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Low-energy extracorporeal shock wave therapy promotes vascular endothelial growth factor expression and improves locomotor recovery after spinal cord injury

Laboratory investigation

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Object. Extracorporeal shock wave therapy (ESWT) is widely used for the clinical treatment of various human diseases. Recent studies have demonstrated that low-energy ESWT upregulates the expression of vascular endothelial growth factor (VEGF) and promotes angiogenesis and functional recovery in myocardial infarction and peripheral artery disease. Many previous reports suggested that VEGF produces a neuroprotective effect to reduce secondary neural tissue damage after spinal cord injury (SCI). The purpose of the present study was to investigate whether low-energy ESWT promotes VEGF expression and neuroprotection and improves locomotor recovery after SCI.

Methods. Sixty adult female Sprague-Dawley rats were randomly divided into 4 groups: sham group (laminectomy only), sham-SW group (low-energy ESWT applied after laminectomy), SCI group (SCI only), and SCI-SW group (low-energy ESWT applied after SCI). Thoracic spinal cord contusion injury was inflicted using an impactor. Low-energy ESWT was applied to the injured spinal cord 3 times a week for 3 weeks. Locomotor function was evaluated using the Basso, Beattie, and Bresnahan (BBB) Scale (open field locomotor score) at different time points over 42 days after SCI. Hematoxylin and eosin staining was performed to assess neural tissue damage in the spinal cord. Neuronal loss was investigated by immunostaining for NeuN. The mRNA expressions of VEGF and its receptor, Flt-1, in the spinal cord were assessed using real-time polymerase chain reaction. Immunostaining for VEGF was performed to evaluate VEGF protein expression in the spinal cord.

Results. In both the sham and sham-SW groups, no animals showed locomotor impairment on BBB scoring. Histological analysis of H & E and NeuN stainings in the sham-SW group confirmed that no neural tissue damage was induced by the low-energy ESWT. Importantly, animals in the SCI-SW group demonstrated significantly better locomotor improvement than those in the SCI group at 7, 35, and 42 days after injury ($p < 0.05$). The number of NeuN-positive cells in the SCI-SW group was significantly higher than that in the SCI group at 42 days after injury ($p < 0.05$). In addition, mRNA expressions of VEGF and Flt-1 were significantly increased in the SCI-SW group compared with the SCI group at 7 days after injury ($p < 0.05$). The expression of VEGF protein in the SCI-SW group was significantly higher than that in the SCI group at 7 days ($p < 0.01$).

Conclusions. The present study showed that low-energy ESWT significantly increased expressions of VEGF and Flt-1 in the spinal cord without any detrimental effect. Furthermore, it significantly reduced neuronal loss in damaged neural tissue and improved locomotor function after SCI. These results suggested that low-energy ESWT enhances the neuroprotective effect of VEGF in reducing secondary injury and leads to better locomotor recovery following SCI. This study provides the first evidence that low-energy ESWT can be a safe and promising therapeutic strategy for SCI.
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Key Words • **spinal cord injury** • **shock wave** • **VEGF** • **Flt-1** • **neuroprotection**

Abbreviations used in this paper: BBB = Basso, Beattie, Bresnahan; ESWT = extracorporeal shock wave therapy; HUVEC = human umbilical vein endothelial cell; NO = nitric oxide; PBS = phosphate-buffered saline; RT-PCR = real-time polymerase chain reaction; SCI = spinal cord injury; VEGF = vascular endothelial growth factor.

Extracorporeal shock wave therapy (ESWT) is widely used for the clinical treatment of various human diseases.[1,7,41–43] We have recently demonstrated that this therapy upregulates the expression of vascular endothelial growth factor (VEGF) in cultured

human umbilical vein endothelial cells (HUVECs) in vitro.[37] In addition, low-energy ESWT increases VEGF expression in ischemic tissues in vivo and promotes angiogenesis and functional recovery in models of chronic myocardial ischemia, myocardial infarction, and peripheral artery disease.[15,22–24,28,37,39,49,54] Moreover, it promotes neovascularization at the tendon-bone junction by increasing VEGF expression.[57]

Vascular endothelial growth factor is known as a stimulator of angiogenesis and a mediator of vascular permeability.[10,12,31,48] Previous studies showed that VEGF can stimulate both endothelial cells and neural cells and can provide neurotrophic, neuroprotective, and neuroproliferative effects.[11,17,40,52] It also suppresses the apoptosis of neuronal cultures, and the blockade of endogenous VEGF signaling increases cell death.[38]

Previous studies have demonstrated the therapeutic potential of VEGF in spinal cord injury (SCI).[11,32,53,60] The administration of a transcription factor engineered to increase VEGF expression suppresses axonal degeneration and apoptosis and promotes vascularity in a model of SCI.[32] In addition, the administration of recombinant VEGF increases the amount of spared tissue and blood vessels and reduces cell death and locomotor impairment after SCI.[60] On the other hand, the endogenous expression of VEGF in the injured spinal cord significantly decreases after SCI.[21] The decreased endogenous VEGF expression can worsen the pathophysiological process in SCI.[21]

If endogenous VEGF expression in the injured spinal cord can be upregulated by a noninvasive procedure, such as low-energy ESWT, it would be very useful for the clinical treatment of SCI. We hypothesized that low-energy ESWT would increase VEGF expression to induce neuroprotective effects and enhance locomotor recovery after SCI. In the present study, low-energy ESWT was applied to a spinal cord contusion model in rats to investigate VEGF expression and its therapeutic effect on SCI.

Methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Tohoku University. All efforts were made to minimize both the number of animals used and the suffering of the animals. Sixty adult female Sprague-Dawley rats (body weight: 250–300 g) were used in the study (CLEA Japan). Six rats were included in each experimental group to evaluate locomotor function, as well as the histological analysis of neural tissue damage via H & E staining. Three rats per group were used to assess neuronal loss by counting the NeuN-stained cells. Four rats in each group at each time point were used to obtain real-time polymerase chain reaction (RT-PCR) results on VEGF and Flt-1 expression. Four rats per group were used for VEGF staining. The rats were housed 3–4 per cage and kept at a temperature of 24°C with free access to water and food before and after surgery.

The rats were randomly divided into the following 4 groups: sham group (laminectomy only), sham-SW group (low-energy ESWT applied after laminectomy), SCI group (SCI only), and SCI-SW group (low-energy ESWT applied after SCI). Random group allocation was performed to prevent bias in the study.

