Original Article
Investigation of dose-dependent neuroprotective effect of human recombinant erythropoietin in acute spinal cord injury induced rats

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Abstract: The aim of this study was to investigate the dose dependent neuroprotective effect of recombinant human erythropoietin (r-Hu-EPO) in acute spinal cord injury induced rats. The rats were allocated into 4 groups of 8 rats each. Spinal cord injury was produced by Yaşargil aneurysm clip at a pressure of 0.7 N for a duration of 60 seconds. Group I (Controls) received laminectomy only. Group II (The trauma-only group) had no medication. In group III postoperative intraperitoneal (IP) EPO, total 2000 IU/kg, were applied in two doses. Group IV received postoperative IP EPO in 3 doses, total 9000 IU/kg. In all groups, neuromotor evaluation using Basso's locomotor grading test was conducted at the 6th and 24th hours, and every day from the 1st to 15th days following surgery. After the fifteenth day, all rats were sacrificed and spinal cord samples were obtained for the assessment of caspase-3 activity. The results showed that caspase-3 activity increased to statistically significant higher levels in the spinal cord after injury comparing to the control group. Caspase-3 enzyme activity levels were significantly reduced in animals treated either with low dose or high dose of r-Hu-EPO. In addition, we observed significant difference about rate of functional recovery between group 3 and group 4. Our study showed that high dose EPO administration in the early period after spinal cord trauma increases neurological improvement to a greater degree and in a more rapid manner.

Keywords: Erythropoietin, caspase-3, functional recovery, spinal injury, neuroprotective

Introduction

Spinal cord injury causes permanent neurological deficits and secondary complications in individuals of all ages [1, 2]. The low chance of full neurological recovery after spinal cord injury arouses more interest in posttraumatic biochemical and pathological processes and secondary autodestructive mechanisms caused by trauma [3-6]. Although the mechanism is not exactly known, it is thought that pathological processes such as edema, inflammation, decreased blood flow, changes in microvascular permeability contribute to secondary injury development, which arises from various pathways including lipid peroxidation and free oxygen radicals [2-6]. Therefore, there are many studies focused on neuroprotective agents that could be used against secondary injury [3-9].

Apoptosis is just one of the complex processes that cause tissue injury that develops after spinal cord damage [10]. Apoptosis is mainly regulated by the caspase enzyme family, which is a cysteine protease [11]. The apoptotic role of caspases in injured spinal cord has been demonstrated in many experimental studies [12-20]. It is reported that apoptosis also occurs in human spinal cord injuries [21].

Erythropoietin (EPO) is a hematopoietic growth factor that is produced in the renal and fetal liver and stimulates the proliferation and differentiation of erythroid precursor cells. Additionally, it is also produced in the central nervous system and exhibits non hematopoietic effects [22-24]. In previous studies, it has been reported that it has trophic effects and neuroprotective effects against experimental brain

injury and ischemia in the cholinergic neurons of rats [25-29]. It has neuron protective effects and a vasoconstriction-preventive effect in subarachnoid hemorrhage [30-32]. The neuroprotective effect of EPO is connected to its inhibition of free radical production and release of excitatory amino acids [28, 33, 34].

The aim of the current study is to investigate dose-dependent neuroprotective effect of EPO in experimental acute spinal cord injury induced rats. In the study, caspase-3 activity was used as a biochemical marker, whereas in the assessment of functional recovery, the locomotor grading scale defined by Basso et al. was used.

Material and method

Spinal cord injury

Thirty-two male Wistar rats, weighing between 230 and 260 g were used in the study. Anesthesia was performed with xylazine (10 mg/kg) and ketamine hydrochloride (60 mg/kg) in rats. Following dorsal incision in the prone position, a T6-T9 laminectomy was performed. Spinal cord injury was produced by the extradural application of a Yaşargil aneurysm clip at a pressure of 0.7 N for a duration of 60 seconds. Following homeostasis the layers were closed with 3/0 silk in accordance with the anatomy. The studies were conducted with the permission of the Istanbul University Experimental Animals Ethics Committee approval.

