Article

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Investigation of efficacy of treatment in spinal cord injury: Erythropoietin versus methylprednisolone

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Abstract

Background: Investigation of the expression of platelet-derived growth factor (PDGF)-β and glial fibrillary acidic protein (GFAP) in rats with spinal cord injury as a marker of neurologic recovery between groups treated with erythropoietin (EPO) and methylprednisolone (MP). **Methods:** Thirty adult female rats were randomly divided into three even groups. A laminectomy was applied to thoracic ninth vertebra and contusion injury was induced by extradural application of an aneurysm clip. Group 1 rats received one-time intrathecal administration of normal saline, group 2 rats received MP, and group 3 rats received EPO. Motor neurological function was evaluated by the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale. Thirty days after the surgery, T8–10 segments of the spinal cords were extracted and the immunohistochemical assay revealed the number of PDGF-β- and GFAP-positive cells. **Results:** Evaluation of the last control animal showed that BBB score in the EPO group showed an increase from 1 to 12 ($p < 0.05$). The immunohistochemical assay revealed that the number of PDGF-β- and GFAP-positive cells was significantly higher in EPO group ($p = 0.000$) when compared to MP and control groups. After studying the effect of PDGF-β expression on the locomotor function, we determined that PDGF-β expression and locomotor function after a spinal injury has a strong relationship ($p < 0.05$). **Conclusion:** EPO seems to better increase the expression of PDGF-β, thus produce better results in locomotor functions when compared to MP.

Keywords

animal study, erythropoietin, GFAP, methylprednisolone, paraplegia, PDGF, spinal cord injury

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Introduction

Traumatic spinal cord injury (SCI) continues to be a serious health problem for all populations at the dawn of the 21st century. Outcomes related to SCI can often restrict ambulation and become debilitating; as a result, SCI can cause further damage in the form of psychological and social problems, which renders life intolerable for many. SCI can be classified into two phases. The primary injury is the result of the direct action of the mechanical force on the spinal cord.[1] This is called the acute phase of the injury and is caused by direct tissue compression and hemorrhage. Starting shortly after the first phase, an ischemic insult

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results from abnormal circulation and edema and causes apoptosis of both neurons and astrocytes, which is a hallmark of the second phase.[2,3] Because no method exists to alter the occurrence of the injury, there is therefore no way to manipulate the first phase. Thus, an ideal treatment must focus on the secondary phase of the injury if any progress is to be obtained toward recovery from a traumatic SCI-derived disability.

However, the current treatment options continue to be rather limited. High-dose methylprednisolone (MP) administration is still used as the only treatment method after acute injury. Although it has been shown that MP reduces chemokine production and the inflammatory reaction after SCI, the exact mechanism of action for glucocorticoids remains unclear.[4,5] Erythropoietin (EPO), a potent inhibitor of apoptosis and a promising therapeutic agent, has been studied in a variety of neurological insult scenarios including traumatic brain injury and SCI.[6,7] Platelet-derived growth factor (PDGF) is synthesized and secreted by multiple types of cells and can bind to its specific receptor (PDGFR) in the central nervous system (CNS). PDGFs are speculated to play a role in CNS development, maintenance, and response to CNS injury.[8] In addition, PDGFs can maintain the reactivity of the neurons and facilitate axonal sprouting. PDGF-β has the capacity to promote mitosis of glial cells and exhibits a neuroprotective effect.[9] Studies have found that EPO stimulates endothelial cells to release PDGF.[8,10] Glial fibrillary acidic protein (GFAP) is produced by fibroblasts and astrocytes. It has been shown to be strongly activated after neurological injury, seemingly as a response to tissue damage, and plays a role in the healing process.[11]

As mentioned above, it has been shown that the intrathecal administration of EPO can alleviate conditions that are related to traumatic SCI and can accelerate neurological recovery in a rat model.[12] However, EPO has not been compared to MP in terms of treatment efficacy. We therefore investigated the expression of PDGF-β and GFAP in rats with SCI as markers of neurologic recovery in animals that were treated with EPO and MP.

