Comparative Study on the Effects of Ceftriaxone and Monocytes on Recovery after Spinal Cord Injury in Rat

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Introduction

As many as 500,000 people suffer a spinal cord injury (SCI) each year. Up to 90% of SCI cases are due to traumatic causes such as road traffic crashes, falls and violence.1,2 However, early interventions can likely spare most tissues and cells, producing a minimum degree of deficit and lead to maximum functional recovery. Monocytes exert anti-inflammatory effects on damaged tissues and overlay the way for the extension of axons. But at the time of SCI, monocyte responses at the site of injury become inefficient, which may be due to reduced numbers of monocytes entering the site of injury from the blood stream. Previous studies have primarily focused on artificial infiltration of monocytes into the site of nerve injury. In recent years, extensive use of anti-inflammatory agents such as methylprednisolone has been implicated in SCI.3,4 However, despite the initial hopefulness, methylprednisolone has not demonstrated a significant clinical efficacy.5,7 Nevertheless, secondary injury after SCI can make post-traumatic inflammatory reactions. As a result, certain inflammatory mediators, namely cytokines, proteases and reactive oxygen species, can trigger the activation of apoptosis executioners like caspases, which will eventually result in neuronal loss and permanent neurological deficit.8,10 Recently, a number of novel important concepts of secondary injury have been proposed. Toxic chemicals released by axons, damaged cells and blood vessels attack to intact neighbor cells. Glutamate as a neurotransmitter plays a critical role excessively disruptive process which has been called excitotoxicity.11 Moreover, one of the glutamate receptors (AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) plays a significant role in oligodendrocyte injury. Glutamate transport is the only known mechanism of extracellular glutamate clearance and glutamate transporter 1 (GLT-1) is the major glutamate transporter of the mammalian brain. Ceftriaxone has recently been discovered to up-regulate GLT-1 expression in the CNS through increasing GLT-1 transcription.12,13 Moreover, ceftriaxone improves neuron protection and functional recovery in rat spinal cord injury models14 and recent studies have extensively focused on molecular and cellular therapeutic interventions. Therefore, the present study has designed to compare the effects of monocytes and ceftriaxone on spinal cord injury in the rats.

Materials and Methods

Animal model
This study was conducted on female Sprague-Dawley rats (10 weeks, 200–245 g) that were obtained from the animal colony at the local institute. Animal experiments conformed to institutional standards. The animals were

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randomly divided into three groups of ten. SCI was performed under general anesthesia, using intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg) injection, by the weight dropping method in which a 10 g metal rod was dropped on the laminectomy area from a height of 5 cm.

**Animal grouping**

Group one received normal saline for seven days post SCI, group two received 2x10⁵ monocytes injected in site of injury on forth day post SCI and group three received ceftriaxone, injected intraperitoneally 200 mg/kg/day.

A Basso, Beattie and Bresnahan (BBB) score test¹⁵ was performed for six weeks. Two weeks before the end of the BBB test, biotin dextran amine (BDA) was injected intracerebrally and tissue staining was performed at the end of the six weeks.

**Extraction and Cell Culture**

In order to monocytes extraction, blood samples were taken from the hearts of several rats and kept on ice in tubes containing EDTA. Equal in volume to the blood sample, ficoll was added to falcon tubes and the blood samples were diluted with Phosphate buffer saline (PBS) and gently added to the falcon tubes so that the samples did not mix with the ficoll. The tubes containing the samples were centrifuged at 400g for 30 min at 18-20°C. In the next stage, the intended layer that contained monocytes was transferred to another tube and 6 ml of PBS was added. These samples were then centrifuged at 100g for 10 min at 18–20°C. The upper layer was discarded and the remaining deposit was suspended with RPMI-1640 medium in 5% fetal bovine serum (FBS). This suspension was incubated at 37°C with 5% CO₂ and then incubated for 2 h. Then the upper mixture was removed and a new cell culture containing 100 ng of monocyte colony-stimulating factor (MCSF) in 1 ml was added to the flask. It can be concluded that most of the attached cells are monocytes because monocytes are able to attach to the bottom of the flask but the other blood cells are unable to attach to the bottom of the flask within 2 h.

