ORIGINAL REPORT

# The Effects of Cyclosporin-A on Functional Outcome and Axonal Regrowth Following Spinal Cord Injury in Adult Rats

**Amrollah Roozbehi[1], Mohammad Taghi Joghataie[2], Mehdi Mehdizadeh[2], Ali Mirzaei[3], and Hamdollah Delaviz[1]**

[1] *Cellular and Molecular Research Center, Faculty of Medicine, Yasuj University of Medical Sciences, Yasouj, Iran*

[2] *Department of Anatomy, Cellular and Molecular Research Center, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Department of Biochemistry, Cellular and Molecular Research Center, Faculty of Medicine, Yasuj University of Medical Sciences, Yasouj, Iran*

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**Abstract-** It has been shown that the immunophilin ligands have the special advantage in spinal cord repair. In this study, the effects of cyclosporine A (CsA) on functional recovery and histological outcome were evaluated following spinal cord injury in rats. After spinal cord hemisection in thirty six adult female Sprague-Dawley rats (200- 250 g), treatment groups received CsA (2.5 mg/kg *i.p.*) at 15min and 24h after lesion (CsA 15min group and CsA 24h group) daily, for 8 weeks. Control and sham groups received normal saline and in sham operated animals the spinal cord was exposed in the same manner as treatment groups, but was not hemisected. Hindlimb motor function was assessed in 1, 3, 5 and 7 weeks after lesion, using locomotive rating scale developed by Basso, Bresnahan and Beattie (BBB). Motor neurons were counted within the lamina IX of ventral horn and lesion size was measured in 5 mm of spinal lumbar segment with the epicenter of the lesion site. The mean number of motor neurons and the mean BBB scale in 3, 5 and 7 weeks in CsA 15min groups significantly increased compared to the control group. Although, the lesion size reduced in rats with CsA treatment compared to the control group, no significant difference was observed. Thus, it can be concluded that CsA can improve locomotor function and histological outcome in the partial spinal cord injury.



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**Keywords**: Cyclosporin A; Recovery of function; Motor neurons; Spinal cord

## Introduction

The secondary damage following spinal cord injury (SCI) may persist for several days and causes the expansion of the lesion to the neighboring cells (1). Secondary degeneration leads to loss of neuron, glia and leaves cysts at the injury site that disrupt the neural conduction (2). Therefore, the inhibition of these deterioration changes to protect survived spinal cells and axonal regrowth remain as challenging questions for further investigations (3). Although, different strategies have been used for this purpose, they were not along with restorative treatment and functional recovery (4). Cyclosporin A (CsA) is an immunosuppressant drug that inhibits the T-cell activation to prevent graft rejection and reduce the inflammatory response (5). After SCI, the blood spinal barrier is disrupted; causes the immune cells and proteins present into the lesion site. These cells contribute to the inflammatory reaction and exacerbate the spinal cord repair (6). So, in most cases external intervention is needed to response to the immune cells to maintain the spinal tissue and functional recovery (7). Thus, the use of appropriate anti-inflammatory drug can decrease the secondary damage and neuronal death and improves the patient outcome after SCI. It is evident that the CsA can create a more satisfactory condition to axon regrowth and reduces delayed brain neuron death following brain trauma injury (8-10), and increases cell survival in transplant regions by inhibition of calcineurin proteins (11). It can meaningfully preserve different allograft including kidney, heart and liver in transplant patients (12). Furthermore, the immunophilin ligands such as, FK506 and CsA can protect the axon and nerve cells after lesion (13,14). Counter reports indicated that some immune cells such as T cells, macrophage and microglia have neuroprotection

**Corresponding Author:** Hamdollah Delaviz
Cellular and Molecular Research Center, Faculty of Medicine, Yasouj University of Medical Sciences, Yasouj, Iran, P.o.Box: 7591994799
Tel, Fax: +98 741 2235153, E-mail: hamdidelaviz@yahoo.com

effect that improves functional outcome (15). This contrasting evidence indicated that the question about functions of the immune cells in SCI has not been answered. Thus, this study was conducted to evaluate whether this drug is suitable to improve functional recovery and histological outcome after SCI in adult rats.