Spinal Cord Contusion Injury

The rats were anesthetized with 1.25% halothane in an oxygen/nitrous oxide (30/70%) gas mixture. During surgery, rectal temperature was monitored and maintained at 37.0 ± 0.5°C using a heating pad (Fine Science Tools Inc.). The skin above the vertebral column was shaved and cleaned with 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 with angled clamps attached to the T-8 and T-12 transverse processes. An SCI was induced with a New York University impactor (W.M. Keck Center for Collaborative Neuroscience).[5,18] A 10-g rod was dropped from 12.5 mm onto the T-10 segment. The impact rod was removed immediately after injury. The muscles and skin were closed in layers. To provide a reference marker for the application of the shock waves, a stitch of nylon thread was placed in the skin at the paravertebral muscle just above the injured spinal cord at T-10. Bladders were expressed twice a day until spontaneous voiding began. The sham-operated animals underwent the same surgical procedures, but no impact injury was inflicted.

Low-Energy ESWT

Low-energy ESWT was performed using a commercially available shock wave generator (DUOLITH-SD1, Storz Medical AG; Fig. 1A). Based on our previous studies,[15,20,22–24,28,37,39,49,50,54] the shock wave was applied to two spots on the injured spinal cord 3 times a week for 3 weeks after SCI. The treatment was performed immediately after wound closure following SCI, as well as 2, 4, 7, 9, 11, 14, 16, and 18 days after injury. Animals were anesthetized during each 5-minute treatment, as described above. The shock waves were 0.1 mJ/mm^2, 4 Hz, 200 shots/spot, with two spots for each treatment, as described previously.[15,22,28,37,39] According to the manufacturer's protocol, the optimal focal point of the shock wave is within a 10-mm width and a 10-mm depth from the tip of the probe (Fig. 1B), and 0.1 mJ/mm^2 (positive energy flux density) is equivalent to 0.25 mJ/mm^2 (total energy flux density). The focal point region was large enough to include the lesion of the spinal cord.

Behavioral Analysis

Locomotor function was evaluated using the Basso, Beattie, and Bresnahan (BBB) Scale (open field locomotor score) for 6 weeks after SCI.[5] The BBB Scale (from 0 to 21 points) can be used to assess locomotor recovery, including joint movements, stepping ability, coordination, and trunk stability. A score of 21 indicates unimpaired locomotion, as observed in uninjured rats. We also analyzed the BBB subscore (from 0 to 13 points), because some animals can show improvements in specific aspects

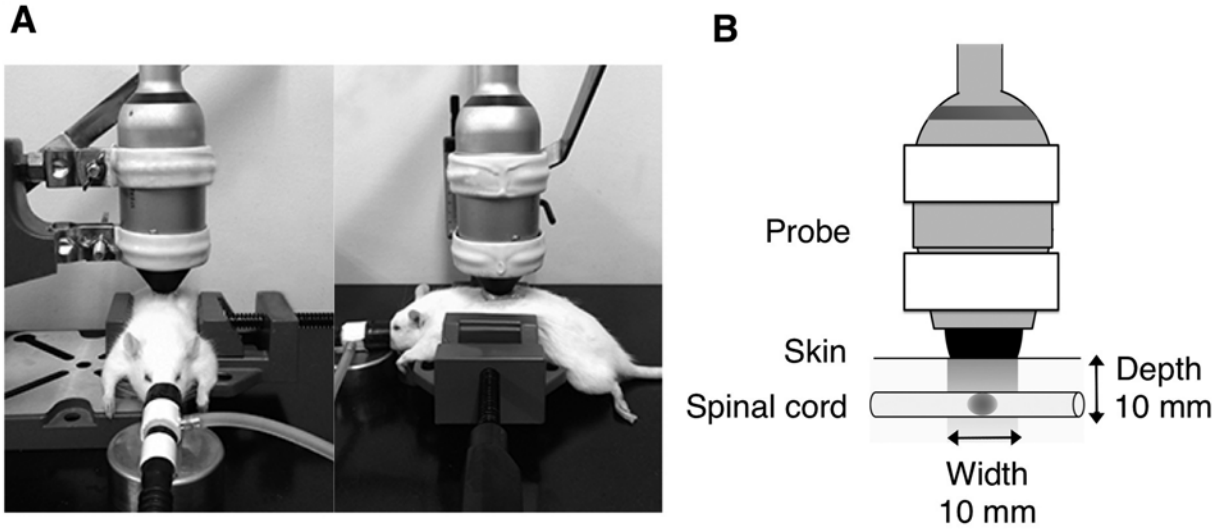

Fig. 1. Application of low-energy ESWT for SCI. **A:** Photographs showing that the shock wave probe was placed on the skin just behind the injured spinal cord between the T-8 and T-12 spinous processes. **B:** Illustration showing the optimal focal point of the shock wave was within a 10-mm width and a 10-mm depth from the tip of the probe.

of locomotion that do not follow a typical pattern of recovery and thus are not reflected in any change in the overall BBB score.[4] For these evaluations, the rats were individually placed in an open field with a nonslippery surface for 4 minutes, and in a blinded manner well-trained investigators rated them using the BBB Scale. Before surgery, the rats were individually placed in the open field for 4 minutes to ensure that all subjects consistently attained the maximum score. The BBB scores were measured at 4 and 24 hours and 7, 14, 21, 28, 35, and 42 days after SCI. An examiner blinded to rat group allocations performed all locomotor assessments.

Tissue Preparation

Seven and 42 days after SCI, the rats (8 and 36 rats, respectively) were overdosed with an intraperitoneal injection of 100 mg/kg of sodium pentobarbital. They were transcardially perfused with normal saline, followed by 4% paraformaldehyde in 0.1 M of phosphate-buffered saline (PBS) at pH 7.4. 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. Twenty-nine sequential sections at 250-μm intervals were collected from each rat, spanning a 7000-μm length in the spinal cord centered at the lesion epicenter. The serial sections were used for histological analyses as described below.

Hematoxylin and Eosin Staining

Serial transverse sections at 250-μm intervals around the lesion epicenter at 7 and 42 days after SCI were stained with H & E. Images of the stained sections were captured using a microscope (BX51, Olympus). For the other histological analyses, the tissue section with the least H & E–stained area was defined as the lesion epicenter for each animal.

To determine whether low-energy ESWT induces detrimental effects on the uninjured spinal cord, we assessed tissue damage in the spinal cord in the sham and sham-SW groups at 7 and 42 days. Given data from a previous study, we, in the present study, investigated the histological findings of tissue damage, such as hemorrhage, vacuole formation, and spindle-shaped changes of neurons in the spinal cord.[25,27,36] Histological analysis was performed using the H & E–stained serial transverse sections including the lesion epicenter. We determined whether the histological findings were present in both the white matter and the gray matter in each section by using microscopy. A pathologist confirmed the histological findings.

