

## Effect of Chondroitinase ABC Enzyme on Glial Fibrillary Acidic Protein, Chondroitin Sulfated Proteoglycans and Chondroitin 4-Sulfate Levels in an Animal Model of Spinal Cord Injury

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### Abstract

**Background:** Following spinal cord injury, reactive astrocytes upregulate chondroitin sulfate proteoglycans (CSPGs) which act as a barrier to neuronal repair and regeneration. Therefore, enzymatic digestion of CSPGs by chondroitinase ABC (cABC) is a key strategy in the treatment of spinal cord injury. Furthermore, cABC has been shown to attenuate post spinal cord injury inflammation and may decrease astrocytes activation. Thus, this study was conducted to investigate the effect of cABC on astrocytes level through measuring glial fibrillary acidic protein (GFAP) in an animal model of spinal cord injury.

**Methods:** spinal cord injury was performed through contusion method. Sixty male rats (220-250 g) were divided into three groups. The first group was just subjected to spinal cord injury. In the second group, phosphate buffered saline (6 µl) was immediately injected in lesion site after spinal cord injury. The third group received cABC (6 µl, 10 U/ml) intrathecally after spinal cord injury. Then, GFAP, CSPGs and chondroitin 4-sulfate (C4S) levels were measured at 4 hours and 1, 3 and 7 days after spinal cord injury using immunohistochemical method.

**Results:** GFAP level increased in all groups until 7 days post spinal cord injury, but no significant difference was observed between enzyme- treated group and two other groups at similar time point ( $p > 0.05$ ). CSPGs level increased significantly in the first and second groups whereas it decreased considerably in enzyme- treated group. In addition, due to enzymatic digestion of CSPGs, C4S level in the third group increased significantly.

**Conclusion:** cABC enzyme decreased CSPGs level through degradation without any..... considerable effect on their source which is reactive astrocytes.

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## Introduction

Spinal cord injury (SCI) is a devastating neurological disorder that affects thousands of individuals each year. More than half of spinal cord injuries are resulted from trauma. Traumatic SCI is characterized by a primary injury followed by secondary damages. Initial injury is resulted from mechanical impact on the spine. Within hours to days after the initial injury, a complex secondary pathologic cascade follows which exacerbates the primary damage. This include alterations in local blood flow, electrolyte homeostasis perturbations, edema, free radicals generation, excitotoxicity and inflammation (1, 2).

Inflammatory response plays a critical role in pathogenesis of acute and chronic SCI. In fact, disruption of blood-spinal cord barrier leads to the recruitment of peripheral immune cells and activation of glial cells and their migration to the lesion site. They abundantly release a range of cytokines which lead to the activation of astrocytes, meningeal cells, and oligodendrocyte precursor cells (OPCs) (3).

Reactive astrocytes are denoted by increased immunoreactivity of glial fibrillary acid protein (GFAP) which is a distinct cellular marker of astrocytes. It has been demonstrated that reactive astrocytes upregulate high levels of chondroitin sulfate proteoglycans (CSPGs) which form an astroglial scar that exert an inhibitory effect on axonal growth. OPCs also contribute to CSPGs production to inhibit neuronal regeneration (4).

Proteoglycans are the major glycanated protein types found in the nervous system. These molecules consist of a core protein linked to glycosaminoglycan (GAG) carbohydrate side chains. There are four known classes of proteoglycans including: heparin sulfate, dermatan sulfate,

keratin sulfate and chondroitin sulfate proteoglycans. CSPGs are the most abundant proteoglycans found in central nervous system (CNS). In adults, they promote brain synapses stability and limit plasticity. Following SCI, CSPGs have been shown to act as barrier molecules in glial scar particularly through the inhibitory influence of their GAG chains on axon growth (5, 6). Therefore, reducing reactive astrocytes or elimination of GAGs can be beneficial in the treatment of SCI (7).

Various therapies have been developed to overcome the inhibitory effect of CSPGs. Amongst them, chondroitinase ABC (cABC) has been repeatedly shown to have beneficial effects on spinal repair following SCI. cABC is a bacterial enzyme that digests GAGs of CSPGs and promotes axon regeneration and plasticity and improves functional recovery in numerous SCI models (8, 9). In addition to these beneficial effects of cABC, in a recent study, intrathecal injection of cABC increased the anti-inflammatory cytokine IL-10 in spinal cord samples. This result reflects the role of cABC in modulating the immune response after SCI (10).

