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Original Investigation

Comparison of Etanercept, Etomidate and Erythropoietin and Their Combinations in Experimentally-Induced Spinal Cord Injury

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ABSTRACT

AIM: The aim of this study was to compare the preventive effects of Etanercept, Etomidate, Erythropoietin and their combination in experimentally induced spinal cord trauma by clinical, histopathological, electrophysiological parameters and biochemical examination.

MATERIAL and METHODS: 85 healthy female Wistar-Albino rats were used in this study. Rats were divided 8 trauma groups that consisted of 10 rats for each, and 5 rats for the sham group. Laminectomy was performed under general anesthesia and the spinal cord was exposed with intact dura mater, and injury was created by the clip compression model. After the spinal cord injury, drugs were administered immediately intraperitoneally or subcutaneously. Except the sham group, all groups received drugs and were observed 24 or 72 hours. At the 72nd hour each group was anesthesized and somatosensorial evoked potentials (SEP) were recorded from the interarcuate ligament from the 2 vertebra proximal to the injured spinal cord and spinal cord tissue samples were taken for histopathological and biochemical evaluation.

RESULTS: Etomidate groups showed a lower effect on spinal cord injury than etanercept and erythropoietin in terms of clinical, SEP and TNF-α. Etanercept and erythropoietin's neuroprotective effectiveness was shown alone or in combination treatments. More meaningful results were achieved with the use of erythropoietin and etanercept combination after spinal cord injury (SCI) than using erythropoietin alone. After SCI, highest Basso, Beattie, and Bresnahan (BBB) scores were achieved in the group which Etanercept and Erythropoietin applied together.

CONCLUSION: The neuroprotective activity of etomidate was suspect. The neuroprotective effect of etanercept and erythropoietin after SCI was shown in individual and combined applications in this study. However, more experimental studies are needed for clinical use.

KEYWORDS: Spinal cord injury, Etanercept, Etomidate, Erythropoietin, SEP, Neural protection, Rat

INTRODUCTION

Spinal cord injury (SCI) is a complex process that causes destruction of neural tissue and vascular structures. The pathophysiology of SCI includes primary and secondary injury mechanisms. The primary damage in spinal cord injury includes neurons, axons, and blood vessels at the affected site and causes hemorrhage, vasoconstriction and ischemia. After the primary mechanical injury, there is a complex secondary injury cascade including vascular disturbances, calcium-mediated cellular injury, free radical generation,

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glutamate-induced excitotoxicity, mitochondrion dysfunction, pro-inflammatory cytocine production and apoptosis (4,9,23, 26,30).

Erythropoietin (Epo) is a glycoprotein that functions as a main regulator of erythropoiesis. During fetal development, Epo is initially synthesized in the liver, but in adults Epo production is shifted mainly to the kidney peritubular cells and in smaller amounts in the uterus and brain. Stressful conditions, like hypoxia, increase Epo synthesis in the brain (10,18,21). Recombinant human EPO (rhEPO) protects neurons at multiple levels, including limiting the production of reactive oxygen species and glutamate, reversal of vasospasm, stimulation of angiogenesis and neurogenesis, attenuation of apoptosis, and modulation of inflammation (3,10,13,18).

Etanercept is a tumor necrosis factor antagonist agent with anti-inflammatory effects. Etanercept was originally developed for rheumatoid arthritis and Crohn disease but in recent years its beneficial activity in other inflammatory diseases, pain and SCI has been documented (15). After SCI, some cytotoxic cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 are produced. These pro-inflammatory cytokines are involved in recruiting leukocytes and activating macrophages and microglia, and such cytokines are upregulated early in the inflammatory response (20). They can be detected in the circulation at the site of injury, TNF-α levels in the cerebrospinal fluid were significantly higher than normal levels one hour after acute SCI (15). Etanercept inhibits TNF activity by competitively binding to receptors and preventing interactions with its cell-surface receptors (7,11).

Etomidate is a strong antiexcitotoxic agent and shows its pharmacologic effect through the stimulation of the gamma aminobutyric acid (GABA) receptors. It has been shown that etomidate treatment after SCI has similar neuroprotection effects as methyl prednisolone (5). It also reduces hippocampal neural injury in rats subjected to the incomplete forebrain ischemia model (31), blocks ischemia-induced increases in extracellular glutamate and glycine in the hippocampus, and attenuates post-traumatic functional and histologic deficits (8, 27).

