Intravenous Granulocyte Colony-Stimulating Factor Administration Can Attenuate Neuropathic Pain Following Spinal Cord Injury in Male Rats

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Abstract- Peripheral neuropathy, regularly expressed as hypersensitivity to painful stimuli, is between the most common complications of spinal cord injury (SCI) that develops in up to 40% of patients and appears to be persistent. Previous studies have demonstrated neuroprotective effects of Granulocyte colony-stimulating factor (G-CSF) on neuropathic pains. We aimed to investigate the antihyperalgesic effect of G-CSF on neuropathic pains following SCI in male rats. Twenty four adult male rats (weight 300–350g) were used. After laminectomy, complete SCI was performed by compression of the spinal cord for 1 minute with an aneurysm clip. Within 30 minutes after the surgery, 200 µg/kg G-CSF was injected intravenously in G-CSF treated groups and then was repeated in 3 consecutive days. Tail flick latency (TFL), acetone drop test scores, BBB test scores, and Von-Frey filament test were performed before surgery and once a week after surgery. Rats in G-CSF treated group showed significantly higher mean TFL, and lower mean score of acetone test compared with those in SCI+veh group 4 weeks after surgery (P<0.05). There was no significant difference between rats in G-CSF treated group and SCI+veh group in BBB and Von-Frey filament tests results. These findings revealed that treatment with systemic administration of intravenous G-CSF would attenuate thermal hyperalgesia, and cold allodynia induced by SCI in rats but has no significant effect on locomotor activity and mechanical allodynia after SCI.

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Keywords: Spinal cord injury; Hyperalgesia; Tail flick; Neuropathic pain; G-CSF

Introduction

Spinal cord injury has profound effects on the body. Despite various treatments, Spinal Cord Injury (SCI) causes severe pain in about 40% of patients that is persistent (1). There are a lot of information about SCI pain mechanisms from experimental models and clinical studies. But treatment remains difficult and insufficient (2).

Recently, it has become clear that inflammatory and immune mechanisms both in the peripheral and the central nervous system play an important role in neuropathic pain. When tissue damage occurs, an inflammatory response develops, triggered by various proinflammatory and pro-algesic mediators activating specialized peripheral pain signaling sensory neurons. The peripheral terminals of Aδ and C fibers transduce and propagate noxious stimuli from peripheral tissues (such as skin, muscles, joints, and viscera) to the dorsal horn of the spinal cord and thereafter to the brain. At spinal and supraspinal sites, the integration of signals from pro-algesic neurotransmitters, environmental and cognitive factors eventually results in the sensation of pain (3). Granulocyte colony-stimulating factor (G-CSF) is a cytokine that promotes survival, proliferation, and differentiation of cells in the neutrophil lineage (4, 5). GCSF can stimulate the bone marrow to produce numerous PMN cells (6). Although the underlying mechanisms are incompletely understood, G-CSF can modulate cytokines (7), chemokines (8) and CD34+ adhesion molecules to affect pain transmission (9). The above findings indicate that G-CSF may play a role in indirect or direct pain inhibition and is a potential mediator in controlling pain. Also, research on GCSF may provide new insights into intrinsic mechanisms of pain control and develop new strategies and alternative

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approaches to treating pain (10).

Materials and Methods

This study was performed on 24 adult male rats (weight, 300-350 g at the start of the experiment). They were purchased from Pasteur Institute, Tehran, Iran. Animals were kept at room temperature of 23±2° C, humidity of 50±5%, and in a 12-hour light/dark cycle (light: 8.00–20.00). They had free access to tap water and a standard pellet chow. They were handled in accordance with the criteria indicated by the "Guide for the Care and Use of Laboratory Animals" (NIH US publication 23-86 revised 1985). This study followed the International Association for the Study of Pain (IASP) guidelines for animal experiments (Zimmermann, 1983, (11). Animal's weight, cases of SCI complications such as autophagia, and mortality were measured and recorded.