Materials and methods

After ethical committee approval was obtained, 30 adult female Sprague-Dawley rats that weighed 200–220 g were randomly divided into three equivalent groups ($n = 10$). After the administration of ketamine–xylazine (0.9 cc/kg intramuscular) and penicillin (10,000 U/kg) for anesthesia and prophylaxis for infection, respectively, all animals were operated on by the same two orthopedic surgeons. After routine preparation and draping of the surgical site, a posterior midline longitudinal incision was utilized to reveal the posterior elements of the ninth thoracic vertebra. A bilateral laminectomy was applied to the ninth thoracic vertebra, which exposed the dura layer of the *medulla spinalis*. A contusion injury was induced via the extradural application of an aneurysm clip (Aesculap® FB435R Glover Mini-Bulldog Clamp, 45 mm, curved, Germany) that exerted pressure on the spinal cord for 1 min. After this point, group 1 rats received a one-time intrathecal administration of 4 ml/kg normal saline, group 2 rats received 3 mg/kg MP, and finally, group 3 rats received 5000 IU/kg EPO.

After the intrathecal injection, the skin incision was sutured and the animals were allowed to recover from anesthesia, after which they were returned to their cages. Motor neurological function of the rats was evaluated with the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale at 1, 24, 48, and 72 h, and then at 1, 2, 3, and 4 weeks after the injury by the same examiner who was blind to the grouping information of the rats. At 30 days after the surgery, all rats were euthanized with a lethal dose of sodium pentothal. T8–10 segments of the spinal cords were extracted from all animals. We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers/animals were followed during the course of this research. Ethical committee approval was given by Acibadem University.

Immunohistochemical assay and hematoxylin and eosin staining

The tissues were fixed in neutral formalin for 24 h and then incubated at 60°C overnight for deparaffinization. The sections were heated in a microwave for 15 min in citrate buffer for antigen retrieval and exposed to hydrogen peroxidase for 15 min to prevent endogenous peroxidase activity. Then, the sections were treated with a blocking serum (Ultra V Block, TP-060-HL; Neomarker, Fremont, California, USA). Afterward, the sections were incubated with primary antibodies to PDGF-β (Thermo, California, USA) and GFAP (SCBT, Dallas, Texas, USA) for 60 min at room temperature and humidity. The antigen-antibody complex was fixed with a biotinylated secondary antibody and a streptavidin–peroxidase complex for 20 min. The sections were incubated with 3-amino-9-ethylcarbazole (Thermo). Finally, the sections were stained with Mayer's hematoxylin and covered with mounting medium. Photos were obtained with a camera attached to an Olympus microscope (CX31, Germany). For each section, 10 fields of 400× magnification were randomly chosen for evaluation. The staining intensity of the slides was assessed semiquantitatively using an immunohistochemical protocol. An *H*-score was calculated for each field using the equation $H\text{-score} = \sum\_Pi\ (i + 1)$, where "*i*" is the intensity of staining, with a value of 1, 2, or 3 (weak, moderate, or strong, respectively), and *Pi* is the percentage of stained cells for each intensity, varying from 0 to 100%. Intensity and uptake scores were calculated for each field. All histometric calculations were performed with image analysis software (Leica QWin V3 Plus Image, Leica, Germany).

Statistical analysis

The statistical analysis was performed using SPSS version 22.0 statistical software for Windows. The Kolmogorov–Smirnov test was utilized to assess the distribution of study parameters between groups, which did not yield a normal distribution; thus, the median and range values were used instead of the means and standard deviations. Comparisons of groups were conducted with a Mann–Whitney U test. The level of significance in this study was $p < 0.05$. In addition, a correlation coefficient was measured with Kendall's τ method to compare PDGF values and 30-day BBB scores.

Results

Initially, 10 rats were included in each group, but one of the rats in the control group died during the surgery. Another rat in the control group died 2 days after the surgery. One rat from each group died the week after the surgery. On the first day after the surgery, all of the rats from all three study groups exhibited dramatic bilateral hind limb paralysis with no significant difference in BBB scores. Later, on the seventh day after the surgery, minimal differences were observed in BBB scores between the control and study groups (EPO or MP), which were not statistically significant ($p = 0.508$). However, the evaluation of the last control animal at 30 days after the surgery showed that the locomotive dysfunction was reproducible, and the BBB score in the EPO group increased from 1 to 12 ($p < 0.05$), whereas the increases in BBB scores for the control and MP groups were not significant (1–3.5 in the control group and 1–4 in the MP group). Figure 1 depicts the differences in locomotive function scores (BBB) for each group between the first hour and 30-day evaluations.

The immunohistochemical assay revealed that the numbers of PDGF-β- and GFAP-positive cells were significantly higher in the EPO group ($p = 0.000$) than in the MP and control groups (Figure 2). PDGF-β expression was apparent in the cell bodies and the cytoplasm of the astrocytes, whereas GFAP expression was stronger in the cytoplasmic projections of the astrocytes[13,14] (Figure 3).