Although many chemical agents have been used for minimizing the devastating effect of spinal cord injury with promising results, none has ability to reverse all negative pathways. Since there are many pathways responsible for secondary injury and all of them have to be reversed, the combination of agents seems crucial. The aim of this study was to combine 3 different agents for which a beneficial effect has been reported for the possibility of potentiating their effect.

MATERIAL and METHODS

Adult female Wistar albino rats weighing 200-250 gr. were used in this study. The rats were housed five per cage in a temperature controlled room (18-21^{o}C) with a 12 hours light: 12 hours dark cycle and free access to food and water. The study protocol was approved by the local ethics committee for animal experiments of Ankara University (Date: 01/07/2009, decision no: 2009-44-204).

The animals were anesthetized by an intraperitoneal injection of 60 mg/kg ketamine and 10 mg/kg xylazine. The animals were positioned in the prone position and surgery was done under sterile conditions. Following T8-11 midline skin incision, paravertebral muscles were dissected and spinous processes removed carefully. Laminectomy was applied to the T9-T10 vertebrae by making sure the dura was left intact. SCI was induced by the extradural application of a Yaşargil aneurysm clip exerting a 0.7N closing force on the spinal cord for one minute. After removal of the clip, the wound was closed. The rats' urinary bladder was expressed carefully twice a day. Etanercept (Enbrel©, Pfizer), Etomidate (Hypnomidate©, Johnson & Johnson), Erythropoietin (Eprex©, Santa Farma) applications and groups formed are presented in the table (Table I). Furthermore, each group was divided into two subgroups, A and B, in two different timeframes of 24th and 72nd hours. To determine the late effects of medications, a 72-hour observation period was planned.

Drug administrations that varied for each group took place just after the SCI, and drug administration was continued to 5 animals from each group at the 24th and 48th hours. In order to prevent urinary infection after the operation, gentamicin sulphate (5 mg/kg/day i.p.) was given and the urinary bladders were manually emptied twice a day. After the operation, 2 ml 0.9% NaCl was administered subcutaneously to all animals to prevent dehydration.

After the 24th hour of SCI, the injured spinal cord segments of 5 animals from each group (group A) were removed under general anesthesia and euthanized by cervical dislocation. A part of the removed sample was stored for histopathological study and the remaining part was frozen in liquid nitrogen for biochemical analysis, with the tissue samples preserved at -80^{o}C. The remaining 5 animals (group B) were administered drugs at the 24th and 48th hours. At the 72nd hour, a clinical examination was performed and somatosensorial evoked potentials (SEP) records were made under general anesthesia. As in the 24th hour group, the injured spinal cord segments were removed and samples were taken for histopathological study and biochemical analysis. Afterwards the animals were euthanized through cervical dislocation.

Motor neurological function of the rats after the surgery was evaluated by using the locomotor rating scale of Basso et al. (2). In this scale, the animals are assigned a score ranging from 0 (no observable hindlimb movement) to 21 (normal gait). The rats were tested for functional deficits at 24 and 72 hours after injury by the same examiner who was blind to the treatment each animal had received. The Medelec/Synergy Oxford 5 channel EMG/EP device was used to take the SEP recordings under general anesthesia. The modified scale by Şirin et al. (29) was used for the qualitative evaluation of the injury potentials in the posttraumatic SEP records (Figure 1).

For histopathological evaluation, 4-5 micron thickness sections were taken from the samples, stained with hematoxylin-eosin (HE) and examined with the light microscope (DM4000B, Leica). The spinal cord injury that occurred after the trauma was examined in three different areas at the same magnification (x100). The lesions detected after the study were ranked in three groups as mild, moderate and severe.

