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Effect of Methylprednisolone and Edaravone administration on spinal cord injury

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Abstract. – BACKGROUND: Spinal cord injury (SCI) is one of the most devastating traumatic conditions that primarily affects young males with an annual incidence of 15-40 cases per million.

AIM: To explore the superior neuroprotective effect of edaravone (ED) on spinal cord injury during maintenance therapy compared with methylprednisolone (MP).

MATERIALS AND METHODS: Sprague-Dawley rat model of spinal cord injury was established by modified Allen's method. Total 114 rats were divided into two groups and then six subgroups individually: A1 (control group, normal saline injection within 8 h), B1 (MP group, MP injection within 8 h), C1(ED group, ED injection within 8 h), A2 (control group, normal saline injection after 8 h), B1 (MP group, MP injection after 8 h), C1 (ED group, ED injection after 8 h). Further, we investigated the changes of histopathology, caspase-3 and Bcl-xL positive cell.

RESULTS: Haemorrhage, swelling, hyperaemia, gliocytes hyperplasia, inflammatory cells infiltration, vacuolar denaturation, and nucleus concentration could be observed, especially in control group. Caspase-3 positive cell was significantly decreased in MP and ED group within 8 h administration, but caspase-3 positive cell was only significantly decreased in ED group after 8 h administration. And B-cell lymphoma extra large (Bcl-xL) was significantly increased in ED group than MP group no matter within 8 h or after 8 h administration.

CONCLUSIONS: More attention should be paid on the time point of MP administration, and ED administration seem to be more effective for maintenance therapy.

Key Words:
Methylprednisolone, Edaravone, Spinal cord injury, Caspase-3.

Introduction

Spinal cord injury (SCI) is one of the most devastating traumatic conditions that primarily affects young males with an annual incidence of 15-40 cases per million[1]. SCI involves in two-step process, including the primary mechanical insult and the subsequent, multi-faceted secondary degenerative response[2]. Primary injury is due to direct tissue detritions, which are the result of external mechanical forces. Secondary injury spreads from the site of initial impact to adjacent, otherwise uninjured tissues, which may result from spinal cord edema, ischemia, free radical damage, electrolyte imbalance, excitotoxicity, inflammatory injury, or apoptosis[3,4]. Therefore, inhibition of secondary injury processes may be one of the most important means of therapeutic intervention[5].

Methylprednisolone (MP) is widely considered as the only effective neuroprotective agent for the treatment of secondary SCI through its inhibition of lipid peroxidation and calcium influx and through its anti-inflammatory effects[6]. Based on the recommendations of National Acute Spinal Cord Injury Studies, a 30 mg/kg bolus of MP should be given over 15 minutes, and followed by a continuous infusion of 5.4 mg/kg/hour. If treatment is initiated within 3 hours after sustaining SCI, the infusion would be for 23 h. If treatment is initiated within 3-8 h then the infusion should be continued for 47 h[7-9]. However, this treatment is still controversial because of unfavourable conclusions from many other authors[10,11]. Moreover, the use of high-dose MP in acute SCI is suggested associated with an increase in complications, such as stomach bleeding, blood glucose elevations[12], myopathy[13], and infectious[14,15]. Therefore, the use of MP is only as an option, and it is indispensable to explore more safe and effective alternative.

Edaravone (ED), as a novel neuroprotective agent, has the ability of scavenging toxic free

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radicals and has also been demonstrated to protect SCI by inhibiting vascular endothelial cell injury, lipid peroxidation, and delaying neuronal death, brain edema, and concomitant neurological deficits. For example, the level of free radical species was lower in ED treated group at 75, 90, and 150 min after the beginning of reperfusion compared with levels seen in non-ED treated group[16]. ED was administered intravenously as a bolus dose of 5 mg/kg at 5 min, 24 h, and 48 h after injury. Six weeks after injury, ED-treated rats showed significantly higher motor score and larger spared white matter area than control rats[17]. These studied indicated ED is effective for SCI therapy. However, no report focuses on the comparison of ED and MP administration in the treatment of SCI. Therefore, in this study, we designed different groups to compare the effect of MP and ED on SCI, with hope to lay a theory foundation for clinical application.

