

Effect of chondroitinase ABC on inflammatory and oxidative response following spinal cord injury

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ABSTRACT

Objective(s): Chondroitinase ABC (cABC) treatment improves functional recovery following spinal cord injury (SCI) through degrading inhibitory molecules to axon growth. However, cABC involvement in other pathological processes contributing to SCI remains to be investigated. Here, we studied the effect of cABC I on oxidative stress and inflammation developed in a rat model of SCI.

Materials and Methods: Male rats (220–250 g) were divided into three groups (n=28) including rats that underwent SCI (SCI group), rats subjected to SCI and received an intrathecal injection of phosphate buffer saline (SCI+PBS group), and rats that underwent SCI and received cABC intrathecally (SCI+E group). Then, the level of TNF- α , IL-1 β , malondialdehyde, nitric oxide, and myeloperoxidase in injured tissues, as well as hindlimb motor function, were measured at 4 hr, 1, 3 and 7 days post-SCI.

Results: Our data showed that cABC treatment reduced the development of inflammation and oxidative stress associated with SCI at all-time points. In addition, functional recovery was improved in rats that received cABC at 7 days post-SCI.

Conclusion: The present findings indicate that cABC treatment can exert its neuroprotective effect through modulation of post-traumatic inflammatory and oxidative response.

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Introduction

Spinal cord injury (SCI) is a devastating neurologic disorder which is characterized by a primary mechanical trauma followed by a secondary degenerative event. In fact, the primary physical injury leads to the death of a number of neurons, which causes the loss of sensory, motor and autonomic functions. However, the secondary neuronal death is determined by a number of cellular, molecular, and biochemical cascades. Hallmarks of secondary injury events are vascular changes such as hemorrhage and vasospasm, inflammation, free radicals formation, disruption of ionic balance, glutamate excitotoxicity, proteases activation, and finally glial scar formation. In such pathological conditions, the lesion extends far beyond the initial area of trauma. Therefore, secondary injury has always been a target in the treatment of SCI (1, 2).

Inflammation is an important physiological mechanism underlying chronic neurodegeneration following SCI. After spinal cord trauma, disruption of blood-spinal cord barrier leads to the recruitment of extrinsic

and resident inflammatory cells. Macrophages and microglia dominate the innate immune response elicited by SCI (3). They produce a range of pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF- α) which contribute to axonal demyelination and cell death (4). Moreover, pro-inflammatory cytokines may potentiate gliosis and induce CSPGs expression in glial cells, which are inhibitory to axon regeneration (5). Furthermore, infiltrate cells release neurotoxic free radicals such as reactive oxygen and nitrogen species which subsequently damage proteins, lipids, nucleic acids, and extracellular matrix proteins such as proteoglycans (6). Among free radicals produced in the area of injury, nitric oxide (NO) is the more studied. Its toxic effects are mainly mediated by its reactive product, peroxy-nitrite (7). Therefore, attenuation of inflammatory and subsequent oxidative response after SCI represents a beneficial strategy to decrease secondary damage (8).

Following damage to the spinal cord and other parts of CNS, glial cells accumulate and surround the

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injury sites. They upregulate expression of chondroitin sulfate proteoglycans (CSPGs) leading to formation of glial scars in order to limit the area of damage. However, glial scar poses a physical and biochemical barrier to axon repair and regeneration. The inhibitory effect of CSPGs can be neutralized by the bacterial enzyme chondroitinase ABC (cABC). It is produced by the bacteria *Proteus vulgaris* which cleaves disaccharides and tetrasaccharides. Many *in vitro* and *in vivo* studies have shown that cABC treatment improves axonal regeneration and sprouting and promotes functional recovery in experimental models of SCI (9-11). Moreover, a sulfated disaccharide derived from CSPGs digestion was shown to be a highly potent compound in regulation of inflammatory response in CNS which protects against neurodegeneration associated with inflammatory conditions (12). Therefore, cABC treatment might have a potential modulating effect on inflammation and subsequent oxidation contributed to neuronal loss in SCI.

To address this issue, the present study was conducted to investigate the effect of cABC on inflammatory and oxidative response as well as motor function recovery in an animal model of SCI. Here we indicated that cABC reduces inflammation, oxidative stress, and motor dysfunction in a contusion model of SCI.