Immunohistochemistry

Immunohistochemical staining for NeuN was performed using the tissue sections obtained 42 days after SCI. In addition, the sections collected at 7 days after SCI were stained for VEGF. The sections were deparaffinized and rehydrated and then 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 of PBS for 2 hours. Tissue sections were incubated with a mouse anti-NeuN antibody (1:100, MAB377, Merck Millipore) or rabbit anti-VEGF antibody (1:50, sc-152, Santa Cruz Biotechnology) 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 on slides with Vectashield containing DAPI to label the nuclei (Vector Laboratories). In each experiment, all of the sections were stained at the same time.

Counting NeuN-Positive Cells

To investigate neuronal loss in the spinal cord, the

number of NeuN-positive cells in the sections was counted. After immunohistochemical staining of NeuN using the spinal cord sections from 42 days after SCI, as described above, each section was scanned using a microscope (BX51). The section at the 1500-μm-rostral, 1000-μm-rostral, 1000-μm-caudal, and 1500-μm-caudal area of the lesion epicenter was chosen for each animal. The scanned image of the transverse section was displayed on a monitor with a grid using the Photoshop Elements software program version 8.0 (Adobe Systems). All of the NeuN-positive cells in each grid were counted using a manual counter and then were added to obtain the total number of positive cells in the entire section. The NeuN-positive cells were defined as cells double-labeled with NeuN and DAPI. The number of NeuN-positive cells in the entire section at the areas 1500 μm rostral, 1000 μm rostral, 1000 μm caudal, and 1500 μm caudal to the lesion epicenter were counted and then compared among the groups at each location. The number of NeuN-positive cells was compared between the sham and sham-SW groups to investigate neuronal cell loss in the uninjured spinal cord after the application of low-energy ESWT. To evaluate the effects of low-energy ESWT after SCI, the number of NeuN-positive cells was also compared between the SCI and SCI-SW groups.

Quantitative RT-PCR

The spinal cord segments (10 mm in length) centered at the injury site were collected and harvested aseptically at 7 and 21 days after SCI and then were homogenized using a POLYTRON unit (Kinematica). Total RNA from the spinal cord was extracted using the TRIZOL reagent (Invitrogen) and cleaned up using a RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA synthesis and quantitative RT-PCR assays were performed to assess the mRNA expression levels of VEGF and its receptor, Flt-1. First-strand cDNA was synthesized using a high-capacity cDNA archive kit (Applied Biosystems). Quantitative RT-PCR was performed using ABI StepOnePlus, Power SYBR Green PCR MasterMix (Applied Biosystems), and 500 nM of each primer. These reactions were routinely run in duplicate. The primers were designed based on sequences in the GenBank database (F: 5′-GAGTTAAACGAACGTACTTGCAGA-3′ and R: 5′-TCTAGTTCCCGAAACCCTGA-3′ for VEGF, F: 5′-CAGTTTCCAAGTGGCCAGAG-3′ and R: 5′-AG GTCGCGATGAATGCAC-3′ for Flt-1, and F: 5′-CCCGC GAGTACAACCTTCT-3′ and R: 5′-CGTCATCCATG GCGAACT-3′ for β-actin). The fractional cycle number at which the fluorescence passes the threshold (Ct values) was used for quantification by using a comparative Ct method. The quantitated Ct values were obtained using the StepOne software program (version 2.1, Applied Biosystems). The Ct values of the gene of interest (Ct[GOI]) were standardized to those of β-actin (Ct[β-actin]). The results are shown as the $-\Delta Ct = -(Ct[GOI] - Ct[\beta\text{-actin}])$.[29]

Immunodensity Analysis of VEGF Staining

To evaluate VEGF protein expression in the injured spinal cord, we quantified the immunodensity of VEGF antibody staining in the spinal cord sections at 7 days after injury.[26] We photographed the entire transverse section at ×10 using a laser microscope with an image-capturing software program (BX51). The section 1500 μm rostral, 1000 μm rostral, 1000 μm caudal, and 1500 μm caudal to the lesion epicenter and 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 whole spinal cord containing the lesion and perilesional areas in each section. Furthermore, we performed automatic thresholding for each image using the ImageJ software program 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.[26]

Statistical Analysis

A repeated-measures ANOVA with a Bonferroni post-hoc test was used to analyze differences in functional recovery between animal groups from 1 to 6 weeks postinjury. The significance of differences in the quantitative RT-PCR, the number of NeuN-positive cells, and the immunodensity of VEGF staining were analyzed using the unpaired t-test. In all analyses, a p value < 0.05 was considered to be statistically significant. All statistical analyses were performed using the GraphPad Prism 5.0a software program (GraphPad Software Inc.).

Results

BBB Locomotor Scores

To evaluate the effect of low-energy ESWT on locomotor function, the BBB score and subscore were measured for 6 weeks. In the sham and sham-SW groups, none of the animals showed any locomotor impairment and had full marks for the BBB score and subscore over 6 weeks (Fig. 2A and B). The SCI-SW group had significant locomotor improvement compared with the SCI group at 7, 35, and 42 days ($p < 0.05$; Fig. 2C). At 42 days after injury, the BBB scores in the SCI-SW group were 14–18 (mean 17 ± 1.6). In contrast, the BBB scores in the SCI group were 12–14 (mean 13 ± 0.9). Except for 1 rat with a BBB score of 14, the other 5 rats in the SCI-SW group achieved consistent plantar stepping and consistent forelimb-hindlimb coordination during gait; the predominant paw position was parallel at the initial contact and lift off at 42 days after injury. On the other hand, 4 of 6 rats in the SCI group did not keep their paws parallel when stepping, and they showed occasional or frequent forelimb-hindlimb coordination. The BBB subscore was significantly higher in the SCI-SW group than in the SCI group at 14, 21, 28, 35, and 42 days after injury ($p < 0.01$; Fig. 2D).