Study groups

Group I: (n=8 rats) only laminectomy was performed.

Group II: (n=8 rats) trauma was produced after laminectomy by a Yaşargil aneurysm clip at a pressure of 0.7 N for a duration of 60 seconds.

Group III: (n=8 rats) following trauma produced after laminectomy by a Yaşargil aneurysm clip at a pressure of 0.7 N for a duration of 60 seconds; early postoperative IP (intraperitoneal) erythropoietin 1000 IU/kg and postoperative IP erythropoietin 1000 IU/kg (2 doses, total 2000 IU/kg) 24 hours later, were applied.

Group IV: (n=8 rats) following trauma produced after laminectomy by a Yaşargil aneurysm clip

at a pressure of 0.7 N for a duration of 60 seconds; early postoperative IP (intraperitoneal) erythropoietin 5000 IU/kg and postoperative IP erythropoietin 3000 IU/kg (3 doses, total 9000 IU/kg) 24 hours later and postoperative IP erythropoietin 1000 IU/kg 48 hours later were applied.

In all rats, neuromotor evaluation using Basso's locomotor grading test [35] was conducted at the 6th and 24th hours, and the 1st , 2nd, 3rd , 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, 12th, 13th, 14th, and 15th days following surgery. After the fifteenth day, anesthesia was performed with ketamine hydrochloride (IP) 60 mg/kg and xylazine (IP) 10 mg/kg; spinal cord samples of rats were then obtained and were held at -80°C in a deep freezer for evaluation of caspase-3 activity. Then all rats were sacrificed by perfusion.

Evaluation of functional recovery

The neurological motor function of each rat was evaluated by using thelocomotor grading scale defined by Basso et al. [35, 36]. With this grading scale, all joints of the hind leg could be evaluated in details. The scale is produced from scores ranging between 0 and 21. While there was no observable movement in the 0 score, the 21 score represents a normal, healthy rat. The locomotor grading scale was applied at the 6th and 24th hours, and the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, 12th, 13th, 14th, and 15th days to all rats used in the current study.

Biochemical assessment

Colorimetric measurement was done with Biovision trademarked caspase-3/CPP 32 ELISA kit. This kit was developed to measure caspase activity recognizing DEVD sequence in an apoptotic pathway. The measurement is based on the detection of light intensity scattered by pNA, which is a chromophore and arises by the degradation of marked substrate DEVD-pNA (p-nitroanilide). The light intensity scattered by p-nitroanilide was read in microtiter plaque reader or in the spectrophotometer at 405 nm wave length. The increase in CPP32 activity was determined by comparing the absorbance of pNA in the apoptotic sample with samples in which apoptosis induction did not occur. The tissue samples in which apoptosis induction has been done were stored at -80°C until the study was done. Homogenization was conducted by studying with kit specific cell