Table I: Drug applications and Groups. Furthermore, Each Group was Divided into Two Subgroups, A and B, in Two Different Timeframes of 24th and 72nd Hours

Group	Name	n	Drug applications
1	Sham	5	Only laminectomy performed without trauma or any medications
2	Control	10	Laminectomy + trauma, no medications.
3	Etanercept (Eta)	10	Laminectomy + trauma + etanercept (1.25 mg/kg SC)
4	Etomidate (Eto)	10	Laminectomy + trauma + etomidate (2 mg/kg IP)
5	Erythropoietin (Epo)	10	Laminectomy + trauma + erythropoietin (1000 IU/kg, IP)
6	Epo+Eta	10	Laminectomy + trauma + erythropoietin (1000 IU/kg, IP) + etanercept (1.25 mg/kg SC)
7	Epo+Eto	10	Laminectomy + trauma+ erythropoietin (1000 IU/kg, IP) + etomidate (2 mg/kg, IP)
8	Eto+Eta	10	Laminectomy + trauma+ etomidate (2 mg/kg IP) + etanercept (1.25 mg/kg SC)
9	Epo+Eto+Eta	10	Laminectomy + trauma + erythropoietin (1000 IU/kg, IP) + etomidate (2 mg/kg IP) + etanercept (1.25 mg/kg SC)

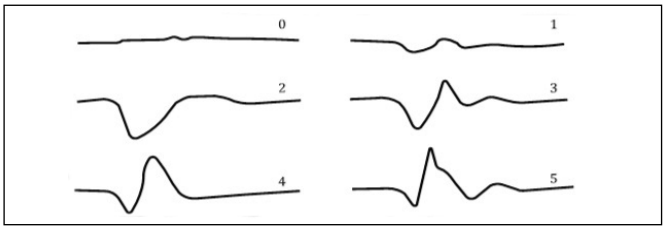

Figure 1: Rating the injury potentials in posttraumatic SEP recordings. **0:** isoelectric line, **1:** major deformation, uncertain response, **2:** complete injury potential, **3:** incomplete injury potential, **4:** morphological change, **5:** normal response (29).

The spinal cord samples taken at the 72nd hour were homogenised in 0.1 M phosphate buffer (pH=7.4) and then centrifuged at 10000 rpm at +4 °C for 10 minutes. After homogenesation, the supernatants were removed and TNF-α levels were measured by using a commercially available rat ELISA kit according to protocol (Invitrogen, catalog no: KRC301). The results were expressed as pg/g protein for TNF-α tissue levels.

Statistical Analysis

In order to control whether data provided the parametric test assumptions, the Shapiro-Wilk and Levene tests were applied. In contrast to the TNF-α measurement values that were obtained by using different animals for each group at the 24th and 72nd hours separately, Student's T test was applied for those groups that provided the parametric test assumptions whereas the Mann Whitney-U test was applied for those groups that did not provide the same assumptions. For the TNF-α measurements that were acquired separately for each timeframe, the Kruskal-Wallis test was applied in order to test the difference between the groups.

For each group, the Paired Sample t-test which is for groups that meet the parametric test assumptions was applied to control the difference between the Basso, Beattie, and Bresnahan (BBB) measurements carried out for the "6th-7th-8th-9th-10th" animals at the 24th and 72nd hours. On the other hand, the Wilcoxon Signed Rank test was applied for the groups that did not meet the parametric test assumptions. At the 24th and 72nd hours, the Kruskal-Wallis test was applied for the BBB measurements taken from all the animals in the study and to control the difference between the groups. One-way variance analysis was used to assess qualitative SEP records. The results were evaluated with a minimal error margin of 5%. The SPSS 14.1 package was used for data analysis.

■ RESULTS

We observed that the operation side of two animals from the 5th and 9th groups were infected at the 72nd hour and three animals from the 4th group nibbled their toes at the 72nd hour. Mild hematuria was present in three cases.

A significant decrease was observed in the trauma groups when compared to the sham groups in BBB scores at the end of the 24th hour (Figure 2). No significant difference was found between the etomidate group and the trauma group ($p<0.001$). A significant increase was found in the BBB scores of other groups except for etomidate in comparison with the trauma group; however, no significant difference was present between the groups ($p<0.001$). Separate or combined administration of etanercept and erythropoietin caused a significant increase in BBB scores ($p<0.001$). However, the combination of the two drugs did not provide additional benefit.

In the statistical data analysis, it was observed that there was no difference in BBB scoring between the etomidate group and the trauma group at the end of the 72nd hour. Drug administration in other groups provided a significant increase in BBB scores but no significant difference was seen between the groups ($p<0.01$) (Figure 2). In the 6th group where

eryhtropoietin and etanercept were used together, BBB scores at the 24th and 72nd hours were higher than in the other groups.