Materials and Methods

Animals and experimental groups

Adult male Wistar rats (220–250 g) used in this study were purchased from Experimental Animal Center of Kerman University of Medical Sciences. Animals were housed in a controlled environment and provided with standard rodent chow and water *ad libitum*. All experiments were approved by the ethics committee of Kerman University of Medical Sciences and performed in accordance with the local guidelines. A total of 84 animals were used in the following groups:

- i. SCI: rats that underwent SCI as described below (n=28).
- ii. SCI + phosphate buffer saline (PBS): rats were subjected to SCI and intrathecal injection of PBS (n=28).
- III. SCI + Enzyme: similar to group ii, but rats received a single injection of cABC intrathecally (n=28).

Spinal cord injury and drug delivery

Animals were anesthetized with an intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine. Under aseptic conditions, dorsal skin was shaved and sterilized. Then, a dorsal laminectomy was performed to expose T9-T10 thoracic spinal cord. The contusion injury was made by dropping a 10 g weight from a height of 25 mm (NYU impactor, USA) immediately after the injury, 6 µl of cABC (10 U/ml; Sigma, USA) or PBS (0.1 M, pH 7.4) was microinjected intrathecally (13). Then, the paraspinal musculature and subcutaneous tissues were

closed using silk sutures. Animals were placed in a warm environment to recover from anesthesia. Then, gentamicin was administered (12 mg/kg, IP) and repeated every other day. Bladder expression was performed twice a day manually until bladder function returned. Animals were kept in a temperature controlled room where food and water were provided *ad libitum*.

Assessment of locomotor functions

Hindlimb motor function was evaluated at 1, 3 and 7 days post-injury using the open field Basso, Beattie, and Bresnahan (BBB) locomotor test which consists of a 21 points scale (14). Rats were observed by a trained observer blinded to the treatments and scored from 0 (complete paralysis) to 21 (normal locomotion).

Sample preparation and biochemical experiments

Animals were sacrificed 4 hr, 1, 3 and 7 days post-SCI (n=7). A segment of spinal cord containing the area of injury (2 cm) was dissected, washed with ice-cold PBS and snap frozen in liquid nitrogen. Then, samples were stored at -80 °C for biochemical analysis.

To determine biochemical parameters, sample homogenization was performed in ice-cold PBS containing 1% (vol/vol) protease inhibitor (Sigma, USA). Then, lysates were centrifuged at 15000 ×g for 20 min at 4 °C and the supernatants were prepared. Both supernatants and pellets were kept at -80°C until analyzed.

Measurement of tissue TNF-α and IL-1β

To investigate the level of inflammation, pro-inflammatory TNF-α and IL-1β were measured in spinal cord tissues. The assay was carried out using a commercial ELISA kit (Abcam, UK) according to the manufacturer's instructions. All measurements were performed in duplicate and the results were presented as pg/mg protein.

Measurement of tissue malondialdehyde

To determine tissue lipid peroxidation, the concentration of malondialdehyde (MDA) was measured based on its reaction with thiobarbituric acid (TBA) (15). Briefly, 150 µl supernatant was mixed with 300 µl trichloroacetic acid (20%; Sigma, USA) and TBA (0.67%; Sigma, USA). Then, samples were heated in boiling water for 60 min. After cooling at room temperature, samples were centrifuged at 3500 ×g for 10 min. The absorbance of the supernatant was measured at 532 nm. Tetramethoxypropane (Sigma, USA) was used to prepare the standard curve. MDA concentrations were reported as nmol/mg protein.

Measurement of tissue nitric oxide

To measure NO as an oxidant in spinal cord samples, 150 µl of supernatant was mixed with sodium hydroxide (0.3 M). After 5 min, 75 µl zinc

sulfate (5%) was added to precipitate the protein. The mixture was then centrifuged at 15000 \times g for 20 min at 4 °C and its supernatant was separated. To 200 μ l of supernatant, 300 μ l vanadium (III) chloride (80 mg VCl_3 in 10 ml HCl 1 M; Sigma, USA) was added. The mixture was then incubated at 37 °C for 45 min. The absorbance was measured at 540 nm. Sodium nitrite was used to plot the standard curve (16). The result was presented as nmol/mg protein.