The animals were randomly divided into 3 groups (6-8 in each group):

Sham+ veh Group: the only laminectomy without SCI was performed. Animals received 1ml normal saline IV (intravenous) injection within 30 minutes before the surgery.

SCI+veh Group: SCI was performed, and animals received 1ml carrier vehicle (normal saline IV injection) within 30 minutes before the surgery.

SCI+ G-CSF Group: Animals received IV injection of 200 µg/kg G-CSF within 30 minutes after SCI which was repeated in 3 consecutive days.

According to the literature, G-CSF has no effect on sham groups, so we did not use sham+ G-CSF groups.

Surgery and drug administration

Rats were anesthetized by ip injection of 50 mg/kg ketamine (Trittau-Germany). Prior to the surgery, the operation site was shaved and disinfected with povidone-iodine solution10%. The surgery was performed using thestandard sterile technique. Rats from each group were randomly selected and handled by a blind observer (blinded to G-CSF and vehicle in SCI groups). An incision was made in the thoracic spine at T7–T12 level. After the incision of dermal and subdermal tissues at the midline, paravertebral muscles were bluntly dissected to expose the lamina bilaterally. Complete laminectomies were performed to expose the spinal cord at T7–T12. SCI was achieved by 1-minute compression of the spinal cord at the T9 level horizontally and extradural with an aneurysm clip. The wounds were then closed with 3/0 silk suture. Within thirty minutes after the surgery, 200 µg/kg G-CSF provided by Kyowa Kirin Pharma, Tokyo, Japan has injected IV as a single dose in G-CSF treated groups for 3 consecutive days.

Postoperative care was performed by controlling the body temperature and prophylactic antibiotic administration to prevent infection (70mg/kg cephazolin for 7 days). SCI rats received manual bladder expression twice daily for 10–14 days until their bladder functions were fully recovered.

Following Wiseman *et al*. experience, we sacrificed rats after 4 weeks for histological studies to confirm SCI site of the spinal cord is correct (12).

Behavioral tests

Measurement of thermal hyperalgesia

Tail flick latency (TFL) was measured with Tail Flick Analgesia Meter (IITC life science model 33t, USA) prior to the surgery and at days 0, 7, 14, 21, 28. After a 45-min acclimatization period, TFL was measured by exposing the dorsal surface of the animal tail to a radiant heat source, and the time taken for the conscious rats to take out their tail from the noxious thermal stimulus was recorded. To reach proper baseline intensity, each control animal was given five test trials, and the strength of the stimulus was adjusted so that tail flick latencies would be between 7 to 8 s. We considered the cut-off time of 8s to prevent tail injury. The mean intensity level was then calculated and used in the following tail flick testing.

Cold allodynia (Acetone drop test)

After the 45-min acclimatization time, the response to cold stimulation was tested by spraying acetone to the plantar surface of the paw (2-3 s) from an estimated distance of 2 cm. The result was classified as 0, no response; 1, startle response without paw withdrawal; 2, brief withdrawal of the paw; 3, prolonged withdrawal (5–30 s); 4, prolonged and repetitive withdrawal (the 30s) along with flinching and/or licking (13). A significant increase in the scores of responses to acetone application was interpreted as cold allodynia (14).

Open field locomotor testing

Locomotor function was recorded using the Basso, Beattie, and Bresnahan (BBB) rating scale (Basso *et al*., 1995) to ensure the reliability of hindlimb somatosensory testing, as well as to assess the motor effects of delivered compounds. Briefly, the BBB is a 21-point ordinal scale ranging from 0, which is no discernable hindlimb movement, to 21, which is

consistent and coordinated gait with parallel paw placement of the hindlimb and consistent trunk stability. Scores from 0 to 7 rank the early phase of recovery with return of isolated movements of three joints (hip, knee, ankle); scores from 8 to 13 describe the intermediate recovery phase with return of paw placement, stepping, and forelimb-hindlimb coordination; and scores from 14 to 21 rank the late phase of recovery with return of toe clearance during the step phase, predominant paw position, trunk stability, and tail position.

