Neuroprotective Effect of Erythropoietin in Experimental Spinal Cord Ischemia-Reperfusion Injury

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ABSTRACT
Spinal cord ischemia and subsequent neurologic deficit are among the most devastating complications of thoracoabdominal aortic surgery. In this study, the role of Erythropoietin in ischemic spinal cord injury model was evaluated. The protective role of Erythropoietin in alleviating neuronal damage in the spinal cord following this type of ischemia in rats was investigated.

Twenty-five rats were used and divided into three groups: Sham, only spinal ischemia (control group), spinal ischemia + Erythropoietin (Erythropoietin group). After spinal ischemia induced by temporary cross-clamping of the abdominal aorta, intraperitoneal saline was given in group 1 (control group), Erythropoietin was administered intraperitoneally as a single dose in group 2 (Erythropoietin group). In group 3 (sham group), abdominal aorta was surgically accessed, but was not clamped, and no substance was applied. Somatosensory evoked potentials (SEPs) were recorded at preoperative and postoperative 24th hour intervals in all rats. Neurological condition was analysed based on Tarlov score at 24 and 48 hours after surgery in all groups. Following sacrifice of the rats, histopathological examinations were investigated.

It was determined that group 2 showed a significant improvement in both Tarlov neurological scores and SEPs after reperfusion than that of group 1 (p<0.001). Histopathological studies revealed less ischemic findings in group 2 than in group 1 (p<0.001).

This study showed that Erythropoietin may hold promise an improvement in clinical, neurophysiological and histological outcomes of ischemia-reperfusion injuries in spinal cord.

Keywords: Erythropoietin, neuroprotective effect, spinal cord ischemia

Introduction
Paraplegia or paralysis, which is reported to occur in 45% iatrogenic causes, is the most devastating and undesired form of spinal cord ischemia–reperfusion injury (SCIRI). It is most seen as a complication of thoracic-abdominal aortic surgery which may lead to life threatening consequences, including death (1-3). Despite advancements in surgical techniques and perioperative strategies for aortic surgery, prevention of SCIRI is still difficult for cardiovascular surgeons, and no consensus guidelines for its treatment (1, 4).

Triggered by the temporary stop or prolonged reduction of spinal cord blood flow during intraoperative aortic clamping, SCIRI causes acute ischaemia in the initial stages. Although some strategies have been developed to diminish this impairment, their capacity to ensure dependable safeguarding against SCIRI is limited (5).

Consequently, diverse pharmaceutical substances have been assessed experimentally in regard to their neuroprotective attributes (1).

Erythropoietin (EPO), a glycoprotein hormone, has neuroprotective effects by reducing glutamate toxicity, increasing neuronal anti-apoptotic factors, promoting neurogenesis and angiogenesis, reducing nitric oxide-associated damage, and exhibiting anti-inflammatory and antioxidant effects (6).

This study aimed to investigate the effectiveness of Erythropoietin for neuronal damage in spinal cord ischemia-reperfusion injury.

Materials and Methods
This study was carried out at the Yuzuncu Yıl University Hospital with local ethical approval (Date: 26.04.2005; Number of approval: 2005/04-
12). Male Sprague-Dawley rats weighing 180 to 200 g were used. The rats were kept under standard conditions in the Experimental Animal Laboratory of Neuroscience Research Unit.

Experimental Design: Three groups were established for twenty-five rats. In group 1 and 2, abdominal aorta was cross clamped by two mini aneurysm clips for 20 minutes, and then clips were released.

Group 1 (n=10): The control group (ischemic) received a single dose of normal saline via intraperitoneal administration after closing the incision following 20 minutes of spinal cord ischemia.

Group 2 (n=10): EPO group received Recombinant Human Erythropoietin (r-hEPO, 2000 IU/kg, Eprex, Cilag AG., Zug, Switzerland) via intraperitoneal injection immediately after the incision was closed following 20 min of spinal cord ischemia.

Group 3 (n=5): Sham group (non-ischemic). A laparotomy was performed, and the abdominal aorta was dissected without clamping.