Measurement of tissue myeloperoxidase

Myeloperoxidase (MPO) activity was measured as an indicator of neutrophil accumulation in spinal cord samples. Tissue pellets were resuspended in ice-cold phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, USA). Homogenates were then frozen in liquid nitrogen and thawed in three consecutive cycles with sonications (10 sec, 4 °C) between cycles. After the last sonication, samples were centrifuged at 15000 \times g for 20 min at 4 °C. Then, the supernatant was utilized for MPO assay. The rate at which a colored product formed during MPO-dependent reaction with o-dianisidine (0.167 mg/ml; Sigma, USA) and hydrogen peroxide (0.003%) was measured at 460 nm. One unit of MPO was defined as the amount that degrades 1 μ mol hydrogen peroxide per minute at 25 °C (17). The results were expressed as mU/mg protein.

Statistical analysis

Data analysis was performed using SPSS software, version 16.0 (SPSS Inc, USA). The results were analyzed using non-parametric Kruskal-Wallis test followed by Mann-Whitney analysis. Data were expressed as mean \pm SD. A *P*-value less than 0.05 was considered significant.

Results

Locomotor function

The recovery of capacity to use hindlimbs in an open field was graded with the BBB scale. Contusion model of SCI resulted in the score of 0 in all animals measured at 1 day post-injury. At 3 days after SCI, no improvement was observed in hindlimb overground walking ability in all groups. However, a slight recovery was seen in the treated group compared to SCI and PBS groups one week after SCI which was statistically significant ($P<0.01$) (Figure 1).

Tissue TNF- α and IL-1 β concentration

TNF- α and IL-1 β were measured in spinal cord tissue as indicators of inflammation. Data showed that the level of TNF- α peaked at 4 hr post-SCI and then was decreased by 1 day after injury. A slight reduction was observed in TNF- α level by day 7 post-surgery. Comparing SCI and PBS groups, no significant difference was found at any time points ($P>0.05$). However, cABC treatment reduced the TNF- α level at

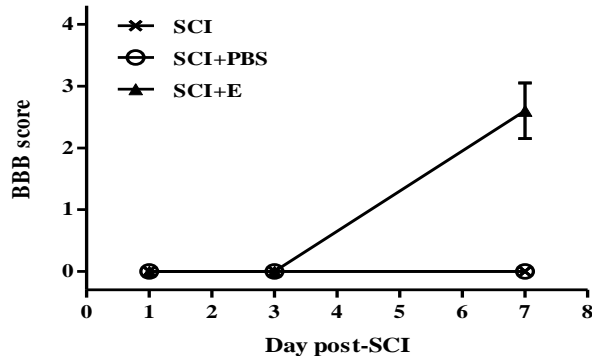


Figure 1. Effect of cABC on behavioral function after SCI. Hind limb locomotor function was assessed using Basso, Beattie and Bresnahan (BBB) criteria at times indicated. Treatment with cABC improved motor function significantly at 7 d post-SCI. Data represents mean \pm SD of 7 rats for each group. The results were analyzed by non-parametric Kruskal-Wallis followed by Mann-Whitney test. * $P<0.01$ versus SCI

4 hr, 1 and 3 day after SCI significantly compared to SCI and PBS groups ($P<0.05$) but the level was not significant at day 7 post-damage ($P>0.05$) (Figure 2A).

The level of IL-1 β reached a peak at 1 day after SCI and then decreased considerably by day 7 post-injury in all groups. Treatment with cABC reduced the IL-1 β level at all time points compared to SCI and PBS groups ($P<0.01$). However, there was no significant difference between SCI and PBS groups at any time points ($P>0.05$) (Figure 2B).