Based on this finding, we postulated that cABC might decrease CSPGs level not only through their digestion but also via reducing their sources, namely reactive astrocytes. Therefore, we measured CSPGs and GFAP levels in an animal SCI model for one week. We also measured the level of chondroitin 4-sulfate (C4S) which is one of the enzyme products to assess whether the enzyme degraded CSPGs or not.

## Materials and Methods

### Experimental groups

In this study, 60 male Wistar rats (220-250 g) were used. All experiments were approved by the Ethics Committee of

Kerman University of Medical Sciences. Animals were grouped as follows:

- 1- SCI: rats underwent laminectomy and SCI as described later (n=20).
- 2- SCI+Vehicle: rats received intrathecal injection of phosphate buffer saline (PBS) following SCI (n=20).
- 3- SCI+Enzyme: rats were subjected to SCI and intrathecal injection of the cABC enzyme (n=20).

#### Laminectomy, spinal cord injury and drug delivery

Animals were anesthetized through intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Then, dorsal skin was shaved and sterilized. Laminectomy was then performed to expose T9-T10 thoracic spinal cord. A contusion injury was made by dropping a 10 g weight from a height of 25 mm. Immediately following SCI, 6  $\mu$ l of PBS or cABC (10 U/ml, Sigma) was microinjected intrathecally (11). Then, the paraspinal musculature and subcutaneous tissues were closed using silk sutures. Animals received gentamycin (12 mg/kg) intraperitoneally every other day. Bladder expression was performed twice a day manually until the return of bladder function.

#### Sample preparation

Five animals of each group were deeply anesthetized at the time points of 4 h, 1, 3 and 7 d following SCI. Then, they were transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde. Immediately after the perfusion, lesion site tissue was dissected (~ 1 cm with the lesion located centrally) and post-fixed in 20% sucrose for 2 days at 4 °C. Then, the tissue was embedded in tissue OCT compound and frozen at -

80°C before cutting. Then, it was cut into 20  $\mu$ m sections transversely for immunostaining (12).

#### Immunohistochemistry

In order to determine the level of GFAP, CSPGs and C4S at the site of damage, 4 spinal cord sections per animal were used. Firstly, spinal cord sections were washed with PBS to remove OCT. Then, sections were blocked with 1% bovine serum albumin and 0.3% triton X-100 in 0.1 M PBS. After 3 washes in PBS, primary antibodies including anti-GFAP (1:400, Abcam), anti-CSPGs (1:200, Abcam) and anti-C4S (1:1000, Abcam) in PBS were applied overnight at 4 °C. Then, sections were rinsed in PBS 3 times. Appropriate Alexa Fluor secondary antibodies (1:500, Abcam) in PBS were applied for 1 h at room temperature followed by an additional 3 times washing in PBS. Then, sections were mounted using glycerin (13).

#### Image analysis of immunohistochemistry

Fluorescence labeling was visualized using an Olympus TH4-200 microscope with a 40x objective and appropriate fluorescent filter sets and photographed using an Optronics (Magnifire) digital camera. Then, the average pixel intensity within the lesion area was measured and quantified using Image J software. This was performed on 4 serial sections centered at the middle of the lesion site and averaged (13).

#### Statistical analysis

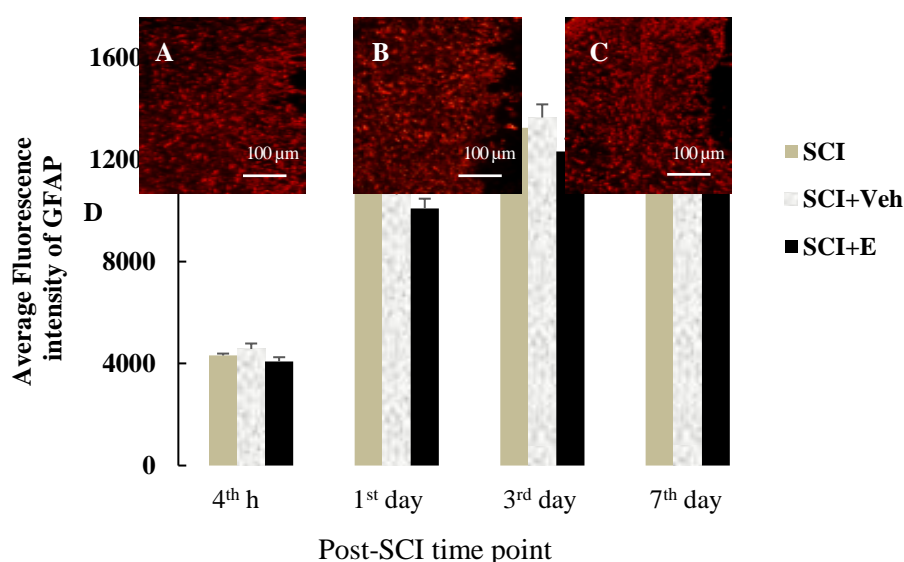
Data analysis was performed through SPSS16 software and using non-parametric Kruskal-Wallis test followed by Mann-Whitney analysis. Data were expressed as mean $\pm$ SD. A p value less than 0.05 was considered significant.

