Title: Evaluation of Chondroitin Sulfate and Dermatan Sulfate Expression in Glial Scar to Determine Appropriate Time of Therapeutic Interventions in Contused Rats

Authors: Sara Rezaei\textsuperscript{1}, Salar Bakhtiyari\textsuperscript{2}, Khairolah Assadollahi\textsuperscript{3\textsuperscript{4}}, Somayeh Heidarizadi\textsuperscript{1}, Ardashir Moayeri\textsuperscript{1}, Monireh Azizi\textsuperscript{1,4*}

1. Department of Anatomy, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
2. Department of Biochemistry, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
3. Department of Epidemiology, Faculty of Health, Ilam University of Medical Sciences, Ilam, Iran
4. Biotechnology and Medicinal Plants Research Center, Ilam University of Medical Sciences, Ilam, Iran

*Corresponding author: Dr Aziz M, Department of Anatomy, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran, P.O. Box: 6939177143
E-mail: azizi.moaz@gmail.com, azizi-m@medilam.ac.ir
Tel: +98 8432235716
Fax: +98 8432235714

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Abstract

**Purpose:** Proteoglycans of extracellular matrix, increases in the glial scar during spinal cord injury and play important role in inhibition of axonal regeneration.

**Methods:** So far, the results of injury therapies have been limited due to lack of identifying the timely therapeutic intervention. The present study aimed to investigate the glial scar chondroitin sulfate (CS) and dermatan sulfate (DS) levels at different times post-injury to determine the appropriate time for therapeutic intervention.

**Results:** By an experimental study 72 Wistar rats were randomly divided into 12 groups: control, sham, injured animals at 1, 2, 4 and 8 days and 2, 4, 8, 12, 16 and 20 weeks post-injury. Animals in the injured groups were contused in the T10 segment of spinal cord. The motor function of animals was assessed using BBB test and the histological assessment was performed using Luxol Fast Blue and Bielshovisky Staining. CS and DS levels of lesions were measured using ELISA method.

**Conclusion:** The motor function assessment indicated a relative recovery over time. Histological results confirmed some regeneration in the injury site at 20 weeks post-injury. The ELISA results demonstrated much higher level of DS than that of CS in the glial scar. Considering high levels of DS compared to CS in the glial scar and its reduction from second weeks after SCI onwards, it seems that second week after SCI is the best time for the therapeutic intervention in terms of the scar permeability.

**Keywords:** Spinal cord injury, Glial scar, Chondroitin sulfate, Dermatan sulfate
1. Introduction

Spinal cord injury (SCI) is one of the most severe disabilities that imposes a large impact on one’s life and causes broad restrictions in life (Devivo, 2003). After severe SCI, CNS astrocytes form the main component of the glial scar by being transformed into reactive astrocytes that are considered as large barrier to axonal regeneration (Matsui and Oohira, 2004; Rhodes and Fawcett, 2004). Rapid proliferation of astrocytes around the lesion, is one of the special characteristics of the injury in all mammals (Fawcett and Asher, 1999). After the injury, the resulting scar is composed by laminin growth promoting molecules and cell adhesion molecules, growth factors and some inhibitory molecules; however, axonal growth is inhibited due to dominance of the inhibitory molecules at the injured site (Shields et al., 2008). Chondroitin sulfate proteoglycans (CSPGs) acts as guidance and signaling molecules during the development and maintains the structural integrity of the intact CNS in specialized areas such as basement membrane, perineuronal nets and nods of Ranvier (Lau et al., 2013).