Materials and Methods

Experimental Protocol

All animal experiments were approved by Animal Center of Central South University. Adult 6-week-old healthy Sprague-Dawley rats (n=144) with an average body weight of 225 g (200-250 g) were used in this study. Rat model of SCI was established according to modified Allen's method[18]. Following that, the SCI rats were included into our later experiment based on the incline-plane test (< 30°-angled incline plane)[19]. The SCI rats were then randomly divided into two groups: medicine administration within 8 h (n=72) and after 8 h (n=72). Further, these two groups were divided into three subgroups: control group (n=24), MP group (n=24), and ED group (n=24). Among them, in the first group (within 8 h), six rats were randomly selected to inject with normal saline (**NS**) (A1 group), 30 mg/kg MP (**B1** group), and 3 mg/kg ED (**C1**) at four time points, 2 h, 4 h, 6 h, and 8 h, respectively. In the second group, six rats were randomly selected to inject with NS (**A2**), 5.4 mg/kg/h MP (**B2**), and 3 mg/kg ED (**C2**) at four time points, 10 h, 12 h, 14 h, and 24h, respectively. Examination of functional recovery after medicine administration 2 days with rats (n=3) was conducted by using the 21-point Basso-Beattie-Bresnahan (BBB) locomotion scale as previously described[20].

Histological Assessments

At 2 h after injections, the cord segments were isolated from the remaining three rats in each group at different time points. Then the injury spinal cord was fixed with 4% paraformaldehyde, dehydrated in a graded alcohol series, and embedded in paraffin. The thin sections (5 um thick) were stained with Hematoxylin-Eosin (HE) and observed the pathological changes under optical microscope.

Caspase-3 and Bcl-xL Staining

The distribution of caspase-3 and Bcl-xL positive cells were analyzed by immunohistochemical staining (IHS) as previous described[21]. Paraffin-embedded spinal cord sections were deparaffinized in two changes of xylene for 2 min each. The sections were rinsed sequentially in 100%, 95% and 80% ethanol before rinsing in phosphate buffered saline (PBS) for 5 min. After aspirating the excess liquid from the slides, the sections were incubated for 10 min in 3% hydrogen peroxidase in methanol to quench endogenous peroxidase activity. The slides were rinsed three times in PBS each for 2 min and then incubated in normal non-specific goat serum for 10 min at room temperature, followed by overnight incubation with primary antibodies (caspase-3 and Bcl-xL) at 37°C. The sections were then rinsed in PBS three times each for 2 min, incubated with biotin-conjugated secondary antibody for 30 min, again rinsed in PBS for three times each for 2 min. The sections were colorized with 3,3'-diaminobenzidine tetrahydrochloride, which results in a brown color in positive reaction.

Statistics Analysis

All data were analyzed by SPSS15.0 (SPSS Inc., Chicago, IL, USA) and the results were measured by average ± standard deviation (±s). ANOVA was used for comparisons between the groups where appropriate. $p < 0.05$ was considered as statistically significant.

Results

Neurologic Function Score

Neurologic function assessments were performed using the BBB locomotor rating scale, a 21-point scale (0-21) based on the observation of hind-limb movements of a rat freely moving in an open field. The results showed that MP administration within 8 h led to a better recovery of

Table I. Neurologic function score of different subgroups at four time points when medicines administration within 8 h.

Group	2 h	4 h	6 h	8 h
A1	0.24 ± 0.02	0.26 ± 0.03	0.27 ± 0.03	0.28 ± 0.04
B1	0.28 ± 0.02	0.29 ± 0.03	0.32 ± 0.05	0.35 ± 0.04
C1	0.27 ± 0.02	0.28 ± 0.03	0.31 ± 0.04	0.33 ± 0.04

Note: Significant difference could be observed between the groups, $p < 0.05$

Table II. Neurologic function score of different subgroups at four time points when medicines administration after 8 h.

