Research Article

The combined effect of granulocyte-colony stimulating factor (G-CSF) treatment and exercise in rats with spinal cord injury

Chan-Hyuk Park¹, Kyung-Lim Joa¹, Mi-Ok Lee², Seung-Hwan Yoon³,
Myeong-Ok Kim¹

¹Department of Physical & Rehabilitation Medicine, School of Medicine, Inha University, Incheon, Republic of Korea, ²School of Medicine, Inha University, Incheon, Republic of Korea, ³Department of Neurosurgery, School of Medicine, Inha University, Incheon, Republic of Korea

Objective: To identify that the combined G-CSF and treadmill exercise is more effective in functional recovery after spinal cord injury (SCI).

Design: Rats were divided into 4 groups: a SCI group treated with G-CSF (G-CSF group, $n = 6$), a SCI group treated with treadmill exercise plus G-CSF (G-CSF/exercise group, $n = 6$), a SCI group with treadmill exercise (exercise group, $n = 6$), and a SCI group without treatments (control group, $n = 6$). We performed laminectomy at the T8–10 spinal levels with compression injury of the spinal cord in all rats. G-CSF (20 μ g/ml) was administered intraperitoneally for 5 consecutive days after SCI in G-CSF and G-CSF/exercise groups. From one week after surgery, animals in G-CSF/exercise and exercise groups received 30 min of exercise 5 days per week for 4 weeks. Functional recoveries were assessed using the Basso, Beattie, and Bresnahan (BBB) scale and the inclined plane test. Five weeks after SCI, hematoxylin and eosin staining for cavity size and immunohistochemistry for glial scar formation and neuro-regeneration factor expression were conducted.

Setting: Inha University School of medicine, Incheon, Korea

Results: Rats in G-CSF/exercise group showed the most effective functional recovery in the BBB scale and the inclined plane test, and spinal cord cavity size by injury were the smallest, and immunohistochemistry revealed expression of higher BDNF (brain-derived neurotrophic factor) and VEGF (vascular endothelial growth factor) and lower GFAP (glial fibrillary acidic protein) than others.

Conclusion: Combined treatment provided more effective neuroplasty and functional recovery than individual treatments.

Keywords: G-CSF, Spinal cord injury, Exercise, BDNF, VEGF, GFAP

Introduction

Spinal cord injury (SCI) destroys neural structures by causing cell death and inflammation, which subsequently trigger a progressive cascade that exacerbates the damage initially caused.¹ Apoptosis is a major component of such secondary injuries and represents a failure of functional recovery after SCI.^{1–3} Many researchers have studied motor function recovery using various treatments.^{4–10} G-CSF (granulocyte-

colony stimulating factor) and GM-CSF (granulocyte-macrophage colony stimulating factor) of treatments induce suppressing glial scar to lead to a failure to regenerate injured neurons because of a mechanical barrier and the productions of inhibitory molecules by astrocytes to express GFAP (glial fibrillary acidic protein) to form a dense network of glial scars.^{4–8,11} Additionally, others have studied immunohistochemical analysis such as BDNF (brain-derived neurotrophic factor) to support role in determining the level of functional recovery, and VEGF (vascular endothelial growth factor) which improves ischemic conditions such as vessel occlusions and decreased

Correspondence to: Myeong-Ok Kim, Department of Physical & Rehabilitation Medicine, School of Medicine, Inha University, Inha University Hospital, 27, Inhang-ro, Jung-Gu, Incheon, 222-332, Korea; Ph: +82-32-890-2480. Email: rmkmo@inha.ac.kr
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blood flow and restore motor function recovery after treatments in SCI.^{9,10,12}

