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Research paper

Low-energy extracorporeal shock wave therapy promotes BDNF expression and improves functional recovery after spinal cord injury in rats

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ABSTRACT

Low-energy extracorporeal shock wave therapy (ESWT) has been used to treat various human diseases. Previous studies have shown that low-energy ESWT promotes the release of various cell growth factors and trophic factors from the cells surrounding the target lesion. The aim of the current study was to determine whether the application of low-energy ESWT upregulates the expression of brain-derived neurotrophic factor (BDNF) and reduces neural tissue damage and functional impairment using a rat model of thoracic spinal cord contusion injury. We found that low-energy ESWT promoted BDNF expression in the damaged neural tissue. The expression of BDNF was increased in various neural cells at the lesion. Additionally, low-energy ESWT increased the area of spared white matter and the number of oligodendrocytes in the injured spinal cord compared with untreated control animals. There were more axonal fibers around the injured site after the application of low-energy ESWT than control. Importantly, low-energy ESWT improved the locomotor functions evaluated by both the BBB scale and ladder rung walking test in addition to the sensory function measured using a von Frey test. Moreover, the electrophysiological assessment confirmed that the conductivity of the central motor pathway in the injured spinal cord was restored by low-energy ESWT. These findings indicate that low-energy ESWT promotes BDNF expression at the lesion site and reduces the neural tissue damage and functional impairment following spinal cord injury. Our results support the potential application of low-energy ESWT as a novel therapeutic strategy for treating spinal cord injury.

1. Introduction

To date, extracorporeal shock wave therapy (ESWT) has been widely used for treatments of various human diseases. Numerous studies have highlighted the positive effects of ESWT on various pathological conditions (Raza et al., 2017; Schaden et al., 2015; Wang, 2012; Young Academic Urologists Men's Health et al., 2017; Zhang et al., 2017). According to the level of energy induced by the shock wave, ESWT is generally classified into two categories: low-energy and high-energy ESWT (Albert et al., 2007; Gerdesmeyer et al., 2003). Recently, low-energy ESWT has been used to treat diseases such as myocardial infarction (Fukumoto et al., 2006; Ito et al., 2009; Kagaya et al., 2018; Kikuchi et al., 2010), acute and chronic wounds (Zhang et al., 2017), peripheral artery disease (Serizawa et al., 2012) and erectile dysfunction (Man and Li, 2018). Furthermore, low-energy ESWT has a therapeutic effect in the treatment of orthopaedic pain syndromes, including calcific tendinitis, epicondylitis and plantar fasciitis (Albert et al., 2007; Gerdesmeyer et al., 2003; Haake et al., 2002; Zimmermann et al., 2008).

Numerous human clinical trials have suggested that ESWT produces therapeutic effects to improve functional outcomes in various diseases. ESWT induces myocardial revascularization and improves clinical symptoms and the left ventricular function in patients with ischemic heart disease (Wang et al., 2015; Zuoziene et al., 2012). It was also reported that ESWT improves the walking ability of patients with peripheral artery disease and intermittent claudication (Harwood et al.,

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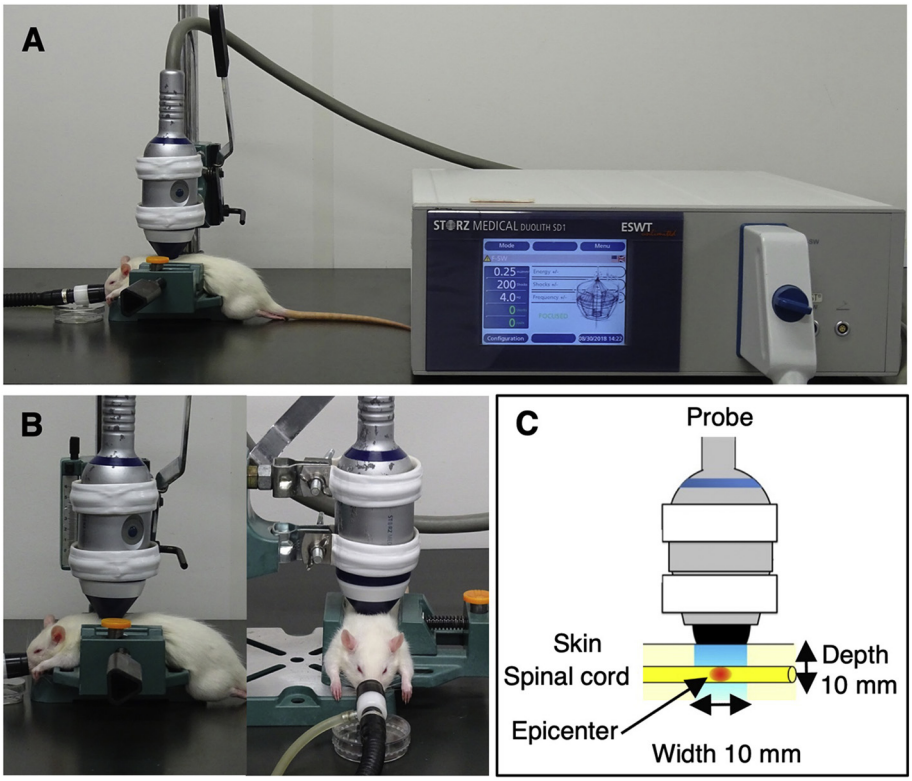

Fig. 1. Application of low-energy ESWT for SCI. (A, B) The shock wave probe was placed on the skin just behind the injured spinal cord between the T8 and T12 spinous processes. (C) The optimal focal point of the shock wave was within 10 mm in width and 10 mm in depth from the tip of the probe.

2018; Serizawa et al., 2012). In addition, ESWT promotes bone healing and provides better clinical outcomes in patients with a long-bone nonunion (Cacchio et al., 2009). ESWT has been shown to improve the symptom severity and functional status of patients with carpal tunnel syndrome (Wu et al., 2016).

High-energy ESWT may damage neural tissues, including the peripheral nerve (Wang et al., 2002), spinal cord (Karatas et al., 2008) and brain (Nakagawa et al., 2003). In contrast, low-energy ESWT has the potential to reduce neural tissue damage and improve functional outcomes in stroke as well as peripheral nerve injury (Hausner et al., 2012; Lee et al., 2016). In addition, low-energy ESWT improves the electrophysiological conductivity of the median nerve in patients with carpal tunnel syndrome (Raissi et al., 2017). Interestingly, low-energy ESWT has been shown to promote the release of various cell growth factors and trophic factors from the cells surrounding the target lesion (Hausner and Nogradi, 2013). Fibroblast growth factor-2 (FGF-2), a multifunctional cell growth factor, was shown to be produced in human fibroblasts and osteoblasts after ESWT (Hausdorf et al., 2011). In addition, low-energy ESWT increased the expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) in ischemic tissues *in vivo* and promoted angiogenesis and functional recovery in chronic myocardial ischemia (Abe et al., 2014; Nishida et al., 2004) and peripheral artery disease (Oi et al., 2008). Furthermore, the expression of neurotrophic factor and the subsequent regeneration of neurons were promoted by low-energy ESWT in a model of crushed sciatic nerve injury (Lee and Kim, 2015).

Many studies have suggested that brain-derived neurotrophic factor (BDNF) exerts a therapeutic effect on spinal cord injury (SCI) (Ikeda et al., 2002; McTigue et al., 1998; Weishaupt et al., 2012). BDNF has neuroprotective functions to prevent the apoptosis of oligodendrocytes and demyelination after SCI (Ji et al., 2015; Koda et al., 2002). Furthermore, BDNF attenuates axonal dieback in the injured spinal cord (Abe et al., 2014; Schwartz et al., 2005). BDNF can help promote functional recovery following SCI (Ikeda et al., 2002; Ji et al., 2015).

