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LABORATORY INVESTIGATION

Low-energy extracorporeal shock wave therapy for promotion of vascular endothelial growth factor expression and angiogenesis and improvement of locomotor and sensory functions after spinal cord injury

Kenichiro Yahata, MD, PhD,[1] Haruo Kanno, MD, PhD,[1] Hiroshi Ozawa, MD, PhD,[1] Seiji Yamaya, MD, PhD,[1] Satoshi Tateda, MD, PhD,[1] Kenta Ito, MD, PhD,[2] Hiroaki Shimokawa, MD, PhD,[2] and Eiji Itoi, MD, PhD[1]

Departments of [1]Orthopaedic Surgery and [2]Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

OBJECTIVE Extracorporeal shock wave therapy (ESWT) is widely used to treat various human diseases. Low-energy ESWT increases expression of vascular endothelial growth factor (VEGF) in cultured endothelial cells. The VEGF stimulates not only endothelial cells to promote angiogenesis but also neural cells to induce neuroprotective effects. A previous study by these authors demonstrated that low-energy ESWT promoted expression of VEGF in damaged neural tissue and improved locomotor function after spinal cord injury (SCI). However, the neuroprotective mechanisms in the injured spinal cord produced by low-energy ESWT are still unknown. In the present study, the authors investigated the cell specificity of VEGF expression in injured spinal cords and angiogenesis induced by low-energy ESWT. They also examined the neuroprotective effects of low-energy ESWT on cell death, axonal damage, and white matter sparing as well as the therapeutic effect for improvement of sensory function following SCI.

METHODS Adult female Sprague-Dawley rats were divided into the SCI group (SCI only) and SCI-SW group (low-energy ESWT applied after SCI). Thoracic SCI was produced using a New York University Impactor. Low-energy ESWT was applied to the injured spinal cord 3 times a week for 3 weeks after SCI. Locomotor function was evaluated using the Basso, Beattie, and Bresnahan open-field locomotor score for 42 days after SCI. Mechanical and thermal allodynia in the hindpaw were evaluated for 42 days. Double staining for VEGF and various cell-type markers (NeuN, GFAP, and Olig2) was performed at Day 7; TUNEL staining was also performed at Day 7. Immunohistochemical staining for CD31, α-SMA, and 5-HT was performed on spinal cord sections taken 42 days after SCI. Luxol fast blue staining was performed at Day 42.

RESULTS Low-energy ESWT significantly improved not only locomotion but also mechanical and thermal allodynia following SCI. In the double staining, expression of VEGF was observed in NeuN-, GFAP-, and Olig2-labeled cells. Low-energy ESWT significantly promoted CD31 and α-SMA expressions in the injured spinal cords. In addition, low-energy ESWT significantly reduced the TUNEL-positive cells in the injured spinal cords. Furthermore, the immunodensity of 5-HT–positive axons was significantly higher in the animals treated by low-energy ESWT. The areas of spared white matter were obviously larger in the SCI-SW group than in the SCI group, as indicated by Luxol fast blue staining.

CONCLUSIONS The results of this study suggested that low-energy ESWT promotes VEGF expression in various neural cells and enhances angiogenesis in damaged neural tissue after SCI. Furthermore, the neuroprotective effect of VEGF induced by low-energy ESWT can suppress cell death and axonal damage and consequently improve locomotor and sensory functions after SCI. Thus, low-energy ESWT can be a novel therapeutic strategy for treatment of SCI.

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KEY WORDS spinal cord injury; extracorporeal shock wave therapy; vascular endothelial growth factor

ABBREVIATIONS BBB = Basso-Beattie-Bresnahan; ESWT = extracorporeal shock wave therapy; PBS = phosphate-buffered saline; SCI = spinal cord injury; VEGF = vascular endothelial growth factor.

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Secondary neural tissue damage after spinal cord injury (SCI) is caused, in part, by ischemia, cellular and tissue edema, and oxidative damage.[19] Compromised blood flow, hemorrhage, cord compression, intravascular thrombosis, and vasospasm induce ischemia, which initiates events that counteract oxygenation, nutrition delivery, and angiogenesis.[63] Secondary neural tissue damage worsens neurological symptoms following SCI.[69] Recent studies have shown that angiogenesis plays a critical role in recovery after SCI.[50] Reducing blood loss, promoting new blood vessel formation, and restoring blood supply to the lesions may contribute to reduction of the secondary neural damage and to recovery from SCI.[13]

Extracorporeal shock wave therapy (ESWT) was first applied to a patient to break up kidney stones in 1980.[8] Shock wave treatment has previously been clinically established as an effective and safe treatment for lithotripsy and chronic plantar fasciitis.[2,52,67] Application of shock waves can induce cavitation (a micrometer-sized violent collapse of bubbles) in the cells.[1] The physical force generated by the cavitation produces localized shear stress on cell surface membranes.[15] The stress to the cells caused by the shock wave may cause various biochemical effects.[9,17,38,39,55,61,66] Low-energy ESWT has been shown to increase vascular endothelial growth factor (VEGF) expression in ischemic tissues in vivo and to promote angiogenesis and functional recovery in models of chronic myocardial ischemia, myocardial infarction, and peripheral artery disease.[16,27–29,33,41,45,57,65] VEGF has been shown to be a potent stimulator of angiogenesis and to affect blood vessel permeability modulated by vascular permeability factor[3,11] via the phosphotyrosine kinase receptors Flt-1 and Flk-1 (VEGF-R1 and -R2).[49,68]

Previous studies have demonstrated the therapeutic potential of VEGF in treating SCI.[9,32,53,58] Administration of a transcription factor engineered to increase VEGF expression suppressed axonal degeneration and apoptosis and promoted vascularity in a model of SCI.[32] In addition, administration of recombinant VEGF increased the amount of spared tissue and blood vessels and reduced cell death and locomotor impairment after SCI.[58] On the other hand, endogenous expression of VEGF in injured spinal cord has been shown to significantly decrease after SCI.[19] A neuroprotective effect of VEGF has been suggested by Oosthuyse et al.,[46] who demonstrated that deletion of the hypoxia-response element in the VEGF promoter caused adult-onset progressive motoneuron degeneration. We have previously demonstrated that low-energy ESWT significantly increased expressions of VEGF and Flt-1 in the spinal cord without any detrimental effect. Our previous study showed that ESWT significantly increased the expression of VEGF at 7 days after SCI.[59]

In our previous study, low-energy ESWT significantly reduced neuronal loss in damaged neural tissue and improved locomotor function after SCI. These results demonstrated that low-energy ESWT enhanced the neuroprotective effect of VEGF and led to locomotor recovery after SCI.[59] However, the effects of low-energy ESWT on the cell specificity of VEGF protein expression and angiogenesis remain unknown. The neuroprotective mechanism in the injured spinal cord and the therapeutic effect on sensory function produced by low-energy ESWT also are unclear. The purpose of this study was to investigate the effect of low-energy ESWT on angiogenesis and cell specificity of VEGF expression in the injured spinal cord. We also examined the neuroprotective effects of low-energy ESWT on neural cell death, axonal damage, and white matter sparing as well as a therapeutic effect for the improvement of sensory function after SCI.

