Research Article

Trehalose attenuates spinal cord injury through the regulation of oxidative stress, inflammation and GFAP expression in rats

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Objective: Inflammation and oxidative stress are implicated in pathogenesis of spinal cord injury (SCI). Trehalose, a nonreducing disaccharide, exhibits anti-inflammatory and antioxidant effects. The present study investigated the therapeutic efficacy of trehalose in the SCI model.

Design and setting: An experimental study was designed using 120 male Wistar rats which were randomly divided into three groups including SCI, SCI + phosphate buffer saline (vehicle) and SCI + trehalose. All rats were subjected to SCI. Immediately after SCI, vehicle and trehalose groups received intrathecal injection of buffer and trehalose, respectively.

Outcome measures: The level of tissue TNF α , IL-1 β , nitric oxide, malondialdehyde, myeloperoxidase, glial fibrillary acidic protein (GFAP) as well as hindlimb function were assessed at 4 hours, 1, 3 and 7 days post-SCI.

Results: Data indicated an early significant decrease in inflammatory and oxidative responses following SCI in trehalose treated group. Moreover, trehalose reduced GFAP expression as soon as 1-day post-trauma. Furthermore, trehalose treatment increased the score of hindlimb function.

Conclusion: Our results indicated that treatment with trehalose reduces the development of secondary injury associated with SCI. This effect likely underlies improved neurological function.

Keywords: Spinal cord injury, Trehalose, Inflammation, Oxidative stress, Reactive astrocytes, Locomotor function

Introduction

Spinal cord injury (SCI) is a life-disrupting health problem due to detrimental effects on social and personal life. Immediately after SCI, neurons, glial cells and blood vessels are mechanically damaged which is called primary injury. Within hours to days after SCI, a complex cascade of secondary injury events including blood-spinal cord barrier permeability, ionic dysregulation, edema, glutamate excitotoxicity, lipid peroxidation and inflammatory reactions initiates. The consequences

of these events are myelin degradation and ongoing necrotic and apoptotic changes.¹

SCI triggers a robust immune response which may play an important role in the secondary damage processes. In fact, disruption of the blood–spinal cord barrier leads to infiltration of immune cells into the injured tissue. They abundantly release a range of cytokines which are mostly pro-inflammatory.² Among them, TNF α and IL-1 β are released significantly faster which recruit neutrophils to the site of lesion.³ Post-traumatic accumulation of neutrophils is associated with an increase in oxidative and proteolytic enzymes.⁴ Moreover, a high concentration of these inflammatory cytokines stimulates the expression of neurotoxic genes including COX-2 and iNOS in different target cells.⁵ Furthermore, TNF α induces proliferation and hypertrophy of astrocytes. Reactive astrocytes

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promote the formation of glial scar which is known to be an inhibitor of axonal regeneration. Therefore, attenuation of inflammatory response after SCI has been shown to be beneficial to reduce secondary damage.⁶

A variety of pharmacological agents have been tested in animal models to suppress inflammation and free radicals production. However, few agents have potential applications to patients with SCI.⁷ The most prescribed agent in treatment of SCI is methylprednisolone (MP). MP has an anti-inflammatory nature and is identified to inhibit lipid peroxidation.⁸ However, treatment with MP does not improve neurite sprouting, remyelination of spared axons and hence functional recovery.⁹ Therefore, the need for new pharmacological agents to alleviate SCI is of clinical priority.

Trehalose, a nonreducing disaccharide, is synthesized by bacteria, yeast, fungi, insects and plants which preserves them against environmental stresses such as heat, freezing, desiccation, dehydration and oxidation.¹⁰ Besides these properties, recent reports showed that trehalose suppresses inflammatory response induced by endotoxic shock *in vivo* and *in vitro*.^{11,12} Moreover, trehalose treatment inhibited oxidative stress and inflammation in an experimental subarachnoid hemorrhage.¹³ Furthermore, trehalose could suppress TNF α production by bone marrow cells in lipopolysaccharide (LPS) injected mice.¹⁴ A recent study indicated that a combination of high dose of trehalose and chondroitinase ABC (cABC) administration promotes functional recovery after SCI.¹⁵ Furthermore, we showed that trehalose protects cABC against oxidative stress and proteolysis *in vitro*.¹⁶ However, the effect of trehalose on inflammatory response and oxidative stress following SCI has not been reported. Therefore, we designed this study to examine whether trehalose can suppress inflammation and oxidative stress induced in SCI. Additionally, we investigated astrocytes activation and locomotor function following trehalose treatment in an experimental model of SCI.