Table 1. Motor function scores according to time

TIME	GROUP I (CONTROL) (avg ± SD)	GROUP II (TRAUMA) (avg ± SD)	GROUP III (LOW DOSAGE EPO) (avg ± SD)	GROUP IV (HIGH DOSAGE EPO) (avg ± SD)	P
6th hour	19.25±0.71	0.00±0.00	0.00±0.00	0.00±0.00	>0.05
12th hour	19.88±0.99	0.13±0.35	1.25±1.04	0.88±0.35	<0.001
1st day	20.50±0.53	1.13±0.64	0.88±0.83	2.88±1.64	<0.001
2nd day	20.75±0.46	1.38±0.92	1.63±0.74	3.63±2.20	<0.001
3rd day	21.00±0.00	1.75±0.89	1.88±0.83	5.00±2.14	<0.001
4th day	21.00±0.00	1.63±0.92	2.50±0.53	5.25±2.25	<0.001
5th day	21.00±0.00	1.63±0.92	3.13±0.83	6.25±2.19	<0.001
6th day	21.00±0.00	2.00±0.76	3.13±0.64	6.50±2.20	<0.001
7th day	21.00±0.00	2.25±0.71	3.88±0.83	7.25±1.98	<0.001
8th day	21.00±0.00	2.50±0.76	4.25±0.89	7.75±1.91	<0.001
9th day	21.00±0.00	2.63±0.74	4.63±0.74	8.38±1.69	<0.001
10th day	21.00±0.00	2.75±0.89	5.00±0.93	9.00±1.85	<0.001
11th day	21.00±0.00	3.00±0.93	5.63±1.06	9.75±1.83	<0.001
12th day	21.00±0.00	3.50±0.93	6.00±0.93	10.63±1.60	<0.001
13th day	21.00±0.00	3.63±0.74	6.38±0.92	11.38±1.85	<0.001
14th day	21.00±0.00	3.88±1.13	7.13±0.83	12.25±1.67	<0.001
15th day	21.00±0.00	4.13±1.13	7.50±0.93	12.75±1.67	<0.001
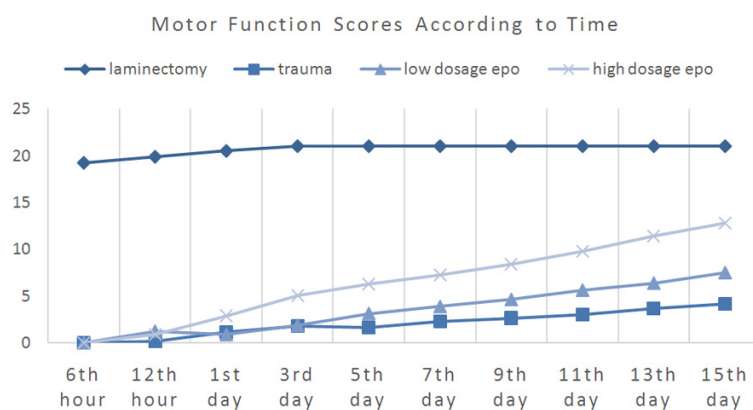
Figure 1. Motor function scores of all groups according to time.

Student's t-test and the Mann-Whitney U-test were used in the comparison of the 1st and 2nd groups; the Mann-Whitney U-test, ANOVA, Turkey HSD, and Kruskal Wallis tests were used in the comparison of the 3rd and 4th groups. P<0.05 was accepted as significant.

Results

Findings of biochemical evaluation

lyses tamponade in a cold environment. The tissue samples were preserved in ice during the study. After homogenization, the tissue samples were centrifuged and a protein analysis of supernatant was done by the biuret method in an Abbott architect trademarked autoanalyzer. Appropriate to the protocol, by adding DEVD-pNA substrate, it was incubated at 37°C for 2 hours. Caspase-3 activity was determined by reading at 405 nm proportional to the amount of protein of the samples.

Statistical analysis

SPSS for Windows 10.0 statistical package program was used in the assessment of the data.

Caspase-3 activities of the groups were determined by obtaining spinal cord samples at the end of the 15th day. Statistically significant differences were found between all groups in terms of caspase-3 activity at the end of 15th day (**Table 2**; **Figure 2**). Caspase-3 activity was significantly higher in the trauma group according to the other groups (P<0.001). The values of the groups in which low and high dose erythropoietin was used were also significantly higher than the laminectomy group (P<0.001). It was found that erythropoietin use significantly decreases caspase-3 activity according to the trauma group (P<0.001). It was concluded that low dose EPO use significantly decreases cas-

pase-3 activity (P<0.05). It was found that high dose EPO use significantly decreases caspase-3 activity at an advanced level (P<0.001). No statistically significant difference was found between low dose EPO use and high dose EPO use in terms of caspase-3 activity (P>0.05).