Group	10 h	14 h	16 h	24 h
A2	0.31 ± 0.03	0.32 ± 0.03	0.34 ± 0.03	0.36 ± 0.03
B2	0.35 ± 0.04	0.37 ± 0.03	0.38 ± 0.04	0.42 ± 0.04
C2	0.36 ± 0.02	0.38 ± 0.03	0.39 ± 0.03	0.43 ± 0.05

Note: Significant difference could be observed between the groups, $p < 0.05$

the motor function compared with NS group and ED group. And significant difference could be observed among them, B1 > C1 > A1 ($p < 0.05$) (Table I). However, ED administration after 8 h seemed to cause a better recovery of the motor function compared with NS group and MP group. And significant difference could be observed among them, A2 < B2 < C2 ($p < 0.05$) (Table II).

Histological Assessments

Usually, the spinal cord contains amounts of neuron and gliocyte, and cells arrange close together under normal condition. However, traumatic injury results in haemorrhage, swelling, and hyperaemia in spinal cord tissue[22,23]. In addition, cell structure was unclear, partial cell showed vacuolar denaturation or only residual necrotic debris, and partial cell nucleus concentrated or even disappeared. In accordance with previous reports, our findings also indicated that debris, residue and some erythrocyte spread in the injury site after traumatic injury 8 h. Besides, patchy bleeding, peripheral gliocytes hyperplasia, inflammatory cells infiltration, neuron swelling, vacuolar denaturation, and cell nucleus concentration could be observed, especially in control group (Figure 1).

Caspase-3 and Bcl-xL Positive Cell

From our data, we could find that caspase-3 positive cell gradually increased and reached the peak value after 6 h of SCI (Table III, Figure 2). Caspase-3 positive cell was the most in A1 group, and the least in B1 group (B1<C1<A1). And significant difference was present among them ($p < 0.05$). Yet, Bcl-xL expression was showed as C1 > B1 > A1 ($p < 0.05$) (Table IV, Figure 4). In second group, caspase-3 positive cell gradually increased and reached the peak value after 24 h of SCI (Table V, Figure 3). And caspase-3 positive cell was

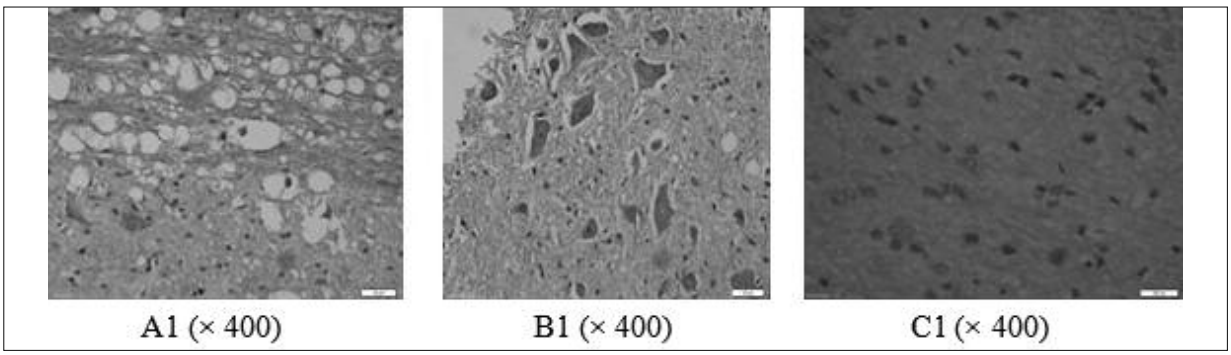

Figure 1. HE staining of spinal cord injury at 8 h.

Table III. Caspase-3 positive cell within 8 h medicines administration ($\bar{x} \pm s$).

