Induction of endogenous neural stem cells by extracorporeal shock waves after spinal cord injury

Dong-Chul Shin, MD, Kee-Yong Ha, MD, Young-Hoon Kim, MD, PhD, Jang-Woon Kim, MD, Yoo-Kyung Cho, MD, Sang-II Kim, MD
Department of Orthopedic Surgery, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea.

Address correspondence and reprint requests to
Young-Hoon Kim, M.D., Ph.D.
Department of Orthopedic Surgery, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea.
222 Banpo-Daero, Seocho-Gu, Seoul, 137-701, Korea
Tel; 82-2-2258-6118
Fax: 82-2-505-9834
E-mail: boscoa@catholic.ac.kr

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Abstract

**Study Design:** Animal experimental study

**Objectives:** The purpose of this study is to investigate the effects of extracorporeal shock waves (ESWs) on endogenous neural stem cells (NSCs) proliferation after spinal cord injury (SCI).

**Summary of Background data:** Exogenous stem cell transplantation for SCI still has many limitations to be addressed such as ideal cell sources, timing of transplantation, and fate of the transplanted cells. Moreover, the efficacy is another issue due to a peculiar pathologic condition in the chronic phase of SCI.

**Methods:** Contusive SCI was made using 24 Sprague-Dawley rats, and ESWs were applied at post-injury 4 weeks in rats. Proliferation and differentiation of endogenous NSCs (DCX, Sox-2) and axonal sprouting (GAP-43 and MAP-2) were observed at 6 weeks after application of ESWs. Differentiation of the activated neural stem cells was also investigated by co-expression of neuronal/glial cell markers (GFAP, Neu N and CC-1). Immunofluorescence staining and western blotting were performed for quantitative analysis, and these results were compared with those in the control group. For clinical assessment, the BBB locomotor rating scale was performed.

**Results:** More proliferation of endogenous neural stem cells was noted in the experimental groups, and these activated cells were mainly founded in the ependymal layer of the central canal and the injured posterior horn. Differentiation into neuronal and glial cells was also noted in a limited number of cells. With respect to axonal regeneration, GAP-43 and MAP-2 expressions in the experimental groups were also significantly higher than those in the control group. During 6 weeks clinical observation following ESWs application, functional improvement of the hindlimb was observed without clinical deterioration by trials.

**Conclusions:** Collectively, these findings indicate that ESWs on the chronic phase of SCI induce activation of endogenous NSCs and consequent functional improvement.

**Key words:** Spinal cord injuries, endogenous neuronal stem cells, Shock waves

**Level of Evidence:** N/A
INTRODUCTION

Cell therapy for regeneration following spinal cord injury (SCI) has been attempted in various ways. However, diverse results preclude active clinical application of these approaches, and several issues such as the type of the transplanted cells, timing and their fate in the host environment still need to be addressed.\(^1\)\(^-\)\(^3\) Recently, alteration of the microenvironment or activation of endogenous stem cells has also been attempted as one of the neuro-regenerative therapies in trials.\(^4\)\(^-\)\(^6\) And these promising results could present a new therapeutic approach without the possible complications of exogenous cell transplantation.\(^7\)

Since the time neural stem cells (NSCs) have been discovered in the hippocampus and the sub-ventricular zone of the forebrain, the possibility of the regenerative potency of the central nervous system (CNS) was raised. Similar to the brain, the presence of NSCs in the spinal cord has also been researched. Also in several studies, some cells lining the central canal of the spinal cord, consisting of the ependymal layer, were found to have the regenerative properties in several studies.\(^8\)\(^,\)\(^9\) Under normal physiologic conditions in adults, these cells are quiescent, and their main physiologic role is to maintain the function of the central canal. However, some pathologic conditions such as SCI cause activation of the stem cell properties of ependymal cells and multi-lineage differentiation into various glial cells. Therefore, alteration or modulation of these endogenous neuro-regeneration processes could provide a new therapeutic approach. Although some experimental studies have shown the possibility of clinical use of endogenous NSCs recruited from the ependymal cells, information on the fate of activated endogenous NSCs is still lacking. Corresponding evidence for induction of these cells into more functional cells such as oligodendrocytes or neurons is also crucial.