Methods

Experimental Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Tohoku University. All efforts were made to minimize the number of animals used and to decrease the suffering of the animals used in the study.

A total of 60 adult female Sprague-Dawley rats (body weight, 250–300 g) were used (CLEA Japan). The rats were randomly divided into the following 2 groups: the SCI group (SCI only) and the SCI-SW group (low-energy ESWT applied after SCI). Random group allocation was performed to prevent bias in the study. Ten rats from each experimental group were used to evaluate locomotor function. Five rats from each experimental group were used to evaluate sensory function. Eight or 10 rats per group were used for CD31 and α-SMA staining. Four or 5 rats from each experimental group were used for 5-HT staining, white matter staining, and TUNEL staining. The rats were housed 2 or 3 per cage and kept at a temperature of 24°C with free access to water and food before and after surgery.

Spinal Cord Injury

The rats were anesthetized with 1.25% halothane in an oxygen/nitrous oxide (30%/70%) gas mixture. During surgery, the rectal temperature was monitored and maintained at 37.0°C ± 0.5°C by a heating pad (Fine Science Tools Inc.). The skin above the vertebral column was shaved and cleaned using an antiseptic. A midline skin incision was made, and the laminae of the T8–12 vertebrae were exposed. The T9–11 vertebrae were laminectomized to expose the dorsal cord surface with the dura mater intact. The vertebral column was stabilized using angled clamps attached to the T-8 and T-12 transverse processes. An SCI was induced using a New York University Impactor (W.M. Keck Center).[6,20] A 10-g rod was dropped from a height of 12.5 mm onto the T-10 segment. The impact rod was removed immediately after injury. The contusion height and velocity were monitored. Animals were excluded immediately when height or velocity errors exceeded 10%.[5,48] The muscles and skin were closed in layers. Bladders were expressed twice a day until spontaneous voiding began.

Extracorporeal Shock Wave Therapy

Low-energy ESWT was performed by using a commercially available shock wave generator (DUOLITH SD1, Storz Medical AG). The animals were anesthetized to receive ESWT. On the basis of our previous study results,[16,23,27–29,33,41,45,56,57,65] the shock wave was applied to

2 spots on the injured spinal cord 3 times a week for 3 weeks after SCI (at 0, 2, 4, 7, 9, 11, 14, 16, and 18 days after injury). The condition of the shock wave was 0.25 mJ/mm^2, 4 Hz, 200 shots/spot, 2 spots for each treatment, as described previously.[16,27,33,41,45] The ESWT was applied from outside the body to the spinal cord lesion after closing the wound.[59] According to the manufacturer's protocol, the optimal focal point of the shock wave was within an area 10 mm wide and 10 mm deep from the tip of the probe.

Locomotor Function

Locomotor function was evaluated using the Basso-Beattie-Bresnahan (BBB) open-field locomotor score for 6 weeks after SCI.[6] Locomotor recovery, including joint movements, stepping ability, coordination, and trunk stability, can be assessed by the BBB score (range 0–21 points). A score of 21 indicates unimpaired locomotion as observed in uninjured rats. For these evaluations, the rats were placed individually in an open field with a nonslippery surface for 4 minutes, and well-trained investigators scored them on the BBB in a blinded manner. Before surgery, the rats were placed individually in the open field for 4 minutes to assure that all subjects consistently obtained the maximum score. The BBB scores were measured at 4 and 24 hours and at 7, 14, 21, 28, 35, and 42 days after SCI.[70] Animals were excluded when the BBB score was > 7 at 24 hours after injury.

Mechanical Allodynia

To evaluate mechanical sensitivity in the hindpaw, the withdrawal threshold was measured using a von Frey filament (0.25–15 g) applied to the plantar surface. A modification of the "up-down" method was used to determine the value at which paw withdrawal occurred 50% of the time.[7,10]

Thermal Allodynia

Thermal allodynia was assessed by measuring the withdrawal latency of the hindpaws from an infrared heat stimulus. On the baisis of Hargreaves' method, the Plantar Test Apparatus (Ugo Basile) was applied through the glass floor to the middle of the plantar surface of the rat's hindpaws.[22] When the animal felt pain and withdrew its paw, the photocell switched off and the reaction time counter stopped. An average of 3 trials was used as the withdrawal latency.

Tissue Preparation

At 7 or 42 days after SCI, the rats were overdosed with an intraperitoneal injection of 100 mg/kg sodium pentobarbital. The rats were transcardially perfused with normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. For immunohistochemical staining, the spinal cord segments containing the injured site were collected, postfixed in the same fixative overnight at 4°C, and embedded in paraffin. Serial 7-μm transverse sections around the injured site were mounted on slides. A total of 15 sequential sections at 500-μm intervals spanning a 7000-μm length in the spinal cord centered at the epicenter were prepared. The sections were used for immunohistochemical staining as described below.

Immunohistochemistry

Immunohistochemical staining for CD31, α-SMA, and 5-HT was performed using the sections obtained at 7 or 42 days after SCI. The sections were deparaffinized, rehydrated, and washed in PBS for 10 minutes, followed by washing with PBS containing 0.3% Tween for 10 minutes and blocking with 3% milk and 5% fetal bovine serum in 0.01 M PBS for 2 hours. The sections were incubated with mouse anti-CD31 antibody (1:100; M0823, Dako) or mouse anti-SMA antibody (1:100; M0851, Dako) diluted in PBS overnight at 4°C. After rinsing with PBS, the sections were incubated with goat anti–mouse IgG Alexa Fluor 488 secondary antibody (1:500; Molecular Probes) or goat anti–rabbit IgG Alexa Fluor 594 secondary antibody (1:500; Molecular Probes) for 1 hour at room temperature. The sections were mounted with Vectashield containing DAPI to label the nuclei (Vector Laboratories). In each experiment, the sections were stained at the same time.