Studies showed that although the glial scar plays a key role in protecting intact tissues by surrounding the injured site (Faulkner et al., 2004; Myer et al., 2006; Rolls et al., 2009; Sofroniew, 2009), preventing inflammation and further degeneration of myelin, but it unfortunately inhibits axonal regeneration (Okada et al., 2006). Very limited regeneration ability of CNS neurons is mainly due to the release of inhibitory molecules in the CNS (Wilson et al., 2013). CSPG secreted by the reactive astrocytes forms the main components that inhibit the axons’ growth (Matsui and Oohira, 2004). The level of CSPG is upregulated and is considered as a strong inhibitor of axonal regeneration after traumatic injury (Lau et al., 2013; Silver and Miller, 2004). Increased CSPG expression level after SCI has been
reported by previous studies (Pasterkamp et al., 2001; Plant et al., 2001). Increased CSPG expression level at the injury site as well as at the sites far from SCI contusion model was reported by Andrews et al. (2012). Researchers also showed that CSPG directly inhibits oligodendrocytes and progenitor cells growth process and their differentiation into mature oligodendrocytes in the in vitro model (Siebert and Osterhout, 2011). Therefore, considering the scar inhibitory effect on axonal regeneration, the regeneration process as well as the reduction of scar extent and destruction of degeneration molecules should be carried out as quickly as possible. But it is important that inhibition of factors causing scar formation must be avoided due to their primary protecting role (Ueno and Yamashita, 2008). Problems such as incomplete transection and knife-cut cause damage to the spinal cord meninges and allow the peripheral cells to invade the injury site. These injuries induce changes such as upregulation of neurocan, brevican, and NG2 in CSPGs expression levels in the injured site (Tang et al., 2003; Massey et al., 2008; Jones et al., 2003), which are responsible for creating a chemical barrier to extend axons in the injury site (Fitch and Silver, 2008). However, little known about changes in the CSPGs expression levels contusion SCI which almost accounts for half of all cases of clinical injuries (Norenberg et al., 2004). Nevertheless, many researchers have achieved no significant recovery in this regard due to lack of timely therapeutic interventions.

Therefore, the main goal of this study was to determine the CS-DC levels in the glial scar at different times post SCI to determine the effective time of therapeutic intervention and reduce the inhibitory effect of the scar.

2. Methods

Animals grouping and SCI induction
A total of 72 male Wistar rats weighing 210 ± 10 gr were used. One week before the start of the experiment and during the whole period of the study, animals were kept at Animals Care Center of the University under standard laboratory conditions, including easy access to water and food, 12h:12h light: dark cycles and temperature of 22°C. The animals were randomly divided into 12 groups (n=6) of control, sham, injured animals at 1, 2, 4 and 8 days and 2, 4, 8, 12, 16 and 20 weeks post-injury. No action was taken in the control group and laminectomy was the only action in the sham group. All spinal cord-injured groups underwent laminectomy and then contusion SCI was created in T-10 segment of the spinal cord by dropping a 10gr weight from a height of 25mm (Wang et al., 2011). It should be noted that injured animals were kept in disinfected cages during the study period. They also received Cefazolin (1mg/kg, IP) and saline (2-5 ml) for five days after injury.

**Motor functional assessment**

To assess the motor function, Basso, Bresnahan and Beattie (BBB) test with a score range of 0-21 was used (Basso et al., 1996). A blind analysis was performed on the locomotor capacity of all groups by two individuals and the final score was reported as the mean score given by these individuals. BBB test was conducted on a daily basis in injured animals at 1, 2, and 4 days post-injury and in other groups after first two days and then on the weekly basis until the end of the survey.

**Transcardial perfusion**

At the end of study period, tissue samples of the injury sites were collected from three rats from each of the groups for histological studies. For this purpose, animals underwent initial perfusion fixation transcardially with normal saline followed by 10% formal saline after
being anesthetized using ketamine / xylazine (60/6 mg/Kg). Then, after removing the injury site, samples were placed in the same fixatives in order to undergo the secondary fixation for 24 hours.

**Histological study**

LFB and Bielshovisky staining methods were used for histological evaluation of tissue samples obtained from the injury site. Perfusion-fixed spinal cord tissue underwent the paraffin molding process after passing step and finally 5μm thick slices were prepared. LFB and Bielshovisky staining methods were used respectively to assess the myelin level and the presence of nerve fibers in the injury site.

**Molecular approach**

**Injured spinal cord preparation for ELISA technique**

The spinal cord was removed after anesthesia with ketamine / xylazine (60/6 mg/Kg) and reopening of the laminectomy area. Then equal rostral and caudal cuts were prepared from all samples at a distance of 1 mm apart from the center of the injury site. The samples were then washed with normal saline and maintained in formaldehyde solution in -80°C freezer. At the end of the study period (twentieth week), all samples were transferred from the freezer to room temperature and the protein extraction was performed.