Especially, the chronic phase of SCI is characterized by a fixed gliosis which consists of reactive proliferation of astrocytes. This process is the initial natural reaction following injury to the CNS. It prevents propagation of the initial injury and aberrant synapse
formation. However, it also has negative effects on regeneration. Several experimental trials have been performed to overcome this limitation of the chronic phase of SCI and to enhance the effects of regenerative trials.\textsuperscript{10,11} As one of these trials, extracorporeal shock waves (ESWs) have been used for the chronic phase of SCI. Enhancing the regenerative process by regulating inflammation or inducing neo-vascularization has been suggested as the main mode of action of ESWs in the medical field.\textsuperscript{12,13} Recently, alteration of the micro-environment and enhancement of engraftment of the transplanted cells in the chronic phase of SCI have been shown in an experimental study using ESWs.\textsuperscript{14} In this study, the authors showed higher expression of stromal derived factor-1 and more engraftment of the intravenously transplanted cells at the injured site. Also, the authors suggested that the alteration of the fixed condition in the chronic phase of SCI by ESWs may be related to these findings. In the present study, we investigated the effects of ESWs on the activation/proliferation of endogenous NSCs in the chronic phase of SCI.

**MATERIALS AND METHODS**

**SCI model and group allocation**

A chronic contusive SCI model was made using 24 Sprague-Dawley rats divided into four groups (6 rats in each group). All animals were kept under standardized conditions (2 rats / cage, 20-24°C, 45-65% humidity and 12 hr of daily light) and given free access to standard food and drinking water. The rats were anesthetized with intraperitoneal injection of ketamine (50 mg/kg) and rompun (2 mg/kg) after body weight measurement. Briefly, their backs were shaved and sterilized with antiseptic betadine. Preoperatively, 5 mg gentamycin was administrated intramuscularly. The spinal cords of the rats were exposed by laminectomy at T9 after exposure of the paravertebral muscles from T8-10. Spinal cord injury was induced
by a 25 g-cm contusion using the MASCIS (Multicenter Animal Spinal Cord Injury Study) impactor (a rod weighing 10g and dropped from a height of 2.5 cm). Postoperatively, 5 mg ketoprofen was administered for 3 days, and the bladder was manually emptied during the experiment. At post-injury 4 weeks, rats were randomly assigned to one of the following four groups; (1) the group I (n=6, SCI + ESWs 500 impulses), (2) the group II (n=6, SCI + ESWs 1000 impulses), (3) the group III (n=6, SCI + ESWs 1500 impulses) and (4) the group IV (n=6, control, SCI only). ESWs were applied at the 0.04 mJ/mm² energy level using the Swiss Dolorcast (EMS, Lyon, Switzerland) with a 10-mm focus application. This energy level was determined based on the findings of the previous study. After observation of neurological improvement using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for 6 weeks after application of ESWs, the rats were sacrificed for laboratory analyses (4 rats for protein analysis and 2 rats for histological analysis in each group).

**Tissue Sampling and Analysis**

Injured spinal cords were extracted and prepared as previously described. Briefly, with respect to tissue extraction for western blot analysis, all injured tissues (~1-cm length) were extracted by a tissue protein extraction reagent (ThermoScientific, Rockford, IL) with 1 protease inhibitor cocktail tablet (Roche Diagnostics, IN, USA). Initially, the lysate was centrifuged at 16,000 rpm for 10 min at 4°C, and afterwards, it was centrifuged at 16,000 rpm for 20 min and the supernatant liquid from samples was separated by SDS-polyacrylamide gel electrophoresis, and later, it was transferred to a nitrocellulose blotting membrane (GE Healthcare LifeScience, Freiburg, Germany) for 1 hr at 4°C. The membranes were blocked with 3% bovine serum albumin for 1 hr at room temperature, and then, they were probed with primary antibodies for 24 hr at 4°C and secondary antibodies for 1 hr at room temperature. Measurement of protein expression was performed with antibody detection system ECL.
(Thermo scientific), and the membrane were exposed to LAS 4000 (BioRad, Hercules, CA). Quantification of protein band intensity was performed by using the multi gauge V 3.0 software (Fuji photo film, Tokyo, Japan) was used.

After sampling and histological analysis, each injured spinal cord was obtained after trans-cardiac perfusion at post-ESWs application 6 weeks. Tissues were fixed overnight in 4% paraformaldehyde and 15, 30% sucrose consequently. Tissues were embedded in optimal cutting temperature compound (OCT, Tissue-Tek), and axially and longitudinally sectioned at 5µm thickness. To detect proliferation, differentiation, and regeneration in the injured tissue, immunofluorescence (IF) staining was performed. Positive cells with co-localization with DAPI, and each antibody markers (green and red) under low- (x70, 200) and high-power (x400, 1000) magnification was confirmed using a LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany). Fluorescence intensity was recorded at emission wavelength of 488 and 543 nm in the region of interest (ROI, 50625 x50625) under x400 magnification. The devices used for evaluation was Axiovert 200 fluorescence microscope (Zeiss, Germany) and panoramic MIDI slide scanner (3DHISTEC Ltd., Budapest, Hungary).