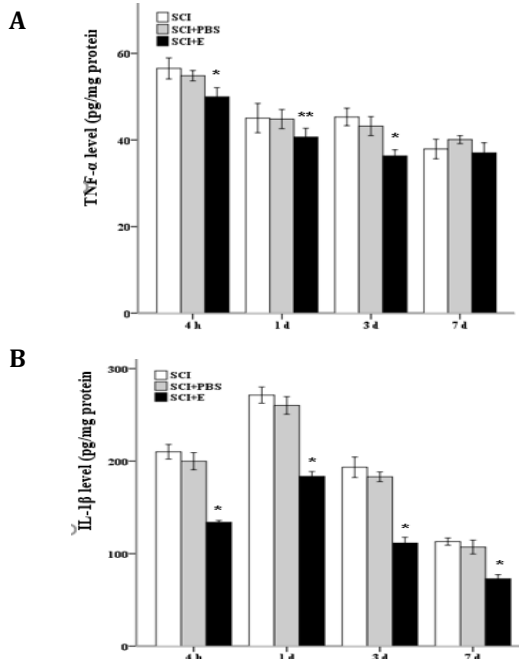


Figure 2. Effect of cABC on pro-inflammatory cytokines after SCI. The levels of TNF- α (A) and IL-1 β (B) were measured at 4 h, 1, 3 and 7 d post-SCI. A considerable increase in TNF- α and IL-1 β was found at 4 h and 1 d post-SCI respectively in spinal cord tissues collected from SCI rats. However, cABC treatment decreased the level of these cytokines significantly. Data are expressed as mean \pm SD of 7 rats for each group. The results were analyzed by non-parametric Kruskal-Wallis followed by Mann-Whitney test. * $P<0.01$ and ** $P<0.05$ versus SCI

