

Thermostabilized chondroitinase ABC Promotes Neuroprotection after Contusion Spinal Cord Injury

Mahboobe Akbari, M.Sc.¹, Shahriar Dabiri, M.D.², Mohammad Mehdi Moeini-Aghtaei M.D.³, Mahdieh Nazari-Robati, Ph.D.⁴

1- Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran

2- Professor, Stem Cells Research Center, Kerman University of Medical Sciences, Kerman, Iran

3- Assistant Professor, Stem Cells Research Center, Kerman University of Medical Sciences, Kerman, Iran

4- Assistant Professor, Department of Clinical Biochemistry, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran

(Corresponding author: Email: mnazari@kmu.ac.ir)

Received: 1 March, 2020

Accepted: 4 August, 2020

ARTICLE INFO

Article type:

Original Article

Keywords:

Chondroitinase ABC

Trehalose

Spinal cord injury

Neuroprotection

Abstract

Background: Chondroitinase ABC (cABC), due to its loosening impact on the extracellular matrix scaffold, has been used to enhance regeneration of injured axonal tracts after spinal cord injury (SCI). However, cABC thermal instability at physiological temperature has limited its clinical application. The disaccharide trehalose has been shown to increase the stability of cABC in body temperature. Therefore, the present study was conducted to assess the effect of combined trehalose and cABC on inflammation, lipid peroxidation and histopathological changes following SCI.

Methods: Male Wistar rats were randomized into six groups (n=12) including sham, SCI, vehicle, trehalose, cABC and trehalose + cABC. In sham group, only laminectomy was performed. Other groups underwent laminectomy followed by contusion spinal cord injury. Twenty four hours after treatment, the level of IL-1 β , Myeloperoxidase (MPO) and Malondialdehyde (MDA) were measured. Histopathological changes were also scored.

Results: Spinal cord injury in rats resulted in severe trauma characterized by increase in inflammation, oxidative stress, neutrophils infiltration, hemorrhage, necrosis and edema. The levels of IL-1 β , MDA and MPO were 260.3 \pm 16.4 nmol/mg protein, 1.2 \pm 0.06 mU/mg protein and 18.9 \pm 0.7 pg/mg protein, respectively in the vehicle group. However, combined treatment with cABC and trehalose reduced the amount of these factors significantly to 142.4 \pm 17 nmol/mg protein, 0.57 \pm 0.03 mU/mg protein and 13.8 \pm 0.4 pg/mg protein, respectively (P<0.05). In addition, treatment with cABC and trehalose improved histopathological alterations.

Conclusions: The present data suggest that coadministration of trehalose and cABC exhibits neuroprotection effect through reducing inflammation and tissue injury events associated with SCI.

Copyright: 2020 The Author(s); Published by Kerman University of Medical Sciences. This is an open-access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Citation: Akbari M, Dabiri SH, Moeini-Aghtaei M.M, Nazari-Robati M. Thermostabilized chondroitinase ABC Promotes Neuroprotection after Contusion Spinal Cord Injury. *Journal of Kerman University of Medical Sciences*, 2020; 27 (5): 369-379.

Introduction

Traumatic spinal cord injury (SCI) causes severe and often permanent neurological deficits due to the loss of motor and sensory axonal pathways. Tissue damage after spinal cord trauma results from both direct mechanical injury and secondary autodestructive reactions that occur over a period of several weeks. The secondary injury contributes largely to the neurological impairment seen in patients. Several mechanisms underlying the secondary injury have been identified including excitotoxicity caused by impaired glutamate homeostasis, free radicals production, calcium overload, neuroinflammation and apoptosis (1, 2).

Immediately after spinal cord injury, innate immune response starts which is characterized by the production of inflammatory cytokines/chemokines by the resident innate immune cells (microglia, and astrocytes) and the subsequent recruitment of infiltrating immune cells (neutrophils and monocytes) (2, 3). Inflammatory cytokines activate astrocytes which upregulate inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) that create a nonpermissive environment for axons to regenerate (4). Therefore, strategies for modulating inflammation and removing axon growth inhibitors following SCI continue to be a topic of great interest (5).

The bacterial enzyme chondroitinase ABC (cABC) manipulates CSPGs by digesting glycosaminoglycans (GAGs) chain from the CSPGs core protein. Several studies have demonstrated that cABC mediated liberation of GAGs chain is useful for overcoming the inhibitory effect of GAGs on axon growth in the injured spinal cord (6, 7). However, *in vivo* application of cABC is limited by its thermal sensitivity as

reported previously (8). To overcome this limitation, we previously thermostabilized cABC *in vitro* using the sugar trehalose (9).