Group	2 h	4 h	6 h	8 h
A1	49.99 ± 5.00	60.06 ± 4.02	57.85 ± 2.98	54.38 ± 4.87
B1	20.72 ± 3.92	24.46 ± 3.59	28.91 ± 4.78	26.12 ± 2.87
C1	29.61 ± 3.87	34.69 ± 2.40	38.72 ± 5.16	36.52 ± 3.64

Note: Significant difference was present among all subgroup ($p < 0.05$).

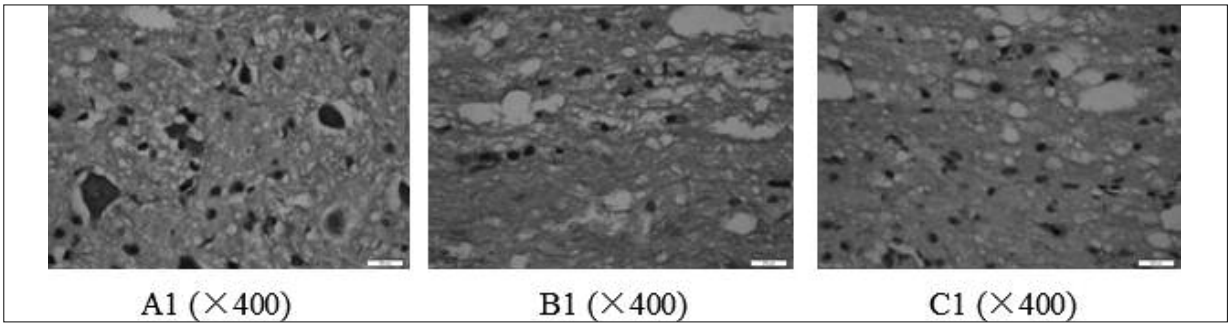

Figure 2. ISH analysis of caspase-3 positive cell at 6 h.

Table IV. Bcl-xL positive cell within 8 h medicines administration ($\bar{x} \pm s$).

Group	2 h	4 h	6 h	8 h
A1	30.00 ± 3.07	27.31 ± 2.57	26.10 ± 3.39	22.26 ± 3.15
B1	48.36 ± 3.76	47.82 ± 3.31	45.20 ± 3.35	40.98 ± 4.87
C1	49.70 ± 2.78	48.32 ± 3.41	47.56 ± 4.23	42.02 ± 7.54

Note: Significant difference was present among all subgroup ($p < 0.05$).

the most in A2 group, and the least in C1 group. No significant difference was observed between A2 and B2 subgroup ($p > 0.05$), but significant difference was present between A2 and C2 subgroup ($p < 0.05$). Bcl-xL expression was showed as C2 > B2 > A2, and significant difference was present among all subgroup ($p < 0.05$) (Table VI, Figure 5).

Discussion

It is now commonly agreed that the secondary SCI results from a cascade of biological processes that include increased oxidative stress, calcium mobilization, glutamate toxicity and inflammatory factors[24,25]. These mechanisms have been proposed to be responsible for neuronal dysfunction and cell

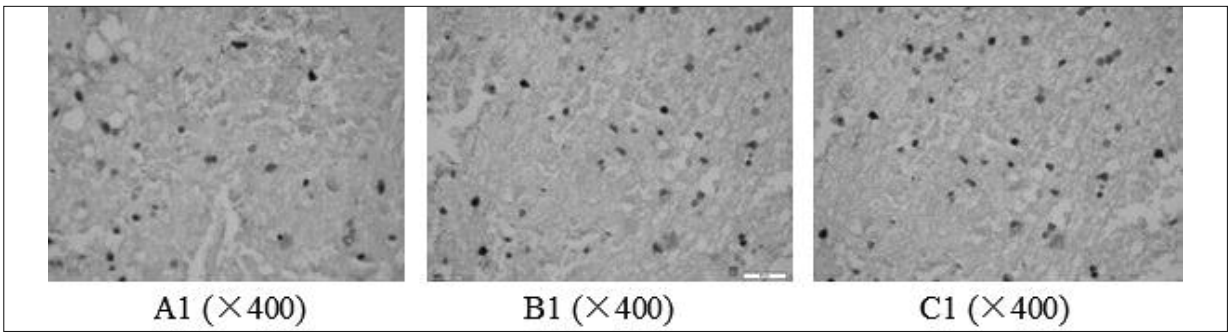

Figure 3. ISH analysis of Bcl-xL positive cell at 6 h.

