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Combined Therapy Following Spinal Cord Injury: Synergistic Neuroprotective Effects of Ceftriaxone and N-acetylcysteine

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Abstract

Background: Spinal Cord Injury (SCI) is one of the leading causes of severe neurological deficits worldwide. The pathophysiology of SCI includes a primary injury followed by a cascade of secondary biochemical and cellular changes. Current pharmacological options are limited for significant recovery from SCI. The β-lactam antibiotic ceftriaxone (CEF) and N-acetylcysteine (NAC) have shown to induce neuroprotection in animal models of neurodegenerative disorders.
Objectives: This study aimed to evaluate the effects of CEF, NAC, and their combination on the functional recovery and histological damage in experimental SCI.
Methods: Rats were randomly divided into four groups (n = 7): Saline, CEF, NAC, and CEF + NAC. Then, SCI was performed on rats under general anesthesia using the Neurosciences Research Center (NSRC) impactor. Locomotor recovery following SCI was monitored using the locomotor rating scale of Basso, Beattie, and Bresnahan (BBB). At the end of the study, all rats were sacrificed, and spinal cord cross-sections were stained with hematoxylin and eosin for histopathological evaluation.
Results: The CEF and NAC administration, either alone or in combination (CEF + NAC), significantly improved locomotor recovery following SCI in rats when compared to the saline group. The histological analysis showed that the severity of histopathological lesion in the spinal cord of rats was significantly lower in the CEF, NAC, and CEF + NAC groups than in the saline group.
Conclusions: Treatment with CEF and NAC, either separately or in combination, promotes locomotor recovery following SCI, which is associated with the effective reduction of the histopathological lesion.

Keywords: Spinal Cord Injury, N-acetylcysteine, Ceftriaxone, Methylprednisolone, Rat

1. Background

Spinal Cord Injury (SCI) as a neurodegenerative disease can lead to sensory and motor defects below the lesion area, depending on the severity of the injury. The traumatic injury to the spinal cord causes neuronal damage, ischemia, hemorrhage, and edema in the affected area. The primary phase of injury initiates a cascade of cellular and biochemical excitotoxicity and inflammatory responses, leading to secondary tissue damage that progressively extends from the injury site to adjacent areas. During this secondary phase, cell death occurs by necrosis and apoptosis (1). Therefore, one of the treatment options for SCI is an attempt to minimize the secondary responses through pharmacological interventions to protect neural cells that have survived the primary injury and to increase the potential for neurological recovery (2).

Methylprednisolone (MP) is the only drug that is clinically used for the treatment of acute SCI. Nevertheless, it has low efficacy in long-term functional recovery. Besides, it may be associated with increased adverse complications, including wound infection and gastrointestinal hemorrhage (3, 4). According to the definitive agreement, methylprednisolone is not recommended for the treatment of SCI (5). Therefore, designing experimental studies of SCI is necessary to approach new therapeutic options with greater efficacy and fewer complications.

Ceftriaxone (CEF) is a β-lactam antibiotic that can enter the spinal cord and the brain through the blood-brain barrier. In addition to antibiotic effects, CEF also has neuroprotective and anti-excitotoxicity features (6). Several studies have shown beneficial effects of treatment with CEF in neurodegenerative diseases, including stroke, Parkinson's disease, and Traumatic Brain Injury (TBI) (7-9). Based on a pre-

vious study, the administration of CEF improves neuronal survival and motor recovery in rats with the weight-drop contusion model of SCI (10).

N-acetylcysteine (NAC) is an antioxidant with neuroprotective and anti-inflammatory properties (11). The administration of NAC immediately after SCI attenuates the short and long-term neuroinflammation, induces neuroprotection, and decreases cell death in the affected area, as previously demonstrated (12, 13). However, no studies have ever examined the therapeutic potency of the combination of CEF and NAC on neurological and functional recovery following SCI.

2. Objectives

This study aimed to determine the effectiveness of CEF, NAC, and co-treatment with these agents on the improvement of histological and functional locomotor recovery following the contusion model of SCI in rats.