Findings of functional evaluation

There was a significant difference between groups in all postoperative periods. In the postoperative 6th hour comparisons, a statistically significant difference was found between Group I and the others (P<0.001). There was no statistically significant difference between Group II, Group III, Group IV at the postoperative 6th hour (P>0.05). Between all traumas groups (Group II, Group III, Group IV) significant differences were initiated beginning from the 3rd day. Upon neurological examination of the groups in which erythropoietin treatment was administered beginning from the 4th postoperative day (Group III, Group IV), the recovery was significantly better than trauma group (Group II) (P<0.001). While a significant recovery was found in the low dose erythropoietin group (Group III) beginning from the 5th day when compared to the trauma group (Group II), a significant recovery began at the 12th hour in the high dose erythropoietin group (Group IV) (P<0.05). When both treatment groups (Group III, Group IV) were evaluated, the researchers observed that the significant recovery in the high dose erythropoietin group (Group IV) (P<0.05) developed on the 3rd day.

Discussion

Protection from secondary injury in acute spinal cord damage is called neuroprotection. For this purpose, many medical and surgical approaches such as correction of tissue oxygenation with drug therapies, suppression of spinal cord compression, and stabilization of the vertebral column are experienced. For this purpose, many studies aimed at the pharmacological protection in spinal cord damage have been conducted during the recent 20 years; however, none of these became the standard treatment that would be used in humans.

Secondary injury is a process that starts within minutes or hours following the primary injury and continues for weeks. It is not possible to treat the primary injury. The purpose of the studies aimed at secondary injury is to find and use pharmacological agents and preventions aimed at the protection of the vigor of the neurons that are still intact after the primary injury, their connections to distal neurons and the neurons in the area of the lesion, and increasing their endurance or terminating the pathological processes that could cause harm [37].

In clinical practice, EPO is widely used in treatment of anemias caused by renal insufficiency, cancer, and surgery [38]. The presence of the neuroprotective effects of EPO has been demonstrated in previous studies [28, 30, 39-42].

Today, the knowledge that is obtained following studies is in support of the finding that endogenously produced erythropoietin plays an important role in the central nervous system [43].

Although the mechanism of action of erythropoietin on the central nervous system is not exact, within today's knowledge, it is thought that it demonstrates the effect on signal systems that are different in hematopoietic and neuroprotective characteristics. The receptor producing neuroprotective effect and the receptor producing hematopoietic effect are different from each other [44].

It is found that the activation of caspase-3 that plays important role in development of apoptosis is substantially decreased with erythropoietin treatment [45].

Furthermore there is a direct anti-inflammatory effect of erythropoietin [29, 46].

Another means of the antiapoptotic effect of erythropoietin is the regulation of gene expression [47]. Wen et al. found that by increasing the Blc/Bax ratio and the Blc_2 level of erythropoietin, it leans toward the antiapoptotic side [48].

Additionally, erythropoietin provides neuroprotection by triggering the nuclear factor-the KB signal pathway with JAK_2 activation [49].

The action mechanisms and clinical results of erythropoietin were studied in different experimental models by the investigators. The current study aimed to functionally and biochemically compare the neuroprotective characteristics of erythropoietin, its contribution on