G-CSF is a 19.6-kDa glycoprotein and a growth factor of hematopoietic progenitor cells,^{8,12} and has commonly used to treat neutropenia and mobilize bone marrow hematopoietic stem cells for transplantation.^{4,12,13} G-CSF induces several pathways in neurons that protect against apoptotic cell death.⁷ Recent studies have shown the presence of the G-CSF/G-CSF-receptor (G-CSFR) system promotes neural tissue repair and improves functional recovery.¹² It has also reported G-CSF can elevate tissue regeneration in brain,^{14,15} and that it significantly promoted motor function recovery in a rat model of SCI.^{7,16,17} Furthermore, those demonstrated that G-CSF could potentially substitute for GM-CSF in the treatment of SCI.⁴

Additionally, exercises, such as treadmill exercises^{9,18,19} and swimming,^{20,21} enhance motor recovery after SCI in rat models. Exercise has neuroprotective effects enhancing neurogenesis, increasing the expression of neurotrophic factors, and inhibiting apoptosis,¹⁹ and treadmill exercise has reported enhancing functional recovery from SCI.^{10,19,22} These results are that BDNF played an active role in mediating the improvement in motor recovery that occurs with appropriate rehabilitative training, and after SCI increased expression of angiogenic cytokine mRNA (VEGF) induced to promote motor function recovery.¹²

However, despite the effects of G-CSF and treadmill exercise treatments on functional recovery, no report has issued of the efficacy of combined G-CSF and exercise. Thus, we undertook to determine whether combined G-CSF and treadmill exercise treatment offers any function recovery advantage over either treatment alone with various assessments and expression of GFAP, BDNF, and VEGF.

Materials and methods

The SCI model

All animal experiments were performed in an authorized animal care facility at Inha University School of Medicine. Twenty-four male Sprague–Dawley rats (Orient Co.), each weighing 280–300 g, were randomly assigned to 4 groups, as follows: an SCI group treated with G-CSF (G-CSF group ($n = 6$)); an SCI group treated with G-CSF and treadmill exercise (G-CSF/exercise group ($n = 6$)); an SCI group that received treadmill exercise but not G-CSF (exercise group ($n = 6$)); and a sham-operated group that underwent SCI but was not treated with G-CSF or exercise (control group ($n = 6$)). All animals were anesthetized

Figure 1 (a) Experimental spinal cord compression injury in a rat showing vascular clipping at the T9 level. (b) Photograph of the treadmill used.

with ketamine (75–95 mg/kg i.p., Yuhan) and xylazine (5 mg/kg of Rompun, Bayer), and we performed a laminectomy at the T8–10 spinal levels and applied compression injury of the spinal cord using a vascular clip (Aesculap) at the T9 level of the spinal cord for 10 min using an occlusion pressure of 0.29–0.35 N in all rats (Fig. 1 (a)). G-CSF (JW Pharmaceutical, 20 µg/ml daily) was administered via intraperitoneally for 5 consecutive days after SCI in G-CSF and G-CSF/exercise groups. Following SCI, urinary bladders were emptied daily for one week. One animal died in each group within one week of surgery.

Training

During the two days before SCI, all 24 rats trained to walk on a treadmill. From one week after surgery, rats in G-CSF/exercise and exercise groups walked on the treadmill for 5 days per week for 4 weeks (Fig. 1 (b)). Rats trained on treadmill exercise for 30 min each day. The daily exercise involved three ten-minute sessions with a five-minute rest between sessions. Exercise speed was 35 rev/min during the first and second weeks and at 47 rev/min during the third to fifth weeks.

Locomotion analysis

Behavior analysis: Locomotor recovery

The 21-point BBB (Basso, Beattie and Bresnahan scale developed by Basso, Beattie, and Bresnahan) locomotion scale was used for the assessment of locomotion function.²³ The assessment was carried out on 1, 7, 14, 21, 28, and 35 days post-SCI. The rats placed in an

open field (40 × 40 cm) on a rough floor and we observed them for 5 min.

Inclined plane test

A rat placed on an inclined non-slip mat in a box (30 × 40 × 20 cm) with its head directed up the inclined plane. The angle of the mat increased to a level at which the rat was only just able to maintain its position for 5 seconds. The test conducted at 1, 7, 14, 21, 28, and 35 days post-SCI.