Trehalose is a non-reducing disaccharide which consists of two molecules of glucose. It is found in many non-mammalian species and protects the integrity of cells against various environmental stresses like heat, cold, dehydration and oxidation (10). Trehalose has been shown to protect proteins against thermal inactivation. Therefore, it is currently used in the biopharmaceutical preservation of labile protein drugs (11). In addition, recent studies have shown that trehalose could prevent oxidative and inflammatory response induced by various cellular stresses *in vivo* and *in vitro* (12, 13). Therefore, trehalose may be considered as a potential therapeutic agent for diseases involving oxidative stress and inflammation.

In the present study, we hypothesized that trehalose-stabilized cABC may limit inflammatory response and oxidative stress and may attenuate histopathological injury after experimental SCI.

Materials and Methods

Thermostabilized enzyme preparation

Trehalose-stabilized enzyme was made by mixing trehalose and cABC at room temperature. The final concentration of trehalose in the mixture was 1 M while the activity of cABC was 10 U/ml. All preparations were done in phosphate buffer saline (PBS, 0.1 M, pH 7.4). Aliquots were placed at -20 °C for the next step (9).

Animals

A total of 72 male Wistar rats (220-250 g) were used in this study. Animals were randomized into six groups:

I. Sham: rats were subjected to laminectomy (n=12).

II. SCI: rats were subjected to spinal cord injury as described afterward (n=12).

III. SCI+PBS (vehicle): rats received PBS intrathecally after SCI (n=12).

IV. SCI+T: rats received trehalose intrathecally after SCI (n=12).

V. SCI+E: rats received intrathecal injection of cABC following SCI (n=12).

VI. SCI+ET: rats received trehalose and cABC mixture intrathecally after SCI (n=12) (14).

All the experimental procedures were approved by the ethics committee of Kerman University of Medical Sciences.

Spinal cord injury and animal treatments

Rats were anesthetized using ketamine (50 mg/kg) and xylazine (5 mg/kg). Then laminectomy was performed at the T9-T10 level, exposing the cord beneath without disrupting the dura. A contusion injury was made by dropping a 10 g weight (NYU impactor) from 25 mm height onto the exposed dura. Immediately after the injury, 6 μ l of PBS, cABC, trehalose or trehalose with cABC was microinjected intrathecally into lesion site according to the groups. After suturing of the muscle and skin, animals were kept in a warm place to recover from anesthesia. Rats received gentamicin intraperitoneally (12 mg/kg) to prevent infection. Bladder evacuation was performed manually until reappearance of the voiding reflex (15).

Tissue preparation and analysis

Animals were sacrificed one day after SCI (n=7 of each group). Then a 2-cm segment of spinal cord with a lesion in the middle was dissected. Tissues were immediately frozen in liquid nitrogen and stored at -80°C until use. For histopathological analysis, five rats of each group were deeply anesthetized. Then animals were perfused with PBS followed by 4% paraformaldehyde in PBS. The injured spinal cords were carefully excised and post-fixed in 4% paraformaldehyde for 24 hours. After being dehydrated using a series of ethanol washes, tissues were embedded in paraffin and cut into 5- μ m sections. Then sections were deparaffinized in xylene and hydrated using a series of ethanol washes before being stained (16).

Measurement of IL-1 β

To assay the level of inflammation, IL-1 β was measured in spinal cord. Tissues were firstly homogenized in cold PBS containing 1% protease inhibitor. Then lysates were centrifuged at 15000 \times g for 20 min at 4°C and supernatants were separated. IL-1 β was assayed in supernatants using a commercial ELISA kit (Abcam, UK) according to the manufacturer's instructions. IL-1 β concentration was presented as pg/mg protein.

Measurement of myeloperoxidase

To assay neutrophil infiltration into injured tissue, myeloperoxidase (MPO) activity was measured. Tissue pellets obtained from homogenized spinal cord were used for assay. After releasing MPO from pellets using 0.5% hexadecyltrimethylammonium bromide, MPO activity was

measured in the presence of o-dianisidine (0.167 mg/ml) and hydrogen peroxide (0.003%) at 460 nm. One unit of MPO was defined as the amount that degrades 1 μ mol hydrogen peroxide per minute at 25 °C. MPO activity was expressed as mU/mg protein (17).

Measurement of malondialdehyde

To assay the level of lipid peroxidation, malondialdehyde (MDA) was measured in the injured tissues. Briefly, supernatants were mixed with thiobarbituric acid and trichloroacetic acid and kept in boiling water for 60 min. After cooling, the absorbance was measured at 532 nm. Then MDA concentration was calculated using a standard curve and reported as nmol/mg protein (18).