3. Methods

3.1. Animals

This study was conducted on 28 adult male Wistar rats (280 - 300 g, age 10 - 11 weeks at the time of surgery) provided by the Experimental Animal Center of Tabriz University of Medical Sciences. Rats were kept under standard environmentally controlled conditions and 12h/12h light/dark cycles with water and food available *ad libitum*.

3.2. Experimental Design and Drug Administration

Rats were randomly divided into four groups (n = 7 per group). Group I (Saline) received normal saline (10 mL/kg, intraperitoneally (ip); once a day for seven days after SCI surgery). Group II (CEF) received ceftriaxone (100 mg/kg, ip; once a day for seven days after SCI surgery). Group III (NAC) received N-acetylcysteine (150 mg/kg, ip; once a day for seven days after SCI surgery). Group IV (CEF + NAC) received ceftriaxone (100 mg/kg, ip) and N-acetylcysteine (150 mg/kg, ip) once a day for seven days after SCI surgery.

3.3. Surgical Procedure of SCI

Spinal cord contusion injury at T10 thoracic level was performed using the Neurosciences Research Center (NSRC) impactor under aseptic conditions. Briefly, rats were anesthetized by the inhalation of the isoflurane-oxygen mixture with an induction dose of 5% for 5 min and a maintenance dose of 2 - 3% during surgery. The animals were subjected to laminectomy at the T10 vertebral level while the intact spinal cord was exposed. Then, they received moderate spinal cord contusion using the NSRC impactor (Home-made code: 90778) (14) with a force of 150 kilodyne (kdyn). Immediately after surgery, 0.05 mg/kg of ketoprofen was injected subcutaneously, which was repeated the next day to limit postoperative pain. In the first week after surgery, rats received daily administration of ciprofloxacin (8 mg/kg/day, ip) in 2 mL saline to prevent infection and dehydration. After SCI, rats were not capable of spontaneous micturition (7 - 14 days post-SCI) and their bladders were manually expressed twice daily.

3.4. Locomotor Assessment

Following SCI, hindlimb motor function was assessed based on the Basso, Beattie, and Bresnahan (BBB) score test (15) weekly for six weeks. In a blinded study, the BBB score was evaluated for each rat according to the movement of each joint, each paw position, coordination, and stepping from 0 (no observable hindlimb movement) to 21 (normal movement, including consistently coordinated stepping with parallel paw position during locomotion).

3.5. Histological Examination of the Spinal Cord

Six weeks after SCI, all rats were deeply anesthetized using a lethal overdose of ketamine and xylazine and perfused intracardially with saline followed by 4% paraformaldehyde in a phosphate-buffered solution (pH 7.4). Spinal cords were carefully dissected. Approximately 1.5 cm of the spinal cord at a thoracic region, including the injury site, was removed immediately after perfusion and placed in the same solution overnight. After fixation, tissue samples were embedded in paraffin, and serial 5-μm thick cross-sections were prepared. These sections were subsequently stained with Hematoxylin and Eosin (H & E). In a blinded study, the histopathological analysis was performed based on tissue injury indices as cytoarchitectural disorganization (lymphocyte infiltration, axonal swelling, spared tissue, pyknotic nuclei, vacuolation, cystic degeneration, and large cystic cavity). The score indicating the severity of histopathological lesion was documented as follows: 0 = none; 1 = very low; 2 = low; 3 = moderate; 4 = severe; and 5 = very severe (16).

3.6. Statistical Analysis

Values were expressed as means ± SEM. The BBB locomotor rating scale scores were compared between groups using two-way repeated measures Analysis of Variance (ANOVA), and the histopathological results were analyzed by one-way ANOVA. Tukey post hoc test was used for multiple comparisons between the groups. The statistical analyses were performed using GraphPad Prism 7.03 software. The P values of less than 0.05 were considered statistically significant.