In the SEP recordings taken from the rats that were applied spinal trauma at the 72nd hour, the rostral injury potential score was found to be low caudally in all the groups. In the one-side variance analyses, the rostral injury potential increase in the 9th group that received three drugs was found to be significant in comparison with the trauma group ($p<0.05$). No other significant change was detected in the other groups (Figure 3).

On histopathological examination, there was no lesion variance among the groups in the spinal cord samples taken from the sham and the trauma groups at the 24th and 72nd hours, though severity variance was detected in some cases. Some neurons were observed to be small, inflated, round or wrinkled in all cases (Figures 4 A-F). It was also seen that the cytoplasm was basophilic and the nucleus was not visible in some neurons. Glial cells (neuronophagy) were detected around the neurons. Nissl granules were quite explicit in some neurons and they were localized near the cell wall (chromatolysis). Demyelinated areas, axonal degeneration and gliosis were detected partly in varying severity.

When compared to the control group, a considerable increase was detected in the posttraumatic TNF-α levels ($p<0.001$). The TNF-α levels of groups that received the drug at the 24th and 72nd hours were observed to decrease a considerable degree in comparison with the trauma group (Figure 5). At the 24th hour, it was statistically observed that the use of erythropoietin and etanercept decreases TNF-α level more than the use of etomidate ($p<0.001$). It was found that the separate use of etomidate or its combination with erythropoietin did not provide a remarkable decrease in TNF-α levels when compared to the trauma group ($p<0.001$). The use of etomidate together with etanercept caused a remarkable decrease in the TNF-α level when compared to the combination of etomidate and erythropoietin ($p<0.001$).

At the 72nd hour, the least decrease in TNF-α level occurred in the etomidate group. In the cases where etomidate was administered separately or together with erythropoietin, the decrease in TNF-α levels was less than the other groups. The smallest TNF-α level occurred with the combination of erythropoietin and etanercept.

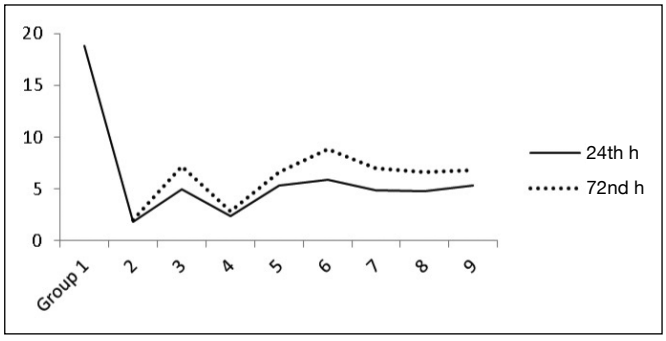

Figure 2: Graphic showing the BBB scores of groups at the 24th and 72nd hour after trauma.

DISCUSSION

More remarkable results were achieved by using erythropoietin and etanercept combination after SCI than the use of erythropoietin alone. The fact that the animals in trauma groups came out of anesthesia with paraplegia after the operation proved the reliability and repeatability of the clip method.

Sprague-Dawley and Wistar rats have mostly been used in experimental SCI studies. In these studies, female rats were used in 39%, male rats were used in 33% and hybrid rats were used at a rate of 28%. Female rats have a shorter urethra than male rats so urination with abdominal palpation is easier in female rats than males after SCI (28). Considering these advantages, Wistar albino female rats were used in the study. No difficulties occurred during urination activities.

Allen's weight-drop model, the aneurysm clip model and the compression model are preferred frequently as the trauma model. In Allen's weight-drop model, good results can not be achieved due to fast spontaneous healing if very little damage occurs in the spinal cord. Likewise, if there is a severe damage in the spinal cord, functional development can not be considered and potential beneficial consequences can be