Hematoxylin and eosin staining (H&E) and spinal cord cavity sizes

Blinded evaluators performed histological examinations on randomized samples. Spinal tissue samples fixed in 10% formalin solution within 48 h, dehydrated through a graded ethanol series, cleared in xylene, and paraffin wax embedded using a routine protocol. The sections were deparaffinized and hydrated by sequential incubations in xylene and ethanol. After washed in distilled water for 5 min, tissue cross sections (4 μm) were stained sequentially with hematoxylin for 2 min and eosin for 15 s. We visualized the images on the slides with an Olympus BX40 light microscope.

Immunohistochemical analysis

4 μm thick cross sections cut in the paraffin block mentioned above. A deparaffinization protocol performed as heat slides in a dry oven at 60°C for 4 h, positioned to allow drainage of melting paraffin. Wash slides with xylene for 10 min, 3 times. Hydrate through an ethanol gradient ending with a distilled water wash. Slides fixed tissue in 10% neutral buffered formalin (NBF) for 20 min followed by a distilled water wash.

Antigen Retrieval (AR) / microwave treatment (MWT) at all cycles rinsed with antigen retrieval 9 (AR9) solution. Rinsed slides blocked with TBST. Glial Fibrillary Acidic protein monoclonal antibody (GFAP, 1:100, MAB360, Merck), VEGF monoclonal antibody (1:100, ab1316, abcam), and BDNF (1:200, ab108319, abcam) incubated for 60 min. Slides washed with 1 X TBST for 3 times at 2 min each. We applied Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at room temperature (RT). Slides were washed with 1X TBST for 3 times, 2 min each. Opal-520 working solution (1:100) for GFAP, Opal-570 working solution (1:100) for VEGF, and Opal-690 working solution (1:100) for BDNF to the tissue and incubated for 10 min at RT was applied, and slides washed with TBST 3 times, 2 min each. Microwave Treatment performed MWT as previously and allowed slides to cool down to room temperature on the bench for at least 15 min.

For spectral DAPI, slides were rinsed in distilled water and then in TBST, and incubated in DAPI solution for 10 min at RT. The slides washed with TBST for 2 min, and then with distilled water for 2 min. Mounting medium for fluorescence microscopy and coverslip applied. Slides were imaged using Vectra imaging software, and the number of pixels measured from the region of the lesion was analyzed using inForm analysis software (both from PerkinElmer) were used for statistic analysis between groups.

Statistical analysis

Quantitative data are presented as means ± standard deviations, and the statistical analysis was performed with SPSS version 22.0 (SPSS, Chicago, IL). The non-parametric Kruskal–Wallis test was used to identify statistical significance to values from BBB, values from the inclined plane test, and the number of pixels representing the expression of GFAP, BDNF, and VEGF measured from immunohistochemical analysis between each group. *Post hoc* analysis used the Mann–Whitney U test. The statistically significant P-values were considered as < 0.05 or < 0.01 (as indicated).

Results

Locomotor assessments using BBB scores

Higher BBB scores indicate better locomotory ability, and were obtained by testing 5 animals per group at 1, 7, 14, 21, 28, and 35 days post-SCI (Fig. 2). BBB scores increased more in G-CSF/exercise group than in the other 3 groups from 7 days post-SCI, and BBB scores were also higher in G-CSF group and exercise group than in control group from 7 days post-SCI. There was a statistically significant relationship between the treatment groups compared to the control group. However, there was no significant difference between G-CSF group and exercise group at any time point. At 5 weeks post-SCI, mean BBB scores in G-CSF, G-CSF/exercise, exercise, and control groups were 11.4 ± 0.9 , 13.8 ± 1.6 , 10.6 ± 1.3 , and 8.25 ± 0.9 respectively (Fig. 2, $P < 0.01$).

Inclined plane test results

Figure 3 showed Inclined plane test results. Mean plane angles increased rapidly in all four groups up to 2 weeks post-SCI, and then increased gradually. The three treatment groups had higher plane angle results from 7 days post-SCI than control groups, and rats in the G-CSF/exercise group managed significantly higher mean plane angles than rats in the other three groups.

