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Effect of Trehalose on *Neurocan* and *Neural-Glial Antigen* 2 Genes Expression in Rats with Spinal Cord Injury

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Abstract

Background: Chondroitin sulfate proteoglycans (CSPGs) are the major cause of axonal regeneration failure at the site of lesion in spinal cord injury (SCI). Inflammation is believed to stimulate the upregulation of CSPGs expression. Recent evidence showed that trehalose reduces the development of inflammation in SCI. The aim of this study was to investigate the effect of trehalose on *neurocan* and *Neural-Glial Antigen 2* (*NG2*) mRNA levels in SCI in rats.

Methods: In this experimental study, male rats were divided into six groups (n=15). Sham (laminectomy), SCI (laminectomy and SCI), vehicle (laminectomy and SCI, treated with phosphate buffer saline), and T10, T100 and T1000 (laminectomy and SCI, treated with 10, 100 and 1000 mM trehalose). Five rats in each group were sacrificed at 1, 3 and 7 days post-injury to measure *neurocan* and *NG2* mRNA levels in lesion. Statistical analysis was performed using Kruskal-Wallis methods followed by the Mann-Whitney test.

Results: Findings indicated that SCI upregulated *neurocan* and *NG2* mRNA levels at all times. No significant difference was observed in *neurocan* and *NG2* gene transcripts between SCI and vehicle groups ($p>0.05$). However, 10 mM trehalose downregulated the mRNA level of both *neurocan* (0.76 and 0.65 fold) and *NG2* (0.75 and 0.70 fold) at 3 and 7 days post-SCI compared to vehicle group ($p<0.05$ and $p<0.01$, respectively).

Conclusion: Collectively, treatment with low dose trehalose showed a decrease in *neurocan* and *NG2* mRNA levels in spinal cord injured rats.

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Introduction

Spinal cord injury (SCI) is a destructive damage which leads to permanent neurological flaw (1). It is estimated that almost 180,000 persons around the world experience some forms of traumatic SCI every year (2). SCI consists of two distinct stages, primary and secondary phases. The initial phase often results from mechanical impaction to the spine. The secondary phase deals with the multifarious pathological mechanisms that may last for days or months. These events include the breakdown of blood-spinal cord barrier, neuro-inflammation, oxidative stress, neuronal injury and ischemic dysfunction (3).

Numerous factors limit the response to treatment in SCI patients which include a decrease in growth factors, limited recovery of neural tissues, an increase in myelin-related outgrowth inhibitors and inhibiting factors associated with glial scars such as chondroitin sulfate proteoglycans (CSPGs) (4). These compounds are major components of extracellular matrix in the central nervous system (CNS) and play a critical role in the development of brain and spinal cord (5). After SCI, various CSPGs, such as aggrecan, neurocan, versican and neural-glial antigen 2 (NG2) are densely placed in the lesion site and contribute to scar formation, which is a major obstacle for the regeneration of the injured spinal cord (6). It is well evidenced that pro-inflammatory cytokines, inflammatory mediators and oxidant species can induce CSPGs synthesis. CSPGs can also mediate the inflammatory responses that influence CNS damage in CNS lesions (7). Therefore, it is likely that by removing these inhibitors, axonal outgrowth increases and functional recovery improves following SCI (6).

As progress is made in our understanding of the pathophysiological events that occur after SCI, neuroprotective and anti-inflammatory agents are being developed for the treatment of SCI (8). Numerous studies indicated that methylprednisolone (MP) is potentially beneficial in acute SCI. A significant improvement in motor function and sensations has been shown in patients treated with MP (9). This is likely due to its classical anti-inflammatory effect. Furthermore, MP acts mainly as a scavenger of free radicals, thus prevents lipid peroxidation mediated tissue damage (10). In addition, MP downregulates CSPGs expression induced by inflammation in reactivated astrocytes (11). Statins elicit numerous favorable effects on injured CNS which lead to neurite outgrowth. It has been suggested that statins induce anti-inflammatory and anti-oxidative effects through reducing inflammatory mediators, thereby attenuating CSPGs level (12).