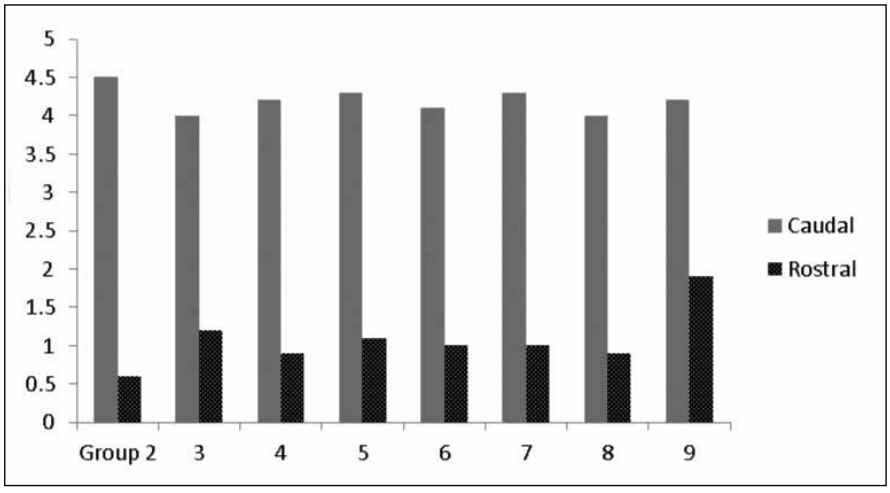

Figure 3: Qualitative SEP findings after spinal cord injury.

masked. Neurological deficits formed after the compression applied by locating the clip on the dorsal and ventral surfaces of the spinal cord and by locating it on the lateral surface are similar (25). We therefore created SCI by compressing the lateral surfaces of the cord with a Yaşargil aneurysm clip.

Various doses were used for erythropoietin administration and they were generally 1000 IU/kg or 5000 IU/kg intraperitoneally or intravenously. Various studies have been conducted on the dose activity (6,14,17). It was observed in these studies that optimal results were obtained with low-dose applications and there ws no difference between intravenous or intraperitoneal use. We administered erythropoietin intraperitoneally at a dose of 1000 IU/kg in accordance with the literature data in this study.

Agnello et al. (1) demonstrated that erythropoietin application decreases inflammatory cell infiltration in the spinal cord in the autoimmune encephalomyelitis model created experimentally in rats. Gorio et al. (14) reported that high dose erythropoietin and methyl prednisolone suppress proinflammatory cytokine, and only erythropoietin reduces microglial infiltration and the formation of scar tissue. In this study, the separate use of erythropoietin in accordance with the literature data reduced

