

Figure 4. An example to grade 1 IL6 staining in a sample taken from an animal in G3 (× 200).

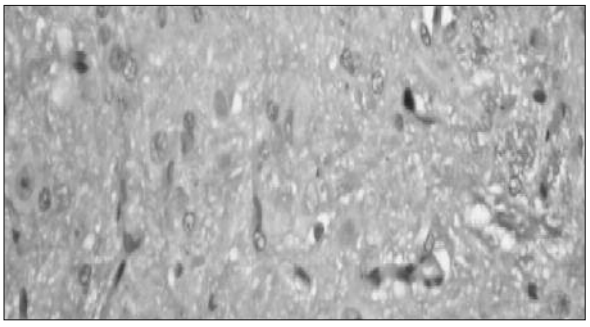

Figure 5. An example to grade1 MPO staining in a sample taken from an animal in G2 (× 200).

dominal aortic surgery is to administer bolus of 100 U/kg heparin and to maintain ACT level around 200 sec. As we could not find any difference between G3 (ACT: 200 sec) and G4 (ACT: 400 sec) groups, keeping ACT level around 200 sec during thoracoabdominal surgery seems both ensure the adequate level of anticoagulation and avoid the adverse effects such as bleeding.

Conclusions

Protection of spinal cord from I/R injury requires a multimodal management. Recent developments in operative techniques, anesthetic management and monitoring, pharmacological medications and postoperative care have decreased the risk of neurologic complications but obviously further investigation is necessary. We should not miss out the importance of adequate anticoagulation in thoracoabdominal and abdominal surgical procedures. Furthermore, the recently discovered anti-inflammatory property of glycosaminoglycans, including heparin, deserves to be investigated.

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