We previously reported that low-energy ESWT increases the expression of VEGF and reduces secondary neural tissue damage after SCI (Yahata et al., 2016; Yamaya et al., 2014). However, whether or not low-energy ESWT affects the expression of neurotrophic factors in the injured spinal cord is unclear. Considering that low-energy ESWT has a potential effect of increasing the expression of various cell growth factors and trophic factors at the target lesion (as described above), we hypothesized that low-energy ESWT could promote the expression of BDNF to induce a neuroprotective effect and improve functional outcomes after SCI. In the present study, we examined whether or not the application of low-energy ESWT upregulates the expression of BDNF and reduces secondary neural tissue damage and functional impairment using a rat model of SCI.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats (body weight, 250–300 g) were used (CLEA Japan, Tokyo, Japan). The animals were randomly divided into the following 3 groups: SCI group (SCI only), SCI-SW group (low-energy ESWT applied after SCI) and Sham group (laminectomy only). The group allocation was performed randomly to prevent bias in the present study. The animals were housed 2 per cage and were kept at a temperature of 24 °C with *ad libitum* access to water and food before and after surgery. All efforts were made to minimize the number of animals used and to decrease the suffering of the animals used in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee of our university.

2.2. Thoracic spinal cord contusion injury

The animals were anesthetized with 4% sevoflurane in an oxygen/nitrous oxide (30/70%) gas mixture. The rectal temperature was

monitored and maintained at 37.0 ± 0.5 °C by a heating pad (Fine Science Tools Inc., Canada) during surgery. A mid-line skin incision was made, and the laminae of the T8-T12 vertebrae were exposed. To expose the dorsal spinal cord surface, the T9–11 vertebrae were laminectomized with the dura intact. Using angled clamps attached to the T8 and T12 spinous processes, the vertebral column was stabilized. Then, SCI was made using a MASCIS Impactor (W.M. Keck Center) (Basso et al., 1995; Gruner, 1992). A 10-g rod was dropped from 12.5 mm onto the T10 segment. The skin and muscles were sutured in layers. Sham animals that received laminectomy without SCI were prepared as normal controls. Until spontaneous voiding began, bladders were expressed twice a day.

2.3. Extracorporeal shock wave therapy

Using a commercially available shock wave generator DUOLITH SD1 (Storz Medical AG) (Fig. 1A and B), low-energy ESWT was performed. According to our previous study protocol (Nishida et al., 2004), the shock wave was applied to 2 spots on the injured spinal cord 3 times a week for 3 weeks (at 0, 2, 4, 7, 9, 11, 14, 16, and 18 days after injury) following SCI. As described previously (Yahata et al., 2016; Yamaya et al., 2014), the condition of the shock wave was 0.25 mJ/mm^2, 4 Hz, 200 shots/spot, 2 spots for each treatment. 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 (Fig. 1C), and 0.1 mJ/mm^2 (positive energy flux density) is equivalent to 0.25 mJ/mm^2 (total energy flux density) (Serizawa et al., 2011). The focal point region was large enough to include the spinal cord lesion.

2.4. Quantitative RT-PCR

The animals were anesthetized with 4% sevoflurane and overdosed with an intraperitoneal injection of 100 mg/kg sodium pentobarbital at 7 or 21 days after injury. The spinal cord segments centered at the epicenter (5 mm in length) were harvested aseptically and then homogenized using POLYTRON (Kineatics). Total RNA was extracted using TRIZOL reagent (Invitrogen) and cleaned up using an RNeasy Mini Kit. A first-strand cDNA synthesis assay and a quantitative real-time polymerase chain reaction (RT-PCR) were performed to assess the mRNA expression of BDNF and its receptor, TrkB. Total RNA from the spinal cord was extracted with an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Using a High capacity cDNA archive kit (Applied Biosystems), first-strand cDNA was synthesized. The quantitative RT-PCR was performed using an ABI StepOnePlus, Power SYBR Green PCR MasterMix (Applied Biosystems), and 500 nM of each primer. These reactions were run routinely in duplicate. These primers were designed based on the sequences in the GenBank database (F: 5′-agcgcgaatgtgttagtggt-3′ and R: 5′-gcaattgtttgcctcttttttct-3′ for BDNF, F: 5′- cgaggttggaacctaacagc-3′, R: 5′-ccttttctggtttgcaatgag-3′ for TrkB). The fractional cycle number at which the fluorescence passes the threshold (Ct values) was used for quantification using a comparative Ct method. The Ct values of the gene of interest (Ct[GOI]) were standardized by that of β-actin (Ct[β-actin]). Results were shown as -ΔCt = −(Ct[GOI] - Ct[β-actin]) (Kishimoto et al., 2009; Yamaya et al., 2014).

2.5. The enzyme-linked immunosorbent assay of BDNF

An enzyme-linked immunosorbent assay (ELISA) was performed to measure the BDNF in the spinal cord tissues obtained at 7 days following injury. The spinal cord segments (5 mm in length) centered at the injured sites were obtained. Tissues were homogenized in lysis buffer, frozen quickly and stored at −20 °C. The specific assay was performed strictly according to kit instructions (Promega Catalog #G7610, RRID:AB_2571723).

2.6. Tissue preparation for staining

At 7 or 42 days following injury, the animals were anesthetized with 4% sevoflurane and overdosed with an intraperitoneal injection of 100 mg/kg sodium pentobarbital. The animals were transcardially perfused with normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. Then, spinal cord segments containing the lesion site were collected, post-fixed in the same fixative overnight at 4 °C. After rinsing overnight in phosphate buffer and cryoprotection in 30% sucrose, they were blocked and frozen in optimal cutting temperature (OCT) compound (Sakura Finetek). Serial 15-μm transverse sections around the injured site were mounted on slides. A total of 7 sequential sections at 500-μm intervals spanning a 3000-μm length of the spinal cord (centered at the epicenter) were prepared. The spinal cord sections were used for immunohistochemical staining and Luxol fast blue staining.

2.7. Immunohistochemistry

Using sections obtained at 7 and 42 days following injury, immunostaining was performed. The sections were washed in PBS for 10 min followed by washing with PBS containing 0.3% Tween for 10 min and blocking with 3% milk and 5% fetal bovine serum (FBS) in 0.01 M PBS for 2 h. The sections were incubated overnight with rabbit anti-BDNF (1:200, Abcam Catalog #ab108319, RRID: AB_10862052), mouse anti-RT97 (1:100, DSHB Catalog #rt97, RRID: AB_528399), and goat anti-Olig2 (1:100, Santa Cruz Biotechnology Catalog #sc-19969, RRID:AB_2236477) antibodies diluted in PBS at 4 °C. After rinsing with PBS, the sections were incubated with goat anti-mouse and donkey anti-goat IgG Alexa Fluor 488 secondary antibody (1:500, Thermo Fisher Scientific Catalog #A32723, RRID: AB_2633275 and A-11055, RRID:AB_2534102) or donkey anti-rabbit IgG Alexa Fluor 594 secondary antibody (1:500, Thermo Fisher Scientific Catalog #R37119, RRID: AB_2556547) for 1 h at room temperature. The sections were mounted with Vectashield containing DAPI to label the nuclei (Vector Laboratories Catalog #H-1200, RRID: AB_2336790). The sections were stained at the same time in each of the experiments.

2.8. Double-staining of BDNF and markers of various cell types

To examine the expression of BDNF in specific populations of cells in the injured spinal cord, transverse sections obtained at 7 days from the SCI-SW group were double-stained for BDNF and markers of various cell types: NeuN for neurons, GFAP for astrocytes, and Olig2 for oligodendrocytes. The sections were incubated overnight with a mixture of rabbit anti-BDNF antibody (1:200, Abcam Catalog #ab108319, RRID: AB_10862052) and goat anti-Olig2 (1:100, Santa Cruz Biotechnology Catalog #sc-19969, RRID: AB_2236477), mouse anti-GFAP (1:50, Agilent Technologies Catalog #M0761, RRID: AB_2109952), or mouse anti-NeuN (1:100, Millipore Catalog #MAB377, RRID: AB_2298772) antibodies diluted in PBS at 4 °C. After rinsing with PBS, the sections were incubated with a mixture of donkey anti-rabbit IgG Alexa Fluor 594 antibody (1:500, Thermo Fisher Scientific Catalog #R37119, RRID: AB_2556547) and either donkey anti-goat IgG Alexa Fluor 488 (1:500, Thermo Fisher Scientific Catalog #A-11055, RRID: AB_2534102) or goat anti-mouse IgG Alexa Fluor 488 (1:500, Thermo Fisher Scientific Catalog #A32723, RRID: AB_2633275) antibodies for 1 h at room temperature. The sections were mounted with Vectashield containing DAPI to label the nuclei (Vector Laboratories Catalog #H-1200, RRID: AB_2336790).