## Results

In this study, a contusion model of SCI in rat was used to investigate the effect of cABC on GFAP, CSPGs and C4S levels at 4 h, 1, 3 and 7 d post-SCI. Intrathecal injection of PBS did not change any of these parameters significantly compared to SCI group at any time point ( $p>0.05$ ).

By comparison, GFAP level increased rapidly during the first post-injury day. The increase was also observed at 3 d post-injury and continued until 7 d post-damage. However, the

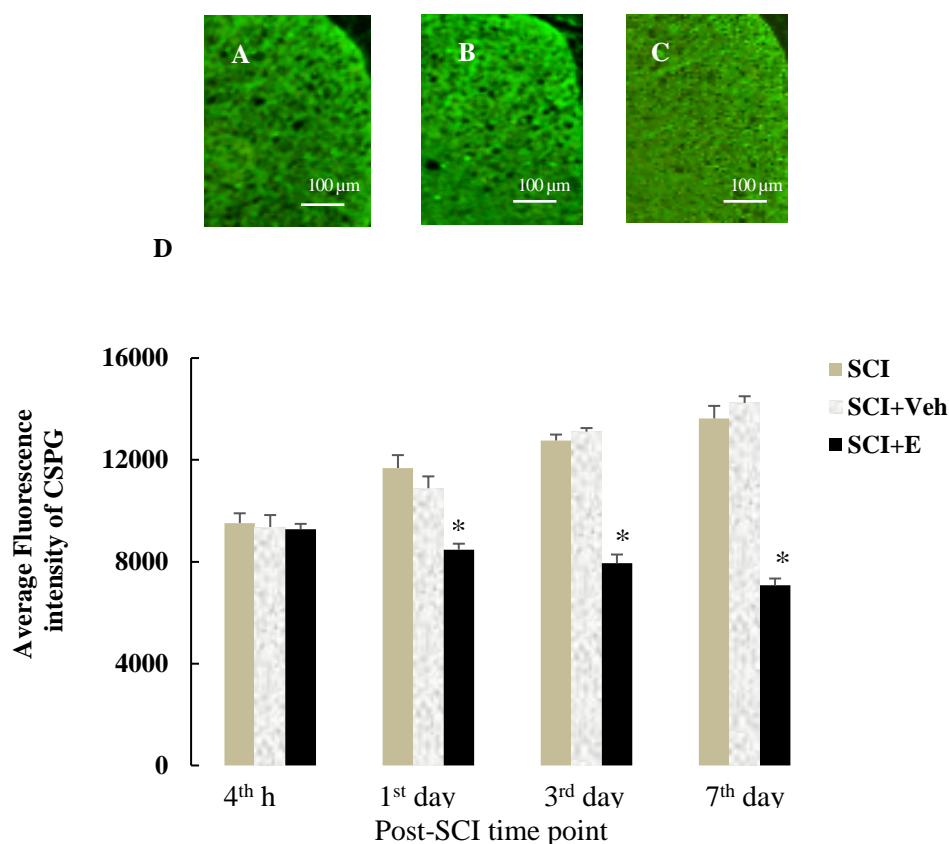
difference between 3 and 7 d post-SCI was not significant ( $p>0.05$ ). These data suggested that SCI led to an increase in GFAP level due to astrocyte activation. Comparison of GFAP level in SCI+E and SCI+Veh groups showed that GFAP immunoreactivity in enzyme-treated animals tended to be lower, but was not significantly different from vehicle-treated animals ( $p>0.05$ ) (Figure 1).



**Figure 1:** Immunohistologic analysis of GFAP in spinal cord sections. (A-C) Representative images of GFAP immunostaining from SCI (A), SCI+Veh (B) and SCI+E (C) groups at the 3<sup>rd</sup> post-SCI day. (D) The average fluorescence intensity of GFAP at post-SCI 4<sup>th</sup> h and 1<sup>st</sup>, 3<sup>rd</sup> and 7<sup>th</sup> days in different groups. Data have been presented as mean±SD. \* denotes  $p<0.05$  compared to the SCI+Veh.