**Total protein extraction from tissue samples**

Total protein of spinal cord tissue was extracted using the protein extraction solution (Cat.No.17081, Bulldog Bi Company). Briefly, 10-20 mg tissue samples were prepared from the injury sites and tissue homogenization was performed in 600 μl of protein extraction solution with protease inhibitor cocktail. Cells lysis was then continued by incubating the
cells in the -20 °C freezer for 20-30 minutes. The samples centrifuged at 13000 rpm (4 °C) for 5 minutes and the supernatant was transferred to 1.5 ml tube. Finally, the protein concentration was determined using the Bradford’s method (Bradford, 1976).

**Measuring DS and CS level of the injury site using ELISA method**

The levels of DS and CS were measured using commercially available kits according to their protocols (Rat Dermatan sulfate ELISA Kit: cat No. MBS263602-MyBioSour, USA and Chondroitin sulfate Kit: SEB141Ra96, Designed by Cloud-Clone Crop, Usen Life Science Inc. tests, USA). Finally, the levels of DS and CS normalized to the levels of total protein.

**Ethical approval:** The present study was approved by the Ethics Committee of Ilam University of Medical Sciences with the following code number: EC/93/A/112. This study was also in accordance with the Helsinki Declaration of 1975.

**Statistical analysis:** Statistical analysis was performed using the Minitab 16 software. Data were reported as mean ± SD at a significance level of p≤0.05. Significant differences between the groups were stated when p ≤0.05. Inter-group comparisons were made using one-way ANOVA with a significance level of p ≤0.05.

3. **Results**

**Evaluation of BBB motor test**

Comparing the BBB test results showed a significant difference in the control and sham groups with injured groups during the study period (P<0.05). But there was no such significant difference between the control and sham groups from beginning to the end of study (twentieth weeks), which shows laminectomy alone does not impair the motor function
in these animals and all animals gained Score 21 from the beginning to the end of study (Fig. 1).

A motor functional recovery with a slow pace was observed in groups with spinal injury at the end of the second to the fifth weeks of the study. The recovery level was increased more quickly after the fifth week until the end of study (week 20) to the extent that the motor score of injured animals reached to the score of 16 at the end of study (Fig. 1).

![Graph showing BBB motor test in various groups from the beginning to the end of the twentieth week of study.](image)

**Fig.1.** BBB motor test in various groups from the beginning to the end of the twentieth week of study.

Results are expressed as mean ± SD and animals in each group were (N = 6). A significant difference in the control and sham groups with injured groups during the study period are observed. The motor’s score of injured animals reached to 16 at the end of study (20 weeks).

**Histological results**
In order to assess the myelin level, LFB staining was applied. The LFB staining results confirmed the intactness of the spinal cord and the safety of laminectomy in sham group (Fig. 2A). The staining results in injured animals, at 20 weeks post injury, also confirmed filled cystic cavity and the presence of some myelination in the injured area (Fig. 2B & C).

Fig. 2. Luxol fast blue (LFB) staining of spinal cord sections at level T10 segment.
(Image A). Intact spinal cord in the sham group with normal integration of white and gray matters, (Images B and C). Injured spinal cord without any treatment intervention in 20 weeks after SCI lesion; injured area in the 20-weeks post injury is filled with some tissue and myelinated axons which probably is a reason for some regeneration observed in these animals. Magnifications of images A, B and C are 10X, 4X and 40X respectively. W and G: indicate the white and gray matters of spinal cord respectively.

In order to assess the presence of axons in the lesion, Bielshovisky staining was used. The results of this staining also confirmed the intactness of the spinal cord tissue and absence of laminectomy injury in the sham group (Fig. 3A). Some of the axons were also observed in injured animals at 20 weeks after the injury (Fig. 3B).
Fig. 3. Bielschowsky staining of intact sham (A) and injured- T10 segment of spinal cord 20 weeks post injury (B).

(Image A). Intact spinal cord is observed in the sham group (Image B). A cavity with some axon growth are observed in the spinal cord section 20 weeks after lesion. The axons existence in the cavity shows some degree of regeneration. Magnification of image A is 10X and image B is 40X.