**Locomotor Assessment**

Following SCI, hind limb motor function was assessed weekly based on the BBB scale, as previously described.¹⁴

**Biotin Dextran Amine Detection**

Biotin dextran amine (BDA) was injected intracerebrally two weeks prior to end of BBB by creating a hole situated 2 mm posterior and 2 mm right of Bregma, according to previous methods.¹⁴ Tissue section preparation and staining was performed at the end of the six weeks. Sections were washed in PBS containing 0.1% Triton X-100, incubated for 1 h with avidin and biotinylated horseradish peroxidase (HRP) (NeuroTrace™ BDA-10,000 Neuronal Tracer Kit, N-7167), washed in PBS and then reacted with 3,3'-diaminobenzidine (DAB) in 50 mM Tris buffer, pH 7.6, 0.024% hydrogen peroxide and 0.5% nickel chloride. Following BDA administration and subsequent staining with DAB, which leads to black deposit formation¹⁶,¹⁷ then ten sequential cross sections, 5 micrometers apart, were randomly prepared. Cross-sections from the rostral-most block were used to determine the extent of corticospinal tract (CST) labeling above the lesion, the number of BDA-labeled axon arbors that entered the gray matter of the thoracic spinal cord at the thoracic vertebrae (T₁₀) and the number of BDA-labeled axon arbors that entered the gray matter of the thoracic spinal cord above the lesion.¹⁶,¹⁷ The axons were counted using software (imaging software for life science microscopy) attached to a microscope (Olympus Bx52, Japan).

**Drug**

Ceftriaxone was dissolved in sterile endotoxin-free 0.9% normal saline at 60 mg/ml and stored at 4°C. This solution was injected intraperitoneally into group three. For intraperitoneal injections, the 60-µg/µl ceftriaxone stock was diluted with normal saline to obtain 30 µg/µl of the drug then 5 µl of the diluted ceftriaxone was injected (227 nmol).

**Cell injection**

Monocytes numbering 2x10⁵ were suspended in 2 µl PBS and stereotactically injected into the caudal border of the lesion through a Hamilton syringe with a 30 G needle.

**Data evaluation and statistical analysis**

Values were expressed as means ± standard deviations. Sample size (n) for each group was 10 rats. Comparisons were performed using analysis of variance (ANOVA) followed by post-hoc Scheffe tests. P values less than 0.05 were considered significant. All statistical analyses were performed using SPSS version 10.0 computer software program for Windows (SPSS Inc, Chicago, IL, USA).

**Results**

**Comparison of the effect of ceftriaxone and monocytes injection on hindlimb motor function after SCI**

Following SCI and ceftriaxone administration, there was significant recovery of locomotion over the first week. There were significant differences for BBB scores between experimental groups in the second (P<0.005), third (P<0.001), fourth (P<0.001), fifth (P<0.004), and sixth (P<0.001) weeks (Figure 1). Pair wise comparisons showed no significant differences between groups in the first week (P = 0.058). Following SCI and administration of monocytes, there was no correlation with the control group (normal saline) but in contrast to the above finding, there was a correlation with the first group (ceftriaxone) (Figure 1).
Repair of Spinal Cord Injury using ceftriaxone, Monocytes

**Effect of ceftriaxone and monocytes injection on axonal regeneration**

Following SCI and either ceftriaxone or monocytes injection, there was a significant increase in axon number in both groups compared to the control group ($P<0.001$). The mean score of BBB in the ceftriaxone group was different from the other groups in the second, third, fourth, and sixth weeks. At the same time, there was a significant increase in axon number in the ceftriaxone and monocytes groups compared to the control group ($P<0.001$), whereas this difference was not significant between the ceftriaxone and monocytes groups (Figure 2).

**Discussion**

Molecular therapies after SCI serve several goals, including protection of neurons from secondary cell death, promotion of axonal growth, and improving nerve conduction. Several goals are achieved by cellular transplantation after SCI, including the filling of cavities or cysts, the generation of new neurons or myelinating cells, and to provide an appropriate environment for regeneration of axons. The nature of the macrophage response has been proposed as the likely cause of the failure of the spinal cord to recover. This is different from the situation observed in the regenerative peripheral nervous system (PNS). After transection and transplantation of activated macrophages incubated with PNS or skin tissue in rats, recovery of hind limb function occurs. Fibers extend across the lesion, and recovered functions were terminated by re-transection of the spinal cord. However, the extent of recovery was similar to the results obtained from the transplant of other cell types and succeeded only in a rat subgroup. On the contrary, tissue survival and hind limb recovery are hindered by the activation of intrinsic macrophages at the lesion site with microinjections of a pro-inflammatory agent. Improvement of Hind limb usage during locomotion, increased white matter sparing, and functional recovery have been the result of macrophage depletion after SCI. Thus, our study investigated whether treatment with monocytes improved ambulatory ability and prevention of paralysis in rat models of SCI.