Another compound that seems to be effective in the treatment of SCI is trehalose. Trehalose is a non-reducing disaccharide in which two glucose units are linked by an α-1,1glycosidic bond. Trehalose has multiple functions including a protective action against stressors such as inflammation and reactive oxygen species (ROS) (13). Trehalose inhibits inflammatory reactions by preventing the activation of nuclear transcription factor-kappa B (NF-κB), thus decreasing the production of pro-inflammatory cytokines (14). In our previous study, we reported that trehalose could reduce oxidative markers including myeloperoxidase, malondialdehyde and nitric oxide as well as pro-inflammatory mediators after SCI (15). These two issues contribute to CSPGs induction. However, it is not obvious if trehalose can reduce CSPGs level in injured spinal cord. Therefore, in this study we examined the effects of different concentrations of trehalose on mRNA levels of two CSPGs genes including *NG2* and *neurocan* in a rat model of SCI.

Materials and Methods

Animals

A total of 90 male Wistar rats (250-300 g) obtained from the Animal House of Kerman University of Medical Sciences were subjects of this study. Animals were divided into 6 groups including:

- Sham group (n=15): rats that only got laminectomy after anesthesia.

- SCI group (n=15): animals that were subjected to SCI after laminectomy.

- Vehicle group (n=15): rats that received SCI and immediately 6 µl phosphate buffer saline (PBS; Sigma, USA) intrathecally.

- Trehalose 10 mM group (n=15): this group received SCI and then 6 µl of trehalose (Sigma, USA) 10 mM in the lesion site.

- Trehalose 100 mM group (n=15): rats received 6 µl of trehalose 100 mM intrathecally following SCI.

- Trehalose 1000 mM group (n=15): animals were subjected to SCI and then immediately 6 µl of trehalose 1000 mM was injected to the lesion site (14, 16).

Animals were then killed at 1, 3 and 7 days post-injury, while 5 rats in each group and time were considered for further studies (17). All experiments were approved by the ethics committee of Kerman University of Medical Science (IR.KMU.REC.96.5). This experimental study was performed in Biochemistry and Physiology departments of Kerman University of Medical Sciences, Kerman, Iran.

Surgery

All surgeries were performed under anesthesia with a combination of 50 mg/kg of ketamine (Alfasan, Netherlands) and 5 mg/kg of xylazine (Alfasan, Netherlands) by an experienced physiologist. After a skin incision, contusion lesions were made by dropping the NYU device rod (10 g) from the distance of 25 mm onto the intact dura exposed after laminectomy at T9–T10 spinal cord level (15-17). Then rats were recovered from surgery in temperature and humidity controlled incubation chambers. Gentamicin (Alborz Darou, Iran; 12 mg/kg) was given intraperitoneally shortly after injury and continued every other day. Bladder evacuation was also accomplished manually until bladder function returned. The animals had free access to food and water and were monitored once per day for infections, general health and mobility throughout the post-injury survival period. After killing animals at specific points in time, 1 cm of spinal cord tissue with a lesion in the middle was dissected and kept at -70 °C (15-17).

Sample Preparation and Real-time PCR

Total RNA was isolated from 100 mg of frozen thoracic spinal cord tissue at 1, 3, and 7 days post-surgery in ice-cold Trizol (Geneall, Korea) following homogenization with a homogenizer (Hiescher, Germany). After DNase (Jena Biosicence, Germany) treatment, RNA was quantified spectrophotometrically (Thermo Fisher Scientific, USA) by measuring the absorbance at 260 nm and RNA purity was determined by measuring the 260/280 nm absorbance ratio (17).