Von Frey filament test

The hind paw withdrawal threshold was determined using von Frey hairs (Bioseb, USA), and was expressed in grams. Hairs ranging from 0.23 to 250 g were used. Testing was blind such that the experimenter was not aware of the kind of rat being tested. The protocol used in this study was a variation of that described by Takaishi *et al.,* (15). A testing session for a particular rat began after 5 min of habituation or as soon as the rat stopped exploring and appeared acclimatized to the testing environment. The series of von Frey hairs were applied from below the customized platform or the wire mesh to the plantar surface of the left hind paw in ascending order beginning with the lowest hair (0.23 g). The application was to the central region of the plantar surface avoiding the foot pads. A particular hair was applied until buckling of the hair occurred. This was maintained for approximately 2s.

The hair was applied only when the rat was stationary and standing on all four paws. A withdrawal response was considered valid only if the hind paw was completely removed from the platform. Although infrequent, if a rat walked immediately after application of a hair instead of simply lifting the paw, the hair was reapplied. On rare occasions, the hind paw only flinched after a single application; as the hind paw was not lifted from the platform, this was not considered a withdrawal response. A trial consisted of application of a von Frey hair to the hind paw five times at 5 s intervals or as soon as the hind paw was placed appropriately on the platform. If withdrawal did not occur during five applications of a particular hair, the next larger hair in the series was applied in a similar manner. When the hind paw was withdrawn from a particular hair either four or five times out of the five applications, the value of that hair in grams was considered to be the withdrawal threshold. Once the threshold was determined for the left hind paw, the same testing procedure was repeated on the right hind paw after 5 min. Second and third testing sessions were run for each of the left and right hind paws. If the withdrawal threshold in the second or third session did not match the withdrawal threshold of the previous testing session(s) in a given hind paw, the next larger hair in the series was tested. This was done until the withdrawal thresholds in three successive trials matched. Only hind paw withdrawal thresholds that remained consistent in the second and third successive trials in the control or cuff implanted rats were used in the data analysis. The baseline withdrawal thresholds of each of the hind paws using von Frey hairs were determined for each rat prior to surgical manipulation (day 0). Measurement of the paw withdrawal threshold was measured next to week 1 and then on weeks 2, 3 and 4.

Statistical analysis

For data analysis, one- way and two-way repeated measure analysis of variance was used followed by Tukey's post-test. $P<0.05$ was considered significant. All data are expressed as mean±SEM. SPSS version18 was used for analysis.

Results

In the start of the study, there was no significant difference between groups for all variables ($P>0.05$). After SCI surgery there was a weight loss in all SCI groups. In SCI+veh group it was more severe (Figure 1). There was no significant difference between the mean weight of SCI+veh group and mean weight of animals treated with 200 µg/kg G-CSF in weeks 2, 3, and 4.

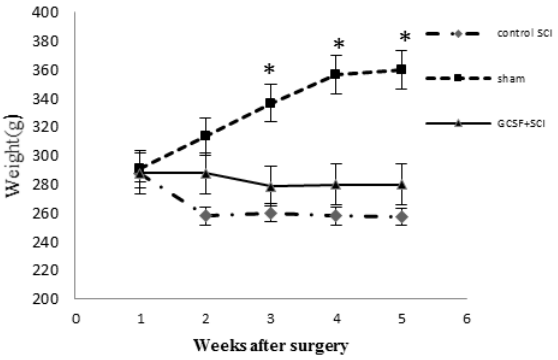

Figure 1. Influence of post-injury administration of G-CSF on rat's weight during 4 weeks of study

G-CSF+SCI indicates SCI rats treated with G-CSF (200 µg/kg i.v). SCI+veh indicates SCI rats received normal saline. The data are presented as mean± SEM (6-8 rats per groups). *: $P<0.05$ indicates a significant difference compared with other groups.