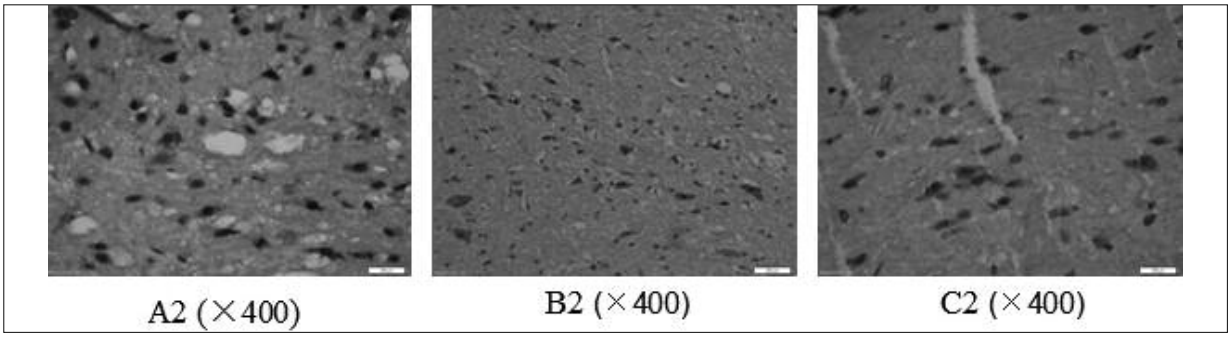

Figure 4. ISH analysis of caspase-3 positive cell at 24 h.

Table V. Caspase-3 positive cell after 8 h medicines administration ($\bar{x} \pm s$).

Group	10 h	12 h	14 h	24 h
A2	54.58 ± 3.19	53.78 ± 4.07	55.52 ± 3.28	56.20 ± 4.56
B2	55.84 ± 2.90	52.98 ± 4.02	56.46 ± 3.06	57.40 ± 3.94
C2	45.03 ± 3.54	42.24 ± 3.25	46.71 ± 2.67	50.38 ± 3.90

Note: Significant difference was present among all subgroup ($p < 0.05$) except that between A2 and B2.

Table VI. Bcl-xL positive cell after 8 h medicines administration ($\bar{x} \pm s$).

Group	10 h	12 h	14 h	24 h
A2	18.94 ± 2.19	17.72 ± 3.35	14.14 ± 2.29	11.28 ± 2.54
B2	20.32 ± 3.64	16.49 ± 3.31	13.72 ± 2.05	10.44 ± 2.35
C2	39.97 ± 3.56	35.08 ± 2.52	33.21 ± 2.47	29.26 ± 3.58

Note: Significant difference was present among all subgroup ($p < 0.05$).

death[26], which was observed in our histological analysis. For example, excess levels of ROS after SCI initiate oxidative chain reactions, damage cellular molecules and ultimately lead to cell death[27]. Elevated concentrations of free radicals further cause lipid peroxidation, which is a process that spreads over the surface of the cell membrane altering polyunsaturated fatty acids, causing impairment of phospholipid-dependent enzymes, disruption of ionic gradients, and even membrane lysis[28]. Following SCI, there is a rapid increase in intracellular free Ca^{2+} levels which activate Ca^{2+}-de-