Figure 2 The therapeutic activities of G-CSF, G-CSF/exercise, and exercise on SCI were shown with the BBB scale analysis. Rats in the G-CSF/exercise group showed the most effective recovery (** $P < 0.01$). There was no significant difference between G-CSF and exercise groups.

This result was statistically significant as compared with the control group as in the BBB score, and statistically significant as compared to the other treatment groups as well as G-CSF/exercise group. There was no significant difference between G-CSF group and exercise group at all points. At 5 weeks post-SCI, mean plane angles in G-CSF, G-CSF/exercise, exercise, and control groups were 41.0 ± 2.2 , 47.0 ± 4.5 , 41.0 ± 4.1 , and 31.3 ± 2.9 degrees respectively (Fig. 3, $P < 0.05$).

H&E and immunohistochemistry results

We performed histological examinations of injured spinal cords using BDNF on neurogenesis, VEGF on angiogenesis, and GFAP on glial cell formation at 5 weeks post-SCI. H&E results showed the destructive nature of the injuries, and G-CSF/exercise group had the smallest spinal cord cavities, and that in G-CSF group was smaller than those observed in exercise and control groups (Fig. 4).

Figure 3 The therapeutic activities of G-CSF, G-CSF/exercise, and exercise on SCI were shown with the inclined plane test. Rats in the G-CSF/exercise group showed the most effective recovery (* $P < 0.05$). There was no significant difference between G-CSF and exercise groups.

Figure 4 H & E stained spinal cord cross sections ($\times 20$) 5 weeks after the injury showed smaller cavity sizes in G-CSF/exercise group (b) than those in G-CSF group (a), exercise group (c) and control group (d). Triangles indicate cavities. Scale bar represents 500 μm . D: dorsal, V: ventral.

The result of immunohistochemical analysis at 5 weeks post-SCI in all groups (Fig. 5), the rats in G-CSF/exercise group had 2 times as many BDNF levels as those in G-CSF group and exercise groups. BDNF protein expression in G-CSF/exercise group was higher 7 times than that in the control group ($P < 0.05$). GFAP expression was strong in the control group, and G-CSF/exercise group showed the lowest levels. The administration of G-CSF and exercise suppressed approximately 70% and 90% of GFAP expression ($P < 0.01$, compared with the control group). Expression of VEGF in G-CSF and exercise groups approximately 10-fold and 20 fold higher than the control group, but and G-CSF/exercise group exhibited less expression than other treatment groups ($P < 0.01$). There was no significant difference between G-CSF group and exercise groups in all expressions, between G-CSF group and G-CSF/exercise groups in the expressions of BDNF and VEGF, and between the expression of BDNF and GFAP.

Discussion

We were experimented to show that combined G-CSF and exercise treatment is the most effective for motor function recovery. The result was that combined treatment made more improved motor function recovery than the individual treatment.

In BBB score and inclined planed test, we showed enhanced motor function improvement in the treatment groups compared to the control group, and the most effective restoring motor function in G-CSF/exercise group. The G-CSF/exercise treatment was more effective in restoring motor function than other treatments as well as the lowest GFAP expression on suppressive glial cell formation and great BDNF expression on neurogenesis than other treatments. Also, Consistent with the previous study,²⁴ the treatment groups showed more recovery from after 7 days post SCI. This study suggested that the period after 7 days post-SCI was essential for motor function recovery, and treatment was necessary for recovery. In other words, in early SCI, combined treatment may be more helpful in motor function recovery than any treatments. However, although cavity sizes were different in the G-CSF group and the treadmill exercise group, there was no significant intergroup difference in locomotion improvement. This result reported that total volume of lesional tissue related more than spinal cord cavity size in the previous study.²⁵

G-CSF group and G-CSF/exercise group showed more expression on BDNF and VEGF, and less expression on GFAP than the control group. G-CSF treatment described positive effects on functional recovery, axonal regeneration, and suppressive effects on glial scar formation because of affected axonal regeneration