Histological Findings in H & E–Stained Spinal Cord Sections

In the histological analysis using H & E staining, no tissue damage was observed in the spinal cord at 7 and

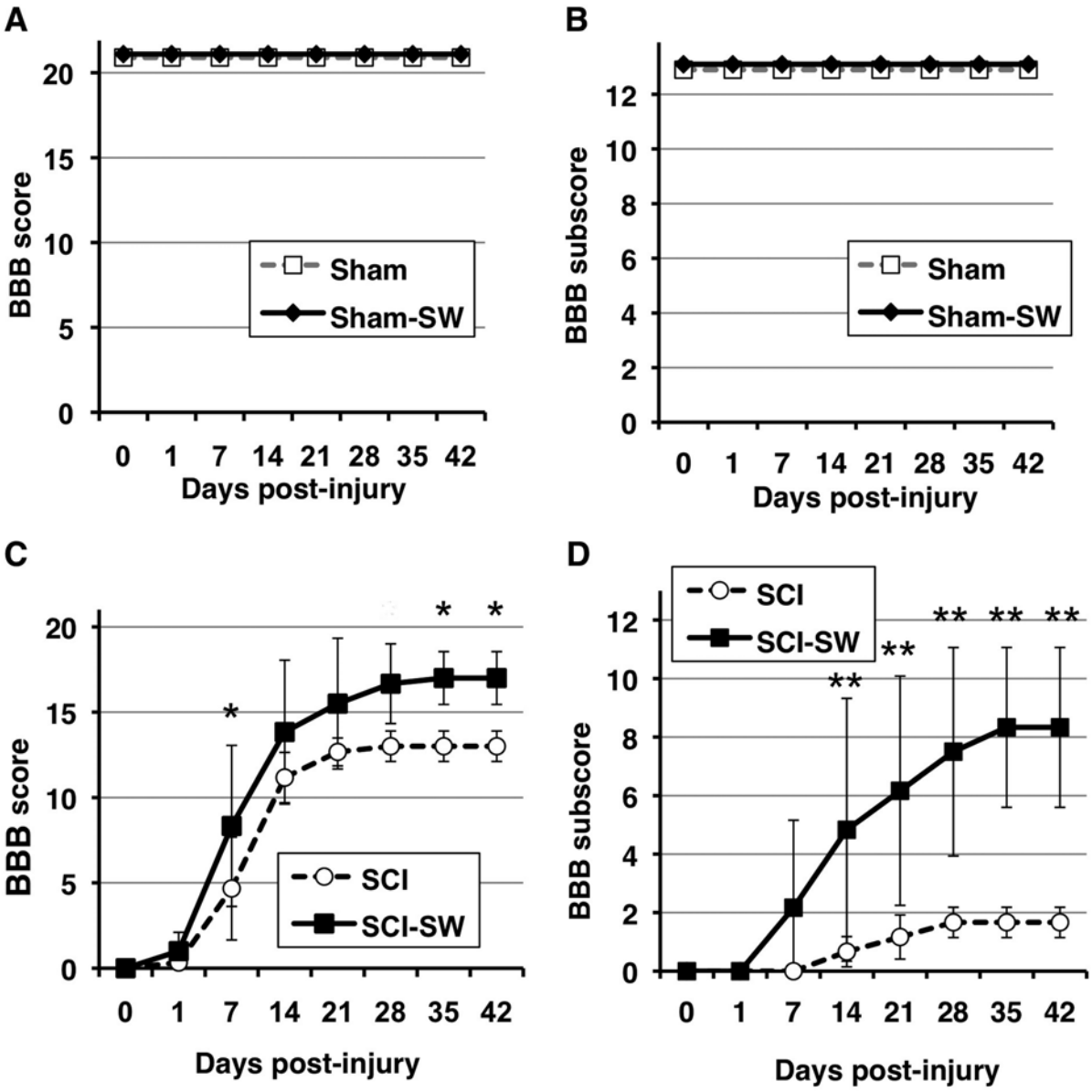

Fig. 2. A and B: In the sham and sham-SW groups, none of the animals showed any locomotor impairment and kept full marks for the BBB score and subscore over 6 weeks. **C:** The SCI-SW group demonstrated significantly better locomotor improvement, as reflected in BBB scoring, than did the SCI group at 7, 35, and 42 days after injury ($p < 0.05$). **D:** The BBB subscore was significantly higher in the SCI-SW group than in the SCI group at 14, 21, 28, 35, and 42 days ($p < 0.01$). Values represent the means ± standard deviation. *$p < 0.05$; **$p < 0.01$. Each group consisted of 6 animals.

42 days in either the sham or the sham-SW group (Fig. 3A–D). The low-energy ESWT caused no evident neural tissue damage, such as hemorrhage, vacuole formation, and spindle-shaped changes of neurons, in the uninjured spinal cord. Histological findings including hemorrhage and vacuole formation as well as cavity formation were observed in both the SCI and SCI-SW groups (Fig. 3E–H).

NeuN-Positive Cells

To investigate the loss of neuronal cells in the uninjured spinal cord after the application of low-energy ESWT, the number of NeuN-positive cells was compared between the sham and sham-SW groups at 42 days after sham injury. Representative NeuN-stained sections showed a similar number of NeuN-positive cells in the sham and sham-SW groups (Fig. 4A–L). There was no significant difference in the number of NeuN-positive cells between the sham and sham-SW groups (Fig. 4N).

To evaluate the neuroprotective effects of low-energy ESWT after SCI, the number of NeuN-positive cells was compared between the SCI and SCI-SW groups at 42 days after injury (Fig. 5A–L). In the SCI group, the NeuN-positive cells were sparsely observed at 1000 μm rostral to the lesion epicenter. In contrast, more NeuN-positive cells were observed in the SCI-SW group. The number of NeuN-positive cells in the SCI-SW group was