The reverse transcription reaction was carried out using 0.5 µg of total RNA according to the protocol of cDNA synthesis kit (Takara, Japan). Gene specific primers were designed and the selected sets of primers were blasted against the GenBank to confirm their species and gene specificity (Table 1). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was

used as the internal control gene. PCR reactions were carried out using cDNA, primers (Pishgam, Iran) and SYBR Green master mix (Ampliqon, Denmark) in 20 µL reaction volume using ABI thermocycler. Real-time PCR was performed with the following thermocycler parameters: 15 min at 95 °C, 40 cycles of 30 sec at 95 °C, 30 sec at 62 °C and 30 sec at 72 °C. PCR product was measured using SYBR Green fluorescence collected during real-Time PCR. All experiments were performed in triplicate. The specificity of amplification was verified by analyzing their characteristic melting curves and by subsequent gel electrophoresis. Then relative gene expression was normalized to *GAPDH* gene. Our method of presenting quantitative real-time PCR data was the comparative threshold cycle (CT) method which is also known as the $2^{-\Delta\Delta CT}$ method. The comparative CT method assumes that both target and internal control genes are amplified with efficiencies near 100% and within 5% of each other. The efficiency of reaction was determined by generating a standard curve for *Neurocan*, *NG2* and *GAPDH*.

Table 1. Primer sets used for real-time PCR

Gene		Sequence	Product size (bp)
Neurocan	F	5′-ACCTGGTAACCCTGGAAGTGA-3′	77
	R	5′-AGCGAAGGTCAACGCATAGC-3′	
NG2	F	5′-TTACCTTGGCCTTGTTGGTC-3′	168
	R	5′-GATGATCTGTTTGGCCTGCT-3′	
GAPDH	F	5′-AACCCATCACCATCTTCCAG-3′	197
	R	5′-GTGGTTCACACCCATCACAA-3′	

Statistical analysis

The statistical significance of differences among groups was first assessed by Kruskal-Wallis test using SPSS software, version 20. Mann-Whitney analysis was used to compare differences between the two groups. Then P values for multiple comparisons were adjusted by Bonferroni correction. Differences were considered significant at $p<0.05$.

Results

Relative expression of neurocan gene

Changes in *neurocan* and *NG2* mRNA levels were quantified in spinal cord tissues which were obtained from sham, SCI, vehicle and trehalose treated groups at 1, 3 and 7 days after injury. At each time point, *neurocan* mRNA level in sham group was lower compared with that of other groups ($p<0.05$). In SCI group, the expression of *neurocan* gene increased significantly at 1 day post-injury compared with sham group (2.63 fold; $p<0.001$). On day 3 after SCI, neurocan mRNA level was significantly higher than sham group (2.70 fold; $p<0.01$) sustained at a high level until 7 days post-trauma (2.14 fold; $p<0.001$). No significant difference was detected in *neurocan* mRNA level between SCI and vehicle groups at any time points ($p>0.05$) (Figure 1).