Materials and methods

Animals

Adult male Wistar rats (220–250 g) were used and housed under a 12 hours light/dark cycle with free access to food and water. All experimental procedures were approved by the ethics committee of Kerman University of Medical Sciences and performed in compliance with the local guidelines. Rats were randomly allocated into the following groups:

- 1- SCI: rats were subjected to SCI as described in the next section ($N = 40$).
- 2- SCI + vehicle (phosphate buffer saline, PBS): rats underwent SCI plus intrathecal injection of PBS ($N = 40$).

- 3- SCI + trehalose: rats were subjected to SCI and treated with trehalose injected intrathecally ($N = 40$).

SCI and treatment

Rats were anesthetized using ketamine (50 mg/kg) and xylazine (5 mg/kg). Then, thoracic region of their backs was shaved and cleansed. A laminectomy was then performed at vertebral T9-T10 segment to expose the underlying spinal cord. SCI was made by dropping a 10 g weight from a height of 25 mm (NYU impactor, USA). Then, 6 μ l of PBS (pH 7.4) or trehalose (1 M) was immediately microinjected intrathecally.¹⁷ The incision was closed and rats received post-operative care including a warm place until recovering from anesthesia and antibiotics (gentamycin, 12 mg/kg, i.p.) every other day. In addition, their bladders were voided by manual compression twice a day until an automatic bladder developed.

Evaluation of locomotor deficit

In order to evaluate behavioral outcome, the functional recovery of hindlimbs was scored using the open field Basso, Beattie and Bresnahan (BBB) scale. This test assessed hindlimb movement, stepping and coordination in an open field and the results were scored from 0 which indicated no observable hindlimb movement to 21 which showed a consistent plantar stepping and coordinated gait.¹⁸ All of the animals were coded and behavioral analysis was performed by two investigators blinded with respect to the groups ($N = 10$ in each group).

Tissue preparation

For biochemical analysis, seven rats from each group were deeply reanesthetized at 4 hours, 1, 3 and 7 days post-SCI. Animals were perfused transcardially with cold PBS and 2 cm spinal cords from T9-T10 segments were removed.

Then, tissues were homogenized in ice-cold PBS containing 1% (vol/vol) protease inhibitor (Sigma, USA). After centrifugation at $15000 \times g$ for 20 minutes at 4°C, supernatants were removed. Both supernatants and pellets were kept at -80°C for next experiments.

For immunohistochemical analysis, anesthetized animals ($N = 3$) were perfused transcardially with ice-cold PBS followed by 4% paraformaldehyde in PBS (Sigma, USA). Around 1 cm section of spinal cord with the lesion at the center was dissected and post-fixed in 20% sucrose for 2 days at 4°C. Then, tissues were embedded in tissue OCT compound (Ted Pella, USA) and frozen at -80°C before cutting. Tissues were then cut into 20 μ m sections transversely for immunostaining.⁷

Cytokines assay

The assay was performed using ELISA kits (Abcam, UK) and according to the manufacturer's instructions. Total protein concentration was measured spectrophotometrically according to the Bradford method.¹⁹ Then, the results were expressed as pg/mg protein.