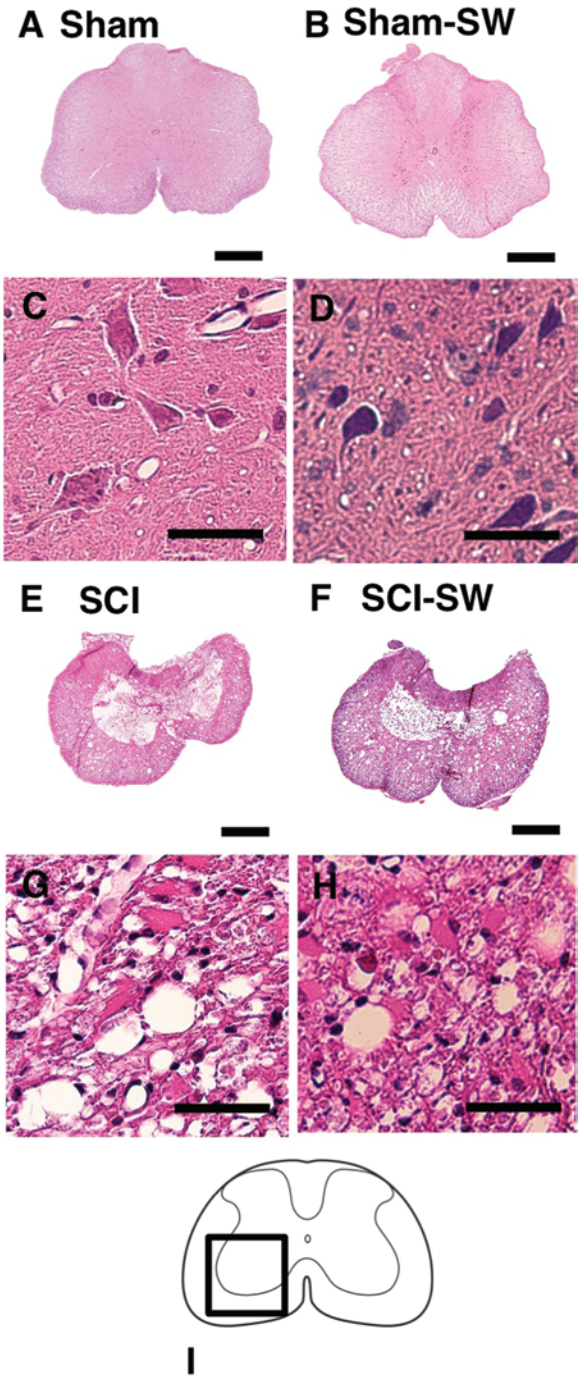

Fig. 3. Results of H & E staining performed to analyze neural tissue damage. **A and B:** In the sham and sham-SW groups, no tissue damage was observed in the spinal cord at 42 days (or 7 days, not shown). Bars = 500 μm. **C and D:** Magnified images showed no neural tissue damage, such as hemorrhage, spindle-shaped changes, or elongation of nuclei, in the spinal cord in either of these groups. Bars = 50 μm. **E and F:** Cavity formation was observed at the lesion epicenter in the SCI and SCI-SW groups. Bars = 500 μm. **G and H:** Magnified images showed the presence of neural tissue damage, such as hemorrhage and vacuole formation. Bars = 50 μm. **I:** Schematic illustrates the location of the micrographs. The histological analysis was performed using 6 animals per group.

significantly higher than that in the SCI group at 1000 μm rostral to the epicenter (313 ± 53.2 vs 78.3 ± 69.9, p = 0.010; Fig. 5N).

mRNA Expressions of VEGF and Flt-1

The mRNA expression levels of VEGF and Flt-1 were significantly higher in the SCI-SW group than in the SCI group at 7 days after injury (p = 0.018 and 0.004, respectively; Fig. 6). These mRNA expression levels in both the SCI and SCI-SW groups increased at 21 days, as compared with levels at 7 days. Expression levels of VEGF and Flt-1 were not significantly different between the groups at 21 days after SCI (p = 0.477 and 0.997, respectively).

Immunodensity of VEGF Staining

To investigate the protein expression of VEGF at 7 days after injury, the immunodensity of VEGF antibody staining was compared between the SCI and SCI-SW groups. The VEGF-positive cells were more frequently observed in the SCI-SW group than in the SCI group (Fig. 7A–L). The immunodensity of VEGF staining in the SCI-SW group was consistently higher than that in the SCI group from 1000 μm rostral to 1000 μm caudal to the lesion epicenter. Immunodensity at the epicenter was significantly different between the groups (p = 0.009; Fig. 7N).

Discussion

In the present study, low-energy ESWT induced no neural tissue damage in the spinal cord and produced no detrimental effect on locomotor function. In addition, it significantly increased the mRNA and protein expression levels of VEGF at 7 days after SCI. This treatment significantly reduced neuronal loss in the injured spinal cord. Furthermore, locomotor function was significantly improved in the animals treated with low-energy ESWT after SCI. These results demonstrated, for the first time, that low-energy ESWT promotes the neuroprotective effects of VEGF and leads to better locomotor recovery following SCI. Thus, low-energy ESWT has significant potential in the treatment of SCI.

The effects of the shock waves on tissues and organs is pressure dependent.[14] High-energy ESWT causes tissue injuries, such as microfracture and hematoma formation, in various tissues and organs.[19,42,45,55] It also causes injury to the neurovascular structures in the spinal cord and brain.[25,27,30] Histological findings of neural tissue damage, such as neuronal loss, contusional hemorrhage, and spindle-shaped changes in neurons, have been detected in the brain and spinal cord after the application of the high- or middle-energy shock waves.[27,30]

In contrast, low-energy ESWT induces no evident neural tissue damage in the spinal cord or brain. This is logical given that some researchers have suggested that the degree of neural tissue damage in the CNS caused by shock waves is pressure dependent.[27,30] In our study, low-energy ESWT caused no evident neural tissue damage, such as hemorrhage or spindle-shaped changes of neurons, in the uninjured spinal cord. In addition, NeuN