2.9. White matter staining

Transverse sections obtained 42 days after injury were used for Luxol fast blue staining to analyze the spared white matter. The images of the stained sections were captured using a microscope, and the

spared white matter area of the spinal cord was analyzed using the Image J 1.50i software program. In the stained sections, the spared white matter appeared dark blue and isocellular, as healthy spinal cord. In contrast, the degenerated or damaged white matter was blanched or replaced by scar tissue that contained clusters of cells with prominent basophilic nuclei (Bao et al., 2011; Steward et al., 1999). This study compared the spared white matter areas between the SCI-SW group and SCI group.

2.10. The immunodensity analysis of BDNF and RT97 staining

Following the immunostaining with BDNF and RT97, each section was scanned using a confocal microscope (BX 51; Olympus,). The sections 1500 μm rostral and caudal to the lesion epicenter were used. For imaging, the appropriate setting for avoiding signal saturation was determined in the first microscopy session and the same setting was used thereafter. The entire spinal cord containing the lesion and perilesional areas was traced in each section using the ImageJ software program. Then, this study performed automatic thresholding for each image using the software program to determine the threshold for a specific signal. Following the threshold setting, the immunodensity above the threshold was automatically calculated (Kanno et al., 2014; Yahata et al., 2016). The default threshold setting was used to maintain the constant levels for all analyses.

2.11. The counting of Olig2-positive cells

To investigate the loss of oligodendrocytes, the number of Olig2-positive cells in the sections at 42 days after injury was counted. Sections 1500 μm rostral and caudal to the lesion epicenter were used. The number of Olig2-positive cells in each section was counted. The Olig2-positive cells were defined as cells double-labeled with Olig2 and DAPI. The cell counting procedure has been described previously (Yahata et al., 2016; Yamaya et al., 2014). The numbers of the Olig2-positive cells were compared between the SCI-SW and SCI groups.

2.12. The BBB locomotor scale

Using the Basso, Beattie, and Bresnahan (BBB) open-field locomotor score, the locomotor function was evaluated for 6 weeks after SCI (Basso et al., 1995). Locomotor recovery, including joint movement, stepping ability, coordination, and trunk stability, can be evaluated using the BBB score (range 0–21 points). For the assessment, the animals were placed individually in an open field with a non-slippery surface for 4 min, and well-trained investigators determined the BBB score in a blinded manner. We confirmed that all animals consistently obtained the maximum score before SCI. In this study, we evaluated the BBB scores at 4 and 24 h and at 4, 7, 14, 21, 28, 35, and 42 days following injury. To obtain a single value per rat per test, the BBB scores were calculated for the left and right hind limbs and were averaged.

2.13. Ladder rung walking test

To further evaluate the locomotor function, a ladder rung walking test was performed at 21 and 42 days. The ladder rung walking task allows the loss and recovery of motor functions to be assessed (Metz and Whishaw, 2002). The horizontal ladder rung apparatus consisted of side walls made of clear Plexiglas and metal rungs (diameter, 3 mm), which could be inserted to create a floor with a minimum distance of 1 cm between rungs. The number of errors in each crossing was counted. Errors were determined based on the foot fault scoring system (Metz and Whishaw, 2009).

2.14. Mechanical allodynia

In order to assess mechanical hypersensitivity in the hindpaw, the withdrawal threshold was evaluated using a von Frey filament (0.25–15 g) applied to the plantar surface at 21 and 42 days (Chaplan et al., 1994). To determine the value at which paw withdrawal occurred 50% of the time, a modification of the “up-down” method (Dixon, 1980) in this study.

2.15. Electrophysiology

Motor-evoked potentials (MEPs) have been widely used in clinical and animal trials to evaluate the neuromuscular function (Garcia-Alias et al., 2006; Wang and Zhang, 2012). MEPs were evoked and recorded by electromyography and an evoked potential response unit (Neuropack2300, Nihon Kohden). After the administration of ketamine (100 mg/kg, intraperitoneal) and xylazine (10 mg/kg, intraperitoneal), short trains of five square-wave stimuli of 0.5 ms in duration with an interstimulus interval of 2 ms were delivered through the occipitocervical area by spiral electrodes and a needle was placed in the right hindlimb. The latency and amplitude were observed.

2.16. Statistical analysis

Significant differences between the SCI and SCI-SW groups were analyzed using the Mann-Whitney *U* test. In the assessment of the latency of MEP, statistical differences were analyzed using a one-way ANOVA. The correlations between the latency of MEP and total BBB score was assessed using a Pearson correlation analysis. A Pearson correlation between the latency and the number of errors in the ladder rung test was also analyzed. In all analyses, p values of < 0.05 were considered to indicate statistical significance, and Pearson's correlation coefficient was reported as an r value. All statistical analyses were performed using the Microsoft Excel (Microsoft) software program and Statcel 4 (OMS Publishing Inc.) an add-on application.

3. Results

3.1. Low-energy ESWT promotes the expression of BDNF following spinal cord injury

As a first step towards addressing whether low-energy ESWT affects the BDNF expression at the lesion site after SCI, we examined the mRNA expression of BDNF in the injured spinal cord using a real-time PCR. As shown Fig. 2A, the mRNA expression of BDNF in the SCI-SW group was significantly higher than that in the SCI group at 7 days following SCI ($p = .036$). The BDNF mRNA expression increased at 21 days in comparison to 7 days in both the SCI group and SCI-SW group. However, the mRNA levels of BDNF in the two groups did not differ to a statistically significant extent at 21 days (Fig. 2A). Next, we examined mRNA expression of TrkB, which is known as a receptor of BDNF. The mRNA expression of TrkB in the SCI-SW group was significantly increased in comparison to the SCI group at 7 days ($p = .008$; Fig. 2 A). Similarly to the mRNA expression of BDNF, the TrkB mRNA expression in both the SCI and SCI-SW groups was higher at 21 days in comparison to 7 days after SCI and the levels of TrkB in the two groups at 21 days did not differ to a statistically significant extent.

To confirm the alternation of the BDNF protein expression, we performed an ELISA of BDNF using injured spinal cord specimens. The ELISA showed that the BDNF protein expression in the SCI-SW group was significantly upregulated in comparison to the SCI group at 7 days ($p = .027$) (Fig. 2B).