Tail flick latency

In general, in SCI rats, the responses to thermal stimuli (tail flick test) showed increased sensitivity relative to pre-surgical tests that were statistically significant (P<0. 001) (Figure 2). Mean tail flick latency in SCI groups significantly reduced one week after surgery, and it remained low until third week (P<0. 0001). In week 3 after surgery, this value showed a significant increase in the group treated with 200 µg/kg G-CSF (P<0. 001). In SCI +veh group, mean TFL decreased from the first week after surgery and continued to decline for 4 weeks. Mean TFL in the sham+veh group didn't change (Figure 2).

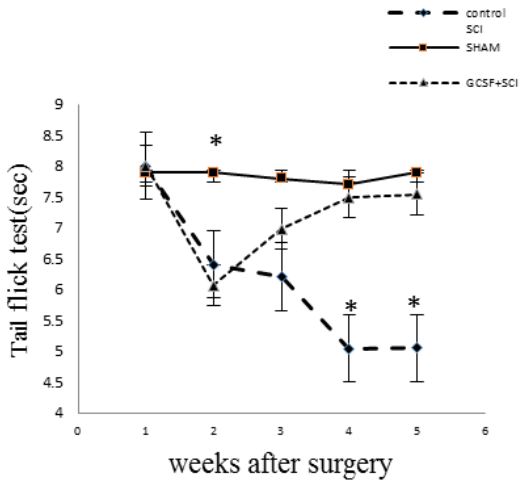

Figure 2. Influence of post-injury administration of G-CSF on the mean tail flick latencies of rats in 4 weeks duration of the study
G-CSF+SCI indicates SCI rats treated with G-CSF (200 µg/kg i.v). SCI+veh indicates SCI rats received normal saline. The data are presented as mean± SEM (6-8 rats per groups). *: P<0.05 indicates a significant difference compared with other groups

Cold allodynia

In SCI rats, the responses to cold stimuli (acetone drop on the paw) showed significantly increased sensitivity compared to pre-surgical tests (Figure 3) (P<0. 001). Mean acetone test score in SCI +veh group increased one week after SCI surgery, and it continued to increase in 4 weeks. About group treated with 200 µg/kg G-CSF, the score picked at the third week then it reduced in the fourth week and remained near the normal in the fifth week. In fourth and fifth weeks mean acetone test score in SCI+veh group was significantly higher than other groups (P<0.01), though differences between other groups were not statically significant (P>0.05) (Figure 3).

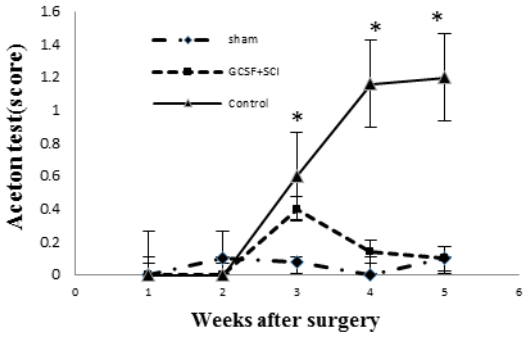

Figure 3. Influence of post-injury administration of G-CSF on the acetone test scores of rats in 4weeks duration of study
G-CSF+SCI indicates SCI rats treated with G-CSF (200 µg/kg i.v). SCI+veh indicates SCI rats received normal saline. The data are presented as mean± SEM (6-8 rats per groups). *: P<0.05 indicates a significant difference compared with other groups

BBB test

In SCI rats, a week after surgery, we witnessed a significant reduction in BBB score from 21 to 0 (Figure 4). Then they started to increase, and there was not any significant difference between SCI control and SCI treated group with 200 µg/kg G-CSF until the eighth week (P>0.05).