According to Fluorescence data, CSPGs level increased steadily until 7 days post-SCI in SCI and SCI+Veh groups. However, the level of CSPGs reduced constantly in enzyme-treated group over this time course. While no significant difference was observed between SCI+Veh and SCI+E groups at 4 h post-injury ( $p>0.05$ ). The enzyme treatment

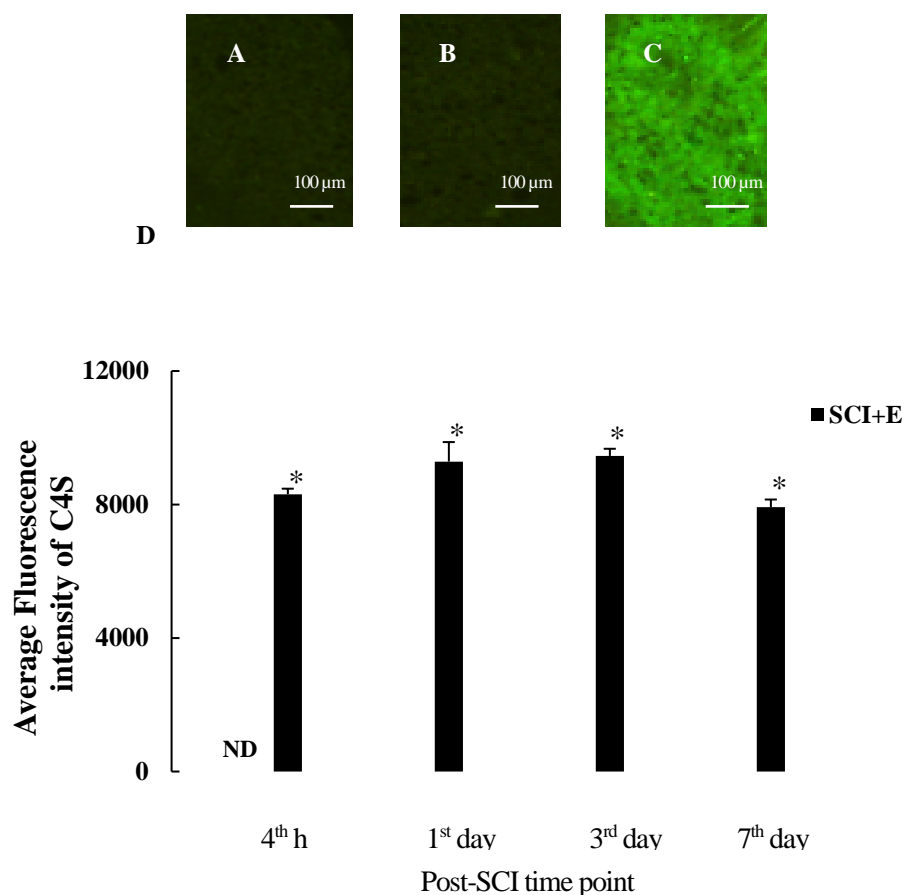
decreased CSPGs level significantly at 1, 3 and 7 d post- SCI ( $p<0.01$ ). In addition, the lowest immunoreactivity of CSPGs was seen at 7 d post-SCI which was around 50% of that in SCI+Veh group. These results proved that CSPGs had been digested by cABC (Figure 2).



**Figure 2:** Immunohistologic analysis of CSPG in spinal cord sections. (A-C) Representative images of CSPG immunostaining from SCI (A), SCI+Veh (B) and SCI+E (C) groups at 3<sup>rd</sup> post-SCI day. (D) The average fluorescence intensity of CSPG at post-SCI 4<sup>th</sup> h and 1<sup>st</sup>, 3<sup>rd</sup> and 7<sup>th</sup> days in different groups. Data have been presented as mean±SD. \* denotes  $p < 0.05$  compared to the SCI+Veh.

In order to test whether the enzyme decreased CSPGs level through enzymatic digestion or reducing the number of reactive astrocytes, C4S level was next measured using anti-C4S antibody. This antibody recognizes unsaturated C4-sulfated glycosaminoglycans stubs upon digestion of proteoglycans with cABC and is a marker for successful CSPG digestion by cABC. Immunostaining of C4S indicated that fluorescence intensity could not be detected for SCI and

SCI+Veh groups. Interestingly, a relatively high level of C4S was observed in enzyme- treated group at 4 h post-trauma showing the effect of cABC on CSPGs. Furthermore, C4S level increased during 3 days after SCI but no significant difference was observed between 1 and 3 d post-trauma groups ( $p > 0.05$ ). However, C4S immunoreactivity decreased at 7 d post-SCI. These data indicated that cABC treatment led to an increase in C4S through CSPGs breakdown (Figure 3).