## Materials and Methods

### Animal groups and surgical procedure

The study protocol was approved by the Ethics Committee for Animal Experiment at Tehran University of Medical Sciences, School of Medicine. Forty eight Spruge-Dawley rats weighing 200-250 g were used in this experiment. Animals were randomly allocated to four groups (n=12/group): sham, control, CsA 15min, and CsA 24h groups. Rats were anaesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) intra peritoneal (IP). A dorsal laminectomy of the T12 vertebrate was performed under an operating microscope. Then, the L1 spinal segment was identified and the hemisection on the left side was performed using a pair of iridectomy scissors. Hemisections were considered complete when they included the dorsal column, Lissaur's tract, lateral and ventral columns, and gray matter (16). The CsA (Sandimmune, Sandoz, Basel, Switzerland) injected (IP) 15min and 24h after lesion in CsA 15min and CsA 24h groups respectively at a dose of 2.5 mg/kg and continued daily for 8 weeks. The control and sham groups received normal saline, 15min after injury daily for 8 weeks. The spinal cords of the sham operated animals were exposed to the same manner, but no damage was done to the spinal cords. The overlying muscles were sutured in layers, and the skin was closed with wound clips. All rats were maintained under the same conditions with free access to food and water after surgery. Postoperative treatments included saline (1.0 cc *s.c.*) for rehydration and penicillin-G (0.35 ml/kg *i.m*) as a prophylactic antibiotic. Their bladders were manually expressed twice a day for the first 3 days.

### Locomotor measurements

Hindlimb motor function was assessed based on the Basso, Beattie, and Bresnahan (BBB) scale (17) at 1, 3, 5 and 7 weeks post-surgery. The BBB Locomotor Rating Scale is a 21-points scale from 0, that is no detectable movement to 21, which is consistent plantar stepping and coordinated gait (17). For BBB assessment the rats were allowed to move individually for 5 minutes on a smooth, nonslip floor in an open field (200 x 100 cm). For each rat, hindlimb motor function was scored from 0 to 21 based on the performance of the ipsilateral hindlimb by an observer who was oblivious of the identity of the groups.

### Motor neuron count and measurements of lesion volume

After 8 weeks, five rats in each groups were deeply anesthetized with an overdose of ketamine (200 mg/kg body weight) and xylazine (20 mg/kg body weight), perfused through their hearts with 0.9% NaCl in distilled water (200 ml) followed by 4% paraformaldehyde in 0.1 M PB, *p*H 7.4 (4°C, 500 ml). A 5-mm-long spinal cord segment with the middle of the lesion site were removed, washed, dehydrated and embedded in paraffin. The segment was cut into 50μm serial horizontal sections (10 sections for each rat). Every horizontal section, from rostral to the caudal of the segment, was mounted onto gelatin-coated glass slides, stained with cresyl violet, dehydrated and cover slipped. The motor neuron counted in the ventrolateral quadrants of the ventral horn, the use of Rexed's laminae (18) which their axons leave the spinal cord in the ventral roots to supply the striated skeletal muscle in lower limbs. For counting motor neurons the ventral horn was divided into four portions, then the number of motoneurons was counted on both operated and contralateral side in lamina IX, in each section as previously described (18). Then, the numbers of each section were summed per rat to obtain the final number of motor neurons in a 5-mm-long spinal cord segment. The only neurons that have intact cell membrane with nuclear and nucleus were counted. In other groups of rats (n=6/group), a computer based imaging system (Olympus Ax70. Dp12 olysia soft imaging system, Japan) was used to determine the volume of lesion spinal tissue of a 5-mm-long spinal cord segment. In each section, the total damaged area was determined and the amount of each section was summed per rat to give the total lesion volume of the 5 mm-long cord segment as used earlier (19).

### Statistical analysis

Data was expressed as a mean±SD, one-way ANOVA followed by post-hoc Tukey's test was used to determine statistical differences between the average number of motor neurons and damage area as determined for each group. A statistically significant difference was accepted at $P<0.05$.