Figure 5 Spinal cord tissues were immunostained for BDNF, VEGF, and GFAP 5 weeks after the injury. (a) BDNF, GFAP, and VEGF immunoreactivities in spinal cord tissues at 5 weeks after injury. Sections were imaged at $\times 200$ using a PerkinElmer Vectra. (b) Areas (pixels) of BDNF, GFAP, and VEGF immunostaining in each group were represented with inForm analysis software (both from PerkinElmer). The lowest GFAP level and the highest BDNF level were observed, although VEGF level in individual treatment groups was higher than those in the G-CSF/exercise group. Scale bar represents $118.7 \mu\text{m}$. * $P < 0.05$, ** $P < 0.01$.

and functional recovery by restricting the expressions of some chondroitin sulfate proteoglycans.⁴ Also, it was promoted expression of BDNF and VEGF in the injured spinal cord in the exercise group and G-CSF/exercise group. Exercise stimulated angiogenesis through the effects of VEGF on endothelial cell proliferation and vessel growth, and axonal regeneration through enhanced BDNF on neurotrophins exercise stimulated angiogenesis through the implications of

VEGF on endothelial cell proliferation and vessel growth, and axonal regeneration through improved BDNF on neurotrophins^{26,27} This also led to enhanced axonal sprouting at injury sites.¹⁹ These mechanisms might explain the better motor recovery in SCI and axonal regeneration in treatment groups than those in the control group. We also found that more reduction of cavity size in treatment groups than the control group by reduced glial scar formation. Because it is

involved in functional recovery after central nervous system injury, it is possible that the reduced glial scar formation in treatments groups resulted in better maintenance of the integrity of the axon fibers.⁴

In this study, we showed which combined G-CSF and exercise treatment at 5 weeks after SCI improved more locomotor recovery using BBB and inclined plane test than other treatments. The combined treatment induced better axonal regeneration with the smallest spinal cord cavity size by suppressive glial cell formation by the lowest GFAP expression and promoting the expressions of neurotrophic factors like BDNF. The result was that the interaction of G-CSF with exercise inhibited more effective glial cell formation, and combined treatment induced more distinguished neurogenesis than individual treatments. In other words, the functional recovery was secondarily boosted in the G-CSF group at a later time point. Our result demonstrated various mechanisms were supporting recovery after rehabilitation, including increased production and release of neurotrophic factors, changes in metabolic status and electrical properties, and reconstruction of neural circuits as the previous studies.²⁸ However, we found lower VEGF levels than those in the G-CSF or exercise groups, which differed from the results of a previous study.²⁹ we could explain our findings as G-CSF promotes angiogenesis first and stimulates neurogenesis later. Thus, neurogenesis was more critical than angiogenesis for motor function recovery, and combined treatment was more effective for motor function recovery because neurogenesis progressed faster than individual treatment. The Further experiment with changes in VEGF and BDNF overtime was necessary. As a result, Treadmill exercise plus G-CSF treatment was synergistic to facilitate functional recovery from SCI.

Conclusion

As in previous studies, we confirmed to promote motor function because the treatments as G-CSF and treadmill exercise suppress glial scar formation and enhance neuroplasticity and angiogenesis. G-CSF treatment with treadmill exercise provides a more effective means of promoting motor recovery than either treatment alone.

For clinical on G-CSF, the intraperitoneal injection should be considered carefully, especially when and how. The reason is that intraperitoneal injection of G-CSF immediately after injury is not applicable in clinical cases. The optimized protocol-based research should be performed to study the clinical applications in humans.

Concerning the limitations of the present study, its scale limited, and larger-scale study is needed to confirm our findings. The BDNF levels and VEGF levels did not completely represent neuroplasticity and angiogenesis, and to establish axonal sprouting, furthermore necessary immunochemistry study need. The limitation of the analysis should be acknowledged and discussed.

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Disclaimer statements

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Conflict of interest No author had any conflict of interest in the completion of this work.

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