Table 2. Caspase-3 activations of all groups

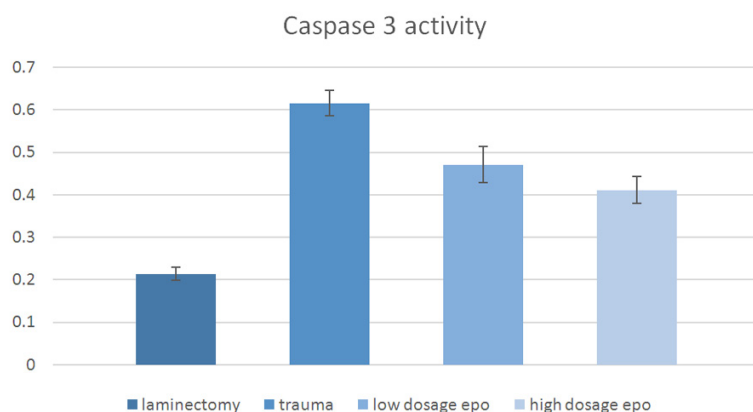|	GROUP I (CONTROL) (avg ± SD)	GROUP II (TRAUMA) (avg ± SD)	GROUP III (LOW DOSAGE EPO) (avg ± SD)	GROUP IV (HIGH DOSAGE EPO) (avg ± SD)	P
Caspase 3 activity	0.214±0.042	0.615±0.082	0.471±0.12	0.411±0.087	<0.001

Figure 2. Caspase 3 activity.

functional improvement, and its antiapoptotic effect using different drug doses. The locomotor grading scale defined by Basso et al. for the assessment of functional recovery was used [35, 36]. Upon evaluation, all joints of the hind limb underwent neurological examination in such a way that it obtained 0-21 points. Antiapoptotic activity was evaluated biochemically by measuring caspase-3 activity in spinal cord samples that were obtained at the end of 15 days.

Upon the evaluation that was done using the locomotor grading scale, it was observed that motor score significantly decreased in all experimental groups that were exposed to trauma when compared to the control group (P<0.001). In all groups that were exposed to trauma (Group II, Group III, Group IV) flask paralysis was detected at the 6th hour and there was no statistically significant difference in terms of motor function assessments that were done at the 6th hour (P>0.05).

It was found that erythropoietin administration after trauma increased motor function scores. In all treatment groups, erythropoietin administration significantly improves motor functions (**Figure 1**; **Table 1**). This finding is parallel to the previous studies that were conducted using erythropoietin [41, 42, 45, 50, 51].

In a similar experimental study, Vasileios et al. administered erythropoietin to two trauma groups with 2×1000 IU/kg and 14×1000 IU/kg (28 days) and they found significant improvement in motor functions on the postoperative 3rd day when compared to the trauma groups [51].

In contusion and clip experiment models that were produced by Gorio et al., they investigated the effectiveness of erythropoietin in acute and subacute spinal cord trauma [42]. They have formed trauma, 1×1000 IU/kg EPO, and 3×1000 IU/kg EPO groups in their mounted clip model and formed trauma, 1×5000 IU/kg EPO, 7×5000 IU/kg EPO, and 7×500 IU/kg EPO groups in a contusion model. In their observations, Gorio et al. detected significant improvement in motor functions in all treatment groups in which erythropoietin was used [42].

In spinal cord trauma model produced by Okutan et al., in the treatment group in which they administered erythropoietin 1000 IU/kg, they found significant recovery in motor functions 24 hours later [45].

In the current study, at the 12th hour a statistically significant recovery was found in motor functions of all trauma groups in which erythropoietin was administered (P<0.05). This result is parallel to the studies of Vasileios et al. and Gorio et al. The recovery of motor functions in the period between the 4th day and the 15th day was statistically significant at the highest degree (P<0.001). Erythropoietin use accelerated motor recovery in the early postoperative period. Gorio et al. demonstrated the recovery of motor functions in the same manner [42].

When compared to the trauma group, a significant recovery in motor functions was achieved starting from the 5th day in the treatment group

in which low dose (2×1000 IU/kg) erythropoietin was administered (P<0.05); whereas in the treatment group, in which high dose erythropoietin (1×5000 IU/kg, 1×3000 IU/kg, 1×1000 IU/kg) was used, a significant recovery in motor functions was found after the 12th hour (P<0.05). It was seen that the improvement in motor functions was at a high degree starting from the 4th day (P<0.001).