Double Staining for VEGF and Various Cell-Type Markers

To examine the expression of VEGF in a specific population of cells in the injured spinal cord, the transverse sections in the SCI-SW group at 7 days were double stained for VEGF and various cell-type markers: NeuN for neurons, GFAP for astrocytes, and Olig2 for oligodendrocytes. The sections were incubated with a mixture of rabbit anti-VEGF antibody (1:50; sc-152, Santa Cruz Biotechnology) and either goat anti-Olig2 (1:100; Santa Cruz Biotechnology), mouse anti-GFAP (1:50; Dako), or mouse anti-NeuN antibodies (1:100; Chemicon) diluted in PBS overnight at 4°C. After rinsing with PBS, the sections were incubated with a mixture of goat anti–rabbit IgG Alexa Fluor 594 antibody (1:500; Molecular Probes) and either donkey anti–goat IgG Alexa Fluor 488 (1:500; Molecular Probes) or goat anti–mouse IgG Alexa Fluor 488 antibodies (1:500; Molecular Probes) for 1 hour at room temperature. The sections were mounted with Vectashield containing DAPI to label the nuclei (Vector Laboratories).

White Matter Staining

The transverse sections cut at the lesion epicenter and at 1500 μm rostral, 1000 μm rostral, 1000 μm caudal, and 1500 μm caudal from the epicenter 42 days after SCI were stained using Luxol fast blue for the myelin. The images of the stained sections were captured using a digital photographic camera, and the spared white matter area of the spinal cord was analyzed using the ImageJ 1.42q software program. After performing Luxol fast blue staining, the spared white matter appeared dark blue and isocellular, as seen in healthy neuronal tissue. The damaged or degenerated white matter appeared to be either blanched or replaced by scar tissue that had clusters of cells with prominent basophilic nuclei.[4,31,60] We analyzed the spared white matter areas in both groups.

Immunodensity Analysis of CD31, α-SMA, and 5-HT Staining

After the immunochemical staining with CD31, α-SMA, and 5-HT, as described above, each section was scanned using a confocal microscope (BX 51; Olympus). Sections 1500 μm rostral, 1000 μm rostral, 1000 μm caudal, and 1500 μm caudal from the lesion epicenter and at the epicenter were chosen for each animal. For imaging, we determined in the first microscopy session the appropriate setting to avoid signal saturation, and then used that same setting thereafter.

Using the ImageJ analysis system, we traced the entire spinal cord containing the lesion and perilesional areas in each section. Furthermore, we performed automatic thresholding for each image using ImageJ to determine the threshold for a specific signal. The default threshold setting was used, and the thresholding values were maintained at constant levels for all analyses. After setting the threshold, the immunodensity above the threshold was automatically calculated.

Counting of TUNEL-Positive Cells

To detect DNA fragmentation caused by cell death in the injured spinal cord at the subacute phase, TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche) for the transverse sections obtained at 7 days after injury. The labeled sections at the lesion epicenter and the sites 1000 μm and 1500 μm caudal and rostral to the lesion were scanned using a BX 51 microscope. The number of TUNEL-positive cells in each section was counted. The TUNEL-positive cells were defined as cells double labeled with TUNEL and DAPI. The cell counting procedure was the same as that described previously. The numbers of the TUNEL-positive cells were compared between the SCI-SW and SCI groups.

Statistical Analysis

Significant differences in the immunodensity of CD31, α-SMA, and 5-HT staining; the number of TUNEL-positive cells; and the spared white matter area were analyzed using the unpaired t-test. The significance of any differences in the BBB scores from Weeks 1 to 6 after SCI was determined by performing repeated-measures ANOVA and a Bonferroni post hoc test. In all analyses, a p value < 0.05 was considered to indicate statistical significance. All statistical analyses were performed using the GraphPad Prism 5.0a software program (GraphPad Software, Inc.).

Results

The BBB Locomotor Scores

To evaluate the effect of low-energy ESWT on locomotor function, the BBB scores were measured for 6 weeks. The SCI-SW group had significant locomotor improvement compared with the SCI group at 14, 35, and 42 days (p = 0.049, 0.013, and 0.001, respectively; Fig. 1A). At 42 days after injury, the BBB scores in the SCI-SW group were 11–17 (mean 13.3 ± 1.8). In contrast, the BBB scores in the SCI group were 10–13 (mean 11.4 ± 1.0). Except for 1 rat with a BBB score of 11, 5 rats in the SCI-SW