Histological analysis

Tissue sections from the damaged area were stained with hematoxylin and eosin (H&E) using a standard protocol. Stained sections were observed under a light microscope by a pathologist who was blinded to the study. A semi-quantitative scoring system was used to score the histopathological changes in spinal cord tissues. Six different parameters including hemorrhage, congestion, necrosis, edema, neuronal loss and inflammation were assessed and scored as follows; 0=absent,

1=mild, 2=moderate and 3=common. Then the sum of the scores of these parameters was reported for each tissue (19).

Statistical analysis

Data analysis was performed using SPSS software, version 20 (SPSS Inc, USA). The mean differences among groups were analyzed by Kruskal-Wallis test. Mann-Whitney analysis was used to evaluate the differences between various groups. A p value less than 0.05 was considered statistically significant.

Results

Tissue IL-1 β level

SCI led to a significant increase in tissue IL-1 β level when compared with sham group ($p < 0.001$). There was no significant difference in the level of IL-1 β between SCI and vehicle groups ($p > 0.05$). IL-1 β values were significantly decreased in tissues treated with trehalose, enzyme or enzyme+trehalose when compared with PBS treated tissues ($p < 0.001$). A combination treatment with enzyme and trehalose decreased IL-1 β values to a lower level compared with enzyme group ($p < 0.01$). However, no significant difference was observed between trehalose and enzyme+trehalose groups ($p > 0.05$) (figure 1).

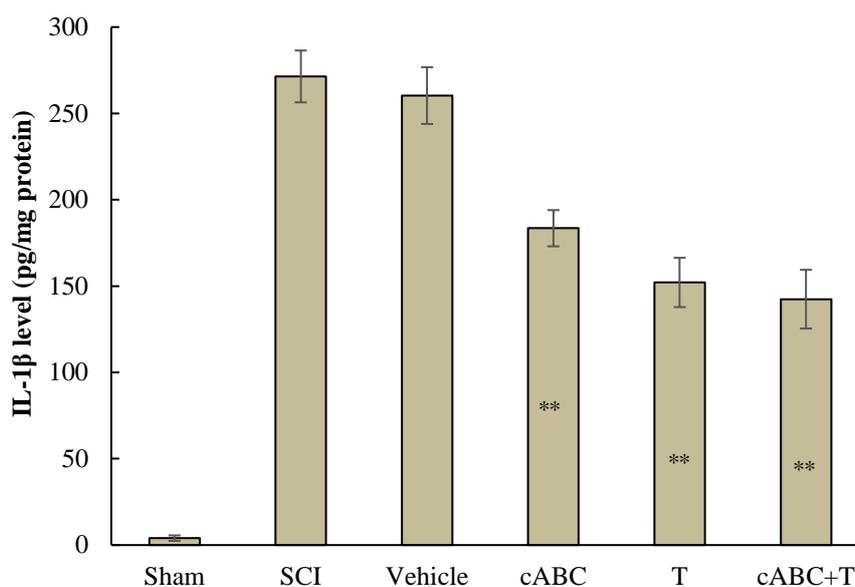


Figure 1. The effect of trehalose and cABC on IL-1 β , 24 hours after the SCI

** $p < 0.001$ compared with the vehicle group, Data represent mean \pm SD.

Tissue MPO level

MPO activity in the injured tissue of SCI group showed a significant increase compared with sham group at the first post-SCI day ($p < 0.001$). No significant difference was observed in MPO activity between SCI and vehicle groups ($p > 0.05$).

Treatment with trehalose, cABC and cABC with trehalose reduced the activity of MPO when compared to the vehicle group ($p < 0.05$). Tissue MPO activity values were found to be lower in enzyme+trehalose group compared with enzyme or trehalose groups ($p < 0.05$) (figure 2).