4. Results

4.1. Effect of CEF, NAC, and CEF + NAC Injection on Hindlimb Motor Function Following SCI

The BBB score was used to compare the progression of functional locomotor recovery following SCI between the groups (Figure 1). In all experimental groups, functional recovery was observed over the weeks. Based on the BBB scores, there was no significant difference between the groups at the end of the first week after SCI. When compared to the saline group, functional recovery in other groups significantly changed from week two until the endpoint at week 6. The highest recovery rate was observed in the CEF + NAC group. Moreover, the post hoc multiple comparisons showed that locomotor recovery was significantly better in rats treated with CEF + NAC than the NAC group from week two onward. These results indicated that the co-administration of CEF and NAC exerted more significant effects than CEF or NAC when administered alone.

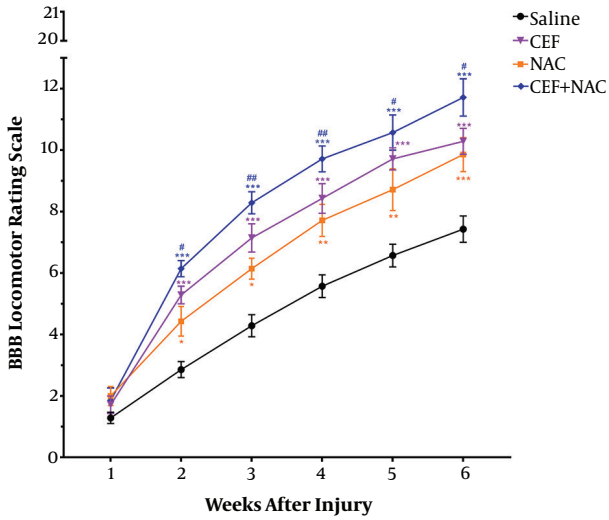

Figure 1. Effect of CEF (100 mg/kg/day, ip), NAC (150 mg/kg/day, ip), and CEF + NAC injection on motor recovery after SCI. Hindlimb motor function was assessed by the BBB score. Each point represents mean ± SEM; n = 7 for each group. Significant changes compared to the saline group; Significant changes compared to the NAC group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). BBB: Basso, Beattie, and Bresnahan. CEF: Ceftriaxone. NAC: N-acetylcysteine

4.2. Effect of CEF, NAC, and CEF + NAC Injection on Histopathological Lesion Following SCI

A histopathological examination was used to evaluate the neuroprotective effect of NAC, CEF, and their combination in rats with SCI (Figure 2A). The severity of histopathological lesion was scored concerning the extension of recommended histopathological parameters, including axonal swelling, spared tissue, pyknotic nuclei, vacuolation, cystic degeneration, and large cystic cavity.

Severe tissue structural damage was observed in the spinal cord of rats in the saline group. When compared to the saline group, histopathological lesions significantly decreased in the CEF and NAC groups ($P < 0.05$). Moreover, co-treatment with CEF and NAC significantly reduced histopathological damage in the CEF + NAC group as compared to the saline group ($P < 0.01$). There was no significant histological difference between NAC, CEF, and NAC + CEF groups. The histological scores are shown in Figure 2B.

5. Discussion

As the main finding, the current study showed that treatment with CEF and NAC either separately or in combination promoted long-term functional locomotor recovery and reduced histological damage following SCI.

The mechanical injury to the spinal cord initiates progressive excitotoxicity and inflammatory events that result in necrotic and apoptotic cell death (5, 17). The inhibition of these processes is thought to be one of the main mechanisms of action for pharmacological agents used for the treatment of SCI (2). Concerning this strategy, we studied the long-term effects of CEF and NAC as potential neuroprotective agents against acute SCI. In this study, the CEF administration following SCI showed to improve the BBB locomotor score when compared to the saline group, and it reduced histopathological damage, as well. In a similar study by Tajkey et al. (2015), treatment with CEF led to the significant improvement of locomotor recovery and axonal regeneration in rats with the weight-drop contusion model of SCI (10). These results, in agreement with the results of our study, demonstrated that CEF could have therapeutic potential to improve functional recovery following experimental SCI. However, its mechanism of neuroprotective action against SCI has not yet been clarified.