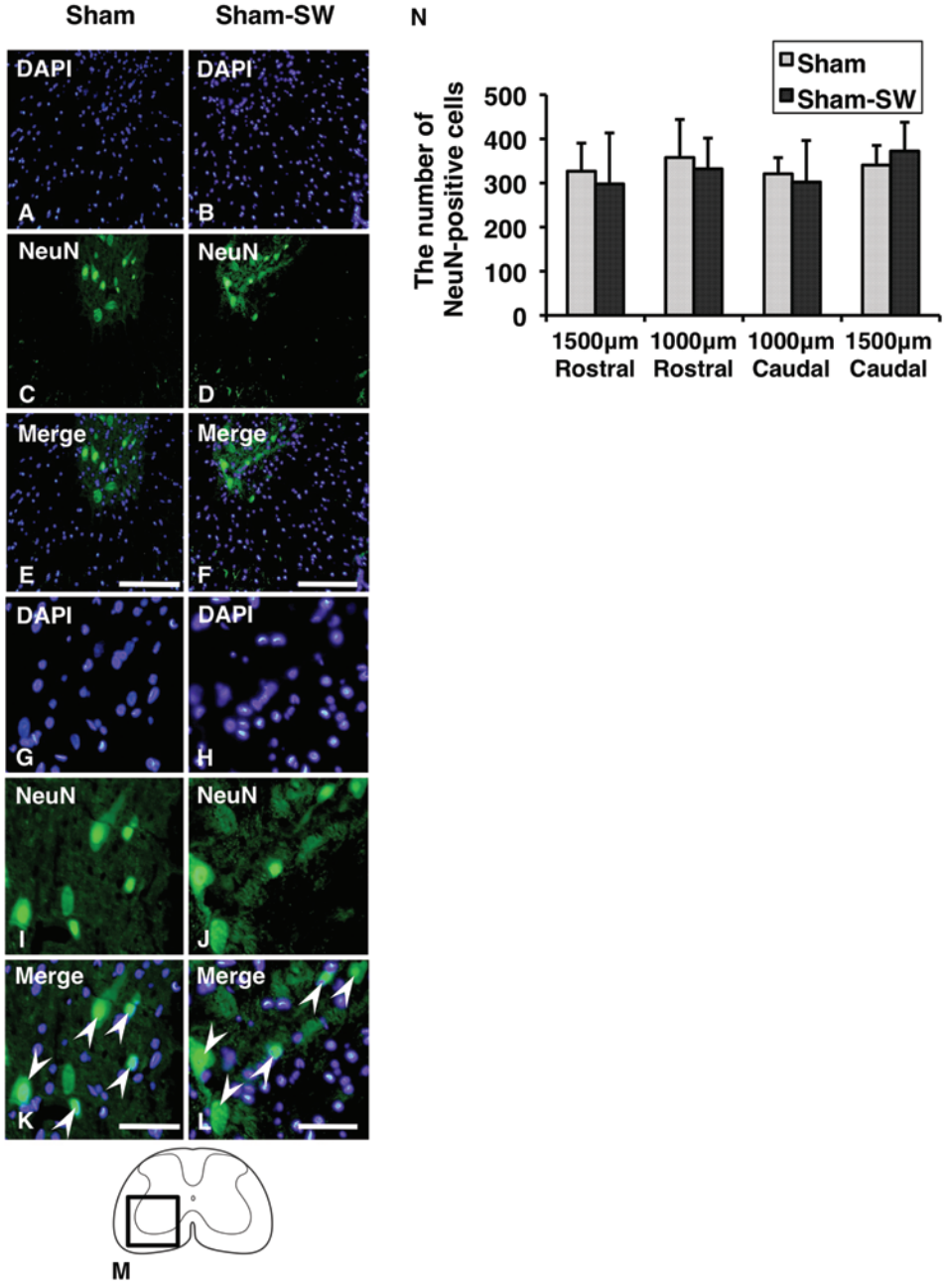

Fig. 4. Immunohistochemical staining of NeuN in the sham and sham-SW groups at 42 days after sham injury. **A–F:** Representative sections showed a similar number of NeuN-positive cells in the sham and sham-SW groups. Bars = 200 μm. **G–L:** Magnified images show NeuN-positive cells *(arrowheads)*. Bars = 50 μm. **M:** Schematic illustrates the location of the micrographs. **N:** The number of NeuN-positive cells was not significantly different between the sham and sham-SW groups. The histological analysis was performed using 3 animals per group. Values represent the means ± standard deviation.

staining revealed that no neuronal loss occurred in the uninjured spinal cord after the application of low-energy ESWT. Furthermore, locomotor function in sham-injured animals was not worsened by low-energy ESWT. These findings indicate that low-energy ESWT induces no detrimental effect on the spinal cord. Our study provides evidence to support the safety of low-energy ESWT for the treatment of SCI.

The application of shock waves can induce cavitation (a micrometer-sized violent collapse of bubbles) in the cells.[2] The physical force generated by cavitation produces localized shear stress on cell surface membranes.[13] The stress to the cells caused by the shock waves may lead to various biochemical effects.[9,16,34,35,47,51,58] We have previously shown that shock waves upregulate the expression of VEGF and its receptor, Flt-1, in various cells and organs.[37,39] Low-energy ESWT can upregulate the expression of both VEGF and Flt-1 in cultured HUVECs.[37] In