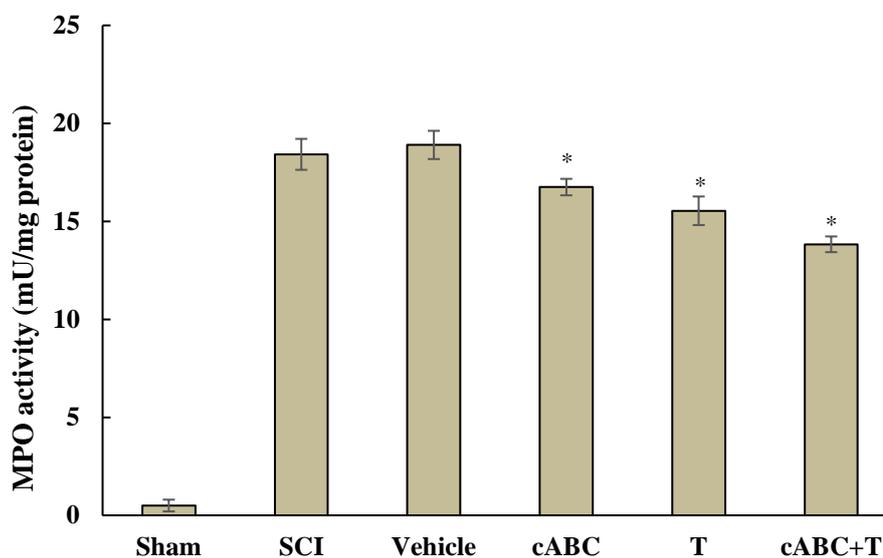


Figure 2. The effect of trehalose and cABC on MPO, 24 hours after the SCI

* $p < 0.05$ compared with vehicle group, Data represent mean \pm SD.

Tissue malondialdehyde level

Tissue MDA level was significantly higher in the SCI group compared with the sham group ($p < 0.001$). Comparing MDA values in the SCI and vehicle groups, revealed no significant difference ($p > 0.05$). Treatment with trehalose,

enzyme or enzyme+trehalose reduced the level of MDA significantly compared with the vehicle group ($p < 0.05$). However, the lowest level was observed in the trehalose treated group when compared with the enzyme or enzyme+trehalose groups ($p < 0.01$) (figure 3).