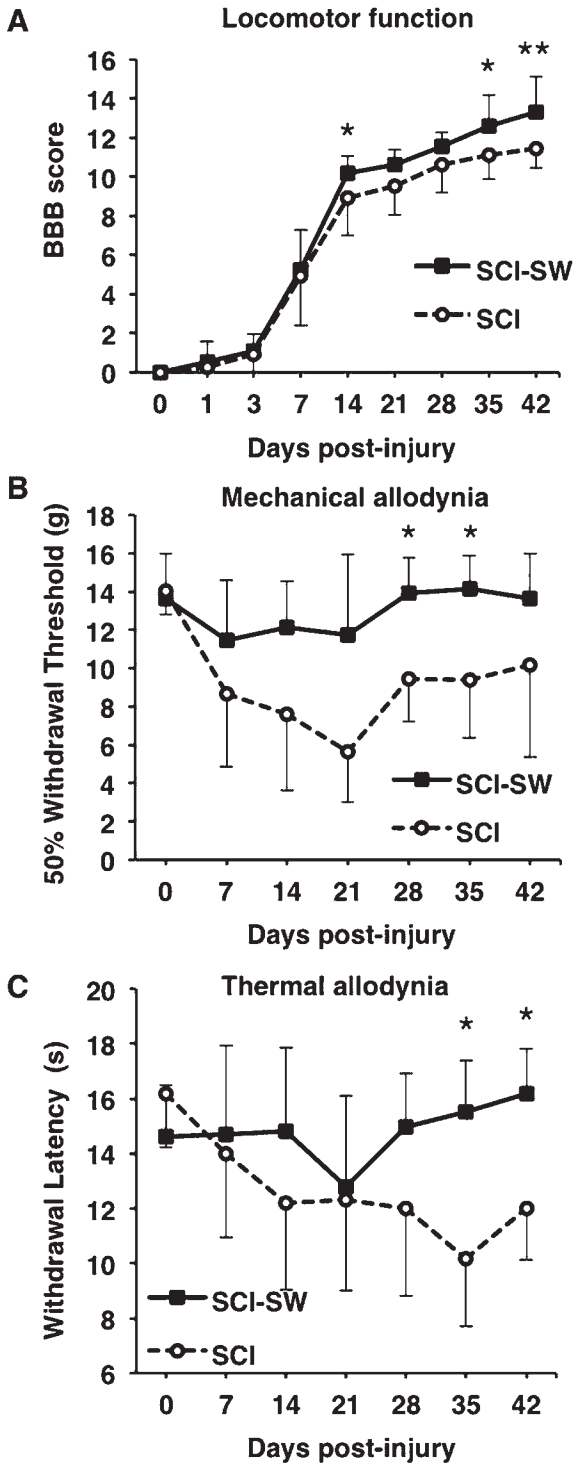

FIG. 1. Graphs showing the locomotor and sensory functions after SCI. **A:** The SCI-SW group demonstrates significantly better locomotor improvement in BBB scoring than the SCI group at 14, 35, and 42 days after injury. The values are expressed as the mean ± SD throughout (*p < 0.05, **p < 0.01, n = 10 per group in panel A). **B and C:** Mechanical (B) and thermal (C) allodynia for 42 days after SCI. The SCI-SW group demonstrates significantly higher withdrawal thresholds for mechanical allodynia at 28 and 35 days, returning to values similar to those at baseline, than the SCI group. In the assessment of thermal allodynia, the SCI-SW group exhibits significantly higher withdrawal latencies than the SCI group at 35 and 42 days (*p < 0.05, n = 5 per group in panels B and C).

group achieved consistent plantar stepping and occasional or frequent forelimb–hindlimb coordination during gait at 42 days after injury. The other 4 rats in the SCI-SW group achieved consistent plantar stepping and consistent forelimb–hindlimb coordination during gait, and the predominant paw position was parallel at the initial contact and lift off at 42 days after injury. In contrast, no rats in the SCI group at 42 days kept their paws parallel when stepping, and they showed occasional or no forelimb–hindlimb coordination.

Mechanical and Thermal Allodynia

Mechanical allodynia was examined using the von Frey filaments. Withdrawal thresholds to mechanical stimuli decreased in all groups after SCI and then gradually increased until 6 weeks (Fig. 1B). Animals in the SCI-SW group demonstrated significantly higher withdrawal thresholds at 28 and 35 days, returning to values similar to those obtained at baseline, than animals in the SCI group ($p = 0.028$ at both 28 and 35 days).

Thermal allodynia was examined using the Hargreaves method. The withdrawal latencies to a heat stimulus decreased in both groups, but animals in the SCI-SW group exhibited significantly higher withdrawal latencies than those in the SCI groups at 35 and 42 days ($p = 0.028$ at both 35 and 42 days; Fig. 1C).

Double Staining of VEGF and Various Cell-Type Markers

To examine VEGF expression in a specific population of cells, including neurons, astrocytes, and oligodendrocytes, the transverse sections were double stained at 7 days after SCI for VEGF and various cell-type markers: NeuN for neurons, GFAP for astrocytes, and Olig2 for oligodendrocytes. In the double staining, expression of VEGF was observed in NeuN-, GFAP-, and Olig2-labeled cells (Fig. 2). The double staining showed that VEGF expression was present in neurons, astrocytes, and oligodendrocytes.

Immunodensity of CD31 and α-SMA Staining

To investigate the effect of low-energy ESWT on angiogenesis in the injured spinal cord, the immunodensities of CD31 and α-SMA antibody staining were compared between the SCI and SCI-SW groups. In representative CD31-stained sections, CD31-positive cells were more frequently observed in the SCI-SW group than in the SCI group (Fig. 3A–G). The immunodensity of CD31 staining was significantly higher in the SCI-SW group than in the SCI group at 1500 μm rostral and 1000 μm caudal to the lesion epicenter and at the epicenter ($p = 0.016$, 0.021, and 0.027, respectively; Fig. 3H). In representative α-SMA–stained sections, α-SMA–positive cells were more frequently observed in the SCI-SW group than in the SCI group (Fig. 4A–G). The immunodensity of α-SMA staining was significantly higher in the SCI-SW group than in the SCI group at the epicenter ($p = 0.041$; Fig. 4H).

Immunodensity of 5-HT Staining

To investigate the 5-HT axons at 42 days after injury, the immunodensities of 5-HT antibody staining were compared between the SCI and SCI-SW groups. In representa-