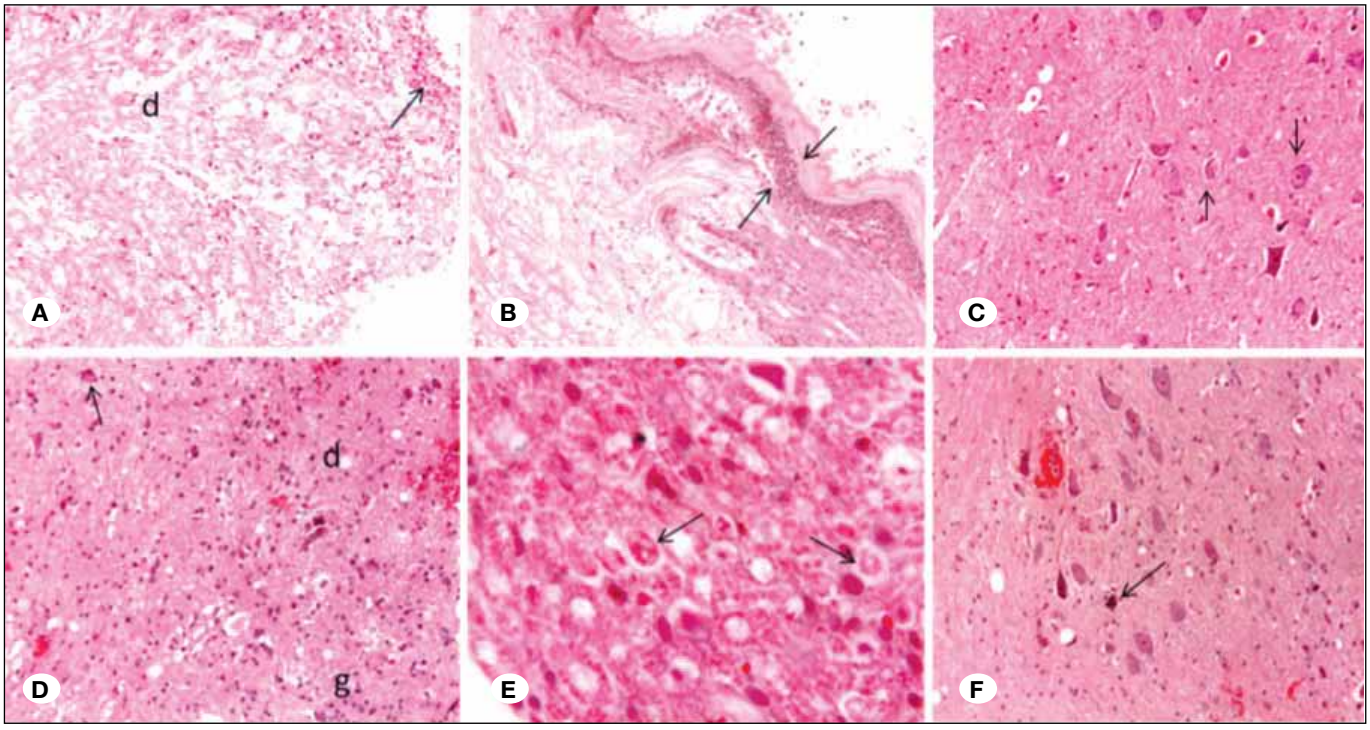

Figure 4: A) Group 2; 24th hour severe bleeding areas (arrow) and demyelination (d). **B)** Group 2; 72nd hour neutrophil infiltration in the substantia alba (arrow). **C)** Group 3; 24th hour moderate neuronal degeneration and peripheral chromatosis (arrow). **D)** Group 3; 72nd hour moderate gliosis (g), neuron degeneration (arrow), and demyelination (d). **E)** Group 4; 24th hour moderate axonal degeneration (arrow). **F)** Group 8; 24th hour moderate neuronal degeneration and neuronophagy (arrow).

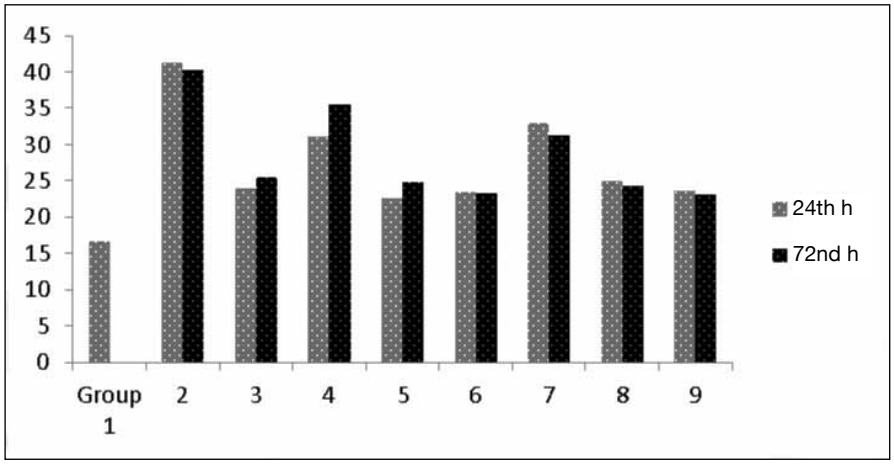

Figure 5: Graphic showing TNF-α levels at 24th and 72nd hours (pg/ml).

the TNF-α level a considerable amount in comparison with the trauma group ($p<0.001$). In the present study, no statistically significant difference was found between the use of erythropoietin alone or its combination with etanercept.

Genovese et al. (12) observed that the combination of dexamethasone and etanercept caused a decrease in neutrophil infiltration in the lesional area after SCI. In the same study, it was also proven that the combination of dexamethasone and etanercept reduced inducible nitric oxide synthase levels, cytokine expression and apoptosis. Marchand et al. (22) demonstrated that the use of etanercept after hemisection in the spinal cord reduces microglial activity and that it also has a protective effect against mechanic hypersensitivity up to 28 days though it does not reverse it. In this study, etanercept administration in accordance with literature data reduced the TNF-α level considerably in spinal cord samples when compared to the trauma group ($p<0.001$). It was observed that when etanercept is used with erythropoietin, it again reduced the TNF-α level considerably in comparison with the trauma group. There was no remarkable difference between the use of etanercept separately and its use with erythropoietin at the 24th and 72nd hours. When TNF-α levels were compared in the groups that only received erythropoietin and etanercept, there was no statistical difference. It can therefore be concluded that erythropoietin is as successful as etanercept in TNF-α inhibition.