Astrocytes in the MP group also showed an increased immunoreactivity and better uptake scores than in the control group. However, the difference was not significant ($p > 0.05$). The EPO group exhibited relatively strong expression of PDGF-β in astrocytes. Figure 3 further summarizes the immunohistochemical assay and hematoxylin and eosin staining results for all groups.

Finally, to study the effect of PDGF-β expression on locomotor function, we calculated the correlation coefficient of the EPO-treated group. We determined that PDGF-β expression and locomotor function after spinal injury exhibited a 60% positive correlation ($p < 0.05$). The correlations between PDGF-β expression and locomotor function variables are shown in Figure 4.

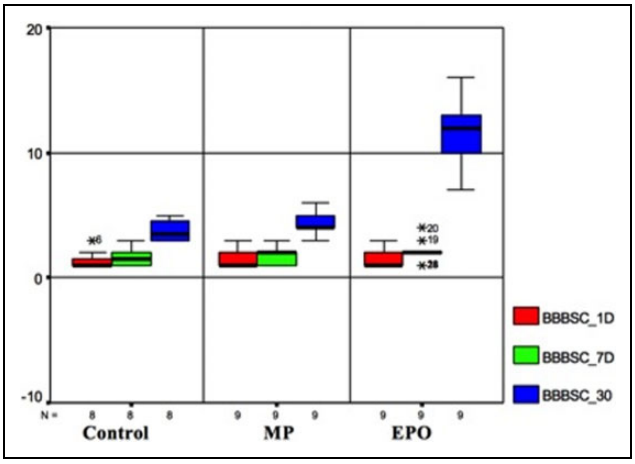

Figure 1. It is shown that difference of locomotive function scales for each group in first and last control.

Discussion

Reducing apoptosis after the hyperacute stage of spinal injury appears to be the only method to improve SCI-related disability. However, no effective intervention can decrease the damage. It is believed that MP sodium decreases secondary damage by reducing lipid cellular metabolism.[15] MP has also been reported to reduce tissue edema, possibly by decreasing pro-inflammatory cytokine production and free radical formation. MP also provides a beneficial modulation of calcium and sodium transcellular fluxes.[4] Although an abundance of evidence from experimental work in animal studies supports the use of MP, these results were not substantiated by clinical trials.

Previously, some studies have demonstrated the neuroprotective effects of EPO and the recovery capacity it confers to locomotor function.[16–18] It has been revealed that the binding of EPO to its receptor induces the activation of JAK2, which leads to the phosphorylation of the inhibitor of nuclear factor kB, resulting in antiapoptotic signals.[19] In addition, the neuroprotective effects of EPO have also been reported to be associated with the common receptor subunit, also known as CD131, which is the signal-transducing component that is used by granulocyte-macrophage colony-stimulating factor, interleukin (IL)-3, and IL-5 receptors.[20] Zhang et al. found that high-dose EPO administration minimizes the increase in iNOS expression in the facial nucleus after facial nerve transection and thus may enhance the survival of facial motor neurons.[21] Hong et al. administered parenteral EPO after SCI, after which they observed high BBB scores and PDGF-positive cell counts, which were correlated with better neurological recovery outcomes.[8] To our knowledge, studies that have investigated the role of EPO in a spinal injury model have utilized parenteral administration of the drug. We chose intrathecal administration to provide faster, direct, and abundant drug action in the traumatized area. We compared the effectiveness of MP and EPO treatments because MP is already one