In order to investigate the distribution of the BDNF protein expression at the lesion site, we performed an immunohistochemical analysis of BDNF using spinal cord sections. Representative pictures of the BDNF-stained sections (Fig. 2C-P) showed that the number of cells expressing BDNF in the SCI-SW group (Fig. 2D, K-P) was remarkably increased in comparison to the SCI group (Fig. 2C, E-J). Furthermore, the immunodensity of BDNF staining in the SCI-SW group was

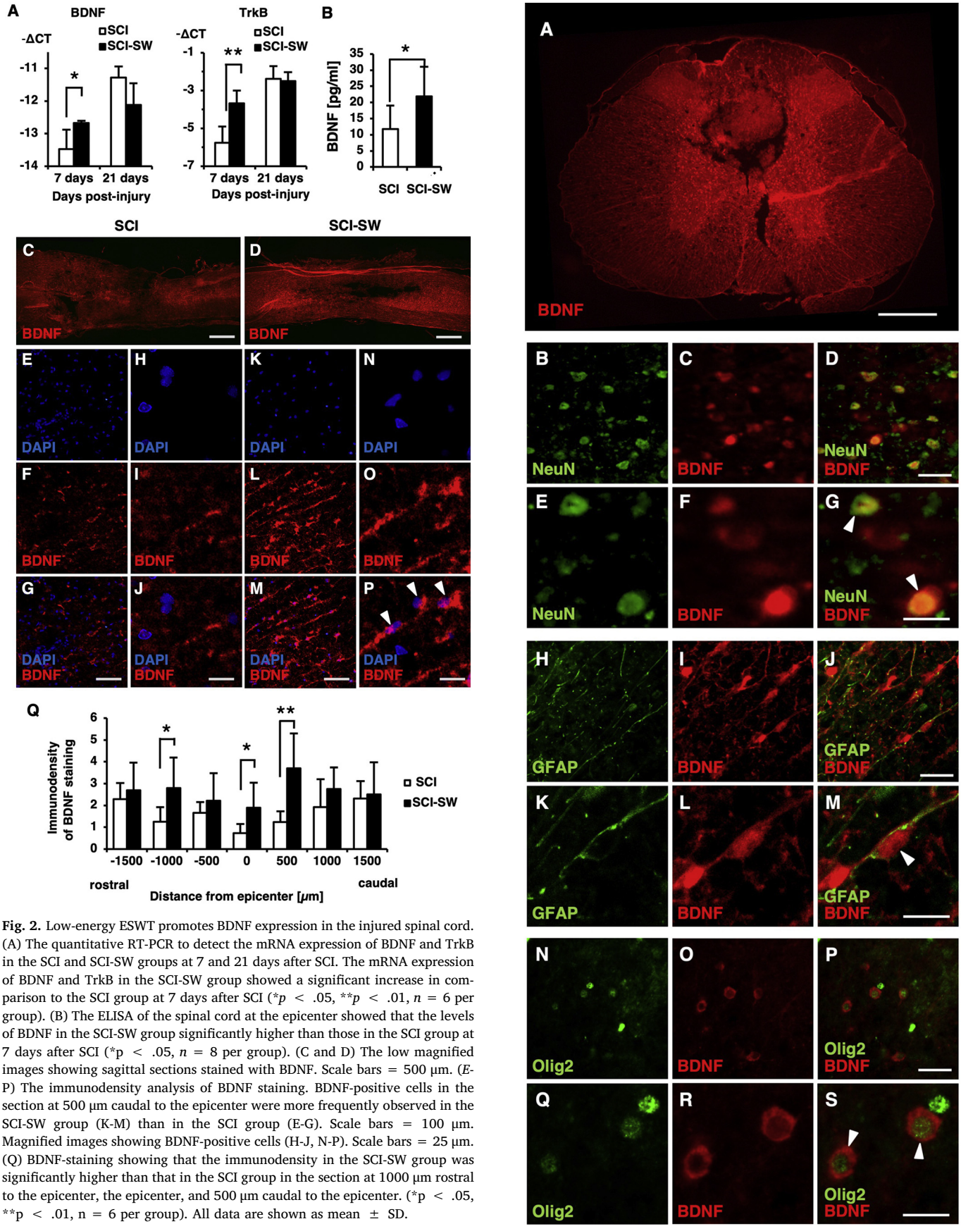

Fig. 2. Low-energy ESWT promotes BDNF expression in the injured spinal cord. (A) The quantitative RT-PCR to detect the mRNA expression of BDNF and TrkB in the SCI and SCI-SW groups at 7 and 21 days after SCI. The mRNA expression of BDNF and TrkB in the SCI-SW group showed a significant increase in comparison to the SCI group at 7 days after SCI (*$p < .05$, **$p < .01$, $n = 6$ per group). (B) The ELISA of the spinal cord at the epicenter showed that the levels of BDNF in the SCI-SW group significantly higher than those in the SCI group at 7 days after SCI (*p < .05, $n = 8$ per group). (C and D) The low magnified images showing sagittal sections stained with BDNF. Scale bars = 500 μm. (*E*-P) The immunodensity analysis of BDNF staining. BDNF-positive cells in the section at 500 μm caudal to the epicenter were more frequently observed in the SCI-SW group (K-M) than in the SCI group (E-G). Scale bars = 100 μm. Magnified images showing BDNF-positive cells (H-J, N-P). Scale bars = 25 μm. (Q) BDNF-staining showing that the immunodensity in the SCI-SW group was significantly higher than that in the SCI group in the section at 1000 μm rostral to the epicenter, the epicenter, and 500 μm caudal to the epicenter. (*p < .05, **p < .01, n = 6 per group). All data are shown as mean ± SD.

(caption on next page)

Fig. 3. The upregulation of BDNF expression in various neural cells at 7 days after SCI. (A) The low magnified image showing transverse sections at 1500 μm caudal stained with BDNF. Scale bars = 500 μm. (B-S) The increased expression of BDNF observed in the NeuN- (B-G), GFAP- (H-M), and Olig2- (N-S) labeled cells in the injured spinal cord demonstrate that the BDNF expression was enhanced by ESWT in neurons, astrocytes, and oligodendrocytes, respectively. Scale bar, 20 μm (B-D, H-J, N-P), 10 μm (E-G, K-M, Q-S).

significantly increased than that in the SCI group at 1000 μm rostral and 500 mm caudal to the epicenter, and at the epicenter (p = .037, 0.004, 0.025, respectively) (Fig. 2Q).

3.2. Low-energy ESWT enhances the BDNF expression in various neural cells in the injured spinal cord

To investigate the BDNF expression in a specific population of neural cells at the lesion site, sections of the spinal cord obtained 7 days after injury from the SCI-SW groups were double-stained for BDNF and various cell type markers. Double-staining clearly demonstrated that BDNF was expressed in NeuN-, GFAP-, and Olig2-labeled cells (Fig. 3).

3.3. Low-energy ESWT reduces myelin damage and oligodendrocyte loss following spinal cord injury

It is well known that BDNF has a neuroprotective function to reduce demyelination after SCI (Ji et al., 2015). Since the expression of BDNF in the injured spinal cord was upregulated by low-energy ESWT, as described above (Fig. 2), we expected that the increased expression of BDNF would attenuate myelin damage. To evaluate the difference in the amount of demyelination after the application of low-energy ESWT, we examined the areas of spared white matter using Luxol fast blue staining (Joshi and Fehlings, 2002). The representative pictures showed that the areas of spared white matter in the sections around the epicenter in the SCI-SW group were markedly larger than those in the SCI group (Fig. 4A-D). As shown in Fig. 4E and F, diffuse microcystic cavitation at the lesion site in the SCI group was more evident than that in the SCI-SW group. Moreover, a quantitative analysis revealed that the average spared white matter areas in sections in the SCI-SW group were significantly larger than those in the SCI group, from 1000 μm rostral to 500 μm caudal to the epicenter (p < .05) (Fig. 4G).

BDNF also has a beneficial role in preventing apoptosis of oligodendrocytes in the injured spinal cord (Koda et al., 2002). To examine the cytoprotective effect of low-energy ESWT on oligodendrocytes, we performed an immunohistochemical analysis of Olig2 using sections obtained 42 days after injury. As shown in Fig. 5, Olig2-positive cells were sparsely observed at the epicenter in representative stained sections of the SCI group (Fig. 5A-F). In contrast, Olig2-positive cells were more frequently observed in the spinal cords of the SCI-SW group (Fig. 5G-L). The number of Olig2-positive cells in the SCI-SW group was significantly higher compared to that in the SCI group at the epicenter and 1000 μm caudal to the epicenter (p = .009 and 0.016, respectively) (Fig. 5M).