**Figure 3.** Immunohistologic analysis of C4S in spinal cord sections. (A-C) Representative images of C4S immunostaining from SCI (A), SCI+Veh (B) and SCI+E (C) groups at the 3<sup>rd</sup> post-SCI day. (D) The average fluorescence intensity of C4S at post-SCI 4<sup>th</sup> h and 1<sup>st</sup>, 3<sup>rd</sup> and 7<sup>th</sup> days in SCI+E group. ND: Not detectable. Data have been presented as mean±SD. \* denotes  $p < 0.05$  compared to the SCI+Veh.

## Discussion

Spinal cord injury leads to secondary tissue damages through inflammatory cytokines production that activates astrocytes and microglia at lesion site (14). The activated astrocytes and OPCs overexpressed CSPGs along with myelin-associated molecules inhibit axonal growth and consequently regeneration (15). Multiple strategies have been developed to remove the axon inhibitory properties of CSPGs at the site of damage. Digestion of CSPGs side chains using cABC enzyme has been established as one approach for over a decade (8, 16). Recently, a study has documented that cABC led to upregulation of anti-inflammatory cytokine IL-10

accompanied by downregulation of pro-inflammatory cytokine IL-12B in injured spinal tissue (10). A similar study has proved that overexpression of cABC is associated with an increase in anti-inflammatory M2 macrophages and reduces tissue pathology (12). The anti-inflammatory effect of cABC has also been demonstrated in our previous study (unpublished data). The neuroprotective effect of cABC may contribute to a sulfated disaccharide derived from CSPGs upon digestion. Rolls et al. have shown that this disaccharide attenuated T cells mobility and decreased secretion of the cytokine interferon- $\gamma$  and tumor necrosis factor- $\alpha$ . It also induced neurite outgrowth and protected against neuronal

toxicity and death *in vitro* (17, 18). In spite of reported anti-inflammatory effect of cABC, our results showed that cABC treatment could not reduce astrocytes activation within 7 days after the injury. Immediately after SCI, astrocytes undergo considerable cellular, molecular and functional changes along with profound alterations in their gene expression. These reactions include astrocytes hypertrophy, proliferation and upregulation of GFAP, vimentin and nestin. These modifications are the hallmark of reactive astrogliosis which is accompanied with increased inhibitory CSPGs production (19). In the present spinal cord contusion model, GFAP expression increased until 3 d post-SCI that indicated a rise in basal astrocytes activation and it remained relatively unchanged until 7 d post-trauma. Similarly, Yang et al. reported an increase in GFAP expression in the injured sites 24 hours after the injury which reached a peak within 3-7 days after SCI (20).

CSPGs were highly upregulated in SCI and SCI+Veh groups within 7 days after SCI. These results were in agreement with GFAP alterations suggesting astrocytes activation and induction of CSPGs expression. However, treatment with cABC decreased CSPGs level. This result along with our observations of increased GFAP immunoreactivity proved that cABC decreased CSPGs level through their degradation not expression. This finding was further supported by C4S results. In the present study, C4S level, a product of CSPGs enzymatic digestion, increased by 3

d post-SCI which indicated the breakdown of CSPGs by cABC. However, the level of C4S decreased at 7 d post-SCI compared to 3 d post-injury which might be a result of C4S removal by immune cells. In addition, another possibility for reduced amount of C4S at this time point is the decreased level of cABC activity at body temperature.

The decreased deposition of CSPGs at 7 d post-damage indicated that the enzyme was still active and could digest its substrate. The method we applied was single microinjection of cABC at the lesion site as described in numerous studies. This approach provides an effective delivery of cABC to the site of injury (21, 22). cABC has been previously shown to remain active for over 10 days (23). This difference is possibly related to the enzyme dosage used and its activity which is suggested to be optimized for clinical applications.

In summary, this study showed that *in vivo* delivery of the cABC enzyme to the injured spinal cord did not change GFAP levels considerably, but decreased the level of CSPGs significantly. Therefore, cABC had no effect on astrocytes activation and astrogliosis but exerted its effect through CSPGs degradation.

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