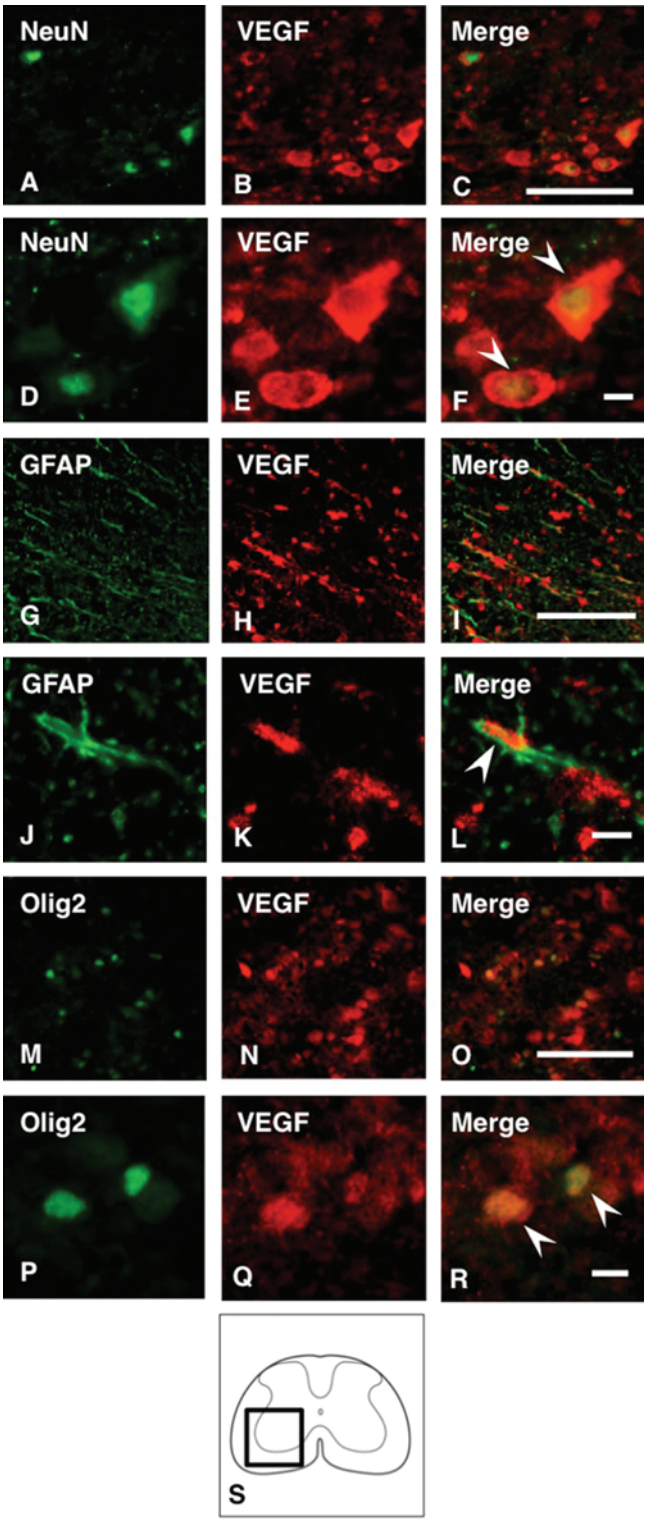

FIG. 2. Double staining of VEGF and cell-type markers on the injured side in the transverse section at 7 days after SCI. The expressions of VEGF observed in the NeuN-, GFAP-, and Olig2-labeled cells *(arrowheads)* demonstrate that VEGF expression increased in neurons, astrocytes, and oligodendrocytes, respectively. Bar = 100 μm **(A–C, G–I, M–O)** and 10 μm **(D–F, J–L, P–R)**. The schematic drawing **(S)** illustrates the location of the micrographs.

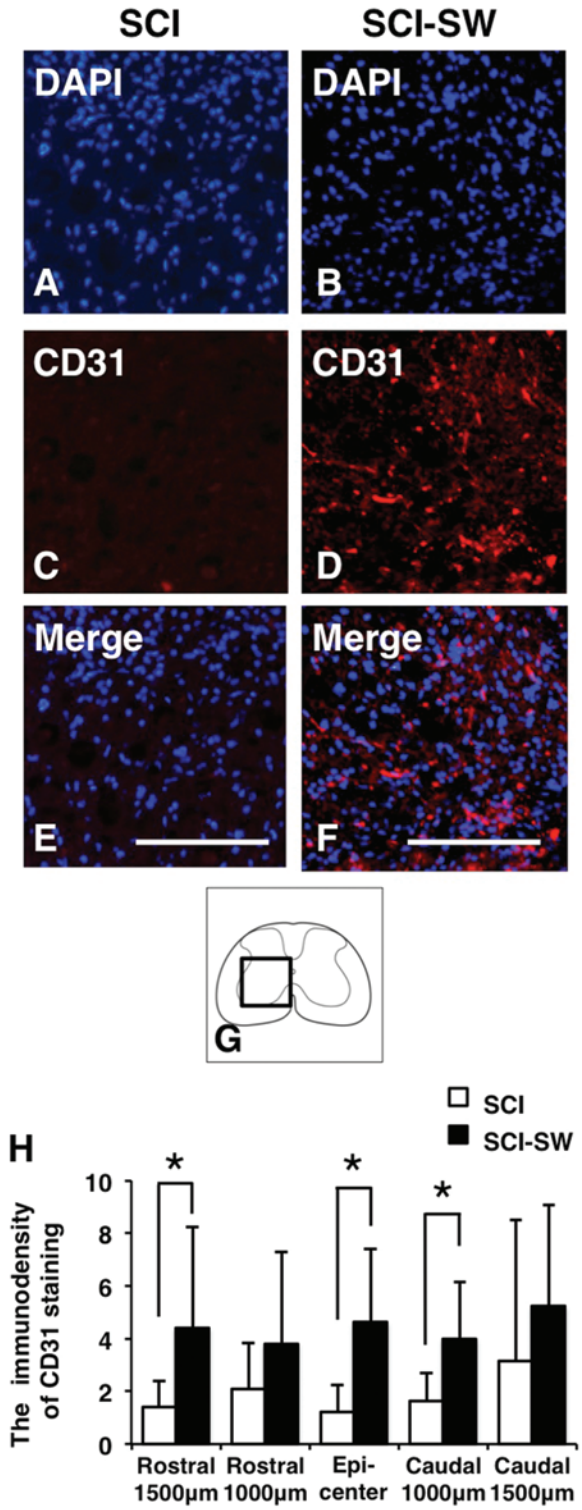

FIG. 3. The immunodensity analysis of CD31 staining. The CD31-positive cells in the section are more frequently observed in the SCI-SW group **(B, D, F)** than in the SCI group **(A, C, E)**. Bar = 200 μm. The schematic drawing **(G)** illustrates the location of the micrographs. **H:** Bar graph of CD31 staining showing that the immunodensity is significantly higher in the SCI-SW group than in the SCI group in the section at 1500 μm rostral to the epicenter, at the epicenter, and at 1000 μm caudal to the epicenter. The values are expressed as the mean ± SD (*$p < 0.05$, n = 8 per group).