Myeloperoxidase activity assay

Tissue pellets were resuspended in ice-cold phosphate buffer (50 mM, pH 6) which contains 0.5% hexadecyltrimethyl ammonium bromide (Sigma, USA). Homogenates were then frozen in liquid nitrogen and thawed in three consecutive cycles with sonication (10 seconds, 4°C) between cycles. After centrifugation at $15000 \times g$ for 20 minutes at 4°C, MPO activity was measured in supernatants. The rate at which a colored product formed during MPO dependent reaction with o-dianisidine (0.167 mg/ml) and hydrogen peroxide (0.003%) (Sigma, USA) was measured at 460 nm. The results were expressed as mU/mg protein.²⁰

Malondialdehyde assay

Malondialdehyde (MDA) level was determined using thiobarbituric acid reactive substances assay. Tetramethoxypropane (Sigma, USA) was used to prepare the standard curve. MDA concentration was reported as nmol/mg protein.²¹

Nitric oxide assay

150 μ l of supernatant was mixed with sodium hydroxide (0.3 M) and 75 μ l zinc sulfate (5%) (Sigma, USA) to precipitate proteins. Then, the mixture was centrifuged at $15000 \times g$ for 20 minutes 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 minutes. The absorbance was measured at 540 nm. Sodium nitrite was used to plot the standard curve. The result was presented as nmol/mg protein.²²

GFAP assay

Spinal cord sections were rinsed 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 (Sigma, USA). After washing with PBS, sections were incubated with mouse anti-glial fibrillary acidic protein (GFAP) (1:400) (Abcam, UK) overnight at 4°C. Sections were rinsed in PBS and mounted using glycerin. A series of images spanning the spinal cord were captured using an Optronics digital camera attached to an Olympus TH4-200 microscope with a 40 $\times$ objective. A minimum of four regions in lesion area were randomly captured and the fluorescence intensity was measured

and quantified using Image J software. This was performed by a blinded investigator on four serial sections centered at the middle of the lesion site from three samples and averaged.²³

Statistical analysis

SPSS software, version 16.0 was used to perform statistical analysis. Data were compared using non-parametric Kruskal–Wallis test followed by Mann–Whitney analysis. Results were expressed as mean $\pm$ SD and the differences between groups were considered significant at $P < 0.05$.

Results

Locomotor function

Following treatment of SCI rats with trehalose, functional improvements in hindlimb locomotion were detected at 3 and 7 days post-trauma when evaluated using the open field BBB scale ($P < 0.05$). On day 3, the BBB score was 1 which represented a slight movement of the hip. On day 7, the score increased to 3.6 which showed a slight movement of hip and knees. However, SCI and vehicle groups displayed no observable movement at any time points after trauma ($P > 0.05$) (Fig. 1).

Tissue cytokines

The highest concentration of TNF α was observed 4 hours after injury in all groups but gradually declined with time. Treatment with trehalose significantly reduced the level of TNF α at 4 hours, 1 and 3 days following SCI by 35%, 27% and 28% in comparison with vehicle group. However, no significant difference was observed between trehalose treated group and vehicle group at 7 days after damage ($P > 0.05$). In addition,

Figure 1 Effect of trehalose on hindlimb locomotor function. The assay was carried out by BBB test. Trehalose treatment significantly improved motor function at 7 days post-SCI. Data are presented as mean $\pm$ SD. $N = 10$.

Figure 2 Effect of trehalose on TNF α and IL-1 β level. Spinal cord TNF α (A) and IL-1 β (B) content were measured at 4 hours, 1, 3 and 7 days post-SCI. A substantial increase was observed in TNF α and IL-1 β at 4 hours and 1-day post-injury, respectively. Treatment with trehalose reduced TNF α and IL-1 β level significantly. Data are expressed as mean $\pm$ SD. $N = 7$, * $P < 0.01$ and ** $P < 0.05$ vs. vehicle.

PBS did not affect TNF α level at any time points when compared to SCI group ($P > 0.05$) (Fig. 2A).

The level of IL-1 β peaked at 1 day after SCI and then decreased remarkably by 7 days post-injury in all groups. After treatment with trehalose, the IL-1 β concentration was significantly reduced by approximately 53% at 4 hours, 3 and 7 days and 41% at 1-day post-SCI compared with vehicle group. Moreover, no significant difference was seen between SCI and vehicle groups at any time points ($P > 0.05$) (Fig. 2B).