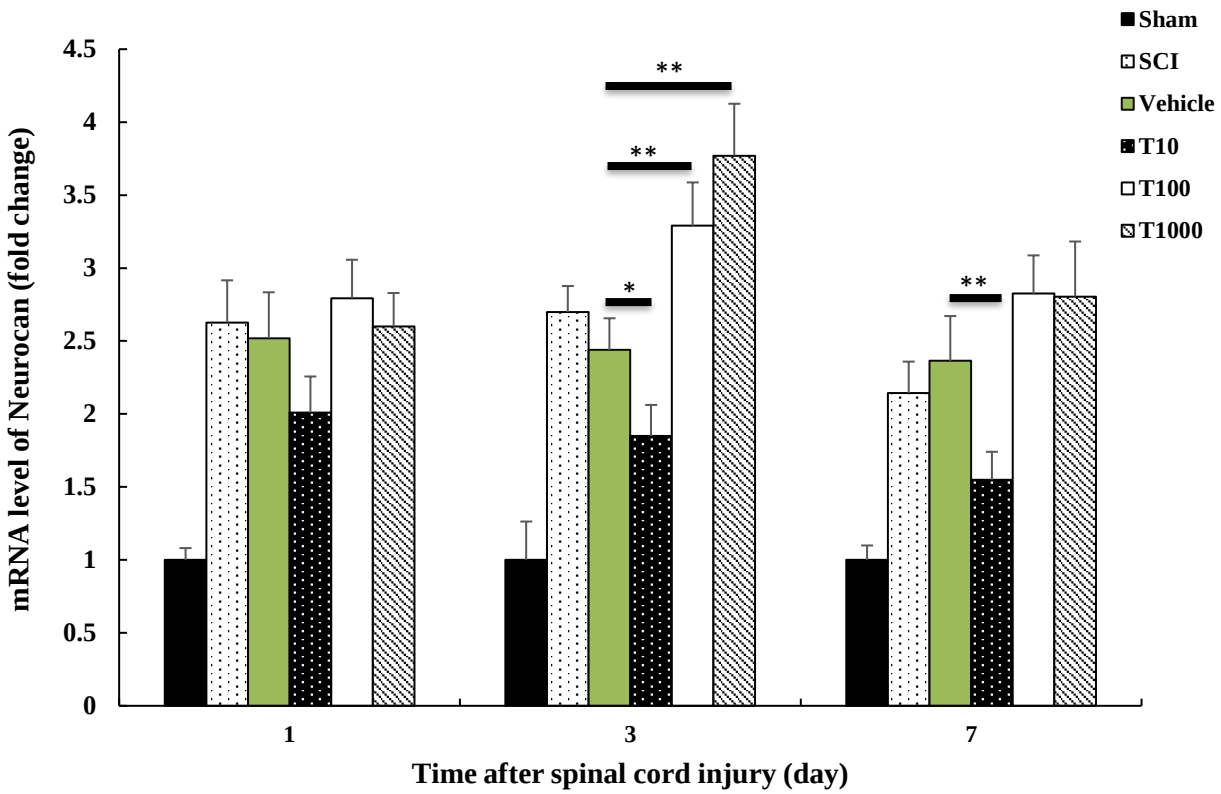

Figure 1. *Neurocan* gene expression level at 1, 3 and 7 days in different groups. Transcripts were measured by real-time PCR, normalized to *GAPDH* mRNA and plotted as fold change from sham group. Data are shown as means ± SEM. *p<0.05; **p<0.01 compared to vehicle group.

At 1 day after injury, *neurocan* mRNA level in trehalose treated groups was not significantly different compared with that in vehicle group ($p>0.05$). Three days after injury, the mRNA level of *neurocan* in T10 group was significantly decreased in comparison to vehicle group (0.76 fold, $p<0.05$). However, in T100 and T1000 groups, *neurocan* mRNA level was increased by 1.35 and 1.55 fold compared to vehicle group ($p<0.01$). After 7 days post SCI, *neurocan* gene expression was downregulated in T10 group compared with vehicle group (0.65 fold, $p<0.001$). However, the level of *neurocan* mRNA in T100 and T1000 groups was not significantly different compared with vehicle group at this time point ($p>0.05$). In addition, no significant difference was observed in *neurocan* gene expression level between groups treated with 100 and 1000 mM terhalose at various times ($p>0.05$) (Figure 1).

Relative expression of NG2 gene

The level of *NG2* mRNA among the groups under investigation was not significant at 1 day following SCI ($p>0.05$). SCI upregulated *NG2* mRNA level by 1.32 fold at 3 and 7 days post-trauma in comparison to sham group ($p<0.01$). However, treatment with PBS did not change the level of *NG2* mRNA compared with SCI at any time points ($p>0.05$). *NG2* gene expression was downregulated significantly in T10 group in comparison to vehicle group on days 3 and 7 after trauma (0.75 and 0.70 fold, $p<0.05$ and $p<0.01$ respectively). Interestingly, *NG2* mRNA level in T10 group remained at

sham level at different times (p>0.05). However, increases in the gene expression were clearly detected in T100 and T1000 groups in comparison to sham group at 3 and 7 days post-SCI (1.4 fold; p<0.01). Furthermore, no significant difference was observed in *NG2* mRNA level between T100 and T1000 groups and vehicle group throughout the experiment (p>0.05) (Figure 2).