3.4. Axonal damage in the injured spinal cord is attenuated by low-energy ESWT

Since BDNF has previously been shown to prevent axonal dieback after SCI (Bretzner et al., 2008; Schwartz et al., 2005; Weishaupt et al., 2012), we hypothesized that the increase in the expression of BDNF after the application of low-energy ESWT could reduce axonal damage in the injured spinal cord. To investigate the amount of axonal fibers in the lesion site at 42 days after injury, we performed an immunohistochemical analysis of neurofilament using RT97 antibodies (Bigbee et al., 2008). As shown in representative pictures (Fig. 6), the RT97-positive fibers were more frequently observed in the SCI-SW group (Fig. 6B, I-N) than in the SCI group (Fig. 6A, C-H). Furthermore, the immunodensity of RT97-positive fibers around the lesion site in the SCI-SW group was significantly higher than that in the SCI group (p < .05) (Fig. 6O). Interestingly, the diameter of the RT97-positive axons in the SCI-SW group appeared to be larger than that in the SCI group (Fig. 6H and N).

3.5. Low-energy ESWT improves the recovery of the locomotor function after spinal cord injury

To evaluate an effect of low-energy ESWT on the recovery of the open field locomotor function, the BBB scores were measured for 6 weeks after injury. Notably, the total BBB score revealed that the animals in the SCI-SW group showed significantly better locomotor improvement in comparison to those in the SCI group from 7 to 42 days after injury (p < .05) (Fig. 7A). At 42 days after injury, the total BBB scores in the SCI and SCI-SW groups were 12.9 ± 1.2 and 15.6 ± 1.8, respectively. To further examine the specific locomotor components (*i.e.*, paw position, trunk control, and tail position), we evaluated the BBB subscores. The BBB subscores (Fig. 7B) showed that the locomotor function of the SCI-SW group was consistently higher than that of the SCI group from 7 to 42 days. Significant differences were observed from 28 days to 42 days (p < .05).

Next, we assessed hindlimb placing and stepping errors using the ladder rung walking test (Metz and Whishaw, 2002). The number of errors in the animals after SCI was increased in comparison to the sham control animals. Importantly, the number of errors in the SCI-SW group was significantly lower than that in the SCI group at 21 days and 42 days (p = .016 and 0.023, respectively) (Fig. 7C).

3.6. Impairment of the sensory function is reduced by low-energy ESWT following spinal cord injury

To address the impairment of sensory function, we examined mechanical hypersensitivity using von Frey filaments. Withdrawal thresholds to mechanical stimuli markedly decreased in the animals following injury in comparison to the sham animals. However, the decrease in the withdrawal threshold in the SCI-SW group was less than that in the SCI group at 21 and 42 days (Fig. 7D). The animals in the SCI-SW group showed a significantly higher withdrawal threshold in comparison to those in the SCI group at 21 and 42 day (p < .001 and p = .001, respectively).

3.7. Low-energy ESWT improves conduction of the central motor pathway through the spinal cord lesion site

To examine conduction of the central motor pathway in the injured spinal cord, MEPs were measured at 42 days after injury (Fig. 8A). The representative data of the MEPs are shown in Fig. 8B-D. There was no significant difference in the amplitude of MEPs between the SCI and SCI-SW groups (Fig. 8E). On the other hand, the latency of MEPs in the SCI group was significantly elongated in comparison to the SCI-SW groups (p = .010) (Fig. 8F), indicating that the electrophysiological conductivity of the injured spinal cord was more preserved in the SCI-SW group. Plotting the latency of MEPs against the total BBB score (Fig. 8G) and number of errors in the ladder rung test (Fig. 8H) revealed significant correlations between a long MEP latency and poor locomotor functions in each animal (Fig. 8G and H).

4. Discussion

The present study demonstrated that low-energy ESWT significantly increased the expression of BDNF in the damaged neural tissue after SCI. This treatment also reduced white matter damage and the loss of oligodendrocytes in the injured spinal cord. In addition, the number of axonal fibers at the lesion site was significantly higher in animals

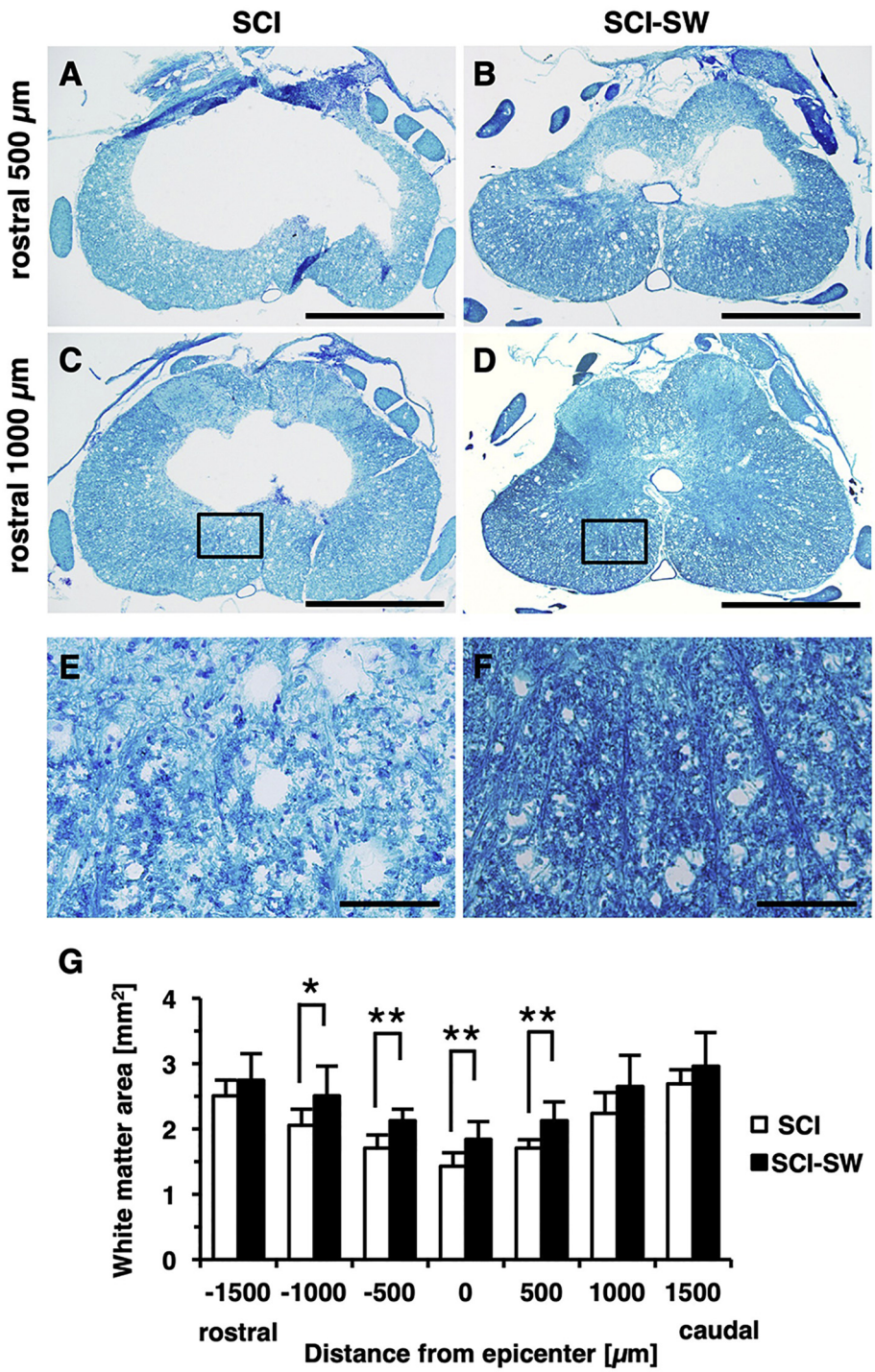

Fig. 4. Low-energy ESWT increased white matter sparing at 42 days after SCI. (A-D) Representative spinal cord sections 1000 μm and 500 μm rostral to the epicenter show that the spared white matter area in the SCI-SW group (B, D) was larger than that in the SCI group (A, C). Scale bars = 1000 μm. High-magnification views of black boxes in (C) and (D) are shown in (E) and (F). Scale bars = 100 μm. (G) The spared white matter area around the epicenter was compared between the SCI and SCI-SW groups. The areas of spared white matter in the SCI-SW group are significantly larger than those in the SCI group from 1000 μm rostral to 500 μm caudal to the epicenter. (*$p < .05$, ** $p < .01$, $n = 8$ per group). All data are shown as mean ± SD.

treated with low-energy ESWT. These findings indicate that low-energy ESWT induces a neuroprotective effect caused by the upregulation of BDNF and prevents secondary neural tissue damage. Our study further revealed that low-energy ESWT markedly improved not only locomotor impairment but also sensory function after injury. Moreover, the electrophysiological assessment confirmed that the conductivity of the injured spinal cord was restored in animals treated with low-energy ESWT. Thus, the present study suggested that low-energy ESWT can be a novel therapeutic strategy for the treatment of SCI.