**Evaluation of CS and DC level at the injured site**

Glial scar is made up different ECM components, the expression levels of which vary at different times after the injury. In this study, DC and CS levels at the injured site were evaluated using ELISA technique at 24 hours to 20 weeks after the injury. The results showed that DS was much higher than the CS in the scar at the above times (Fig. 4). As the results show, the DS level was increased on the first and second days, and the decreasing trend was later observed to the extent that the minimum level in the twelfth week. DS level was equal and less than that of the control group in the sixteenth and twentieth weeks, respectively (Figure 4A). Maximum CS level was observed in the injury site in the second day after the injury that was decreased with a fluctuating trend and reached to the same level as the normal control group in the twentieth week (Fig. 4B).
Fig. 4. DS (A) and CS (B) levels at the injury site based on ELISA technique results in all study groups.

The number of assessed animals in each group was (N = 3), and results are expressed as Mean ± SD. The DS levels are much higher than the CS levels in the scar whole the times. The DS level was increased on the first and second days, and the decreasing trend was later observed to its minimum level in the twelfth week. Maximum CS level in the injury site is seen in the second day post-injury, then decreases with a fluctuating trend and reaches to the same level as the normal control group in the twentieth week.

4. Discussion

Motor test and axonal regeneration

In this 20 weeks study, a relative recovery in motor function was observed over time so that the motor score of 16 was obtained for spinal cord-injured animals at the end of study (week 20). The histological results confirmed the axonal regeneration and remyelination, though limited, in the injury site.

In a study on photochemical model of spinal cord injury, Verdu et al. (2003) reported a BBB score of 18 for injured animals at the end of 12th weeks. Garcia-Alias et al. (2004) also obtained a BBB score of 15 for acute photochemical SCI model in injured rats at the end of
12\textsuperscript{th} week. However, in our study, BBB scores of 6.5 and 16 were obtained at the end of 12\textsuperscript{th} week and 20\textsuperscript{th} week, respectively. This discrepancy may be due to differences in the severity of SCI since the severity of the photochemical SCI used in the study by Verdu and Garcia-Alias was milder than the contusion injury created in the present study. Fouad et al. (2005) also reported a score of 2.1 as the average motor score in injured adult rats with complete severing of the spinal cord in T7-T9 region. Lower motor recovery rate reported by Fouad compared with the present study is probably due to the extent and severity of injury created in Fouad's study.

Richter et al. (2005) attributed post-injury limited regeneration to weak internal regeneration and angiogenesis capacity of neurons. Mothe and Tator (2005) stated that the number of cells stimulated in response to the injury was different depending on the injury model in such way that stem / progenitor cells responses are distributed locally in the spinal cord transection model and systematically throughout the rostral and caudal regions of the spinal cord in the contusion/compression model. This broader response is probably due to the fact that the wider region of spinal cord tissues was affected in the two injury models mentioned above. It has been recently stated that the injury is somehow recovered when some of the stem/progenitor cells in the ependyma are recruited to the injury site and the proliferation power is increased (Widenfalk et al., 2001). However, cell proliferation does not always lead to restoration of the spinal cord function and sometimes fills only the created cavities (Ropper, 2001). The various forms of plasticity have been reported in neurons of the cortex, brainstem and spinal cord, which can somewhat play a role in increasing post-injury compensation recovery (Weidner et al., 2001). However, Raineteau et al. (2002) reported reorganization of descending motor tracts in rats with SCI. The current study revealed an improved motor function, axonal regeneration and remyelination without any therapeutic intervention that may be associated with the presence of stem / progenitor cells in the injured...
area, weak regeneration by the neurons at the injured site, angiogenesis, reorganization of intact circuits and even plasticity changes which has also been reported by Widenfalk et al. (2001).