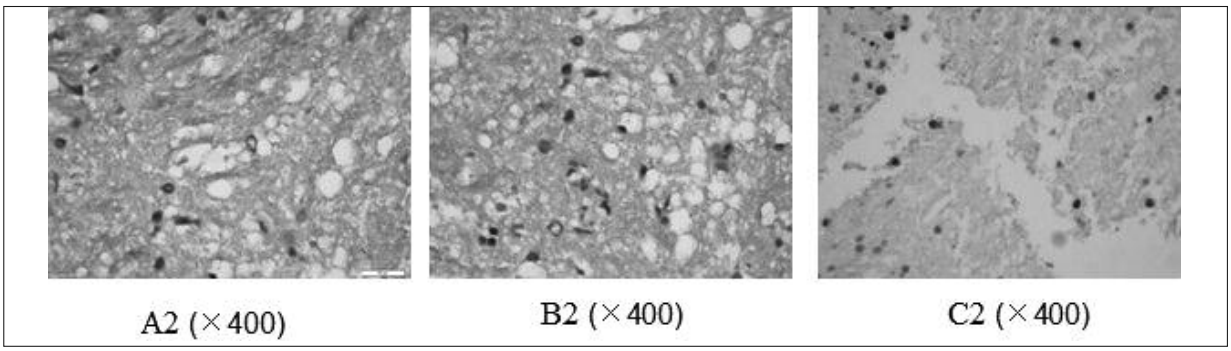

Figure 5. ISH analysis of Bcl-xL positive cell at 24 h.

pendent enzymes including calpain. Upon activation calpain, a Ca^{2+}-dependent cysteine protease, degrades a number of key cytoskeletal and membrane proteins making the cells to succumb to PCD[29,30]. Extracellular excitatory amino acid concentrations increase to neurotoxic levels within minutes following SCI. The observed rise in glutamate is responsible for excessive activation of glutamate receptors in the central nervous system resulting in neuronal cell death[31]. SCI initiates a robust immune response characterized in part by the synthesis of cytokines and chemokines and a coordinated infiltration of the damaged site by peripheral leucocytes. SCI-induced inflammation may result in further reduction in functional recovery because of the development of scar tissue, as well as necrosis or apoptosis of neurons and oligodendrocytes[24].

The mammalian apoptotic cell death is regulated by posttranslational activation of a class of cysteine proteases known as caspases, which have the unique property of cleaving proteins on the carboxyl side of aspartic acid. In particular, caspase-3 has been shown to be important to neuronal development and injury by inducing fragmentation of nuclear DNA[32,33]. Bcl-xL is the most robustly expressed anti-apoptotic protein in adult central nervous system to control cell survival. Bcl-xL protein level was found decrease in the cytoplasm and mitochondria 2 h after SCI and persisted for 24 h[34,35]. Therefore, in this study, we attempted to characterize the effect of MP and ED on secondary SCI through detecting the changes of caspase-3 and Bcl-xL positive cell.

Our findings indicated that MP administration led to a significant decrease in caspase-3 positive cell compared with control group. However, this effect was only present under the condition of MP administration within 8 h. No significant difference of caspase-3 positive cell was observed between B2 and A2 group, indicating little protective effect on SCI after 8 h MP administration. Hall[6], Hsu and Dimitrijevic[36] predicted that it was maybe by the reason that MP could not inhibit lipid peroxidation or scavenge prostaglandin and thrombus.

ED has the ability of scavenging toxic free radicals and, therefore, it is suggested as a novel neuroprotective agent for the treatment of SCI. Our results also indicated ED administration caused an improvement in the motor function. In addition, our results also indicated that ED administration led to a significant decrease in caspase-3 positive cell compared with control group. Interesting, our results found that caspase-3 positive cell in MP group (B1) was significantly less than that in ED group (C1) when medicine administration within 8 h, suggesting the protective effect of MP on SCI is superior to ED. However, caspase-3 positive cell in ED group (C2) was significantly less than that in MP group (B2) when medicine administration after 8 h. Importantly, Bcl-xL was found a significant increase in ED treatment than MP treatment no mater within or after 8h medicine administration, indicating the mechanism of ED on SCI was scavenging toxic free radicals and promoting Bcl-xL expression.

Conclusions

More attention should be paid on the time point of MP administration, and ED administration was more effective after SCI 8h for maintenance therapy.

Conflict of Interest

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