Tissue myeloperoxidase

MPO activity was remarkably enhanced after trauma, reached a peak at 1 day and was reduced by 7 days

following SCI in all groups. The level of MPO activity was significantly decreased at 4 hours, 1, 3 and 7 days after SCI by 26%, 18%, 35% and 75%, respectively, when compared with vehicle group. However, no significant difference was observed between SCI and vehicle groups statistically ($P > 0.05$) (Fig. 3A).

Tissue malondialdehyde

The results indicated that lipid peroxidation was greatly enhanced post-injury, peaked at 3 days and then was decreased by 7 days after trauma. Comparing SCI and vehicle groups, no significant difference was found at any time points ($P > 0.05$). However, MDA concentration was greatly reduced to 86% and 74% at 4 hours and 1 day, respectively, and 46% at both 3 and 7 days following trauma in trehalose treated group compared with vehicle group (Fig. 3B).

Tissue nitric oxide

Comparing all groups at different time points, the highest level of NO was observed at 3 days post-SCI which was then reduced by 7 days after trauma. Whereas no significant difference was observed between SCI and vehicle groups at any time points ($P > 0.05$), treatment with trehalose decreased NO concentration significantly ($P < 0.01$). In fact, trehalose reduced the level of NO at 4 hours, 1, 3 and 7 days post-injury to 47%, 59%, 70% and 35%, respectively, compared to vehicle group (Fig. 3C).

Tissue GFAP

Data showed that GFAP signal was greatly elevated after injury, reached a peak at 3 days and remained at a high level by 7 days post-SCI in all groups. However, trehalose reduced the level of GFAP at 1-day post-injury to 25% and at both 3 and 7 days after damage to 15% compared to vehicle group which confirmed the inhibitory effect of trehalose on activation of astrocytes. Furthermore, GFAP expression level in the vehicle group was not significantly different at any time points compared with SCI group ($P > 0.05$) (Fig. 4).

Discussion

The pathophysiology of SCI consists of a primary injury along with a cascade of secondary events. Inflammatory responses are a major component of secondary injury which play a key role in neuronal damage and control of regeneration following SCI. Therefore, attenuation of early inflammatory responses may limit the extent of tissue lesion and functional deficit.^{24,25}

Trehalose is a natural disaccharide found in many organisms such as fungi, invertebrates and plants. It accumulates considerably during various stresses in these species to protect the integrity of cells against

Figure 3 Effect of trehalose on MPO, MDA and NO level. Spinal cord MPO (A), MDA (B) and NO (C) content were measured during 7 days post-SCI. The level of MPO, MDA and NO were significantly increased at 1, 3 and 7 days following SCI, respectively. However, treatment with trehalose reduced MPO, MDA and NO level considerably. Data are presented as mean $\pm$ SD. $N = 7$, * $P < 0.01$ and ** $P < 0.05$ vs. vehicle.

Figure 4 Effect of trehalose on GFAP level. GFAP immunoreactivity was measured in SCI (A–D), vehicle (E–H) and trehalose treated (I–L) groups at 4 hours, 1, 3 and 7 days post-injury. A substantial increase was found in GFAP level at 3 and 7 days following damage (M). However, trehalose treatment reduced GFAP immunofluorescence significantly. Data are expressed as mean $\pm$ SD. $N = 3$, * $P < 0.01$ vs. vehicle.

damages.¹⁰ Trehalose has also been described as an anti-inflammatory and antioxidative agent, autophagy enhancer and chemical chaperone.^{13,26,27} Furthermore, trehalose stabilizes cell membranes against reactive oxygen species (ROS).²⁸ In addition, trehalose is used as an additive to protect proteins and other biological macromolecules at high temperature. We recently used trehalose to increase thermal stability of the enzyme cABC. Notably, the highest protection was found to be at 1 M trehalose.²⁹ Furthermore, another study showed that delivery of combined cABC and 1 M trehalose to traumatic spinal cord leads to an improvement in functional recovery following SCI.¹⁵ Therefore, 1 M trehalose was used in this study.