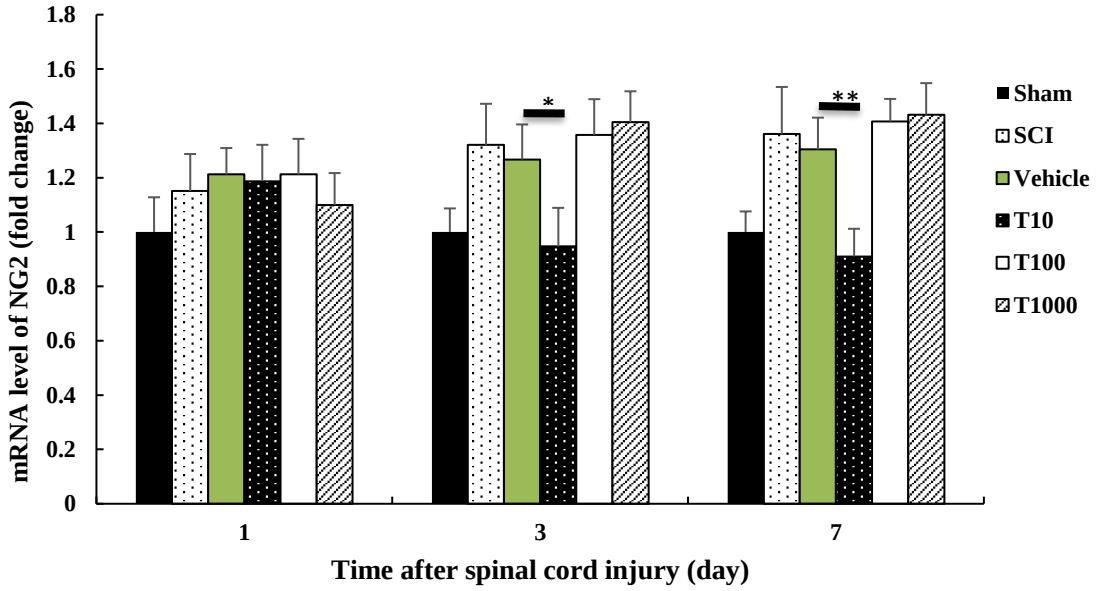

Figure 2. *NG2* gene expression level at 1, 3 and 7 days in different groups. Transcripts were measured by real-time PCR, normalized to *GAPDH* mRNA and plotted as fold change from sham group. Data are shown as means ± SEM. *p<0.05; **p<0.01 compared to vehicle group.

Discussion

This study showed that mRNA level of *neurocan* and *NG2* genes was upregulated following SCI. Our findings indicated that treatment with trehalose at 10 mM concentration reduced *neurocan* and *NG2* gene transcripts at 3 and 7 days post-SCI compared to vehicle group. However, treatment with higher concentrations of trehalose exhibited an opposite effect.

SCI is the most devastating clinical problem which decreases patients' quality of life (18). Studies in several CNS experimental lesion models demonstrated that the scar tissue formed after SCI inhibits axonal regeneration. Scarring is the result of a multicellular response to injury involving astrocytes, microglia, macrophages, oligodendrocyte progenitors, fibroblasts, leptomeningeal and schwann cells. Among the molecules known to contribute to scarring at sites of CNS injury are chondroitin sulfate proteoglycans (CSPGs), which are a family of putatively inhibitory molecules. Thus, inhibition of CSPGs upregulation is thought to play a significant role in improving axonal regeneration and functional recovery (19). In this study, we found a high mRNA level of *neurocan* and *NG2* at 1 day after SCI in rats which remained elevated after 7 days post-injury. The dynamic variation of CSPGs in the rat spinal cord has been previously reported (20). Massey et al. showed that *NG2* and *neurocan* mRNA were dramatically upregulated in the dorsal column nuclei (DCN) 2 days following denervation by SCI (21). This difference may be explained by

the trauma model used by Massey et al. which was a C3-C4 transection model compared to the contusion method used in our study (21). At protein level, upregulation of CSPGs and other inhibitory proteoglycans occurs as early as 1 day after SCI and is sustained for periods up to 6 months after trauma (22). In addition, CSPGs have been shown to be upregulated in other models of injuries such as brain stroke, hypoxic-ischemic injury or nigrosriatal lesion. Harris et al. demonstrated an elevated *versican* mRNA level in the injury core region by 4 days after traumatic brain injury (TBI) which remained upregulated for 2 weeks. However, *neurocan* mRNA level increase was not significant compared to sham group (23). Furthermore, NG2 was significantly upregulated on day 3 after controlled cortical impact model of brain trauma and peaked at 5 days post-injury (24). In addition, the expression of each CSPG increased with a specific time course following cortical stroke (25). *Neurocan* was elevated early after stroke peaking at 3 days post-stroke, then returned to the control level on day 28. However, *NG2* was transiently raised on day 14 after stroke (26). Therefore, CSPGs response to injury is quite heterogeneous and may change as a function of injury model or clinical disease state or temporal factors (23).