Many studies have shown that low-energy ESWT promotes expressions of various cell growth factors, such as VEGF (Nishida et al., 2004), eNOS (Mariotto et al., 2005), proliferating cell nuclear antigen (PCNA) (Zhao et al., 2018), Transforming growth factor-β1 (TGF-β1) (Chen et al., 2004), FGF-2 (Hausdorf et al., 2011), (Bone Morphogenetic Protein-2) BMP-2 (Wang et al., 2014) in different pathological conditions both *in vivo* and *in vitro*. It was been also suggested that the low-energy ESWT promotes expressions of various neurotrophic factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3) and BDNF in damaged neural tissues. These neurotrophic factors can bind Trk receptors and contribute to various molecular functions. The low-energy ESWT stimulated the expression of BDNF through the activation of the PERK/ATF4 signaling pathway in a model of bilateral cavernous nerve crush injury (Wang et al., 2017). In addition, a previous study suggested that the expression of NT-3 was enhanced by low-energy ESWT after injury in a model of crushed sciatic nerve injury (Lee and Kim, 2015). Low-energy ESWT has been shown to upregulate the expression of NGF

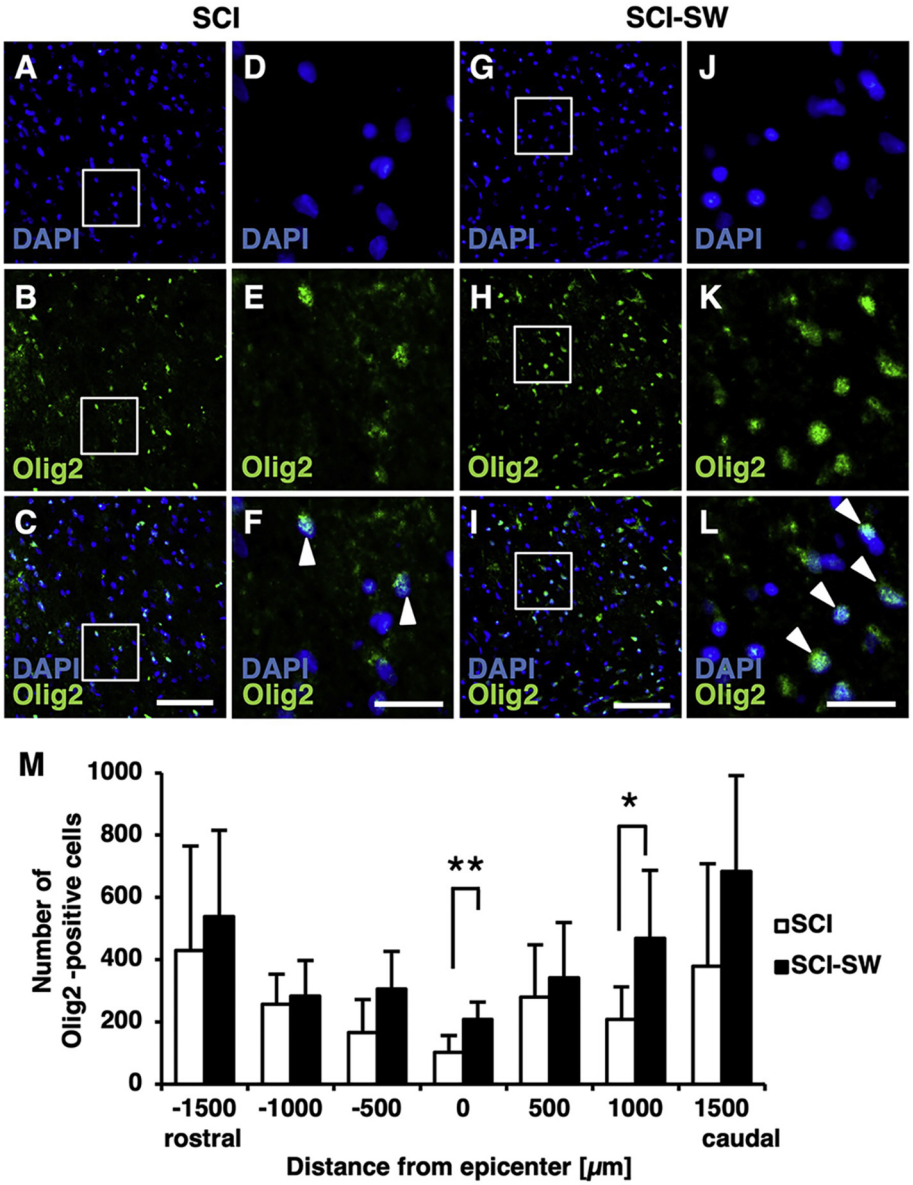

Fig. 5. The Olig2-staining in SCI and SCI-SW groups at 42 days after SCI. (A-C, G-I) Representative sections 1000 μm rostral to the epicenter show that there were more Olig2-positive cells in the SCI-SW group (G-I) than in the SCI-group (A-C). Scale bars = 100 μm. (D-F, J-L) High-magnification views of white boxes. The magnified images show Olig2-positive cells. Scale bars = 20 μm. (M) The number of Olig2-positive cells in the SCI-SW group was significantly higher than that in the SCI group at the epicenter and 1000 μm caudal to the epicenter. (*$p < .05$, ** $p < .01$, $n = 8$ per group). All data are shown as mean ± SD.

in stroke (Lee et al., 2016). These findings suggest that low-energy ESWT has an important effect in increasing the expression of various neurotrophic factors in damaged neural tissues. In the present study, we found that low-energy ESWT significantly upregulated both the mRNA and protein expressions of BDNF in the spinal cord at 7 days after injury. Additionally, the mRNA expression of TrkB (a BDNF receptor) in the injured spinal cord was increased by low-energy ESWT. Furthermore, an immunohistochemical analysis revealed that the BDNF expression was increased in various neural cells, such as neurons, astrocytes and oligodendrocytes, at the lesion site. Our findings indicate that low-energy ESWT contributes to the upregulation of the BDNF expression in damaged neural tissue following SCI.

BDNF is one of the most extensively tested neurotrophins in experimental models of SCI. Many studies have shown that BDNF produces neuroprotective effects in the injured spinal cord (Ikeda et al., 2002; Weishaupt et al., 2012). A previous study demonstrated that the increased expression of BDNF inhibits inflammation and demyelination after SCI in mice (Ji et al., 2015). BDNF suppresses the delayed apoptosis of oligodendrocytes after SCI in rats (Koda et al., 2002). In addition, it has been reported that exogenously applied BDNF reduces the atrophy of cortical, rubrospinal and spinal neurons (Harvey et al., 2015). One of the functions of BDNF is the attenuation of axonal damage in the injured spinal cord (Bretzner et al., 2008; Sayer et al., 2002; Schwartz et al., 2005). Furthermore, the delivery of BDNF has many beneficial effects, including the enhancement of neurite outgrowth, axonal regeneration (Fouad et al., 2013), and the promotion of myelination (Boyce et al., 2012). Importantly, in the current study, we found that the expression of BDNF was upregulated and that the spared white matter area and the number of oligodendrocytes in the injured spinal cord were increased by low-energy ESWT. Our data also revealed that amount of the RT97-stained axonal fibers in the lesion was significantly greater in animals treated by low-energy ESWT. Taken together, these data suggest that the increased expression of BDNF induced by low-energy ESWT provides the neuroprotective effect in damaged neural tissue after SCI.