**Proliferation, Differentiation and Axonal Sprouting**

Proliferation of endogenous neural stem cells in the injured spinal cord was evaluated using IF staining and western blot at post-injury 10 weeks. DCX-2 and Sox-2 markers were used to investigate proliferation of endogenous neuronal stem cells. Positive cells that showed co-localization by expressing DAPI and NSCs markers (DCX and Sox-2) under high power magnification were confirmed using a confocal microscope. Western blot was also performed to investigate the expression of these markers in the injured spinal cord. Additionally fluorescence intensities in the region of interest of each specimen were also obtained for quantitative analysis (4 tissues from each group). Differentiation of endogenous neural stem
cells (DCX, Sox-2 positive cells) were evaluated using double IF staining with neuronal (NeuN), astrocyte (GFAP) and oligodendrocyte (CC-1) markers. Axonal sprouting was evaluated by the expression of GAP-43 and MAP-2 markers in the axial and longitudinal sections at the epicenter of the injured spinal cord. The protein expression of markers of axonal sprouting was evaluated via western blot. IF staining and western blot were performed in accordance with the procedures described in the previous studies\textsuperscript{1,2} and details regarding primary antibodies are summarized in Table 1.

**Clinical Behavioral Outcomes**

The Basso, Beattie, and Bresnahan (BBB) locomotor rating scale were recorded every week. Two researchers independently recorded the BBB rating scale scores after a 2-minutes observation. All data for each week were recorded and analyzed.

**Statistical analysis**

All results presented in the figures and text is expressed as means ± S.E.Ms. The results were analyzed by Kruskal-Wallis analysis followed by Mann-Whitney analysis for an inter-group comparison. A $P$ value of less than 0.05 was considered statistically significant.

**RESULTS**

**Expression of DCX in the injured spinal cord**

DCX positive cells were mainly located in the ependymal cells of the central canal, and scanty DCX positive cells were also found in the posterior horn at the epicenter of the
spinal cord in all groups (see figure S1 in supplementary appendix for comparison with the normal spinal cord). A greater number of DCX positive cells in the experimental groups was noted on the IF staining. Western blot also showed a significantly higher expression of DCX in the injured tissue of rats in the experimental groups (relative optical densities by western blot in group I: 0.12±0.01, group II: 0.12±0.03 and group III: 0.12±0.04) compared to that in the control group (0.07±0.02) (P<0.05) (Figure 1A-C). However, there was no significant difference among the experimental groups. Fluorescence intensities in the ROI were as follows: group I: 254.2±763, group II: 328±43.5, group III: 379.8±17.5 and control group: 169.4±53.2. Although fluorescence intensities in the experimental groups were higher than that of the control group, there was no statistically significant difference among the experimental groups.

Expression of Sox-2 in the injured spinal cord

Sox-2 expression was also noted in the ependymal cells of the central canal; however, there were less number of Sox-2 positive cells in the other areas of the injured spinal cord (see figure S2 in supplementary appendix for comparison with the normal spinal cord). A higher number of Sox-2 positive cells were found in the experimental groups than in the control group. Western blot for Sox-2 also showed significantly higher expression in the experimental groups (group I: 0.07±0.01, group II: 0.07±0.02 and group III: 0.05±0.01) as compared to the control group (0.03±0.004) (P<0.05). There was no significant difference among the experimental groups (Figure 2A-C). Fluorescence intensities of Sox-2 in the ROI were as follows: group I: 93.4±36.2, group II: 248.5±30.4, group III: 209.6±1.31 and control group: 52.2±23.9. Only the fluorescence intensity of Sox-2 in group II showed a statistically significant difference when compared to that in the control group (P<0.05).
**Differentiation of activated NSCs**

On the co-staining using neuronal and glial markers for differentiation of activated sox-2 positive cells, co-expression of GFAP was commonly observed in all groups. However, some NeuN and CC-1 co-positive cells were also noted around the ependymal layer of the central canal. Quantitative analysis was not performed as the number of these positive cells was limited (Figure 3A-C) (magnified images are provided as supplementary appendix).

**Axonal sprouting**

More profound expression of GAP-43 was noted in the experimental groups compared to the control group on IF staining in both the axial and longitudinal examination. Western blot also showed a higher expression of GAP-43 in the experimental groups (group I: 0.75±0.02, group II: 0.69±0.1, group III: 0.55±0.08) than in the control group (0.34±0.08) \((P<0.05)\) (Figure 4AB). Another examination using MAP-2 showed more axonal sprouting in the experimental groups (group I: 0.11±0.02, group II: 0.08±0.01) than in the control group (0.04±0.01) \((P<0.05)\) (Figure 5AB). However, on western blot analysis, group III (0.05±0.01) did not show a significantly higher expression compared to that in the control group.