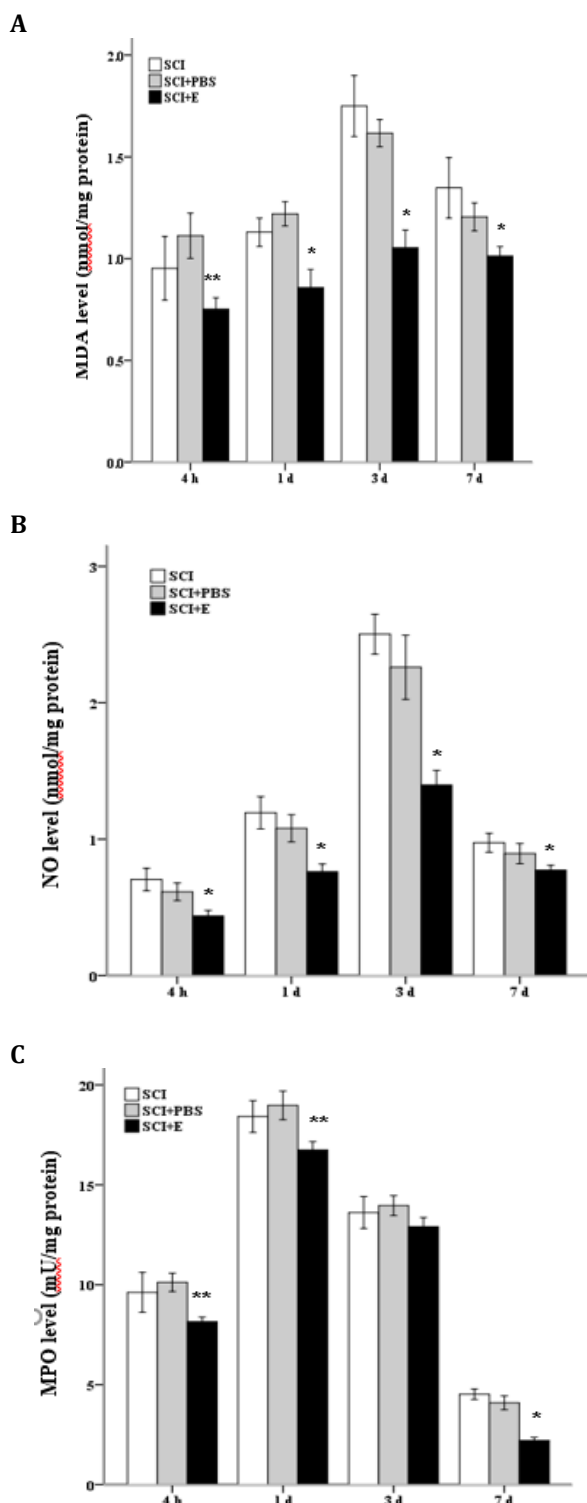


Figure 3. Effect of cABC on oxidative stress markers after SCI. The levels of malondialdehyde (MDA) (A), nitric oxide (NO) (B) and myeloperoxidase activity (MPO) (C) were measured at 4 h, 1, 3, and 7 d post-injury. A substantial increase in MDA, NO and MPO was found at 3, 3 and 1 d post-SCI respectively in spinal cord tissues collected from SCI rats. Whereas treatment with cABC reduced the level of these markers significantly. Data represents mean \pm SD of 7 rats for each group. The results were analyzed by non-parametric Kruskal-Wallis followed by Mann-Whitney test. * $P < 0.01$ and ** $P < 0.05$ versus SCI

Tissue malondialdehyde concentration

To determine whether cABC influences lipid peroxidation in the area of injury, the level of MDA was measured in spinal cord tissues. The level of MDA was increased slightly by 1 day after injury, peaked at day 3 and then was reduced by 7th day post-damage, but it was still higher than those of 4 hr post-SCI group. No significant difference was observed between SCI and PBS groups statistically ($P > 0.05$). However, treatment with cABC reduced the level of MDA significantly at all time points compared with two other groups ($P < 0.05$) (Figure 3A).

Tissue nitric oxide concentration

NO was measured in SCI samples as an indicator of oxidative stress. After SCI, NO level was increased rapidly, reached a peak at day 3 and considerably was decreased at day 7 post-injury in all groups. In addition, data showed that cABC treatment reduced NO level significantly compared to SCI and PBS groups ($P < 0.01$). However, a considerable reduction was observed at day 3 in the cABC treated group ($P < 0.001$). Moreover, no significant difference was seen between SCI and PBS groups at any time points ($P > 0.05$) (Figure 3B).

Tissue myeloperoxidase activity

In order to investigate the level of inflammation, MPO activity was determined. Comparing all groups at different time points, the highest level of MPO was observed at 1 day after surgery which was then reduced to the lowest level at day 7 post-SCI. Whereas no significant difference was observed between SCI and PBS groups at any time points ($P > 0.05$), the level of MPO in the treated group was significantly lower at 4 hr, 1 and 7 days after injury compared with two other groups ($P < 0.05$). However, no statistical significance was found among cABC, SCI, and PBS groups at day 3 post-SCI ($P > 0.05$) (Figure 3C).

Discussion

SCI initiates a sequence of events which lead to secondary neural cell damage. Evidently, early inflammatory response induces tissue damage in an acute SCI (18). The acute inflammatory response to SCI involves recruitment of neutrophils and extrinsic macrophages to the site of injury (19). In addition, microglial cells become activated within minutes to hours after SCI and are transformed to macrophages (20). These cells produce inflammatory cytokines including TNF- α , IL-1 β , and IL-6 at the lesion site and regulate the precise cellular events after SCI (21). In this study, we demonstrated that TNF- α and IL-1 β peaked at 4 hr and 1 day after SCI, respectively. As reported earlier transcripts of TNF- α and IL-1 β are upregulated within the first hours post-SCI. While the expression of IL-1 β is upregulated at 24 hr

post-injury, TNF- α gene expression is nearly downregulated by the 24th hour (6, 22). Both TNF- α and IL-1 β share pro-inflammatory effects such as induction of other cytokines, oxidants production, promotion of neuronal apoptosis, stimulation of astrocyte proliferation, and glial cell activation (6).

Reactive astrocytes provide an inhibitory environment at the lesion site, which is characterized by high level of CSPGs. The use of the enzyme cABC to breakdown CSPGs has beneficial effects on spinal repair as reported in numerous studies (9, 23). Here we demonstrated that cABC treatment was effective in alleviation of inflammation through reduction of TNF- α and IL-1 β level in a model of thoracic contusion injury. This is possibly due to generation of new compounds following enzymatic digestion of CSPGs. There is evidence that the disaccharide CSPG-DS derived from CSPGs modulated inflammatory responses associated with neurodegenerative diseases. This effect was attributed to a reduction in numbers of infiltrating T cells and microglia activation (12). In addition, CSPGs induced neurite outgrowth, prevented neuronal death and axonal collapse both *in vitro* and *in vivo*. Therefore, it would promote CNS system repair (24). Moreover, treatment with high dose of cABC upregulated anti-inflammatory cytokine IL-10, whereas downregulated pro-inflammatory cytokine IL-12B in injured spinal cord tissue. Furthermore, viral overexpression of cABC led to an increase in anti-inflammatory M2 macrophages, which limited secondary inflammatory injury and improved tissue repair (25).