Creation of SCIRI Models: The spinal cord ischemia/reperfusion injury models were created as described in the literature (7,8). The rats were anesthetized with Ketamine (50 mg/kg, Ketalar, Eczacıbası, Istanbul, Turkey) injected into the intraperitoneal cavity. The rats were then fixed in a supine position on the operating table under anesthesia (Figure 1). A maintenance dose of 25 mg/kg Ketamine was administered. After the operating field was covered with a sterile drape, laparotomy was performed with a midline incision on the anterior abdominal wall of rats. Once the bowel was retracted to the right, the retroperitoneal incision was made and access to the abdominal aorta was obtained. After determining the location of the left renal artery, the adjacent structures were separated. The abdominal aorta was dissected and cross-clamped with two mini aneurysm clips just below the left renal artery and at the level of the iliac bifurcation for 20 minutes (Figure 2). It was confirmed that the pulsation of the artery was absent below the site of aortic clamping. Blood flow was stopped for 20 minutes and then was allowed to flow by removing the clips. At the end of the procedure, surgical incision was sutured with 4/0atraumatic silk. The aorta was left unclipped in rats from the sham group. During the postoperative period, the rats were placed in their cages, and fed on a standard diet and water.

Drug Administration: Group 2 rats were administered a solitary intraperitoneal dose of 2000 IU/kg recombinant human Erythropoietin (r-hEPO, Eprex® 2000 IU/0.5ml, Eprex, Cilag AG., Zug, Switzerland) at the onset of reperfusion, immediately after closing the incision. Group 1 rats were given an equal volume of saline intraperitoneally at the initiation of reperfusion, after incision was closed. The sham group was received no medication.

Physiological and Biochemical Parameters: Examples of basic data for the arterial blood gases pH, arterial partial pressure of oxygen (PaO2) and arterial partial pressure of carbon dioxide (PaCO2), blood glucose (Glc) and hematocrit (Htc) were recorded.

Neurologic Assessment: The neurological condition was evaluated at preoperative, postoperative at 24th hr and 48th hr after SCIRI and graded according to the method of Tarlov (9).

SEP Recordings: SEP monitoring was conducted both pre and post abdominal aorta occlusion to measure neurological recovery, utilizing electromyography (EMG) device (NeuroPack 2, Nihon Kohden). Recording electrodes were placed on the scalp over the parietal lobe and sciatic nerve at the stimulation site. For the recording SEPs, sciatic nerve was stimulated with bipolar electrodes at 2 Hertz (Hz) for 50 milliseconds (ms) at an intensity of approximately 12 milliampere (mA). The recordings were obtained from the contralateral parietal lobe. The average value of two hundred responses was computed and documented. Mean latency and amplitude values in SEP recordings were compared prior to the beginning of ischemia as well as 24 hours post-procedure.

Histopathological Examination: Forty-eight hours after reperfusion, the rats were sacrificed. L5-level specimens of the spinal cord were fixed in 10% formalin for a period of 72 hours before being embedded in paraffin. Successive sectional slices were stained with hematoxylin and eosin, then followed by examination under a light microscope. In the region of the ventral gray matter of the spinal cord section, the presence of eosinophilic cytoplasm, loss of Nissl bodies and pycnotic nuclei were investigated as markers of ischemic neurons (10-13). Motor neurons with these conditions were categorized as 'dead' cells. 'Live' cells were those that did not meet all three criteria. Based on the presence or absence and quantity of ischemic motor neurons located in the ventral gray matter of the spinal cord, the motor...
Fig. 1. Preoperative preparation of the rat neurons were classified as non-ischemic, mild ischemic, and severe ischemic.

**Statistical Analysis:** In this study, NCSS (Number Cruncher Statistical System) 2007 statistical software (NCSS LLC, Kaysville, Utah, USA) was used for statistical analyses. In addition to descriptive statistical methods such as mean, standard deviation, median, frequency and ratio, the Shapiro-Wilks test and box plot graphs were used to assess the conformity of the data to the normal distribution. Kruskal-Wallis test was utilized to evaluate variables that did not exhibit a normal distribution among three groups. Dunn test was conducted to determine which group caused the difference. Intragroup evaluations were performed using the Wilcoxon signed-rank test. As a result, differences in distal latency before and 24 hours after surgery within groups were analyzed using the Wilcoxon Signed Rank test. In turn, paired comparisons in groups of amplitude values in SEP recordings were conducted using the Post Hoc Dunn’s test. The quantity of normal motor neurons in the anterior spinal cord of group 1 was less than those of the group 3 (p=0.001), and those of group 2 (p=0.001). The number of normal motor neurons in group 2 was also lower than that in group 3 (p=0.366) (Figure 4: a, b, c).

**Results**

**Physiological and Biochemical Parameters:** pH, arterial blood gases, hematocrit (Hct), and mean blood glucose (glc) levels did not differ significantly between amongst the 3 groups (p>0.05) (Table 1).