**Clinical outcomes**

At the time of application of ESWs, the BBB locomotor rating scale scores did not show any significant differences among the groups (group I: 1.14±1.46, group II: 1.14±1.21, group III: 1±1.07, control group: 0.71±0.76). However, the experimental groups (group I: 3.67±2.73, group II: 3±2.97, group III: 3.83±2.14) showed a significant improvement in the BBB locomotor rating scale scores compared to the control group (1.17±0.98) at the post-injury 10 weeks \((P<0.05)\) (Figure 6).
DISCUSSION

There is a growing body of neuro-regenerative research, which has been performed to overcome SCI. Although promising results of clinical trials using transplantation of various stem cells have been reported, many prerequisites still need to be addressed for their safe and effective clinical application. To overcome these limitations of exogenous cell transplantation, endogenous cell proliferation or alteration of the microenvironment has been attempted for treating SCI.\textsuperscript{4,6,15} Ependymal cells of the central canal which are descended developmentally from embryonic neuroepithelial cells and located in the ventral neural tube are a reservoir of these endogenous stem cells for the spinal cord. Although the regenerative potency of ependymal cells is relatively low in mammals compared to lower vertebrates, recent studies have shown reactive proliferation and expression of neural stem cell properties following SCI.\textsuperscript{8,9,16} In general, the chronic phase of SCI is characterized by reactive astrocytosis, glial scar formation and low neuro-regenerative potency. This fixed condition is mostly responsible for limited regeneration following injury to the central nervous system. Alteration of the microenvironment or induction of endogenous neural stem cells has also studied to overcome these issues arising from exogenous cell transplantation.\textsuperscript{7}

In a previous study, enhanced engraftment of the cells intravenously transplanted into the injured spinal cord by ESWs has been shown in the chronic model of SCI.\textsuperscript{14} Alteration of the microenvironment with enhanced expression of stromal derived factors or neurotrophic factors has been suggested as the mechanism for these findings. Besides this first ESW trial for SCI using ESWs, enhanced regeneration of peripheral nerve and neuro-modulatory effectshave also been reported in experimental trials of ESWs.\textsuperscript{13,17} Even though the mechanism contributing to the regenerative effects of ESWs is not clear at present, several mechanisms have been proposed. Neovascularization, anti-inflammatory effect and induction of growth factors have been demonstrated following application of ESWsto diverse tissues.\textsuperscript{18-20} These
findings can be explained by the concept of mechano-transduction, which means the conversion of physical forces into biochemical signals under microenvironmental conditions. Alteration of the cellular mechanism and cellular signaling by physical forces has been proposed as the mechanism. In this study, significant induction of endogenous neural stem cells originating from the central canal was observed in the experimental groups. Moreover, increased axonal sprouting was also noted. These findings could provide experimental evidence that some physical force such as ESWs might induce alteration of the microenvironment in the chronic phase of SCI. As there was no sufficient evidence for not only possible mechanism but the proper energy level for ESWs on spinal cord, we tried using various frequencies of ESWs in this experiment. And there were no significant differences in proliferation of endogenous NSCs between the experimental groups. However, further studies including long-term observation and various assessments for its efficacy and safety should be needed for more relevant evidences and clinical trials.

In terms of the fate of induced endogenous neural stem cells, the major differentiation pathway of the activated ependymal cells is known to be an astrocyte despite the multilineage potential of these cells in vitro. Similar to this report, astrocyte differentiation was mainly observed in our study even though some cells expressing NeuN and CC-1 along neural stem cell markers were noted. However, as restoration of the neural circuit and remyelination are considered to be the desirable means of regeneration in trials, only activation of ependymal cells in the central canal could not catch up with functional restoration. Therefore, some pharmacological or genetic engineering methods should be attempted to induce specific differentiation of the recruited ependymal-derived neural stem cells. Indeed, pharmacological manipulation (intraventricular administration of pro-oligogenic factors) of the adult brain demonstrated enhanced oligodendrocyte differentiation of neural stem cells. Activation of the oligodendrocyte lineage-specific genes such Olig 1 and Olig 2 in the
activated ependymal cells could provide another means of specific differentiation. Therefore, combination approaches that induce recruitment of the ependymal-derived neural stem cells from the central canal and manipulation of the fate of these activated cells could provide more effective therapies. In one of these trials, ESWs showed the possibility of use as a non-invasive tool for re-activation of the ependymal cells in the chronic phase of SCI.