**Glial scar**

In the present study, CS and DS levels in the glial scar, which is the main axonal regeneration barrier, was measured, using the ELISA method, at 24 hours to 20 weeks after the injury. The results showed that the scar's DS level was much higher than that of CS and their levels reached to maximum rate at the first day after injury, which indicated that these proteoglycans play a main role in preventing axonal regeneration during the acute phase of glial scar formation. The glial scar contains different extracellular matrix components, which have various expressions' levels at different times. In a study on the expression level of CSPGs (NG2, neurocan, phosphacan, brevican and versican V2) and tenacin-c during spinal cord scar, transformation from the acute to the chronic phase within 24 hours to six months after injury, Tang et al. (2003) showed that the axonal growth was inhibited by these macromolecules. These researchers found a sharp increase in the level of neurocan, tenascin-C and NG2 within 24 hours, and stated that these molecules play a role in preventing the axonal regeneration in the glial scar formation during its acute phase. In Tang's study, the maximum level of phosphacan, brevican was observed one month after the injury. In contrast, versican V2 level was reduced sharply even in comparison with its levels in the intact tissue during the study period. Six months after the injury, neurocan, brevican and NG2 levels in chronic scar tissue remained significantly higher than the control groups that showed these CSPGs and tenascin-C in the extracellular matrix could be considered as important inhibitors of axonal regeneration during acute to chronic maturation of spinal cord scar tissue (Tang et al., 2003).
In the present study, the highest levels of CS and DS were observed, using ELISA method, within 24 and 48 hours after the injury respectively, leading to inhibition of axonal regeneration in acute phase of the injury. In contrast to Tang’s study, our study showed that CS level was reduced sharply after 48 hours, and following a fluctuation trend, its level reached to the level of control group at the end of 20th week (Fig. 4B). In a study on spinal dorsal column transection model in C3 of adult female Fischer rats, Jones et al. (2002) stated that NG2 was the most important upregulated proteoglycan among CSPGs family members after SCI. The immunohistochemical results of Jones’ study showed an upregulation of NG2, 24 hours after the injury, and this maximum level was kept until about 7 weeks after the injury. However, an average upregulation was observed in proteoglycans brevican, versican and neurocan, 7 days after the injury and the phosphacan level downregulated unlike other proteoglycans. These researchers stated that NG2 was more expressed in the caudal region of the injury than its rostral region; so, they introduced NG2 as a main component for inhibitory molecules in the ECM that likely inhibits axonal regeneration. The discrepancy between results reported by Tang and Jones with our findings may be due to differences in the methods, the type and severity of injury and even animals species. These researchers used western blotting and immunohistochemical methods, dorsal column transection at C1-C2 or C3 segments of adult female rats of Sprague and Fischer rats. However, Andrews et al. (2012) used the western blotting and immunohistochemical techniques to measure the expression of agrecan, neurocan, brevican and NG2 proteoglycans in severe contusion SCI model in the spinal segments far from the injury site (cervical and lumbar areas) of adult female Sprague rats 3,7,14 and 28 days after the injury. They observed an increase in neurocan level inside and outside of the injury location, while there was a sharp decline in agrecan and brevican levels at the injury site and remained unchanged in the distal segments.
These researchers also showed that NG2 level remained unchanged in the injured site and cervical region and was increased in the lumbar region.

Some researchers showed that neurocan and NG2 levels have slightly increased in segments surrounding the injury in rodent compression models (Iaci et al., 2007). GAG staining (Lemons et al., 1999) and immunoreactivity of NG2 (McTigue et al., 2006) increased after the contusion injury. Upregulation of versican V2, aggregan and neurocan as CSPGs family members, following multiple sclerosis, was also reported by Sobel and Ahmed (2001). According to the results of present study, which showed a higher concentration of DS than CS, it seems that DS plays a major inhibitory role than the CS, in the glial scar of wistar rats with contused spinal cord.

On the other hand, considering the high concentration of these proteoglycans, by the end of the second week after the injury, it seems that unsatisfactory success in therapeutic interventions by previous studies, in which interventions have been conducted early, may be due to the impermeability of glial scar caused by the high concentration of its inhibitors. However, in order to reach an exact conclusion, further studies with larger sample sizes and use of other animal species and other complementary techniques such as western blotting and immunohistochemistry are needed.

4. Conclusion

Histological results of this study showed that the relative motor function recovery observed in the groups over time, though limited, can be partly due to axonal regeneration and remyelination.

Reduced injury DS and CS levels observed by ELISA technique also confirm the permeability of glial scar at the end times of study. So, it seems that the resulting motor functional recovery over time is partly due to reduced levels of CS and DS.
inhibitory molecules, especially DS in the glial scar. Also, considering the high level of DS compared with CS in the scar as well as a reduction in its level from the second week onwards, the second week after the injury is the best time for the therapeutic intervention in terms of scar permeability and earlier interventions will yield no satisfactory results due to high concentration of the above inhibitory factors.

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Conflict of Interest

All authors declared that they have no conflict of interest for this work.

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