There is limited literature showing the neuroprotective activity of etomidate (5,32). Although there have been studies about the decrease of functional and histologic deficits by etomidate administration after SCI (8,27,31), in this study these useful benefits were not reflected on the clinical practice. When compared to the trauma group, it was seen that the use of etomidate alone did not reduce TNF-α levels considerably ($p<0.001$). In the posttraumatic examinations carried out at the 24th hour (group A) and 72nd hour (group B), it was seen that the combination of etomidate and etanercept reduced TNF-α level more than the combination of etomidate and erythropoietin. Moreover, there was no remarkable difference between the use of etomidate together with etanercept and the use of erythropoietin separately ($p<0.001$).

After SCI, the behavioral outcome in rats is important for evaluating the severity of injury and treatment effectiveness. Locomotor, sensory, sensory-motor, reflex response based, autonomic or electrophysiological behavioral tests can be used for this purpose. The BBB test that developed by Basso, Beatie and Bresnahan is the most commonly used locomotor function test in experimental SCI studies (28).

In locomotor evaluations by using the BBB scale, it was observed that the motor score decreased considerably in all traumatised groups when compared to the control group as in previous studies ($p<0.001$). Many researchers have found improvement of the BBB score and improvement in movement on swimming tests as a result of erythropoietin administration (19). Gorio et al. observed progress in the motor functions of all treatment groups that received erythropoietin in the trauma model on which they studied acute and subacute activity (13). Okutan et al. observed considerable progress in the BBB scores at the end of the 24th hour in the treatment groups that received 1000 IU/kg erythropoietin (24). In this study, there was considerable progress in all groups in the evaluations made at the end of the 24th hour in BBB score except for the 4th group that received only etomidate. There was not a remarkable difference between the etomidate group and the trauma group. Although the highest level in BBB score occurred with the combination of etanercept and erythropoietin, there was no difference between the groups statistically. In the statistical evaluation of the BBB scores at the end of the 72nd hour, there was no difference between the etomidate group and the trauma group. Drug applications in other groups led to a considerable progress in the BBB score; however, there was no significant difference between the groups.

Sirin et al. (29) made a qualitative evaluation of the injury potentials in the SEP records by evaluating them between 0 and 5. As in the previous study, injury potentials were classified between 0-5 in this study and the posttraumatic rostral injury potential score was found lower than caudal injury potentials. No injury potential was found in the physiological SEP recordings taken from the sham groups. Although there was no significant difference among the groups, the increase in the rostral injury potential of the 9th group that received erythropoietin, etomidate and etanercept together was considered significant in comparison with the trauma group ($p<0.05$). Thus, it is possible to conclude that the combination of these three drugs protects somatosensory functions. In spite of this, longer studies need to be conducted to evaluate SEP recordings as in motor function evaluations.

Severe inflammation is observed if hemorrhagic necrosis occurs in the spinal cord after SCI. Hemorrhagic necrosis is most frequently seen in the substantia grisea, and the adjacent posterior tractus (16). In histopathological examinations, multifocal bleeding areas were detected in substantia alba and grisea, around the nerve plexus and/or fasciculi. Necrosis was detected in all cases; however, since it could not be calculated quantitatively, no result was found regarding the differences between the groups. Moderate axonal degeneration was seen at the 72nd hour of the etomidate group and at the 24th hour of the erythropoietin+etomidate combination. Mild axonal degeneration was observed in the last group that received the combination of the three drugs. In the erythropoietin group, neuron degeneration and demyelination were more severe at the 72nd hour. In the group that received erythropoietin and etomidate together, neuronal degeneration was mild and demyelination did not develop. However, this positive effect was not reflected on SEP, TNF-α and clinical recovery.

■ CONCLUSION

The neuroprotective activity of etomidate was found to be suspect according to the functional test and TNF-α results. However, it was found that it has a positive effect on demyelination and neuronal degeneration in the histological evaluation. The neuroprotective effect of etanercept and erythropoietin was shown in individual and combined applications in this study. The combination of different drugs looks promising for minimizing secondary injury.

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