Recently, basic research in several studies has demonstrated that

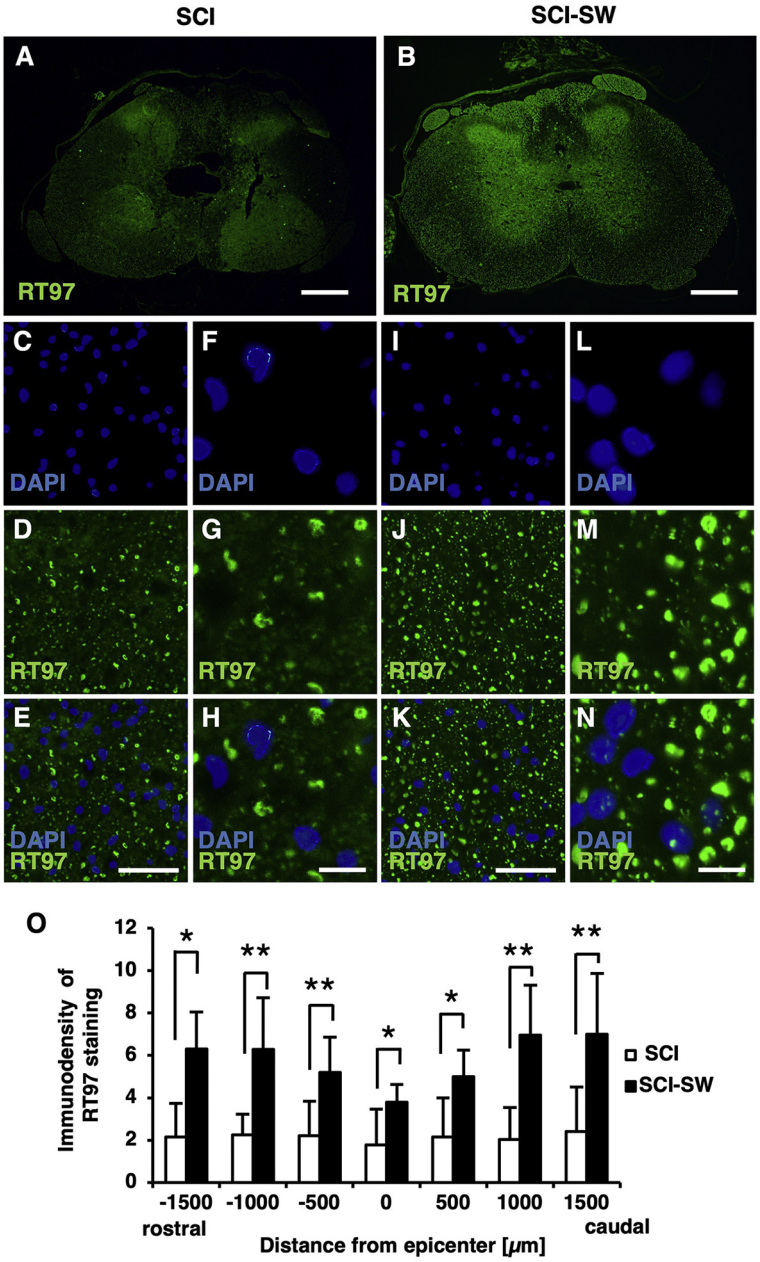

Fig. 6. The immunodensity analysis of RT97-positive axons in the injured spinal cord. (A and B) The low magnified image showing transverse sections at 1500 μm caudal stained with RT97. Scale bars = 500 μm. (C-E, I-K) RT97-positive axons were more frequently observed in the sections of the SCI-SW group (I-K) than in those of the SCI group (C-E). Scale bars = 50 μm. (F-H, L-N) Magnified images showing RT97-positive axons. Scale bars = 10 μm. (O) The immunodensity of RT97-staining in the SCI-SW group was significantly higher than that in the SCI group at all sections. (*$p < .05$, ** $p < .01$, $n = 8$ per group). All data are shown as mean ± SD.

low-energy ESWT has the potential to attenuate neural tissue damage and improve functional recovery in various diseases of the peripheral nerve and CNS. Low-energy ESWT was shown to increase the neurotrophin expression and enhance motor performance in a rat model of crushed sciatic nerve injury (Lee and Kim, 2015). Furthermore, low-energy ESWT effectively attenuated the brain infarct volume and improved the neurological function in rats after acute ischemic stroke (Kang et al., 2017; Yuen et al., 2015). Importantly, our observation revealed that low-energy ESWT significantly increased the expression of BDNF to provide a neuroprotective effect and consequently improved the recovery of the locomotor and sensory functions after SCI. Moreover, the electrophysiological assessment of MEPs showed that the conduction of the central motor pathway in the injured spinal cord was restored by low-energy ESWT, which objectively confirmed that the motor function was significantly improved. It is well-known that the therapeutic effects of BDNF can contribute to promoting functional recovery after SCI (Ikeda et al., 2002; Ji et al., 2015; Weishaupt et al., 2012). Taken together, these data indicate that the upregulation of the BDNF expression induced by low-energy ESWT may play a crucial role in enhancing the functional recovery after SCI. Thus, the present study provides evidence to support that low-energy ESWT may be a promising candidate as a treatment to reduce the functional impairment in patients with SCI.

Several limitations associated with the present study should be mentioned. First, the low-energy ESWT potentially has a function to promote expressions of various other molecular factors that improve neurological outcomes after SCI. Therefore, not only BDNF but also other factors might affect the results in this study. Further studies, such as the comparison between low-energy ESWT and administration of BDNF, may help to clarify the issue. Second, in the clinical use of low-