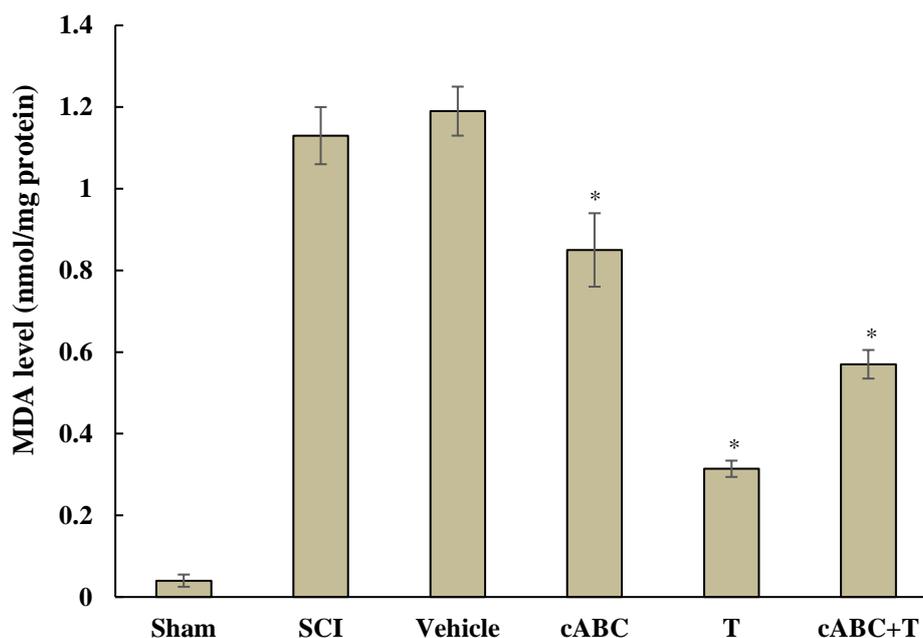


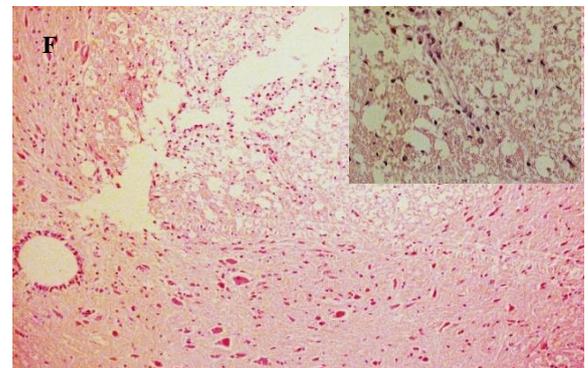
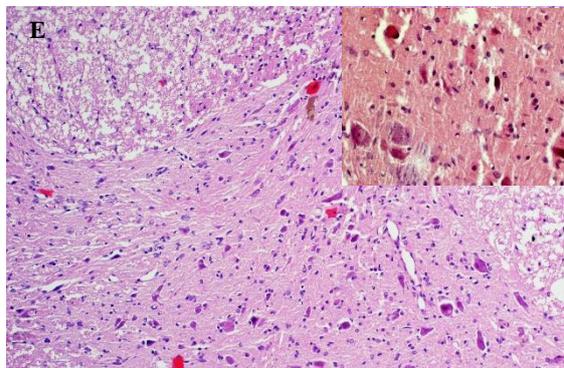
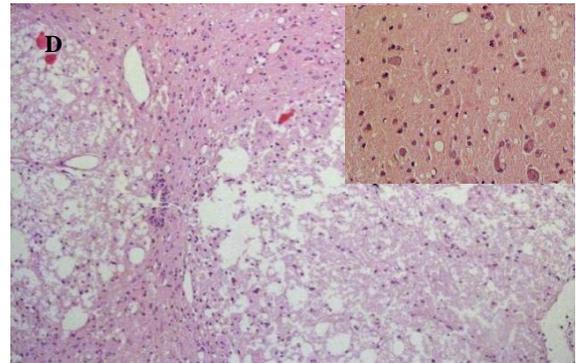
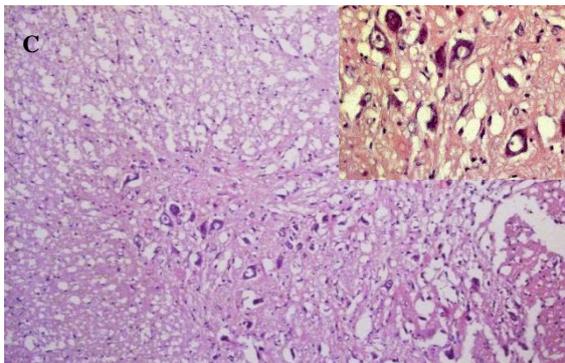
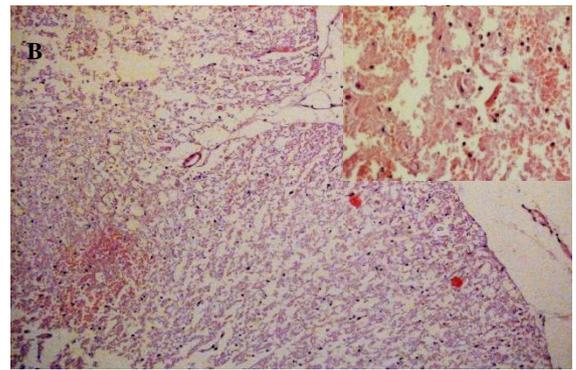
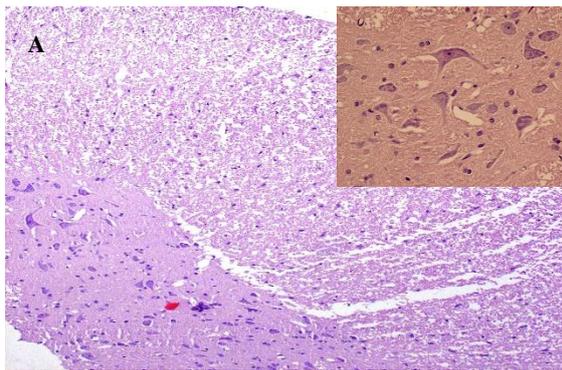
Figure 3. The effect of trehalose and cABC on MDA, 24 hours after SCI

* $p < 0.05$ compared with vehicle group, Data represent mean \pm SD.

Histological analysis

Microscopic analysis of spinal cord tissue in sham group revealed nothing remarkable. However, diffuse hemorrhage and congestion in the gray matter as well as marked neuronal necrosis and degeneration in the gray and white matter were observed in the damaged tissues of the SCI group. Moreover, in the injured area, infiltrating neutrophils and scattered macrophages were observed. Similar histological changes were observed in the tissues of the vehicle group. Therefore, comparing the pathological scores, no significant difference

was observed between the SCI and vehicle groups. However, treatment with enzyme or trehalose reduced histopathological scores significantly when compared with the vehicle group ($p < 0.05$). In these two groups, white matter underwent degeneration and macrophages were the major cells which populated the white matter. When compared the scores, there was no significant difference between the enzyme and trehalose groups ($p > 0.05$). Nevertheless, coadministration of cABC and trehalose decreased histopathological score to a lower level compared with the vehicle, enzyme or trehalose groups which was statistically significant ($p < 0.05$) (figure 4).