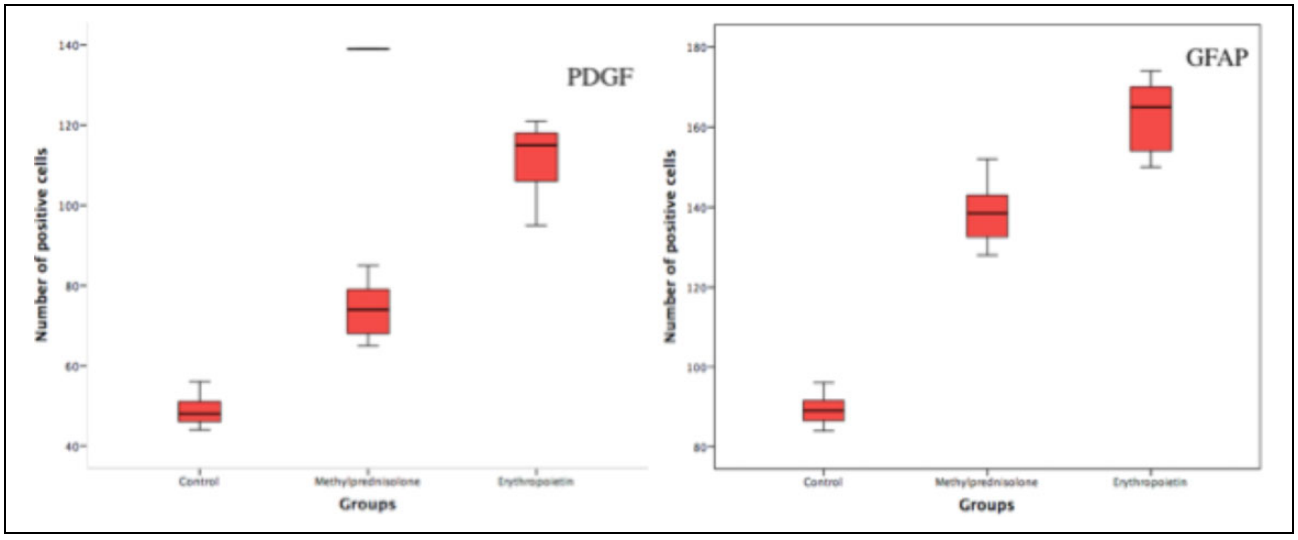

Figure 2. It is shown that distribution of positive cell number for PDGF-β and GFAP in all groups. PDGF: platelet-derived growth factor; GFAP: glial fibrillary acidic protein.

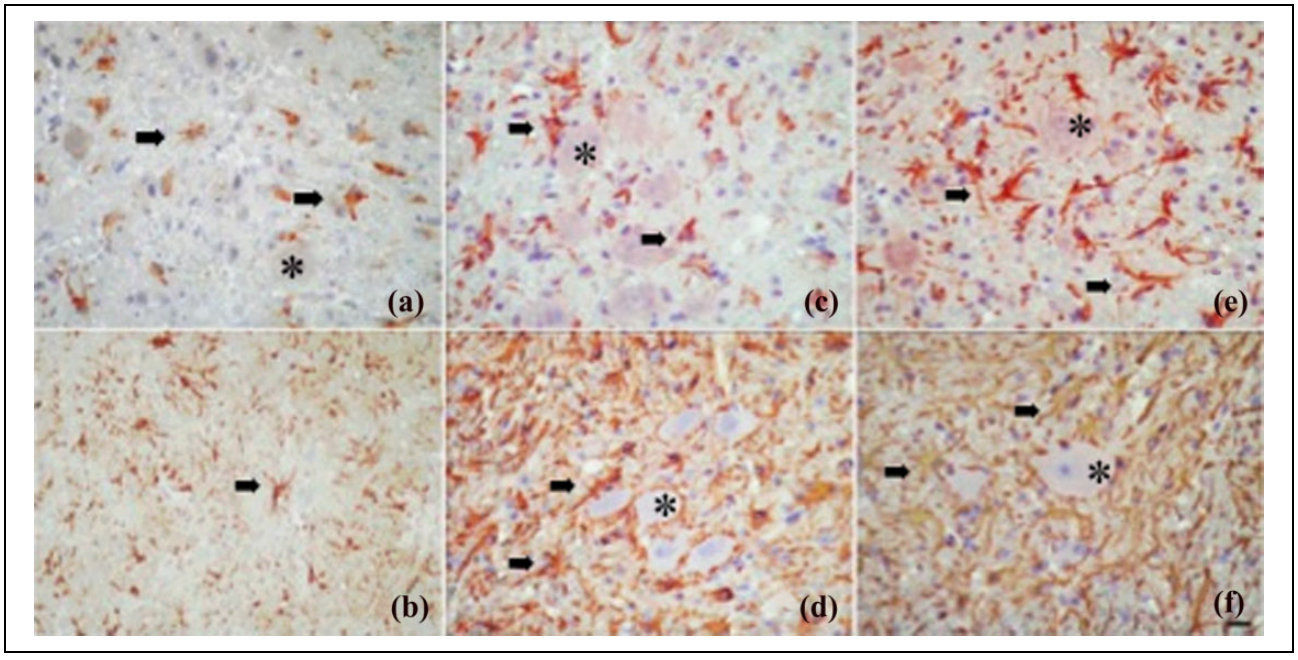

Figure 3. In spinal cord PDGF-β (1) positive GFAP (2) immunohistochemical expression. ➡: Positive immune reactive astrocytes. Saline TSCI group (a, b), MP TSCI group (c, d), and EPO TSCI group (e, f). ×400 Bar = 10 μm. Mayer's hematoxylin staining. PDGF: platelet-derived growth factor; GFAP: glial fibrillary acidic protein; MP: methylprednisolone; TSCI: traumatic spinal cord injury; EPO: erythropoietin.

of the most widely used treatment agents and EPO is a promising agent that has generated considerable excitement as a treatment option in recent years.