Spinal cord injury leads to secondary tissue damage including inflammatory cytokines production and oxidative stress which results in upregulation of CSPGs (27). Therefore, agents which attenuate inflammation and oxidative damage may likely decrease CSPGs production after injury. In the present study, we showed that 10 mM trehalose decreased *CSPGs* mRNA level post-trauma. Trehalose is a natural sugar of invertebrates, fungi and many plants. It protects the integrity of cells against several stress attacks (28). In addition, trehalose was found to attenuate lipopolysaccharide (LPS) induced release of cytokines in BV-2 cells dose dependently. In fact, trehalose reduced two important transcriptional factors including NF-κB and activator protein (AP)-1 which are involved in regulating the expression of pro-inflammatory mediators (14). It also reduced the tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in mouse peritoneal macrophages induced by LPS (29).

We have recently utilized the enzyme chondroitinase to degrade CSPGs at the injured site following SCI in rats (16). Our findings indicated that trehalose which was used to stabilize the enzyme at the lesion site attenuated the release of TNF-α, IL-1β and oxidants (15). Little is known about the mechanisms regulating *neurocan* and *NG2* gene expression. However, cytokines are directly involved in the upregulation of gene expressions and function in response to CNS injuries (30). Therefore, trehalose likely contributed to the reduction of *neurocan* and *NG2* mRNA levels by suppressing pro-inflammatory mediators. Interestingly, the inhibitory effect of trehalose on *neurocan* and *NG2* gene expression was indicated to be dose dependent. We found that 100 and 1000 mM trehalose did not alter *NG2* mRNA level significantly compared to vehicle group at any time points. However, a transient increase in mRNA level of *neurocan* was observed on day 3 post-SCI in 100 and 1000 mM treated groups. We hypothesized that high concentrations of trehalose might have changed the cellular and molecular environment of injury which contributed to different results obtained at *neurocan* and *NG2* mRNA levels compared to 10 mM trehalose. This suggests that additional mechanisms are involved in trehalose-induced alterations of CSPGs genes at transcription level.

CNS lesions are characterized by astroglial scar formation which induces physical and biochemical barrier to axon

regeneration. Several agents have been shown to induce neurite outgrowth. Methylprednisolone (MP) is a first-line drug used in SCI. It has been suggested that MP induces anti-inflammatory and anti-oxidative effect (9). Interestingly, Liu et al. demonstrated downregulation of astrocyte reactivation and astrogliosis by glucocorticoid MP. Furthermore, MP treatment attenuated neurocan and phosphacan at both mRNA and protein levels in reactivated astrocytes which improved the neurite outgrowth of rat dorsal root ganglion neurons (11). Fujiyoshi et al. showed that intraperitoneal administration of interferon-γ (IFN-γ) decreased mRNA level of *neurocan* and reduced the accumulation of CSPGs following SCI. In addition, IFN-γ promoted functional recovery post-injury (31).

Numerous studies have indicated that microinjection of the enzyme chondroitinase ABC into CNS lesion effectively removes CSPGs following SCI and stroke which induces sensorimotor recovery and plasticity (21,32).

Conclusion

In summary, this animal study demonstrated that SCI increased *neurocan* and *NG2* mRNA levels. However, treatment with low dose trehalose reduced gene transcripts. The present study has some limitations. First, we investigated the mRNA level of two major CSPGs, including *neurocan* and *NG2* post-SCI. It is suggested to evaluate the variation of other CSPGs gene transcripts. Second, CSPGs were not measured at protein level in this study. Since CSPGs are major impediment to neural regeneration after SCI, it is recommended to analyze them at both mRNA and protein levels.

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Conflict of interests

The authors have no conflicts of interest to declare.

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