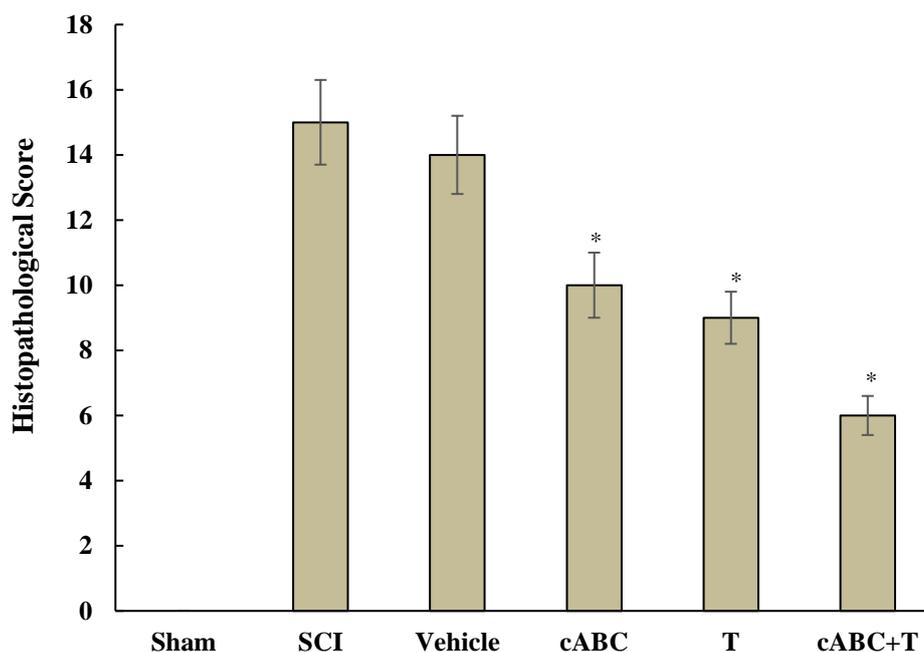


Figure 4. H&E-stained spinal cord tissues of different groups at 100 and 400 magnification for large and small pictures, respectively, (A) sham group; (B) SCI group; (C) vehicle group; (D) cABC group; (E) trehalose group; (F) cABC+ trehalose group

* $p < 0.05$ compared with vehicle group. Data represent mean \pm SD.

Discussion

Traumatic spinal cord injury is followed by a progressive secondary process of tissue damage arising from the inflammatory response. Hallmarks of the inflammatory reaction to SCI are changes in vascular permeability, activation of glial cells and astrocytes as well as leukocytes infiltration to damaged tissue. Leukocytes produce reactive free radicals leading to CNS injury through a mechanism known as oxidative burst. Therefore, a therapeutic intervention for SCI should be directed at limiting the inflammatory response (20, 21).

Glial scars formed by activated astrocytes are a prominent feature of CNS trauma. After SCI, various CSPGs are densely deposited in the lesion site and contribute to limiting axon regeneration. Enzymatic digestion of CSPGs by chondroitinase ABC (cABC) has been shown to increase nerve fibers

regeneration and plasticity (22, 23). However, cABC activity is reduced at body temperature which limits its application (8).

Previously, we reported that the disaccharide trehalose increases thermal stability of cABC (9). In the present study, we found that treatment with cABC or trehalose reduced the level of IL-1 β . However, the reduction was more considerable using trehalose alone or in combination with cABC. IL-1 β plays a critical role in spinal cord damage after SCI. It induces the upregulation of other proinflammatory cytokines such as TNF- α and IL-6. It also enhances vascular permeability and induces neuronal apoptosis. IL-1 β downregulation limits lesion development and neuronal loss, promotes axonal plasticity and improves neurological outcome (24, 25). Therefore, it is likely that trehalose reduced caspase-3 gene expression and apoptosis in injured tissues as was found in our previous study through attenuating inflammatory response post-SCI (14).

MPO is an oxidative enzyme present in neutrophil granules contributing to its oxygen-dependent bactericidal activity. MPO is a marker of neutrophil infiltration and activation (21). Infiltrating neutrophils are major sources of reactive oxygen species which can induce neurotoxicity and axonal degeneration. Therefore, blocking neutrophil accumulation contributes to attenuation of secondary neuronal damage and improvement in neurological recovery after SCI (26, 27). In the present study, we found that neutrophils infiltration into the injured spinal cord was clearly reduced by the treatment with trehalose or coadministration of trehalose and cABC.