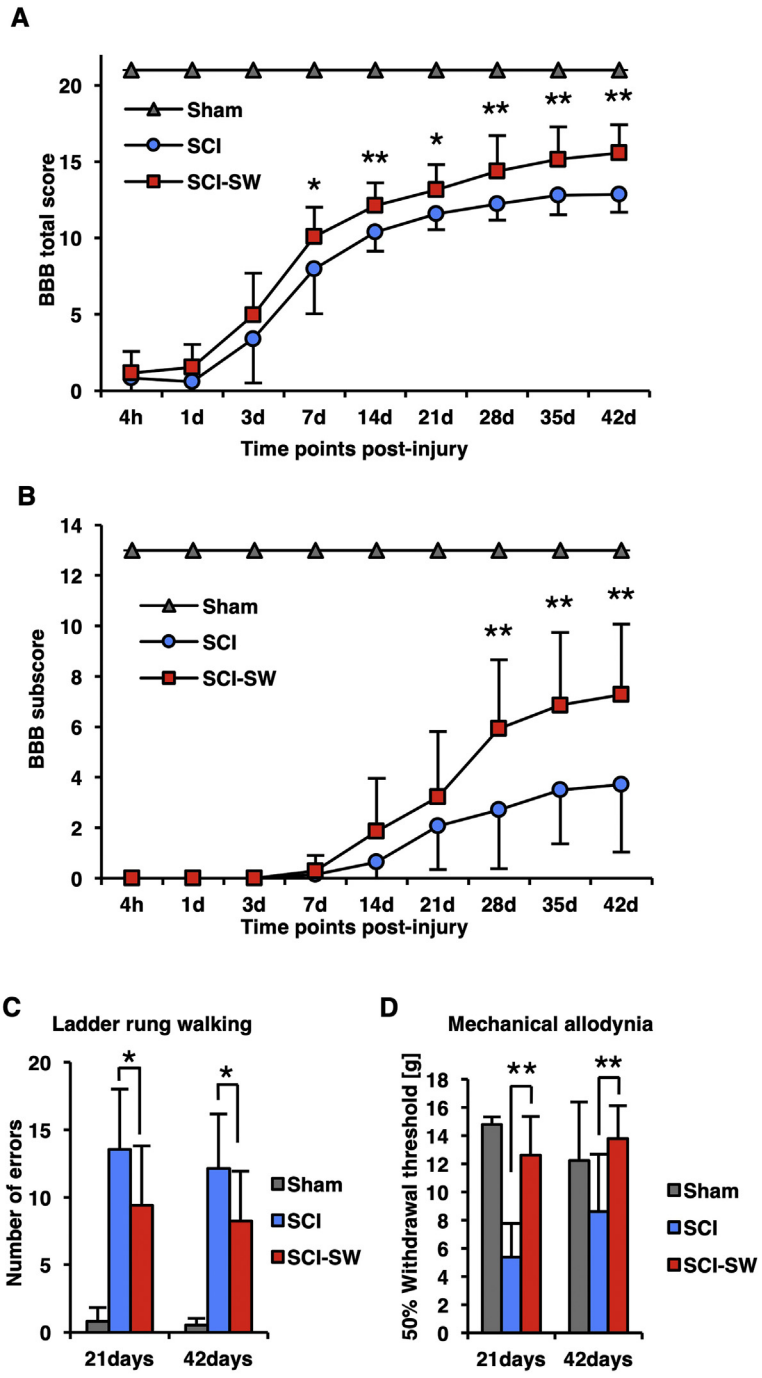

Fig. 7. Low-energy ESWT improved locomotor and sensory functions after SCI. (A) The SCI-SW group showed significantly better locomotor improvement as assessed by the BBB score than the SCI group from 7 to 42 days after injury. (B) The BBB subscores in the SCI-SW group were significantly higher than those in the SCI group from 28 to 42 days after injury. (C) Ladder rung walking tests at 21 and 42 days after SCI revealed that the number of errors in the SCI-SW group was significantly lower in comparison to the SCI group at 21 days and 42 days. (D) In the assessment of mechanical hypersensitivity following SCI, the SCI-SW group showed a significantly higher withdrawal threshold in comparison to the SCI group at 21 and 42 days. (*$p < .05$, **$p < .01$, $n = 14$ in the SCI and SCI-SW group, $n = 10$ in the Sham group). All data are shown as mean ± SD.

energy ESWT for human SCI, the depth from the body surface to the injured spinal cord and surrounded lamina might reduce the therapeutic effect of this treatment. Further studies are needed to verify the clinical usefulness of the low-energy ESWT in spinal cord-injured persons.

Low-energy ESWT is well accepted as a less-invasive and safe procedure. High-energy ESWT can cause injury to the neurovascular structures of the spinal cord (Karatas et al., 2008). In contrast, previous studies demonstrated that low-energy ESWT induced no evident neural tissue damage in the spinal cord or brain (Lee et al., 2007; Yamaya et al., 2014). A major advantage of low-energy ESWT is considered to be associated with a lower risk of adverse effects and procedural complications in comparison to other more-invasive treatment options, such as surgery, cell transplantation (Rosenfeld et al., 2008) and drug treatment (Chikuda et al., 2014). To date, low-energy ESWT has been widely used for many human diseases; thus, there are unlikely to be ethical problems associated with its medical use. Additionally, in the clinical treatment of patients with SCI, if needed, we can repeatedly apply low-energy ESWT to the injured site because no additional procedures such as anesthesia or drug administration, are required. Thus, low-energy ESWT may be a novel therapeutic strategy for safely and effectively treating patients suffering from SCI.

5. Conclusions

We observed that low-energy ESWT enhanced the expression of BDNF in the damaged neural tissue and induced a neuroprotective effect to prevent demyelination and axonal damage after SCI. Furthermore, low-energy ESWT enhanced the recovery of the locomotor and sensory functions following injury. The present study

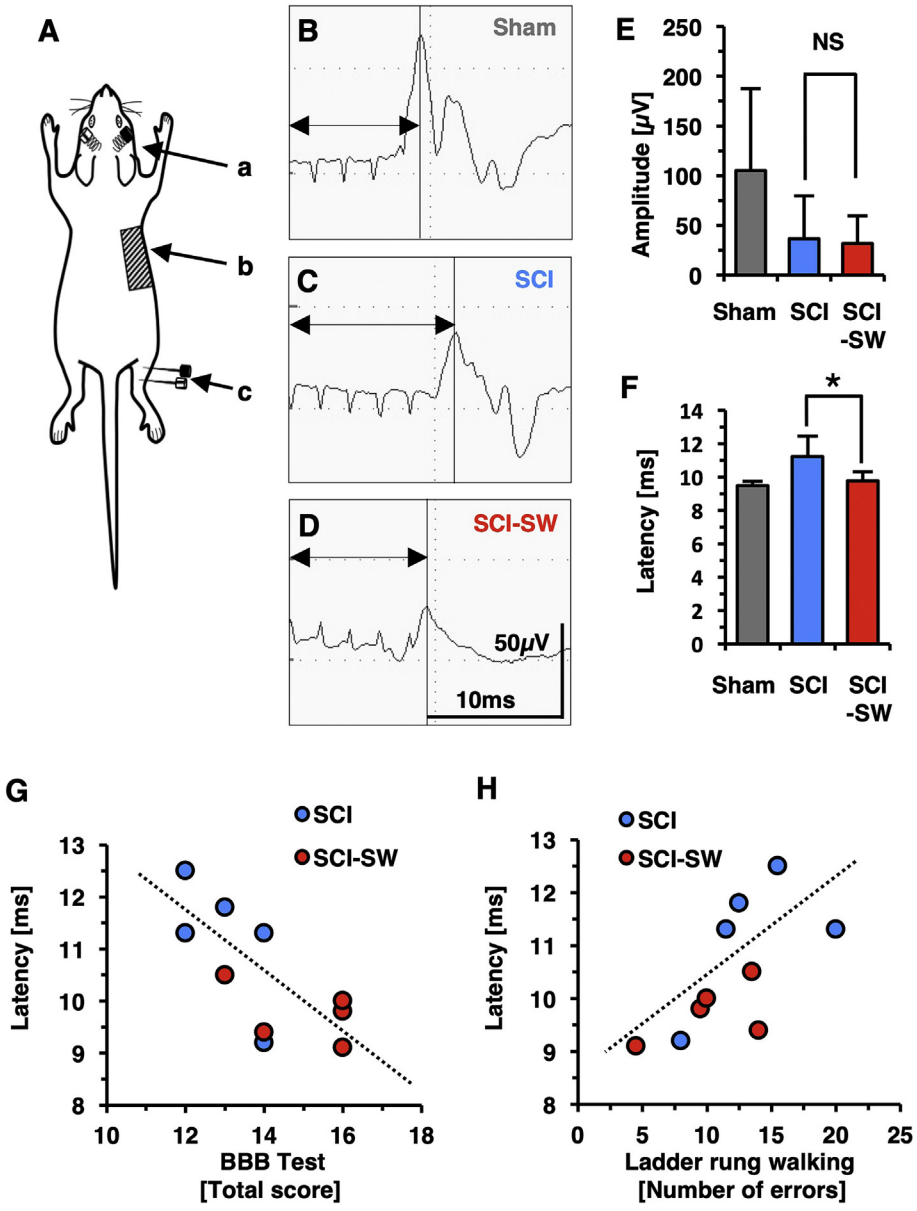

Fig. 8. Electrophysiological assessment of motor-evoked potentials at 42 days after spinal cord injury. (A) A schematic illustration showing the placement of electrodes. Spiral electrodes were placed in the occipito-cervical area (A-a) and needle electrodes were placed in the right hindlimb (A-c). The ground electrode was placed in the trunk (A-b). (B-D) Representative MEP data from each experimental group. (E) There was no significant difference in amplitude among the three groups. (F) The latency of the MEPs in the SCI group was significantly elongated in comparison to that of the SCI-SW group (*p $<$ .05, n = 5 per group). All data are shown as mean $\pm$ SD. (G) There was significant correlations between the latency and the BBB score (r = 0.728, p $<$ .05). (H) There was significant correlations between the latency and the number of errors (r = 0.656, p $<$ .05).

provides evidence supporting that low-energy ESWT may be a strong candidate as a novel therapeutic strategy for treating patients with SCI.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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