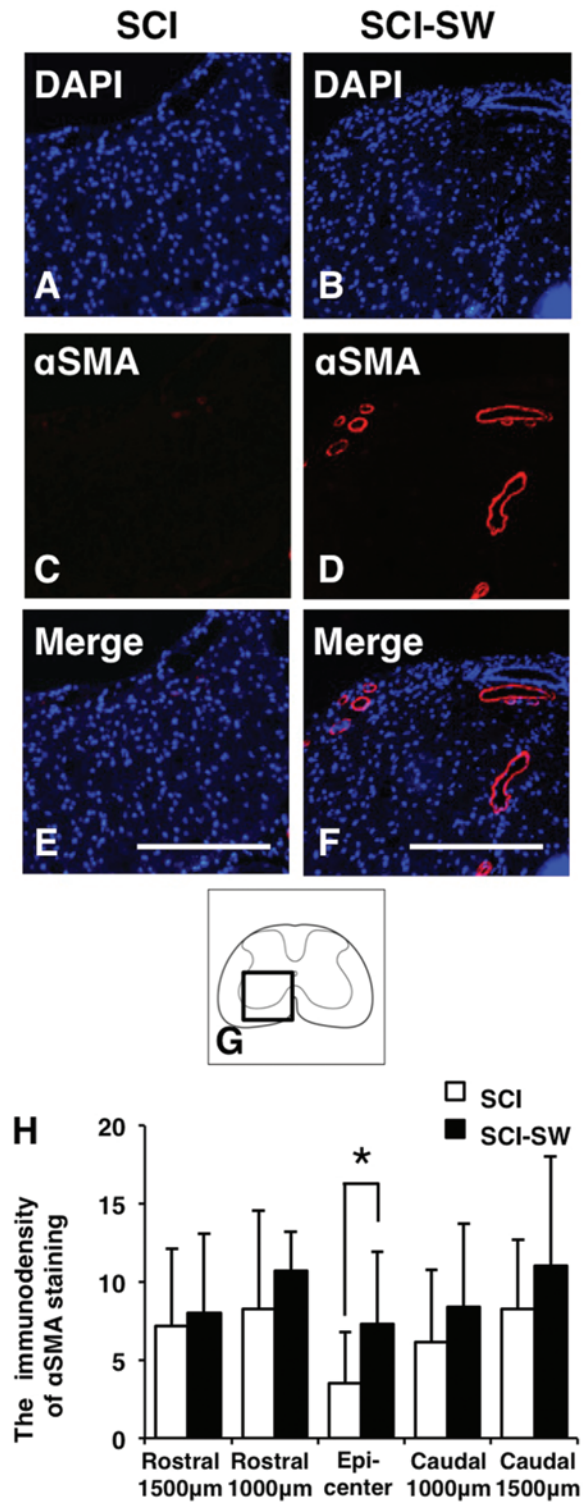

FIG. 4. The immunodensity analysis of α-SMA staining. The α-SMA–positive cells in the section are more frequently observed in the SCI-SW group **(B, D, F)** than in the SCI group **(A, C, E)**. Bar = 200 μm. The schematic drawing **(G)** illustrates the location of the micrographs. **H:** Bar graph showing that the immunodensity of α-SMA staining is significantly higher in the SCI-SW group than in the SCI group in the section at the epicenter. The values are expressed as the mean ± SD (*$p < 0.05$, n = 10 per group).

tive sections, 5-HT–positive axons were more frequently observed in the SCI-SW group than in the SCI group (Fig. 5A–G). The immunodensity of 5-HT staining was significantly higher in the SCI-SW group than in the SCI group at 1000 μm rostral to the lesion epicenter ($p = 0.047$; Fig. 5H).

Areas of Spared White Matter

To investigate the differences in the amounts of demyelination at 42 days after the injury, the spared white matter areas were compared between the SCI-SW and SCI groups by using Luxol fast blue staining. The areas of spared white matter were obviously larger in the SCI-SW group than in the SCI group in the sections around the epicenter (Fig. 6A and B). The white matter area was more preserved in the SCI-SW group than in the SCI group, especially in the dorsal side of the spinal cord. In quantitative analysis of the spared white matter area, the averages of the spared white matter areas were consistently larger in the SCI-SW group than in the SCI group at the sides 1000 and 1500 μm rostral and 1000 and 1500 μm caudal from the epicenter and at the epicenter (Fig. 6C).

Number of TUNEL-Positive Cells

To investigate the effect of low-energy ESWT on cell death after SCI, the number of TUNEL-positive cells was compared between the SCI and SCI-SW groups. In the TUNEL-stained sections obtained 7 days after injury, the number of TUNEL-positive cells had obviously decreased in the SCI-SW group compared with those in the SCI group (Fig. 7A–G). The number of TUNEL-positive cells was significantly lower in the SCI-SW group than in the SCI group at the sides 1000 μm rostral and 1000 μm caudal from the epicenter and at the epicenter ($p = 0.021$, 0.021, and 0.043, respectively; Fig. 7H).

Discussion

The present study demonstrated that low-energy ESWT significantly increased VEGF protein expression in various neural cells and promoted CD31 and α-SMA expression in the injured spinal cord. These findings suggest that low-energy ESWT can enhance angiogenesis regulated by VEGF in damaged neural tissue after SCI. In addition, this treatment significantly improved not only locomotion but also mechanical and thermal allodynia. Interestingly, low-energy ESWT significantly reduced the number of TUNEL-positive cells in the injured spinal cord. Furthermore, the immunodensity of 5-HT–positive axons was significantly higher in rats that were treated with low-energy ESWT than in those that did not receive this treatment. These results suggested that the neuroprotective effect of VEGF induced by low-energy ESWT may suppress cell death and damage to 5-HT axons and consequently improve locomotor and sensory functions following SCI. Thus, low-energy EWT can be a novel therapeutic strategy for treatment of SCI.

Previous studies have shown that VEGF can be expressed in various types of neural cells and can produce neuroprotective effects in the CNS.[34,35,37,40,53,62,69] After SCI, endogenous expression of VEGF from neural cells has been shown to decrease significantly in the injured

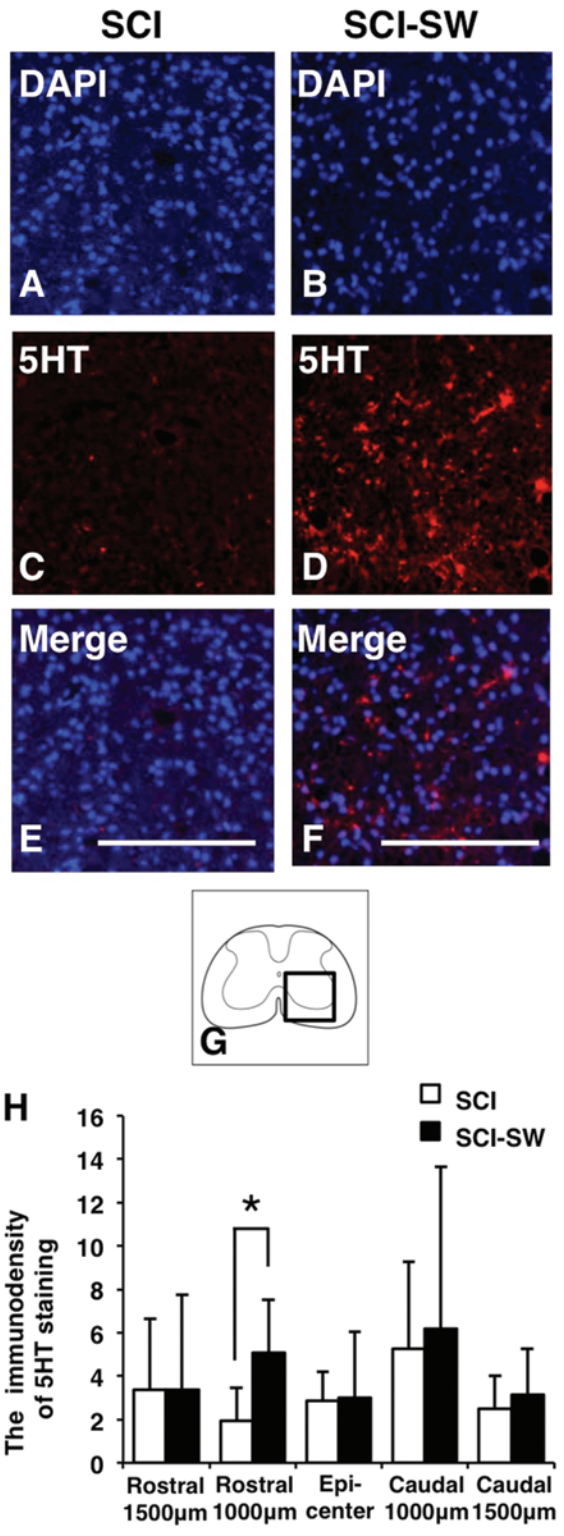

FIG. 5. The immunodensity analysis of 5-HT staining. The 5-HT–positive axons in the section are more frequently observed in the SCI-SW group **(B, D, F)** than in the SCI group **(A, C, E)**. Bar = 200 μm. The schematic drawing **(G)** illustrates the location of the micrographs. **H:** Bar graph showing that the immunodensity of 5-HT staining is significantly higher in the SCI-SW group than in the SCI group in the section at 1000 μm rostral to the epicenter. The values are expressed as the mean ± SD (*$p < 0.05$, $n = 5$ per group).