Reactive oxygen species (ROS) play a key role in mediating secondary injury after SCI. ROS can cause cytotoxicity by damaging lipids, proteins and nucleic acids. MDA is the most abundant aldehyde resulting from lipid peroxidation and is commonly measured to evaluate the level of oxidative stress and ROS (28). Our study provides evidence for formation of MDA following SCI which demonstrates the involvement of lipid peroxidation. Both cABC and trehalose protected spinal cord from lipid peroxidation by lowering MDA level. Interestingly, the antioxidative effect of trehalose was reduced when combined with cABC. As previously mentioned, neutrophils are one of the main sources of ROS. Therefore, we attributed the antioxidative effect of trehalose and trehalose-stabilized cABC to their anti-inflammatory activity.

The putative effect of trehalose against inflammation and oxidative stress was reported in several investigations. In a recent study, we showed that trehalose protects PC12 cells against oxidative stress induced by hydrogen peroxide through the upregulation of heat shock protein 70 (HSP70) gene (29). Trehalose also suppressed LPS-induced IL-1 β and TNF- α production in mouse peritoneal macrophages by inhibiting NF-

κ B activation (30). It also suppressed the production of various pro-inflammatory cytokines including TNF- α , IL-6, IL-1 α and IL-1 β and reduced oxidative stress induced by experimental subarachnoid hemorrhage (12).

The consequences of inflammation are often related to ischemia, tissue edema, oxidative damage, myelin degradation and ongoing necrotic and apoptotic changes which all are responsible for substantial increases in lesion size after the initial injury (21). In this study, histological findings were also associated with the level of secondary damage after SCI. Traumatic injury caused diffuse hemorrhage, congestion, marked edema and necrosis in both white and gray matter. In the damaged area, infiltrating polymorphonuclear leukocytes, plasma cells and lymphocytes were observed which indicates an inflammatory response to the injury. However, a combined treatment with trehalose and cABC showed lower histopathological score which may be related to the anti-inflammatory effect of trehalose-stabilized cABC.

In conclusion, treatment with trehalose-stabilized cABC reduced inflammatory response after SCI which was consistent with the idea that combined trehalose and cABC treatment decreases infiltration of inflammatory cells into the injured spinal cord and reduces free radicals formation and lipid peroxidation. A detailed study of these mechanisms might lead to the introduction of therapeutic strategies into clinical practice.

Acknowledgment

This work was supported by funding from Neuroscience Research Center (Grant No. 93-58), Kerman University of Medical Sciences.

Conflict of interests

The authors have no conflict of interest to declare.

References

1. Ahuja CS, Wilson JR, Nori S, Kotter MR, Druschel C, Curt A, et al. Traumatic spinal cord injury. *Nat Rev Dis Primers* 2017; 3:17018.
2. Putatunda R, Bethea JR, Hu WH. Potential immunotherapies for traumatic brain and spinal cord injury. *Chinese Journal of Traumatology* 2018; 21(3):125-36.
3. Gensel JC, Zhang B. Macrophage activation and its role in repair and pathology after spinal cord injury. *Brain Res* 2015; 1619:1-11.
4. Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol* 2008; 209(2):294-301.
5. Holmberg E, Zhang SX, Sarmiere PD, Kluge BR, White JT, Doolen S. Statins decrease chondroitin sulfate proteoglycan expression and acute astrocyte activation in central nervous system injury. *Exp Neurol* 2008; 214(1):78-86.
6. Huang WC, Kuo WC, Cherng JH, Hsu SH, Chen PR, Huang SH, et al. Chondroitinase ABC promotes axonal re-growth and behavior recovery in spinal cord injury. *Biochem Biophys Res Commun* 2006; 349(3):963-8.
7. Kanno H, Pressman Y, Moody A, Berg R, Muir EM, Rogers JH, et al. Combination of engineered Schwann cell grafts to secrete neurotrophin and chondroitinase promotes axonal regeneration and locomotion after spinal cord injury. *J Neurosci* 2014; 34(5):1838-55.
8. Lee H, McKeon RJ, Bellamkonda RV. Sustained delivery of thermostabilized chABC enhances axonal sprouting and functional recovery after spinal cord injury. *Proc Natl Acad Sci USA* 2010; 107(8):3340-5.
9. Nazari-Robati M, Khajeh K, Aminian M, Fathi-Roudsari M, Golestani A. Co-solvent mediated thermal stabilization of chondroitinase ABC I form *Proteus vulgaris*. *Int J Biol Macromol* 2012; 50(3):487-92.
10. Richards AB, Krakowka S, Dexter LB, Schmid H, Wolterbeek AP, Waalkens-Berendsen DH, et al. Trehalose: a review of properties, history of use and human tolerance, and results of multiple safety studies. *Food Chem Toxicol* 2002; 40(7):871-98.
11. Jain NK, Roy I. Effect of trehalose on protein structure. *Protein Sci* 2009; 18(1):24-36.
12. Echigo R, Shimohata N, Karatsu K, Yano F, Kayasuga-Kariya Y, Fujisawa A, et al. Trehalose treatment suppresses inflammation, oxidative stress, and vasospasm induced by experimental subarachnoid hemorrhage. *J Transl Med* 2012; 10:80.
13. Cejka C, Kossl J, Hermankova B, Holan V, Kubinova S, Olmiere C, et al. The healing of oxidative injuries with trehalose in UVB-Irradiated rabbit corneas. *Oxid Med Cell Longev* 2019; 2019:1-10.
14. Nasouti R, Khaksari M, Mirzaee M, Nazari-Robati M. Trehalose protects against spinal cord injury through regulating heat shock proteins 27 and 70 and caspase-3 genes expression. *J Basic Clin Physiol Pharmacol* 2019; 31(1):1-8.
15. Barritt AW, Davies M, Marchand F, Hartley R, Grist J, Yip P, et al. Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. *J Neurosci* 2006; 26(42):10856-67.
16. Zhang D, Ma G, Hou M, Zhang T, Chen L, Zhao C. The neuroprotective effect of puerarin in acute