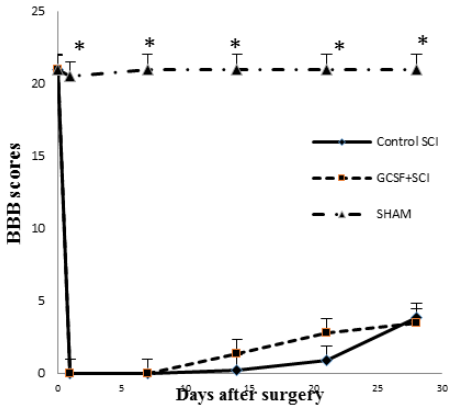

Figure 4. Influence of post-injury administration of G-CSF on the BBB test of rats in 4 weeks duration of the study
G-CSF+SCI indicates SCI rats treated with G-CSF (200 µg/kg i.v). SCI+veh indicates SCI rats received normal saline. The data are presented as mean± SEM (6-8 rats per groups). #: P<0.05 indicates a significant difference compared with other groups

Von Frey filament test

Prior to the SCI surgery, rats showed little to no paw withdrawal response to von Frey stimulation of over 250g filaments (Figure 5). Postoperatively, responses increased over time, and the increase was statistically

significant compared to intra-animal presurgical and sham control values (P<0.05). There was no significant difference between average values in control SCI groups, and G-CSF treated SCI group in any measurement (P>0.05).

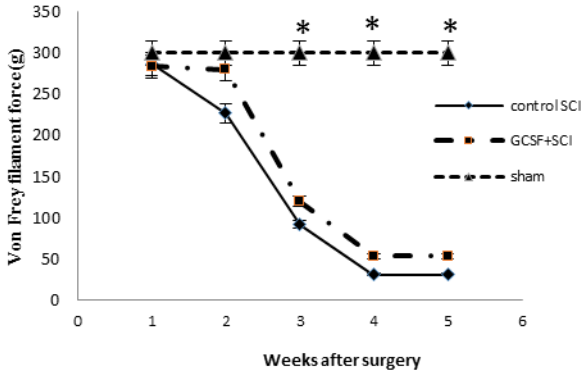

Figure 5. Influence of post-injury administration of G-CSF on the Von-Frey filament test of rats in 4weeks duration of the study G-CSF+SCI indicates SCI rats treated with G-CSF (200 µg/kg i.v). SCI+veh indicates SCI rats received normal saline. The data are presented as mean± SEM (6-8 rats per groups). *: P<0.05 indicates a significant difference compared with other groups

Discussion

The present study revealed that G-CSF could attenuate thermal hyperalgesia, cold allodynia, and locomotor activity but it has no effect on mechanical allodynia induced by SCI in an adult rat model. Previous studies have shown SCI causes chronic neuropathic pain (1,2,16,17).

Granulocyte-colony stimulating factor (G-CSF) that has originally been identified several decades ago, is a growth factor. As a hematopoietic factor, the clinical use of G-CSF is mainly for the generation of neutrophilic granulocytes . More recently, the role of G-CSF in the brain has been revealed as a growth factor for neurons and neural stem cells, and as a factor involved in the plasticity of the vasculature (18). G-CSF also mobilizes peripheral blood stem cells (PBSCs) derived from bone marrow. Transplantation of G-CSF-mobilized PBSCs has advantages for treatment of spinal cord injury in the ethical and immunological viewpoints (19). Studies showed exogenously applied G-CSF is effective in alleviating thermal hyperalgesia and mechanical allodynia in rats with CCI mainly through the activation of leukocyte-derived endogenous opioid secretion, downregulation of IL-6 and TNF-a inflammatory cytokines, and decreased microglial cell activation in the spinal dorsal horn (20).

The present study showed a moderate weight loss in SCI groups for two weeks, which was significantly lower in G-CSF treated group compared with SCI+veh group. It supposed to be due to the pain and disability after SCI, and complexity in food and water consumption. Two weeks after SCI, mean weight of SCI rats remained constant until the third week, and then it started to enhance afterward.