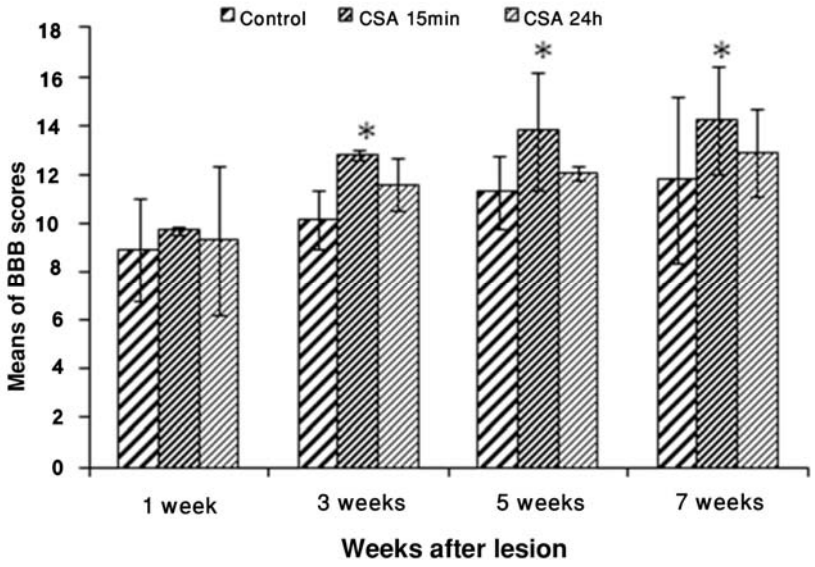


**Figure 1.** Graph shows the improvement of BBB Locomotor Rating Scale, after spinal cord hemisection in different groups. Cyclosporin A has the beneficial effect on promotes motor recovery in left hindlimbs of treated rats. Values represent means ± SD, n=12, **P*<0.05 compared to the control group.

## Results

### Hindlimb locomotor outcome

One week after spinal cord hemisection, the ipsilateral hindlimb motor function in the CsA 15min groups was observed with sweeping of the hindlimb without weight support (9.71±0.3). At this time, the control group showed the extensive movement of the three joint (8.9±1.1). However, a significant difference has not seen between groups during the first week. At the third week, progressive motor recovery has seen in the CsA 15min group with plantar placement compared with the control and CsA 24h groups. At 7$^{th}$ week the average BBB scores in rats with spinal cord injury were 11.8±3.4, 14.2±2.2 and 12.9±1.8 for control, CsA 15min and CsA 24h groups, respectively. At 3$^{rd}$, 5$^{th}$ and 7$^{th}$ weeks, one-way ANOVA followed by Post-hoc Tukey's test determined a significant difference ($P$<0.05) of functional recovery in CsA 15min treatment group compared to the control group (Figure 1). Results showed that the daily administration of CsA immediately after SCI has a therapeutic effect on functional recovery.

### Location and morphology assumed for motor neurons

To identify the motoneuron in the IX lamina the ventral horn was divided into four parts (Figure 2A) and motor neuron in the ventrolateral quadrants of the ventral horn were counted. The stellate morphology, nissl granules with prominent nucleus were considered to confirm the motoneuron cells (Figure 2B).

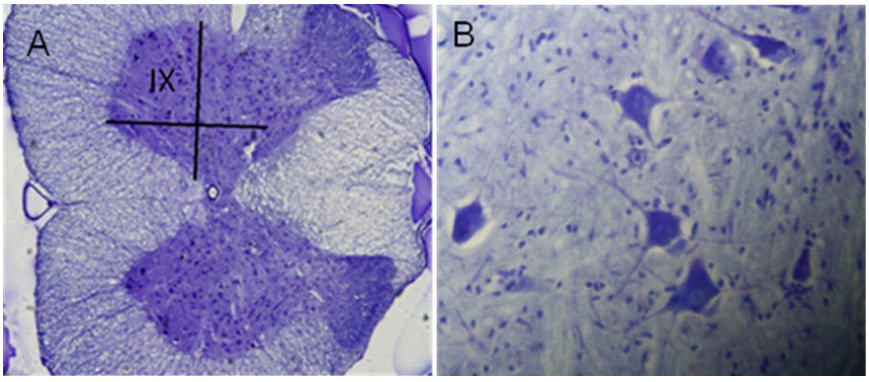


**Figure 2.** Lamina IX contained the motor neuron in the lateral motor column (A). These cells have several dendrites, round nucleus and multipolar shape (B). Cresyl violet staining. Scale bars= 50 µm in A, 20 µm in B.

**Table 1.** The mean ± SD number of motor neuron in different groups 8 weeks after SCI.

| Group | No. | Right (intact) | left (injury) | Ratio R/L |
|---|---|---|---|---|
| Sham | 5 | 791.7±3.5 | 780.4±2.1[#] | 98.6 |
| CsA 15min | 5 | 718.3±1.5 | 284.8±2.3[*] | 39.55 |
| CsA 24h | 5 | 812.5 ±3.5 | 231.2 ±.5 | 28.44 |
| Control | 5 | 804.6 ±3.3 | 210.5±1.4 | 26.11 |

*A significant difference compared with the control group ($P$<0.05).