**Figure 1.** Effect of Ceftriaxone (200 mg/kg/day/ip) and Monocytes injection on hindlimb motor function after the spinal cord injury. Each bar represents mean ±SEM (n = 10) per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different from the normal saline group. (BBB): Beattie and Bresnahan score

**Figure 2.** Distribution and density of CST axons in a PBS injected normal saline (A), Monocyte treated (B), Ceftriaxone (C).
Following treatment with ceftriaxone, there is a significant recovery rate in treated animals in subsequent weeks (p<0.001).

Ceftriaxone administration significantly promoted axonal regeneration in experimental and control groups (P<0.001, Figure 3), showing an important role of ceftriaxone in the regulation of axonal growth.

The secondary injury phase of SCI involves autodestructive events like reactive oxygen species-induced lipid peroxidation, caspase-3 activation, and glutamate production. These findings suggest that compounds that can protect cells from excess glutamate can limit spinal cord destruction. It is also believed that the injury cascade of neurodestructive events will extend when secondary injury increases because of delayed treatment. The first scientifically grounded pharmacological treatment for SCI dates back to the 1990s. Although a clinical study showed that a high dose of the steroid methylprednisolone decreased disability when administered within 8 h of trauma, treatment with a high dose of methylprednisolone was later reported to be associated with complications, including wound infection and increased frequency of gastric bleeding; methylprednisolone treatment remains controversial in many countries. Furthermore, although treatment with this drug might result in the reduction of swelling, inflammation, glutamate release, and free-radical accumulation, the specific mechanism of action remains unclear.

In a similar study, experimental drugs including monosialoganglioside sodium (GM-1 ganglioside), naloxone, and tirilazad were tested in multicenter clinical trials, but the desired results were not achieved. However, significant improvement in functional recovery (BBB) after SCI was reported in another study in which minocycline was administered early (0.5–24 h). The results of another similar study suggested that drugs that impede AMPA-type glutamate receptors turn out to be efficacious in keeping lesions and disability to a minimum. Specific AMPA-receptor antagonists have also been tested in patients with SCI in recent years. A large number of studies assert that glutamate and its structural analogues could have both short and long-term poisonous impacts on cortical and motor neurons. The exposure of neurons to abnormally high concentrations of glutamate results from the defective clearance of glutamate from the extracellular space. Glutamate neurotransmission is greatly regulated, mainly via glutamate transporters. The glutamate transporter GLT-1 is principally responsible for glutamate clearance in the spinal cord. Down-regulation of GLT-1 can happen in activated astrocytes, and is associated with increased extracellular glutamate and neuroexcitation. During other conditions, astrocyte activation occurs subsequent to spinal cord destruction. Recently, glutamate transporters have emerged as a potential therapeutic target in a wide range of acute and chronic neurological disorders, owing to their novel mode of action. The modulation of GLT-1, a primary glutamate transporter, provides neuroprotection in different models of ischemic injury and motoneuron degeneration. Therefore, an attempt was made to explore the neuroprotective potential in spinal cord injury using ceftriaxone, a GLT-1 modulator. In the present study, treatment with ceftriaxone resulted in significant differences in BBB scores compared to the control group. Furthermore, ceftriaxone also promoted axon regeneration and impeded neuronal damage and eventual cell death, and improved motor function.

In spite of a lack of difference in axon counting following monocytes administration, further study is needed to investigate the reasons for increasing cell count and low locomotor improvement, in contrast to what is seen by ceftriaxone administration.

**Conclusion**

In conclusion, our study shows that ceftriaxone improved the functional recovery in the injured rats following SCI more effective than monocytes administration. Further investigations with new procedures such as co-administration of ceftriaxone with monocytes or gene therapies approaches to improve spinal cord injury are required.

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**Ethical Issues**

“Principles of laboratory animal care” (NIH publication No.85-23, revised 1985) was followed, as well as specific national laws where applicable. All experiments have been examined and approved by the ethics committee of Tabriz University of Medical Sciences.

**Conflict of Interest**

Authors declare no conflict of interest.

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