In addition, this study could not provide sufficient data for possibility of clinical application. ESW-treated rats regained motor function before cell proliferation becomes evident in the spinal cord. Although this might imply some mechanism other than spinal cord regeneration by endogenous neural stem cell proliferation, we think this could be solved by more sophisticated measurement tool than BBB and consecutive analysis for histologic changes after ESWs. However, experimental application of ESWs on the chronic phase of SCI induced some microenvironment changes in the fixed condition, and confirmed re-activation of neural stem cells from the ependymal cells. Further studies on combined approaches which lead to specific differentiation of the activated cells could provide more relevant evidences for a new regenerative method; thus, avoiding the risk of exogenous cell transplantation.
REFERENCES


FIGURE LEGENDS

Figure 1. Enhanced expression of the neural stem cell marker, Doublecortin (DCX) in the injured area.

(A) Immunofluorescence image of the epicenter. A higher DCX (green) expression was noted in the extracorporeal shock wave-applied groups compared to the control group. DCX expression was mostly noted around the ependymal cell layer and the posterior part of the injured spinal cord.

(B) Western blot showed higher expression of DCX in the experimental groups compared to the control group with statistical significance. However, there was no difference between the experimental groups (different impulses of ESW, n=4 for each group).

(C) Fluorescence intensities in the region of interest indicated a similarly higher expression with little statistical significance (n=4 tissues for each group).
Figure 2. Enhanced expression of the neural stem cell marker, Sox-2 in the injured area.

(A) Immunofluorescence image of Sox-2 expression (green) at the epicenter. A higher expression was noted in the ESW-applied groups compared to the control group. Co-stained cells were mostly found in the ependymal cells. However, scanty positive cells were also noted around the central canal of the spinal cord.

(B) Western blot showed a higher expression of Sox-2 in the experimental groups compared to the control group with statistical significance. However, there was no difference between the experimental groups (different impulses of ESW, n=4 for each group).

(C) Fluorescence intensities in the region of interest indicated a similarly higher expression with little statistical significance (n=4 tissues for each group).
Figure 3. Differentiation of activated neuronal stem cells into functional cells.

Co-staining was performed to observe differentiation of activated neural stem cells.

(A) NeuN was used as a neuronal marker, and Sox-2 and NeuN positive cells were noted around the ependymal cell layer. Although the control group showed co-stained cells, the experimental groups showed a higher numbers of positive cells (quantitative analysis was not performed).

(B) CC-1 was used as an oligodendrocyte marker, and Sox-2 and CC-1 positive cells were noted around the ependymal cell layer in the experimental groups.

(C) GFAP was used for an astrocyte marker, and Sox-2 and GFAP positive cells were noted around the ependymal cell layer in the experimental groups. The experimental groups showed a higher number of positive cells compared to the control group.

Box indicates the magnified region of interest.
Figure 4. Axonal sprouting in the injured spinal cord.

(A) Immunofluorescence staining using GAP-43 (red) showed more axonal sprouting in the epicenter of the injured spinal cord in the experimental groups than in the control.

(B) Western blot for GAP-43 in the injured tissue showed a higher expression in the experimental groups than in the control group with a statistically significant difference (n=4 for each group).

Box indicates the magnified region of interest. A and L indicate the axial and longitudinal examinations.* indicates statistically significant differences.
Figure 5. Axonal sprouting in the injured spinal cord.

(A) Immunofluorescence staining using MAP-2 (red) showed more axonal sprouting in the epicenter of the injured spinal cord in the experimental groups than the control group.

(B) Western blot for MAP-2 in the injured tissue showed a higher expression in the experimental groups than in the control group with a statistically significant difference (n=4 for each group).

Box indicates the magnified region of interest. A and L indicate the axial and longitudinal examinations. * indicates statistically significant differences.
Figure 6. Clinical observation of the hindlimb function.

During the whole experimental period, the BBB locomotor rating scale scores were used for clinical observation. At the time of ESW application, there were no statistically significant differences among the groups. However, a significant improvement in the BBB scores was noted in the experimental groups compared to the control group at post-injury 10 weeks (post-ESW 6weeks, n=6 for each group).
### Table 1 List of antibodies, sources and dilutions

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<th>Primary antibody</th>
<th>Host</th>
<th>Dilution</th>
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Dilution: * Dilution of immunofluorescence staining  
† Dilution of western blot
Figure S1
Figure S2

[Image of five groups labeled Group I, Group II, Group III, Control, and Normal. Each group shows a different pattern or structure.]

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