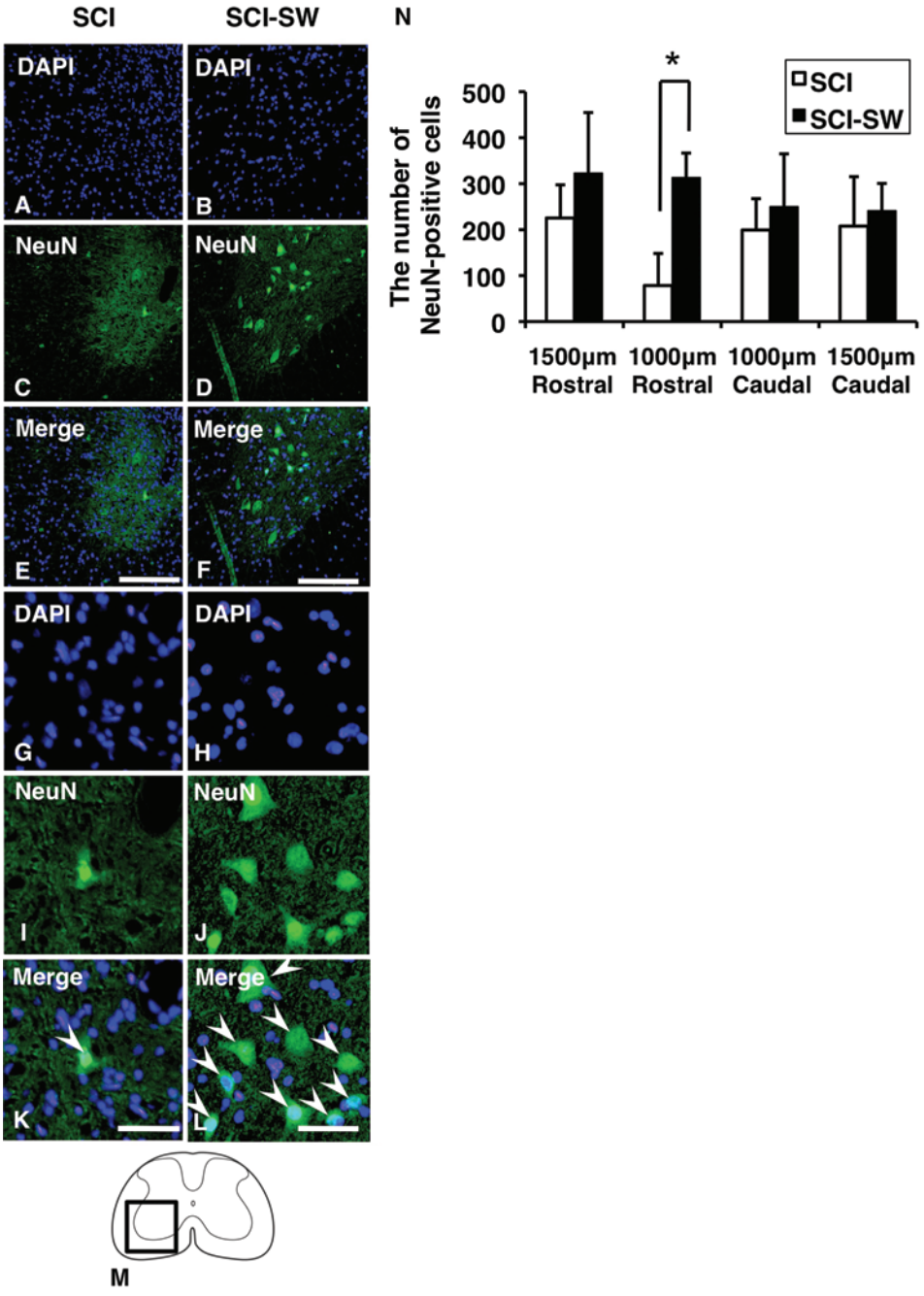

Fig. 5. Immunohistochemical staining of NeuN in the SCI and SCI-SW groups at 42 days after SCI. **A–F:** Representative sections at the position 1000 μm rostral from the lesion epicenter showed that there were more NeuN-positive cells in the SCI-SW than in the SCI group. Bars = 200 μm. **G–L:** Magnified images show the NeuN-positive cells *(arrowheads)*. The NeuN-positive cells were sparsely observed in the SCI group. Bars = 50 μm. **M:** Schematic illustrates the location of the micrographs. **N:** The number of NeuN-positive cells in the SCI-SW group was significantly higher than that in the SCI group at 1000 μm rostral from the epicenter ($p = 0.010$). The histological analysis was performed using 3 animals per group. Values represent the means ± standard deviation. *$p < 0.05$.

addition, low-energy ESWT increases the expression of VEGF in the ischemic tissues in chronic myocardial ischemia, acute myocardial infarction, and peripheral artery disease.[37,39] It has also enhanced lymphangiogenesis in a rat model of secondary lymphedema and accelerated skin wound healing in diabetic mice.[20,50] Data in the present study demonstrated that low-energy ESWT significantly upregulates mRNA expression levels of VEGF and Flt-1 in the spinal cord at 7 days after injury. Moreover, immunohistochemical analysis confirmed that there was a significant increase in the protein expression of VEGF in the injured spinal cord. These results indicate that low-energy ESWT can enhance the biological effects of VEGF in damaged neural tissue following SCI.

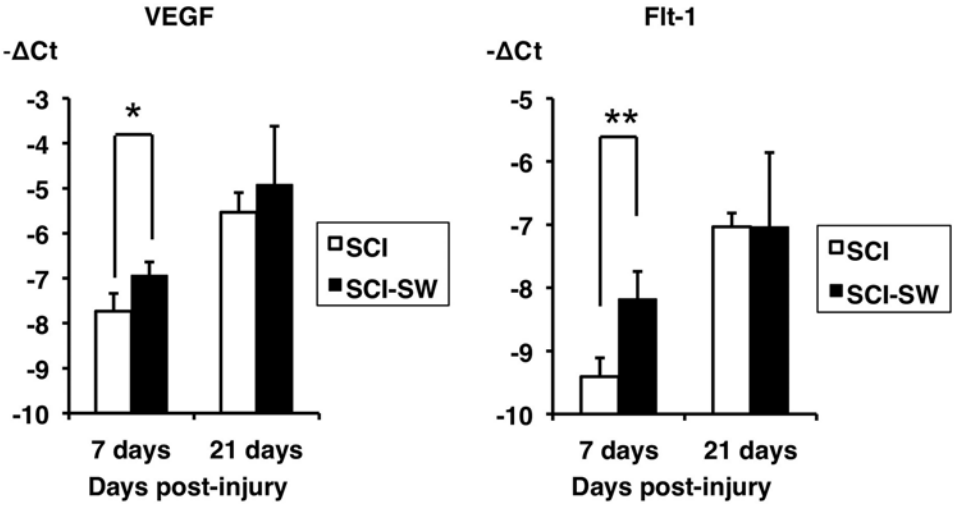

Fig. 6. The mRNA expression levels of VEGF and Flt-1 showed significant increases in the SCI-SW group compared with the SCI group at 7 days after SCI ($p = 0.018$ and 0.004, respectively). These mRNA expression levels in both groups were increased to the same or close to the same level at 21 days after the injury. This analysis was performed using 4 animals per group at each time point. $-\Delta Ct$ = a change in Ct values, which refers to the fractional cycle number at which the fluorescence passes the threshold. Values represent the means ± standard deviation. *$p < 0.05$; **$p < 0.01$.

In the developing nervous system, VEGF plays a pivotal role in vascularization and neuronal proliferation, as well as in the growth of coordinated vascular and neuronal networks. After injury to the nervous system, activation of VEGF and its receptors can restore the blood supply and promote neuronal survival and repair.[6] The upregulation of VEGF expression produces neuroprotective effects to reduce secondary injury and promotes vascularity as well as locomotor recovery after SCI.[32,60] Adenoviral VEGF administration promotes the regeneration of corticospinal tract axons in rats following transection of the spinal cord.[11] A previous report has suggested that the cytoprotective effects of VEGF are mediated through an antiapoptotic pathway.[62] Vascular endothelial growth factor stimulates nitric oxide (NO) release from the vascular endothelium and acts synergistically with NO to induce angiogenic and antiapoptotic effects.[33,44] Vascular endothelial growth factor can also perform multiple repair roles in the nervous system, including angiogenic, blood-brain barrier permeabilizing, and neurotrophic actions.[44,46] In the present study, expression levels of VEGF and Flt-1 in the injured spinal cord were significantly increased by the low-energy ESWT at 7 days after SCI. This treatment significantly reduced neuronal loss in the injured spinal cord and promoted locomotor recovery after SCI. Therefore, the increased expression of VEGF induced by the low-energy ESWT may provide neuroprotective effects that improve locomotor function after SCI.

Previous studies have demonstrated that the mRNA and protein expression levels of VEGF in the spinal cord are decreased in the acute and subacute phases (from 12 hours to 14 days) after SCI.[3,21,46,56] The decreased level of VEGF expression may cause a reduction in the endogenous neuroprotective function and worsen secondary damage after SCI.[21] Importantly, our results revealed that low-energy ESWT significantly upregulates VEGF and Flt-1 expression levels in the injured spinal cord at 7 days after injury. Previous studies have shown that the upregulation of VEGF expression starts to be seen within 7 days after the application of low-energy ESWT in various disease models.[51,57,61] These results suggest that the application of low-energy ESWT in the acute phase of SCI can reinforce the neuroprotective effect of VEGF and reduce the secondary damage following SCI.