**Neurological Outcomes:** There was no observed neurological deterioration following ischemia in group 3. The median Tarlov score in group 3 was higher than both group 1 (p=0.001) and group 2 (p=0.395). When compared both injured groups (group 1 versus group 2), median Tarlov score of group 2 was significantly higher than score of group 1 (p=0.003) (Graph 1).

**SEP Results:** There was normal SEP in group 3 cases. Group 2 cases showed abnormal SEP with prolonged latency and decreased amplitude, and group 1 cases demonstrated absent response in SEP recording (Figure 3: a, b, c). When the data were analyzed statistically significant differences were observed between the isotonic and EPO groups (group 1 and group 2) in terms of mean values of distal latency and amplitude in SEP recordings at 24 hours after ischemia (p<0.001) (Graph 2: a, b).

When comparing SEP recordings taken 24 hours after ischemia to preoperative recordings, group 1 showed a higher prolongation in mean distal latencies than group 2 (p=0.001), but there was no significant difference in group 3 (p=0.538).

Mean amplitude levels in group 3 were higher than both group 1 (p=0.001) and group 2 (p=0.001). Comparing the injured groups, group 1 had lower amplitude levels (p=0.001) than group 2 (p=0.001).

**Histopathological Features:** 100% of the subjects in group 3 and 60% of those in group 2 were identified as non-ischemic, whereas 40% of the rats in group 2 indicated mild ischemia. Moreover, 80% of the rats in group 1 exhibited severe ischemia, while 20% had mild ischemia (Graph 3). The quantity of normal motor neurons in the anterior spinal cord of group 1 was less than those of the group 3 (p=0.001), and those of the group 2 (p=0.001). The number of normal motor neurons in group 2 was also lower than that in group 3 (p=0.366) (Figure 4: a, b, c).
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Fig. 3: a) SEP in group 3 at 24th hr. Typical tracing depicting the latency (in milliseconds) and amplitude (in microvolts) of the SEP. A: Amplitude (yellow arrow); L: Latency (blue arrow); b) SEP in group 2 at 24th hr; c) SEP in group 1 at 24th hr

Fig. 4: Photomicrographs of tissue sections of the spinal cord from the following groups. HE. X 200. a) Group 3 displays typical spinal cord parenchyma (non-ischemic). b) Group 2, few motor neurons show ischemic features (mild ischemia). c) Group 1, most motor neurons exhibit the ischemic criteria (severe ischemia).

Graph 1: Comparison of the Tarlov scores of the groups at 24, and 48 hours following spinal cord ischemia using Kruskal Wallis test (p<0.001). Note that paired comparisons using the Post Hoc Dunn's test revealed higher median Tarlov score in group 3 when compared to group 1 (p=0.001) and group 2 (p=0.395).

Discussion

SCIRI is a rare complication of aortic or other major blood vessels surgery (14-17). Preventing or minimizing such injury is a critical goal in these procedures. Numerous protective modalities and strategies have been tried to reduce the risk and severity of this injury (18, 19). Nevertheless, an effective treatment management has yet to be developed.

EPO is a key haematopoietic growth factor that stimulates erythroid precursor cells to proliferate and differentiate (20). In addition, research have shown potent neuroprotective activity of EPO against a variety of potential brain and spinal cord injuries (SCIs) (21-23). This property of EPO has been examined in many experimental studies involving traumatic, ischemic, and inflammatory animal models imitating SCI (24-34).

Effectiveness of EPO against traumatic contusion type of SCI has been reported (26,29-36). These reports revealed reduction in lesion severity and increased in neuronal regeneration. Gorio et al. (26) reported that a single dose, and early administration of intraperitoneal EPO application in two different traumatic spinal cord lesion models (temporary compression or blunt trauma) in rats significantly improved motor functions compared to placebo. These were attributed to inflammation reduction, which was associated
Table 1: Comparison of the physiological and biochemical parameters of the three groups (p>0.05) using (Kruskal Wallis test)

<table>
<thead>
<tr>
<th>Group</th>
<th>PH</th>
<th>PaCO2</th>
<th>PaO2</th>
<th>Htc</th>
<th>Glc</th>
</tr>
</thead>
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<tr>
<td>Group 1</td>
<td>7.43±0.04*</td>
<td>57.30±16.32</td>
<td>46.20±21.40</td>
<td>33.74±2.70</td>
<td>134.71±64.49</td>
</tr>
<tr>
<td>Group 2</td>
<td>7.40±0.05</td>
<td>46.90±10.73</td>
<td>52.70±21.19</td>
<td>34.58±3.13</td>
<td>126.49±18.79</td>
</tr>
<tr>
<td>Group 3</td>
<td>7.38±0.05</td>
<td>57.00±9.97</td>
<td>35.60±9.93</td>
<td>32.65±2.98</td>
<td>131.79±29.69</td>
</tr>
</tbody>
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*: Mean±SD (Standard Deviation)  p= p-values