Neuroinflammation is a prominent response to trauma in SCI. The first cells which participate in the inflammatory response are microglia and neutrophils. They are likely to contribute to tissue damage by releasing pro-inflammatory mediators such as TNF α and IL-1 β .³⁰ After experimental SCI, TNF-positive cells can be seen over the injured spinal cord segment within 30–45 minutes.³¹ In addition, transcripts of both pro-inflammatory cytokines, TNF α and IL-1 β , are upregulated within the first-hour post-SCI. Then, the expression of TNF α is nearly downregulated by 24 hours.³² In this study, the highest level of TNF α and IL-1 β was observed at 4 hours and 1-day post-SCI, respectively, which was decreased by 7 days following trauma. However, trehalose greatly reduced TNF α and IL-1 β level at all time points when compared with vehicle groups.

The protective anti-inflammatory effect of trehalose is in agreement with previous studies. In a recent report, trehalose was found to attenuate LPS-induced release of cytokines including TNF α , IL-1 β and IL-6 in BV-2 cells dose-dependently.³³ Another study indicated that trehalose reduced LPS-induced TNF α and IL-1 β production in mouse peritoneal macrophages in a dose-dependent manner.³⁴

Neutrophils are recruited to the lesion site in order to phagocyte cellular debris or possible pathogens. Neutrophils infiltration is assessed by measuring MPO activity which is present in neutrophils granules. The enzyme activity is correlated with the number and activation of neutrophils recruited.³⁵ Here, we found that MPO activity peaked at 1-day post-injury which demonstrated the highest number of neutrophils infiltrated to the lesion site. Typically, neutrophils accumulation is increased 3 hours and remains elevated up to 3 days after SCI.³⁶ Comparing MPO activity and IL-1 β level, it was found that they changed in parallel chronologically which indicated the contribution of neutrophils in IL-1 β production. Moreover, our study showed that trehalose treatment reduced the level of MPO activity through decreasing neutrophils recruitment. This effect of trehalose was also reported in a rat model of endotoxin shock. Treatment with trehalose reduced neutrophils accumulation in liver and lung, and thus decreased edema.¹¹

Generally, the activity of oxidative species is balanced by cellular antioxidants. However, during traumatic injuries and post-traumatic reperfusion, the balance between free radicals and scavenging capacity of antioxidants is altered in favor of free radicals leading to oxidative stress.³⁷ When undergoing an oxidative stress, neutrophils and macrophages are major cells that produce reactive species by enzyme systems such as myeloperoxidase and NADPH oxidase.³⁸

Central nervous system (CNS) is particularly sensitive to lipid peroxidation due to its high level of polyunsaturated fatty acids. Their peroxidation leads to the formation of reactive aldehydes such as malondialdehyde (MDA) which is typically measured as a marker of lipid peroxidation.³⁹ Post-traumatic lipid peroxidation initiates as early as 5 minutes after injury and continues within the first week.⁴⁰ In our experimental SCI, the highest level of lipid peroxidation was found to be at 3 days post-injury which was not in agreement with MPO activity. We also revealed that trehalose treatment reduced the level of MDA remarkably at all time points following SCI. Trehalose inhibited lipid peroxidation in RAW 264.7 cells treated with hemolysate. Furthermore, trehalose suppressed H₂O₂-primed arachidonic acid release from cultured cells. These data suggested that trehalose reduces lipid peroxidation induced by ROS.¹³

Following SCI, inflammatory cytokines such as TNF α and IL-1 β induce nitric oxide synthase (iNOS) in various cells to generate NO.⁴¹ Physiologically, NO is produced in CNS by vascular endothelium and neurons in small amounts. However, when both NO and O₂ are formed at high concentrations as in the case of SCI, peroxynitrite might be synthesized which can oxidize lipids, DNA and proteins. NO is generated immediately after SCI, peaks at 24 hours and gradually decreases by 12 days after injury.⁴² The results of this study indicated that NO level increased post-SCI and reached a peak at 3 days which was similar to the time course of neutrophils infiltration. Typically, macrophages enter the lesion site 2 days after SCI and achieve their highest density 5–7 days post-trauma.³⁰ Therefore, they might be a major source of NO at 3 days after SCI. Notably, the time course of NO generation was the same as MDA production indicating the role of NO in lipid peroxidation following SCI.