- spinal cord injury rats. *Cell Physiol Biochem* 2016; 39(3):1152-64.
17. Bradley PP, Priebe DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982; 78(3):206-9.
 18. Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Method Enzymol* 1990; 186:407-21.
 19. Kahveci R, Gokce EC, Gurer B, Gokce A, Kisa U, Cemil DB, et al. Neuroprotective effects of rosuvastatin against traumatic spinal cord injury in rats. *Eur J Pharmacol* 2014; 741:45-54.
 20. Ahmed A, Patil AA, Agrawal DK. Immunobiology of spinal cord injuries and potential therapeutic approaches. *Mol Cell Biochem* 2018; 441(1-2):181-9.
 21. Bao F, Chen Y, Dekaban GA, Weaver LC. Early anti-inflammatory treatment reduces lipid peroxidation and protein nitration after spinal cord injury in rats. *J Neurochem* 2004; 88(6):1335-44.
 22. Cheng CH, Lin CT, Lee MJ, Tsai MJ, Huang WH, Huang MC, et al. Local delivery of high-dose chondroitinase abc in the sub-acute stage promotes axonal outgrowth and functional recovery after complete spinal cord transection. *PLoS one* 2015; 10(9):e0138705.
 23. Bartus K, James ND, Bosch KD, Bradbury EJ. Chondroitin sulphate proteoglycans: key modulators of spinal cord and brain plasticity. *Exp Neurol* 2012; 235(1):5-17.
 24. Burke SJ, Lu D, Sparer TE, Karlstad MD, Collier JJ. Transcription of the gene encoding TNF-alpha is increased by IL-1beta in rat and human islets and beta-cell lines. *Mol Immunol* 2014; 62(1):54-62.
 25. Boato F, Rosenberger K, Nelissen S, Geboes L, Peters EM, Nitsch R, et al. Absence of IL-1beta positively affects neurological outcome, lesion development and axonal plasticity after spinal cord injury. *J Neuroinflammation* 2013; 10:6.
 26. Neirinckx V, Coste C, Franzen R, Gothot A, Rogister B, Wislet S. Neutrophil contribution to spinal cord injury and repair. *J Neuroinflammation* 2014; 11:150.
 27. Lee SM, Rosen S, Weinstein P, van Rooijen N, Noble-Haeusslein LJ. Prevention of both neutrophil and monocyte recruitment promotes recovery after spinal cord injury. *J Neurotrauma* 2011; 28(9):1893-907.
 28. Hall ED, Wang JA, Bosken JM, Singh IN. Lipid peroxidation in brain or spinal cord mitochondria after injury. *J Bioenerg Biomembr* 2016; 48(2):169-74.
 29. Nazari Robati M, Norouzi A, Nasouti R. Effect of Trehalose on the Expression of Heat Shock Protein 70 Gene in PC12 Cells Treated with Hydrogen Peroxide. *J Mazandaran Univ Med Sci* 2019; 29(172):1-9. [In Persian].
 30. Taya K, Hirose K, Hamada S. Trehalose inhibits inflammatory cytokine production by protecting IkappaB-alpha reduction in mouse peritoneal macrophages. *Arch Oral Biol* 2009; 54(8):749-56.