Graph 2: a) Comparison of values for mean latency (msec) in SEPs before and 24th hr after the ischemic procedure in rats using Wilcoxon Signed Rank test (p<0.001). b) Comparison of values for Mean amplitude levels (%) in SEPs at 24 hours after spinal cord ischemia in groups using Kruskal Wallis and Post Hoc Dunn tests (p<0.001).

Graph 3: Histopathological evaluation for 3 groups using Kruskal Wallis test (p<0.001). Under the Post Hoc Dunn's test for pairwise comparisons, the number of normal motor neurons in the anterior spinal cord of group 3 was higher than both group 1 (p=0.001) and group 2 (p=0.366). When compared both injured groups (group 1 versus group 2), normal motor neurons of group 2 were significantly higher than group 1 (p=0.001).

with reduced cavitation within the cord. In other study, Vitellaro-Zuccarello et al. reported alterations in the astrocytic response to injury (34).

According to Dame et al, EPO not only affects apoptosis of motor neurons, but can also modulate many other processes that contribute to cell necrosis and inflammation, such as amino acid excitotoxicity, excessive free radical production, and elevated nitric oxide levels (35). Another injury model is ischemic model. In current study, spinal ischemia-reperfusion was demonstrated using an established model, which closely mimics the real clinical scenario resulting in spinal cord ischemia and the potential effects of Erythropoietin on neuroprotection and functional recovery was evaluated in this ischemia model.

The timing of medical treatment in ischemic model is important. Using the similar methodology to an ischemic spinal cord injury model, Celik et al. (36) demonstrated a noteworthy reduction in neurological damage within one hour of treatment, with no histological signs of injury. Hwang and colleagues (37) discovered that the administering of EPO 24 hours prior to the ischemic insult resulted in neurological and histopathological improvements.
Sonmez et al. (38) conducted a study that confirmed histopathological and electrophysiological changes occurring in neural tissue after the application of EPO following an ischemic spinal cord injury with a same method. The current study examined neurophysiological aspects of injury and response to EPO therapy. The findings confirmed preservation of SEP waveforms in group 2 with some decrease in latencies and reductions in amplitudes. No typical waveforms were observed in the control group.

Upon histopathological examination of the spinal cords of rats in this study, it was confirmed that EPO has a neuroprotective effect on spinal motoneurons against ischemic damage. This effect was found to correspond with the observed functional recovery results. The histopathological examination of group 1 revealed the presence of all ischemic criteria. In group 2, neuronal damage due to ischemic injury of spinal cord was dramatically reduced and significantly better results were obtained in all histopathological examination parameters with EPO. These findings show that using EPO is beneficial in maintaining the morphological structure of the spinal cord.

According to a recent systematic review, EPO may facilitate the recovery of motor function in rats with SCI (39). In the review it was reported that EPO possesses antioxidative qualities. Subgroup analyses reported that the most effective dose was 5,000 UI/kg, but the effect did not differ significantly from that of 1,000 UI/kg. Additionally, it was explained that neuroprotective properties of EPO in SCI were not affected by differences in rat species, animal models, or application method (single or multiple doses).

Although the obtained data hold promises for the use of EPO in neuroprotection against spinal cord ischemia resulting from open or endovascular surgical interventions targeting thoracic or thoraco-abdominal aortic pathologies, it must be acknowledged that there are some limitations in this study. The limited number of subjects also restricts the generalizability of this study. Clinical applications of Erythropoietin therapy for neuroregeneration deserve further investigation with larger samples. To this end, additional experimental research, and meticulously planned randomized controlled studies are necessary to examine its impact on neural recovery.

There exist various injury models for the spinal cord, and one of them is the ischemic model, which may behave and respond to treatment regimens differently from the others. Published data supports that Erythropoietin's efficacy in cases of ischemic spinal cord damage. Our study additionally reveals an improvement in clinical, neurophysiological, and histological conditions of the spinal cord after administering Erythropoietin following such traumas. Thus, this study has shown that Erythropoietin is effective in reducing neuronal damage caused by this type of injury.

References

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