The application of shock waves can regulate VEGF expression as well as induce various other biochemical effects, such as nonenzymatic NO synthesis, Ras activation, and expression of several chemokines and matrix metalloproteinases to provide anti-inflammatory effects.[9,34,35,51,58] A previous study showed that ESWT enhances endothelial NO synthase activity and intracellular NO production in HUVECs and produces an anti-inflammatory effect.[35] Extracorporeal shock wave therapy downregulates NF-κB activation and NF-κB–dependent gene expression, such as the expression of inducible NO synthase and tumor necrosis factor-α, and consequently induces an anti-inflammatory effect.[9] Additionally, ESWT can enhance the expression of growth factors, including BMP-2 and transforming growth factor-β, at treated sites.[8,59] Therefore, VEGF as well as other factors may induce a neuroprotective effect after the application of low-energy ESWT following SCI. It is unclear which type of cell in the spinal cord expresses VEGF in response to low-energy ESWT. The molecular mechanisms underlying the upregulation of VEGF expression induced by the low-energy ESWT needs to be elucidated in future studies. Further analysis of this therapy may lead to a detailed understanding of the molecular and biochemical mechanism and clinical application of this treatment for SCI. In the present study, the protocol used for ESWT application was based on those in previous studies, and only a single application of the shock waves was used. A different protocol for ESWT application may induce better effects on the injured spinal cord.

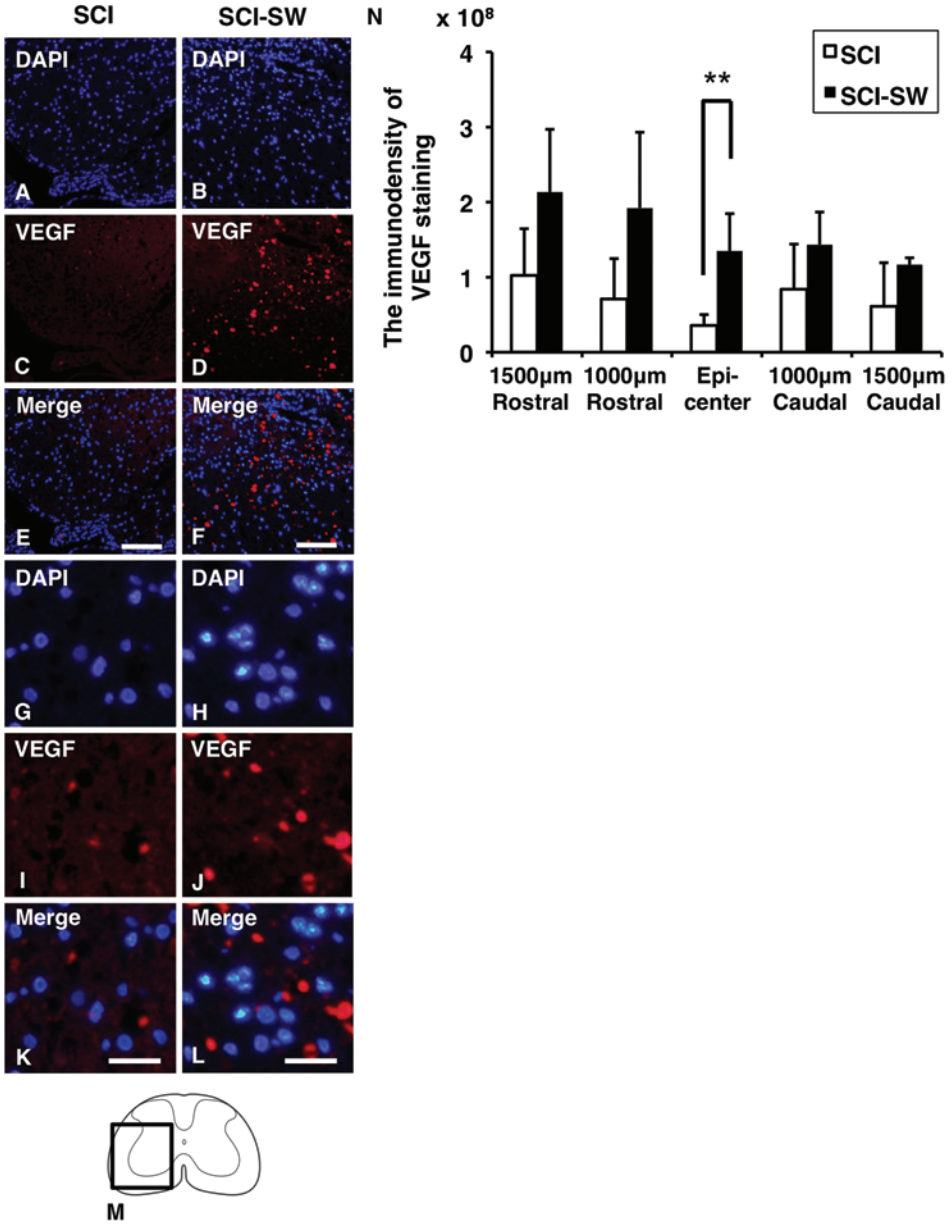

Fig. 7. Immunohistochemical staining of VEGF after SCI. **A–F:** In representative spinal cord sections at the lesion epicenter, the VEGF-positive cells were more frequently observed in the SCI-SW group than in the SCI group. Bars = 100 μm. **G–L:** Magnified images show the VEGF-positive cells. Bars = 25 μm. **M:** Schematic illustrates the location of the micrographs. **N:** The immunodensity of VEGF staining at the lesion epicenter was significantly higher in the SCI-SW group than in the SCI group ($p = 0.009$). The histological analysis was performed using 4 animals per group. Values represent the means ± standard deviation. $^{**}p < 0.01$.

In the clinical treatment of SCI, low-energy ESWT would be applied to the spinal cord from the dorsal side of the patients. Because bone as well as metal can interfere with the effects of shock waves, this extracorporeal treatment is suitable for patients after decompression surgery, such as laminectomy, at the lesion site. In patients treated with spinal fixation, the instruments should not be located between the probe delivering the shock wave and the lesion site on the spinal cord. A major advantage of low-energy ESWT is that it is noninvasive and safe, without any adverse effects or procedural complications.[15,37,54] If necessary, patients with SCI can undergo low-energy ESWT repeatedly, and the procedure is easy to perform because it does not require the induction of anesthesia, catheter intervention, or drug administration.

Conclusions

Findings in the present study showed that low-energy ESWT significantly increases expressions of VEGF and Flt-1 in the spinal cord without any detrimental effect. Furthermore, low-energy ESWT significantly reduced neuronal loss in damaged neural tissue and improved lo-

comotor function after SCI. These results demonstrated that low-energy ESWT enhances the neuroprotective effect of VEGF and leads to better locomotor recovery following SCI. This study provides the first evidence that low-energy ESWT can be a safe and promising therapeutic strategy for SCI.

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Disclosure

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