The disruption of glutamatergic homeostasis is one of the main players in the pathophysiology of CNS neurodegenerative disorders, including cerebral ischemia, TBI, and SCI (8, 18). An excessive concentration of extracellular glutamate and subsequent overexcitation of glutamatergic receptors can increase the generation of Reactive Oxygen Species (ROS) and lipid peroxidation, leading to neuronal dysfunction and excitotoxicity-related cell death (17). It has been demonstrated that Glutamate Transporter-1 (GLT-1) is the predominant transporter of glutamate, which is responsible for the regulation of glutamatergic homeostasis by the reuptake of excess extracellular glutamate into the intracellular space of the astroglia (19). There is growing evidence that CEF selectively induces the expression of GLT-1 in the brain and spinal cord, thereby preventing

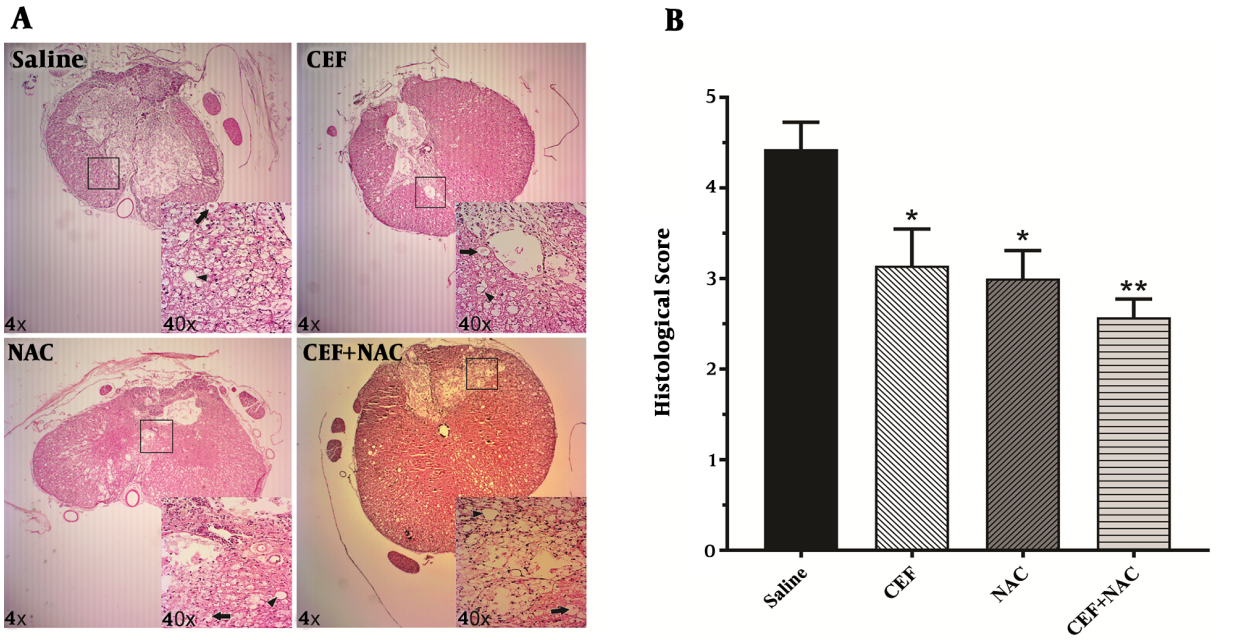

Figure 2. Effect of CEF (100 mg/kg/day, ip), NAC (150 mg/kg/day, ip), and CEF + NAC injection on histopathological changes six weeks after SCI. (A) Histomorphological alterations and cytoarchitectural disorganization of spinal cord tissue were evaluated on the Hematoxylin and eosin-stained (4x and 40x magnification) horizontal cross-sections. Spared tissue (boxed area), Vacuolation (arrowhead), Axonal swelling (arrow). (B) Quantitative analysis of spinal cord lesion. Data are presented as Mean ± SEM, n = 7 for each group. *P < 0.05, *P < 0.01 significantly different from the saline group. CEF: Ceftriaxone, NAC: N-acetylcysteine.

glutamate-induced neurotoxicity in animal models of TBI, stroke, cerebral ischemia, and amyotrophic lateral sclerosis (20, 21). It appears that the clearance of excess extracellular glutamate by up-regulated GLT-1 might be a possible neuroprotective mechanism of CEF administration in experimental SCI.