The literature reveals several documented methods to track neuroprotective effects in the CNS after traumatic injury.[11,12] Our study examined PDGF-β- and GFAP-positive cells that showed signs of healing after the spinal injury. In SCI, as well as in a variety of other traumatic and neurodegenerative diseases of the CNS, astrocytes respond to tissue damage by undergoing rapid hypertrophy and hyperplasia.[11]

Reactive gliosis caused by astrocyte proliferation appears to be necessary to limit neural damage. This is thought to be a result of the ability of glial cells to form a barrier to isolate intact tissue from the lesion. This in return helps to control the concentration of ions in the extracellular matrix, decrease inflammation, and promote the repair of the blood–brain barrier, all of which result in the

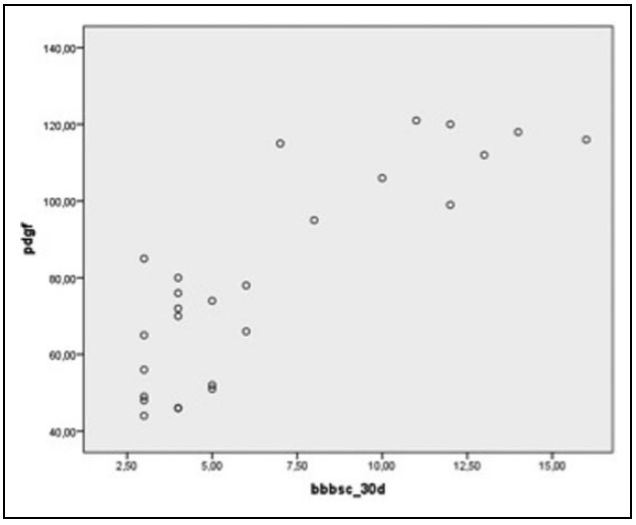

Figure 4. It is shown that there is a strong correlation between improvement of BBB scores and increasing number of PDGF-β-positive cells. BBB: Basso, Beattie, and Bresnahan; PDGF: platelet-derived growth factor.

improved survivability of neurons.[22] Astrocyte reactivity can be measured via GFAP immunoreactivity, which is an acknowledged marker of astrogliosis. Some studies have demonstrated that an increase in GFAP expression facilitates neuroprotective effects in SCI models.[23,24] A study by Vitellaro-Zuccarello et al.[22] indicated that EPO significantly reduces the density of GFAP immunoreactivity in the gray matter of rats. This finding is inconsistent with our results; we found GFAP immunoreactivity to be significantly higher in EPO-treated rats than in MP-treated and control animals. Members of the PDGF family play multiple roles during embryogenesis and in a variety of pathological situations in the adult. PDGF-β is compatible with normal CNS development and the astroglial response to injury.[25] In our experience, exogenous PDGF was able to rescue dopaminergic neurons in the substantia nigra from Tat-induced neurotoxicity.[26] Furthermore, recent studies indicate that PDGF-β protects neurons via the suppression of the NMDA-evoked current and the translocation of the glutamate transporter to the cell membrane.[27] Similar to other studies in the literature, we found that higher levels of PDGF-β-positive cells correlate with better locomotor functional recovery in EPO-treated rats.[8,9,17] In addition, we found that an increase in PDGF-β expression was directly associated with BBB scores.

Conclusion

Despite the fact that modern medicine has made tremendous progress with regard to neurological diseases and disorders in recent decades, spinal injury is still a serious, debilitating problem as well as a continued threat to public health. The catastrophic results of spinal injury in one's life continue to motivate scientists to seek out more efficient drugs. In such an effort, we investigated the efficacy of EPO for neurological recovery after traumatic SCI and found promising results. EPO appears to better increase the expression of PDGF-β, thus producing better results for locomotor function than MP. However, the effectiveness of EPO needs to be further studied in trials that compare EPO and other alternatives.

Declaration of conflicting interests

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