Here we showed that MDA, an indicator of oxidative stress, increased following SCI. There is a large amount of evidence that neutrophils and macrophages are mainly involved in oxidative stress by generating reactive oxygen species (ROS) in the injured spinal cord tissue (26). Detrimental effects of ROS commence immediately after mechanical damage to CNS, which causes cytotoxicity through oxidizing lipids, proteins, and nucleic acids (27). However, polyunsaturated fatty acids which are found in high concentration in the CNS are main targets for ROS. Their peroxidation initiates as early as 5 min post-SCI and continues within the first week after injury. The major product of polyunsaturated fatty acids is MDA, which is considered a reliable biomarker of oxidative injury (28,29). In this study, MDA level peaked at 3rd d post-trauma, which demonstrates the highest level of lipid peroxidation in this contusion model of SCI. However, cABC treatment reduced the MDA level, which indicates its protective effect against lipid peroxidation.

In this investigation, we observed that NO level increased after SCI. NO is a diffusible highly reactive gas that is produced from arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) have been

identified (30). Physiologically, NO is produced in small amounts by endothelial and neuronal cells in CNS. However, immediately following SCI, NO level increases considerably. It then reacts with O₂⁻ to form the reactive oxidant, peroxynitrite (ONOO⁻), which can directly oxidize lipids, proteins, and DNA. In addition, peroxynitrite itself leads to neuronal loss and locomotor dysfunction in SCI (31). High concentration of NO is produced by iNOS, which is mostly expressed in astrocytes and microglial cells. Evidently, iNOS is induced by TNF- α and IL-1 β , which is known to play an important role in the progression of SCI (32). Our data showed that cABC decreased NO level significantly. This result in agreement with another observation indicated a relation between inflammation and NO production, which were both reduced in the presence of cABC (31).

This study demonstrated that MPO activity was increased following SCI and peaked at day 1 post-injury. MPO is a specific enzyme present in neutrophil granules. MPO activity is correlated with the number of neutrophils infiltrated into the lesion site and their activation. Therefore, MPO activity is used as a marker to quantify the post-traumatic accumulation of neutrophils (33). Typically, MPO activity is increased within 3 hr and remains elevated up to 3 days after SCI (19). The enzyme catalyzes a reaction between hydrogen peroxide and the chloride ion and produces hypochlorite which can react with polyunsaturated fatty acids and results in lipid peroxidation (34). However, in this study, the time course of MDA production and MPO activity did not follow the same pattern, which may contribute to other sources of oxidation. Moreover, our results provide evidence that cABC decreased MPO activity which confirms the anti-inflammatory effect of this enzyme.

The current study found that a single injection of cABC enhanced functional recovery in the contusion SCI model used here at day 7 post-injury. The efficacy of cABC treatment in improvement of locomotion has been demonstrated in numerous studies using various models of SCI (9, 35). Recent investigations have indicated that degradation of CSPGs, which act as inhibitors to neuronal growth provides a more permissive environment for axon regeneration and promotes functions (36). The other possibility to explain the functional outcome of this treatment might be related to the effect of cABC in reducing inflammation and oxidative stress level at the site of injury. Immediately after SCI, macrophages and microglia recruited to lesion site produce a variety of cytokines which are contributed to axonal demyelination and cell death (4, 37). Moreover, these cytokines can stimulate the expression of CSPGs inhibitory to regeneration (5). Therefore, cABC treatment may protect neurons and potentiate axonal regeneration by reducing inflammatory response induced in SCI.

Conclusion

Here, we presented novel data demonstrating the potent effect of cABC on reducing secondary injury through modulating inflammation and oxidative stress developed in SCI. Moreover, this neuroprotection effect was associated with motor function recovery in a clinically relevant model of SCI. Therefore, a better understanding of these mechanisms might lead to the introduction of this therapeutic strategy into clinical practice.

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