While in the 1×5000 IU/kg and 7×5000 IU/kg treatment groups, Gorio et al. found motor recovery that is significant to a high degree, in the 7×500 IU/kg treatment group they found motor recovery that is significant at an intermediate degree at the 4th day of the experimental contusion model [42]. Similarly, in the experimental clip model, the difference between erythropoietin 1000 IU/kg and erythropoietin 3×1000 IU/kg gained significance at the 7th day. The treatment group in which erythropoietin 3×1000 IU/kg was administered achieved better recovery in motor functions. This difference disappeared on the 2nd week.

Throughout the study until the 19th day, Vasileios et al. found motor function scores higher in the group in which erythropoietin 14×1000 IU/kg was administered and this difference reached the highest level with 2 points on the 13th day. On the 19th day, erythropoietin 1×1000 IU/kg and erythropoietin 14×1000 IU/kg treatment groups showed equal recovery in motor functions. After this date until the 41st day, higher motor function values could be observed in the erythropoietin 1×1000 IU/kg group. On the 41st day, motor recovery was significantly higher in the low dose erythropoietin group when the two groups were compared (**Figure 1**; **Table 1**).

Upon the evaluation of the treatment groups in which low dose erythropoietin and high dose erythropoietin were administered, the recovery in motor functions gained a significant difference on the 3rd day (P<0.05). Between these two groups, a statistically significant difference at a high degree was found from the 6th until the 15th day (P<0.001). High dose erythropoietin treatment provided faster recovery in motor functions in the early periods.

The current study revealed that erythropoietin has significant contributions to the recovery of motor functions. When compared to low dose erythropoietin use, high dose erythropoietin use improved motor functions more and in a significantly faster manner. The erythropoietin and erythropoietin receptors in the central nervous system reach peak level on the 8th day, after which they gradually decrease and continue until the 14th day [52]. The administration of high dose erythropoietin during this time period may provide further contribution to the recovery of motor functions.

The caspase-3 activity obtained in the biochemical evaluation at the end of the 15th day according to groups was found to be: 0.21±0.042 in Group I, 0.615±0.082 in Group II, 0.471±0.12 in Group III, and 0.411±0.087 in Group IV (**Figure 2**; **Table 2**). There was a significant difference between experiment groups in terms of caspase-3 activity (P<0.001). Trauma also significantly increased caspase-3 activity. This finding was similar to the findings of Okutan et al. [45]. Similarly, the spinal cord trauma model developed by Yoshiya et al. demonstrated that apoptosis develops in oligodendrocytes and there is caspase-3 activation in these cells [53].

In the study by Okutan et al., caspase-3 activity significantly decreased after the administration of erythropoietin 1000 IU/kg (P<0.05) [45]. Yoshiya et al. administered erythropoietin 5000 IU/kg and found the decrease in caspase-3 activity statistically significant at a high degree (P<0.001). In the current study, it was observed that low dose erythropoietin administration decreased caspase-3 activity (P<0.05). The high dose erythropoietin group also decreased caspase-3 activity at a high degree (P<0.001). Our findings are convenient with Okutan and Yoshiya [45, 53]. When the caspase-3 activity of high dose and low dose erythropoietin groups were examined no statistically significant difference was found between groups (P>0.05).

In the current study as already mentioned in the previous literatures we observed that trauma increases caspase-3 activity. Erythropoietin use decreases caspase-3 activity after trauma. Decreasing caspase-3 activity is an effective step in apoptosis cell formation and is an important step in the prevention of apoptosis. If the significance of different doses of erythropoietin in terms of caspase-3 activity is investigated in a larger population of groups it would give more accurate results.

Conclusion

High dose erythropoietin administration in the early period after spinal cord trauma increases neurological improvement to a greater degree and in a more rapid manner. With our present knowledge, for erythropoietin to be an agent that could be used in the management of neural trauma, there is a need to study the management of dose and time of treatment in a larger series and for a longer period.

Disclosure of conflict of interest

None.

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