In the second pharmacological attempt, we found that the administration of NAC induced long-term neuroprotective effects following SCI, which appeared as the improvement of locomotor function and reduction of tissue damage when compared to the saline treatment group. In physiological conditions, the elimination of ROS is controlled by activities of endogenous antioxidants, including superoxide dismutase, catalase, and glutathione. However, following SCI, an imbalance occurs over time between the ROS formation and the antioxidative capacity of the cell, and consequently, the overproduction of mitochondrial ROS leads to extensive oxidative damage (22).

N-acetylcysteine, as a glutathione precursor, is well known for its neuroprotective effect, which can promote free radical species scavenging, resulting in cell survival (23). Some studies have demonstrated that immediate treatment with NAC attenuates neuroinflammation and reduces the death of motor neurons in the ventral horn of the hemisection model of SCI (13, 24). Besides, it was tested as an early neuroprotective treatment following ischemic SCI that provided significant improvement in motor dysfunction (25). A previous study by Patel et al. (2014) demonstrated that treatment with N-acetylcysteine amide (NACA), a modified form of NAC, significantly reduced the severity of histopathological damage and improved hindlimb motor function that was associated with normalized glutathione levels following contusion SCI (26). Therefore, the neuroprotective effects of NAC in the present study are likely to be due to the antioxidative and anti-inflammatory effects (27).

For the first time, we demonstrated that combined therapy with CEF and NAC following SCI is more effective than the administration of each one alone. In the CEF + NAC group, more significant locomotor recovery was associated with substantial histological neuroprotection. Taken together, the present results address the hypothesis that co-treatment with CEF and NAC synergistically reduces excitotoxicity-induced cell death following SCI, which is directly related to the potential for functional locomotor recovery. The neuroprotective effects of CEF and NAC in a synergistic manner could be explained by their complementary role in minimizing oxidative damage caused by the overproduction of ROS and a failure to eliminate it.

Both CEF and NAC are FDA-approved drugs with no reported toxic effects on the CNS at the therapeutic dose. Furthermore, both quickly pass through the blood-brain barrier (6, 13). Thus, CEF and NAC can be attractive drug candidates for SCI. However, follow-up studies are necessary to confirm our results and determine the underlying action mechanisms of CEF and NAC in SCI.

5.1. Conclusion

In conclusion, the results of our study showed that CEF and NAC administration, either separately or in combination, led to locomotor recovery after SCI that was associated with reduced tissue damage in the affected area. Moreover, combined treatment with CEF and NAC exerted more significant neuroprotective effects than CEF or NAC when administered alone. Since both pharmacological agents have been used in clinical practice for many years, they represent a suitable neuroprotective strategy for the treatment of human SCI.

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Footnotes

Authors' Contribution: Study concept and design: Bohlool Habibi Asl, Omid Salimi, and Aliasghar Mohammadvand; analysis and interpretation of data: Omid Salimi, Bohlool Habibi Asl, and Aliasghar Mohammadvand; drafting of the manuscript: Omid Salimi and Bohlool Habibi Asl; critical revision of the manuscript for important intellectual content: Abbas Ebrahimi Kalan and Mohammad Charkhpour; statistical analysis: Javad Mahmoudi; supervision: Bohlool Habibi Asl and Abbas Ebrahimi Kalan.

Conflict of Interests: The authors declare no conflict of interest.

Ethical Approval: All the experimental procedures were approved by the Ethics Committee for Animal Research of Tabriz University of Medical Sciences (Ethics Committee Approval Code: IR.TBZMED.REC.1397.56).

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