[#]A significant difference compared with the other groups ($P$<0.001).

**Number of motor neurons in the ventral horn**

Eight weeks after the hemisection, there were significant difference of the mean number of the motor neurons on the ipsilateral side compare to the contralateral side of the injured rats ($P$<0.05). The mean number of motor neurons in the contralateral intact side (right) in animals with spinal cord (SC) hemisection was not a considerable difference compared with the sham group (Figure 3). The mean number of motor neuron in the left side of the sham group that did not receive the lesion considered 100% and the other groups were compared to this group. The number of motor neuron cells decreased in lesion side (left) in experimental groups. In comparison with the sham group, 26%, 36% and 29% of the survival motor neurons were seen in control, CsA 15min and CsA 24h groups respectively (Table 1). One way ANOVA followed by Tukey's test detected a significant difference in the increase of mean motor neurons in CsA 15min group compared to the control group eight weeks after lesion ($P$<0.05). Result showed that the CsA was efficient on the survival of motor neurons when injected 15min after spinal cord injury.

**Spinal cord histology and lesion volume analysis**

Histological study of a 5-mm-long spinal cord segment with middle of the lesion site showed that the spinal cord hemisection caused a large degeneration of spinal tissue including white and gray matter at the lesion site, which expanded toward the craniocuadal of the injury. Statistical analysis demonstrated a reduction size of the cavity in animals that were treated with CsA 15min after lesion compared with the other groups (Figure 3).

The total lesion vlume in a 5-mm-long spinal cord segment in different groups which received spinal hemisection were 6.3±0.2 $mm^3$, 5.1±0.1 $mm^3$ and 5.4±2.3 $mm^3$ for control, CsA 15min and CsA 24h groups, respectively (Figure 4). Although, the mean injury size was reduced in rats treated with CsA, statistical analysis revealed no significant difference compared with the control group.

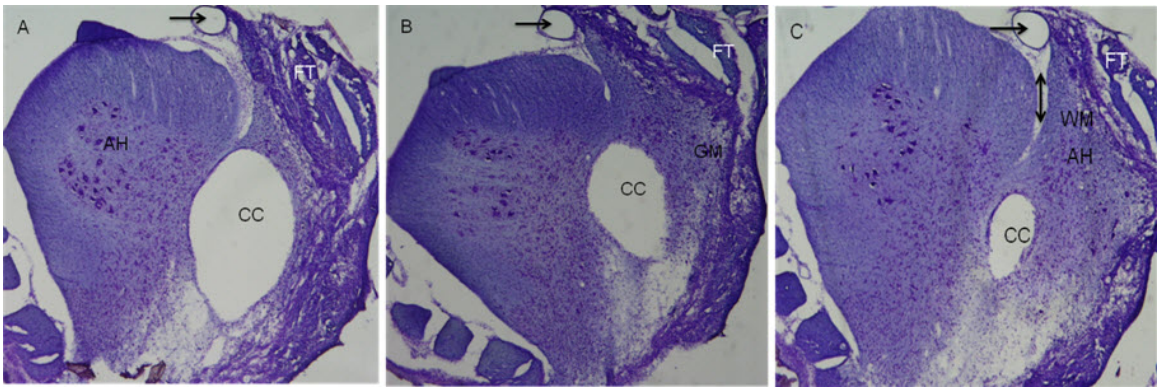


**Figure 3.** Photomicrograph of the spinal lumbar segment in cross section (50 µm) 8 weeks after injury. In the control group a large cavity was seen in the central canal (CC) without remarkable grey and white matter in the ipsilateral side (A). Grey matter (G) was present around the dilated central canal (CC) in CsA 24h group (B). In CsA 15min group the ipsilateral and contralateral sides were divided by anterior median fissure (double arrow). Also, white matter (w) was seen with border zone of the grey matter in the anterior horn. At the peripheral of the lesion site some fibrous tissues (FT) have been seen in different groups. Transverse section (50 µm for all section), central canal (CC), anterior horn (AH), white matter (WM), grey matter(GM), fibrous tissue (FT), Arrow indicated the anterior median vein and the double arrow indicated anterior median fissure. Scale bar=300 µm in A and B, 200 µm in C.