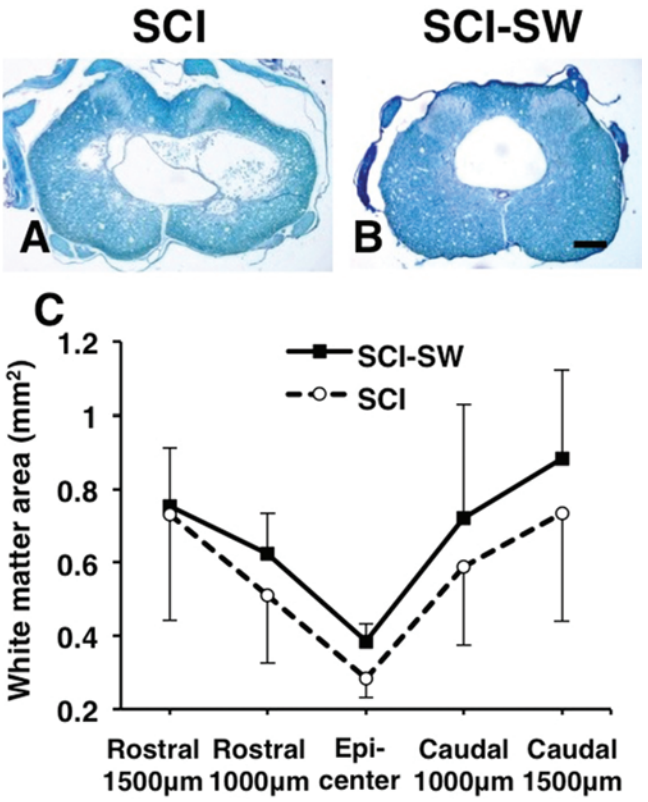

FIG. 6. White matter sparing in the SCI and SCI-SW groups at 42 days after SCI. Representative spinal cord sections at the 1000-μm caudal side from the epicenter show that the spared white matter area is relatively smaller in the SCI group **(A)** than in the SCI-SW group **(B)**. Bar = 1000 μm. The spared white matter area from the epicenter to 1500 μm on the rostral and caudal sides is compared between the SCI and SCI-SW groups **(C)**. The areas of spared white matter are consistently but not significantly larger in the SCI-SW group than in the SCI group at the 1000- and 1500-μm rostral and 1000- and 1500-μm caudal sides from the epicenter and at the epicenter. The values are expressed as the mean ± SEM (n = 4 per group). Figure is available in color online only.

spinal cord.[24] Decreased endogenous VEGF expression can worsen the pathophysiological process in SCI.[24] We recently reported that low-energy ESWT significantly increased expression of VEGF in the injured spinal cord.[70] However, it has not been known which type of cells express VEGF in the injured spinal cord after application of low-energy ESWT. The present study demonstrated that low-energy ESWT promoted VEGF protein expression in various neural cells—such as neurons, astrocytes, and oligodendrocytes—after SCI. Therefore, this treatment may prevent reduction of endogenous VEGF expression following SCI, and it may improve the pathophysiological condition of the injured spinal cord.

As a proangiogenic growth factor that can also promote neurogenesis,[18,30] VEGF has been investigated for its ability to promote axonal repair. In one study, VEGF stimulated axonal regeneration in preparations of sciatic nerves in vitro,[25] and adenoviral VEGF administration promoted regeneration of corticospinal tract axons in rats following transection of the spinal cord.[12] In addition, VEGF has been shown to provide a neuroprotective effect against neuronal cell death induced by serum withdrawal, expo-