Analgesic and anti-hyperalgesic effects of G-CSF have been demonstrated in several studies (20,21). Formerly it has been shown that SCI rats have significantly lower TFLs compared with normal rats (22,23). Observing this fact in the present study, there was significantly higher mean tail-flick latency in G-CSF treated SCI group compared with SCI+veh group after 4 weeks which indicates lower heat hyperalgesia than that in SCI+veh group. Similar to the present study, a study showed that exogenously applied G-CSF is effective in alleviating thermal hyperalgesia in rats with CCI mainly through the activation of leukocyte-derived endogenous opioid secretion, downregulation of IL-6 and TNF-a inflammatory cytokines, and decreased microglial cell activation in the spinal dorsal horn (20). It is demonstrated that G-CSF inhibits inflammatory cytokines that contribute to inflammatory pain; and (24) G-CSF significantly decreases microglial cell activation in the spinal dorsal horn compared to vehicle treatment (20).

Similar to the previous studies showing SCI can cause cold allodynia (25), present results show after SCI, there was cold allodynia in SCI groups, which was attenuated in the fourth week. A previous study also showed exogenously applied G-CSF is effective in alleviating cold allodynia in rats with CCI (20).

Our data showed G-CSF significantly didn't improve the recovery of hind limb motor function in rats with a spinal cord compression lesion. However, another study confirms that G-CSF may be effective on locomotor recovery after SCI (26). They used rats with a balloon-induced spinal cord compression lesion and administered a subcutaneous injection of G-CSF from day 7 to 11 post-injury, but we administered single dose of 200 µg/kg G-CSF which was injected IV for 3 consecutive days. Also, another study showed that G-CSF significantly improves the rats' motor function after SCI (27). The administration route may be the origin of this difference in results. In their study, rats with SCI received a bolus of G-CSF via i.t. injection (10 µg/23 µL;).

Our study showed no significant effect of G-CSF on

mechanical hyperalgesia after SCI. However, one study showed attenuation of mechanical hyperalgesia (28), another one showed that G-CSF increases mechanical hypersensitivity after injection (29). In the first-mentioned study, they used intraperitoneal recombinant human G-CSF (15.0 μg/kg) for 5 days three weeks after SCI and reported that G-CSF attenuates mechanical allodynia induced by SCI in the contusive injury model. Three weeks after the injury, there were no significant differences between the average paw withdrawal threshold for rats in the vehicle and G-CSF groups. The administration of G-CSF caused a marked attenuation of mechanical allodynia (28). Difference in results shows the neccesity of further research in this area.

Several peripheral endogenous antinociceptive mechanisms are involved in counteracting inflammatory hyperalgesia (30-32). Under inflammatory conditions, leukocytes secrete opioid peptides that bind to opioid receptors on peripheral sensory neurons and mediate antinociception (33-36). G-CSF is potentially important for the development of the immune and nervous systems, but its' effects on neuropathic pain have not been fully elucidated. We suggest that high doses of G-CSF via different routes might be able to deliver its neuroprotective effects in vivo. Moreover, G-CSF treatment can increase the number of leukocytes (37) and enhance T-cell cytokine secretion (38,39). G-CSF also exerts some neuroprotective actions through the inhibition of apoptosis and inflammation as well as through the stimulation of neurogenesis (40), which may also contribute toward alleviating neuropathic pain. Alternatively, G-CSF modulates the micro-environment in inflammatory sites, including cytokine expression profiles, resulting in enhanced cell survival, proliferation, and differentiation into cells of the neural lineage of bone marrow-derived stem cells that migrate into the lesion site (41).

Whatever the mechanism may be, this study indicates that G-CSF could be a safe drug for antinociception. Endogenous G-CSF is usually generated at the site of inflammation and acts as an endocrine hormone to mobilize immune cells from the bone marrow to replace the inflammatory cells consumed in an inflammatory reaction.

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