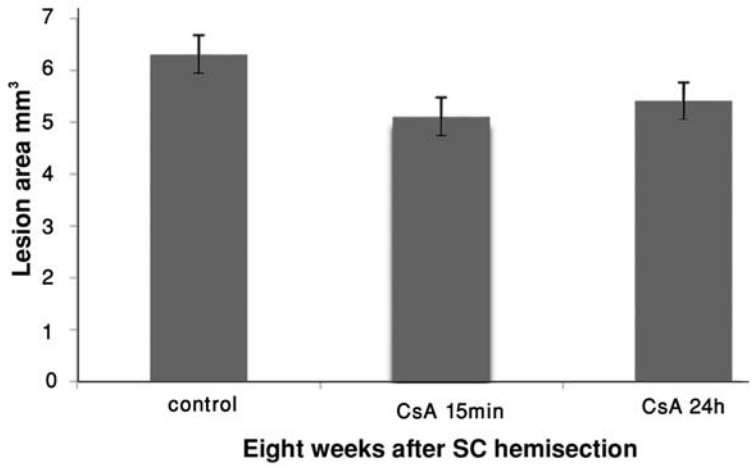


**Figure 4.** Mean lesion volume of differentt groups eight weeks after SC hemisection. No significant difference has seen between the studied groups ($P$>0.05), n=5.

## Discussion

This study confirmed that CsA has neuroprotective effect on the injury site and could improve the locomotor recovery following spinal cord hemisection at the L1 level. The increase of survival motoneuron and locomotor outcome in rats treated with CsA compared to the control group indicated that CsA has counteracting effect on some degeneration elements. The major findings of this study showed that the neuroprotection feature of CsA was time dependent. Our findings were in accordance with other results that demonstrated the drug action of CsA 15min after brain trauma was more efficient for myelinated axons (20). It is also evident that the injection of CsA, 15 min or 1h compare to the 6h after SCI is more neuroprotective in decreasing lesion area (21). The narrow therapeutic window of CsA remained unknown, but it may be correlated with the penetration of CsA in blood-brain barrier after brain injury (22). In this regard, the timing of the activated immune response after SCI is very important to control inflammation.

Accumulating evidence has demonstrated that CsA ameliorated cortical damage after traumatic brain injury (9, 10) and restores neural tissue following spinal cord injury (23). CsA administration, immediately after spinal cord trauma, may inhibit the scar formation at the lesion site (24). In our study the improvement of motor function and neuroprotection in CsA 15min group compared with the CsA 24h group are due to the effect of CsA on early inflammatory events. It has been reported that interleukin IL-1A and cytokines increased within 15 min to 3 h after SCI injury (25) while after 24 h the elevation of these molecules was transient (26). There is also document which shows that primary phase of neuroinflammation after SCI has detrimental effects, whereas the later phase is beneficial (27). The second phase of immune cells reaction is prominent in protection of nerve cells and functional recovery (28).

CsA can cross the plasma membrane and binds to different receptors such as cyclophilins that alter the cell function and behavior (29) and prevents the activation and proliferation of T-lymphocyte (28). It has been suggested that the stimulation of T-cells leads to axonal injury and motor neuron loss at the injury site in the CNS lesion (30). Inflammatory cells such as, macrophages produce cytokines and neurotoxic materials that cause damage to the CNS (31). In contrast, it has been reported that growth factor from the T-cell is important to neuroregeneration (32). So, study on property of cellular immune response is needed for effective treatment. Furthermore, our results demonstrated that, there is a correlation between the locomotor outcome and survival of motor neuron in the ventral horn of spinal cord. In parallel with these observations, cyclosporin increases axonal regeneration, tissue protection and functional recovery following transverse section in SCI (33,34). These neuroprotective effects of CsA, thought to be reducing inflammatory cytokines, control the cell population and lipid peroxidation following acute SCI (19). In this study, the decrease in total lesion volume in CsA 15min compared to the other groups may be related to the increase in spared white matter which reduces delayed motor neuron at the injury site. In other study, the increase of spinal cells and oligodendrocytes has been seen with CsA treatment compared to the untreated animals (34). However, contrasting report seemed to show that the

CsA therapy failed to improve the functional recovery and spinal tissue sparing following the SCI (35). Anyway, the methods in their study were different from ours. In our study, the rats received hemisection and IP injection of CsA continued for 8 weeks after injury. Whereas, in their report the rats received mild contusion and CsA injected only one week after surgery (35). Thus, different results from two studies are possible. In conclusion, it is worthy to mention that the administration of cyclosporin-A immediately after SCI has beneficial property in limiting lesion volume and improvement of locomotor outcome. Further works on CsA accompanying with other therapies will provide better restorative treatments for spinal cord repair.

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