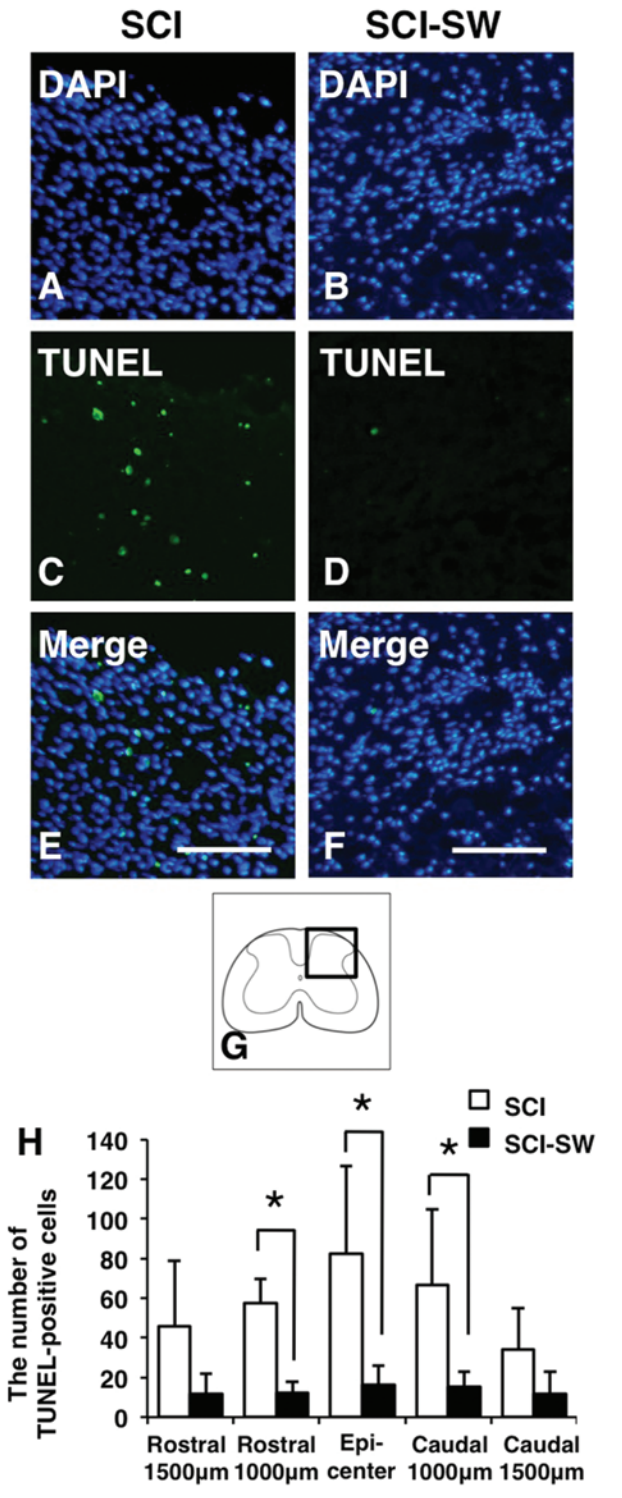

FIG. 7. TUNEL staining in SCI and SCI-SW groups at 7 days after SCI. Representative sections at the epicenter show that there are obviously fewer TUNEL-positive cells in the SCI-SW group **(B, D, F)** than in the SCI group **(A, C, E)**. Bar = 200 μm. The schematic drawing **(G)** illustrates the location of the micrographs. **H:** Bar graph showing that the number of TUNEL-positive cells is significantly lower in the SCI-SW group than in the SCI group at the 1000-μm rostral side, the epicenter, and the 1000-μm caudal side. The values are expressed as the mean ± SD (*$p < 0.05$, n = 4 per group).

sure to hypoxia, or excitotoxic stimuli in vitro.[72] Following SCI, treatment with recombinant VEGF also was shown to cause improvement in recovery associated with reduced apoptosis in the lesion area.[69] In this study, low-energy ESWT increased VEGF expression and 5-HT–positive axons and reduced cell death in the injured spinal cord. The ability of VEGF to regenerate axons and suppress cell death may be enhanced by low-energy ESWT following SCI.

Angiogenesis is an important part of healing in various tissues, including the CNS, after lesions. Previous studies have indicated that angiogenesis has a critical role in SCI repair.[50] Lack of local vascular tissue at the injury site hinders the ability of the body to self-heal and limits the use of treatment measures. Reducing blood loss, promoting new blood vessel formation, and restoring blood supply to the lesions may contribute to the recovery from SCI.[13] Recent studies have indicated that angiogenesis has a very important role in axonal regeneration after SCI. In addition, nerve fiber regeneration and synaptic reconstruction, tissue repair, and functional recovery after SCI require nutritional support provided by blood vessels to nourish damaged tissues.[47] Intervention by drugs or cells for improving angiogenesis has been shown to promote functional recovery.[21,44] Treatment with recombinant VEGF improved functional recovery associated with increased vessel density after SCI.[69] The present study demonstrated that low-energy ESWT significantly increased VEGF expression and angiogenesis in the injured spinal cord and promoted functional recovery. The results of this study suggested that the therapeutic effect of low-energy ESWT for SCI is associated with enhancement of angiogenesis.

Neuropathic pain described as burning, stabbing, and electric-shock like occurs in 48%–96% of patients with SCI.[58,71] Neuropathic pain seriously affects quality of life and causes further incapacity. Treatment to attenuate neuropathic pain is important for improving the quality of life for patients with SCI.[14,64] Interestingly, numerous studies have reported that administration of neuroprotective therapy during the acute or subacute phase after SCI can improve neuropathic pain in the chronic phase.[19,36,51] The present study demonstrated that applying low-energy ESWT from the acute to subacute phase actually improved mechanical and thermal allodynia in the chronic phase after SCI.

The descending pain modulatory system plays a critical role in homeostasis and pain control. One of the main neurotransmitters implicated in descending pain control is serotonin (also called 5-HT).[59] A previous study suggested that increased 5-HT fiber density immediately rostral to the SCI lesion site could reduce mechanical allodynia via actions at the 5-HT1 and/or 5-HT2 receptors.[43] In addition, selective 5-HT receptor agonists inhibited SCI-induced hyperalgesia.[26] The present study showed that low-energy ESWT significantly increased 5-HT–positive axons in the rostral spinal cord and attenuated mechanical and thermal allodynia in the hindpaw following SCI. These results suggested that low-energy ESWT may promote the descending pain modulatory system associated with 5-HT axons and consequently reduce neuropathic pain after SCI. Therefore, this treatment may be a useful therapeutic strategy for reducing not only locomotor impairment but also neuropathic pain following SCI.

Promising candidates that may provide effective treatment for SCI repair may involve medication and cell transplantation.[32,42] However, any medication essentially involves adverse effects. Cell transplantation into the injured spinal cord can be an invasive procedure and may pose ethical, logistical, and safety problems.[54] In contrast, a major advantage of low-energy ESWT is that it is noninvasive and safe, with no adverse effects or procedural complications.[16,41,65] If necessary, patients with SCI can undergo low-energy ESWT repeatedly, and the procedure is easy to perform because it does not require induction of anesthesia, catheter intervention, or drug administration. Thus, low-energy ESWT has a great advantage over other treatments, and it has significant therapeutic potential for patients with SCI.

Conclusions

The present study demonstrated that low-energy ESWT promoted VEGF expression in various neural cells and enhanced angiogenesis in the injured spinal cord. In addition, this treatment significantly reduced cell death and axonal damage after SCI. Furthermore, locomotor and sensory functions were significantly improved by low-energy ESWT. These results suggested that low-energy ESWT can be a novel therapeutic strategy for treatment of SCI.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Kanno, Yahata, Ozawa, Yamaya, Ito, Shimokawa, Itoi. Acquisition of data: Kanno, Yahata, Yamaya, Tateda. Analysis and interpretation of data: Kanno, Yahata, Tateda, Ito, Shimokawa. Drafting the article: Kanno, Yahata. Critically revising the article: Kanno, Yahata. Reviewed submitted version of manuscript: Kanno, Yahata, Ozawa, Yamaya, Ito, Shimokawa, Itoi. Approved the final version of the manuscript on behalf of all authors: Kanno. Statistical analysis: Kanno, Yahata. Administrative/technical/material support: Yahata, Ozawa, Yamaya, Tateda, Ito, Shimokawa, Itoi. Study supervision: Kanno, Ozawa, Yamaya, Ito, Shimokawa, Itoi.

Correspondence

Haruo Kanno, Department of Orthopaedic Surgery, Tohoku University School of Medicine, 1-1 Seiryo-machi Aobaku, Sendai 980-8574, Japan. email: kanno-h@isis.ocn.ne.jp.