NO has been implicated in microglia-dependent demyelination and apoptosis of neurons.⁴³ Our study provided evidence that trehalose decreased NO level at all time points following SCI. In agreement with this finding, trehalose has been shown to reduce NO content of liver and lung in endotoxin shock induced male rats.¹¹ Moreover, trehalose attenuated NO production in LPS-stimulated BV-2 cells, rat peritoneal macrophages, astrocyte and oligodendrocyte cell cultures.^{12,33,44} In addition, trehalose inhibited NO synthesis in hemolysate treated macrophage-like cells.¹³

A major feature of cellular response to SCI is astrogliosis. Reactive astrogliosis can be detected immunohistochemically by measuring GFAP which is an intermediate filament protein. GFAP expression increases early within 24 hours after SCI and persists

for weeks following trauma.⁴⁵ In the present study, GFAP level increased significantly 1-day post-SCI, reached a peak at 3 days and remained elevated by 7 days following injury. Glial scar is well documented to inhibit axonal regeneration.⁴⁶ This study provided evidence that trehalose decreased astrocytes activation at early time points remarkably. Most likely, trehalose reduced astrogliosis by decreasing TNF α , IL-1 β and NO level. In fact, these signaling molecules as well as INF- γ , IL-6, TGF- β upregulate astrocytes activation and scar formation.⁴⁷

Acute SCI is followed by a secondary process of tissue degeneration and functional deficit arising from inflammation.¹ In the present study, evaluation of motor function with the BBB scale demonstrated that SCI led to a considerable decrease in BBB score. However, trehalose treatment indicated a slight improvement in hindlimb function by 7 days post-trauma. Inflammatory responses after SCI cause considerable damage to surrounding neurons and glia. The extent of damage and motor alterations correlate with the maximum peak of neutrophils migration.³⁵ Therefore, early therapeutic interventions targeting inflammation will lead to motor function recovery post-SCI. It is likely that the neuroprotective effect of trehalose was exerted through reducing cytokines and reactive species after trauma. In addition, trehalose may improve neurological function by decreasing reactive astrocytes which make barriers to neuronal regeneration.

There are some limitations associated with this study. First, the concentration and dosage of trehalose were used according to previous studies. Therefore, the most appropriate concentration and dosage should be determined. Second, this study was conducted in the early time course following SCI and a single injection of trehalose was used. Thus, delayed biochemical and behavioral assessment as well as multiple injections of trehalose are suggested. Third, intrathecal injection of trehalose is considered as a barrier to its clinical application. In addition, the presence of trehalase limits its oral administration. Therefore, the study of peritoneal injection of trehalose is recommended. Fourth, in the present investigation, the efficacy of trehalose was not compared with other therapeutic agents. Thus, providing comparison is proposed in future clinical studies.

Conclusion

In summary, this study confirmed the finding that trehalose treatment reduces secondary damage after SCI. In fact, trehalose decreased inflammation and lipid peroxidation induced by SCI at early time points. In addition, treatment with trehalose alleviated reactive astrocytes

which are regarded as detrimental to functional outcome after SCI. The neuroprotective effect of trehalose was also supported by improvement in motor function post-SCI.

Disclaimer statements

Contributors All authors have contributed significantly to the design, analysis and writing of this manuscript. This study represents original work and has not been published elsewhere.

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Conflicts of interest None.

Ethics approval The ethics committee of Kerman University of Medical Sciences approved the experimental procedures.

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