

Extracorporeal shockwave therapy enhances peripheral nerve remyelination and gait function in a crush model

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Conflict of interest

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Abstract

Background. Conservative treatment, such as electrical stimulation and steroid injection, have been employed in an attempt to improve symptoms after peripheral nerve injury, without significant success. Although non-invasive and safe extracorporeal shockwave therapy (ESWT) can be a practical alternative, the therapeutic effects of ESWT on peripheral nerve remyelination has not been established.

Objectives. To investigate the effects of ESWT on peripheral nerve remyelination and gait function for 5 weeks in a sciatic nerve crush model.

Material and methods. In total, we divided 97 rats into 5 groups: group 1 – a healthy negative control group; group 2 – 3 weeks after sciatic nerve crush and 3 sessions of ESWT; group 3 – 5 weeks after crush injury with 3 sessions of ESWT; group 4 – 3 weeks after crush injury with no ESWT; and group 5 – 5 weeks after crush injury with no ESWT. The focused ESWT was applied to the unilateral sciatic nerve injury site. One session consisted of 1,500 stimuli, and the session were performed at intervals of 1 week.

Results. The degree of myelination and expression of myelin basic protein at the distal part of the injured sciatic nerve tended to increase in the ESWT groups compared with the no-ESWT groups 3 and 5 weeks after crush injury. Regarding the functional gait recovery, the print width and area of the injured leg in the ESWT groups was significantly larger than that in the no-ESWT groups 3 and 5 weeks after crush injury.

Conclusions. The ESWT may enhance peripheral nerve remyelination and gait function in a nerve crush model. Long-term follow-up after ESWT and investigation of molecular mechanisms will be needed to confirm these therapeutic effects.

Key words: peripheral nerve injuries, extracorporeal shockwave therapy, myelin basic protein, remyelination, gait

Introduction

Trauma or entrapment, herniated intervertebral discs, and cancer metastasis can cause damage to the peripheral nervous system, resulting in various neuromuscular disorders, depending on the location and extent of the lesion. These conditions present focal neuropathy, plexopathy and radiculopathy, all of which are associated with neuropathic pain and muscle paralysis. The clinical importance of peripheral neuropathy is increasing because of the rising number of neuromuscular diseases due to aging of the society. The aim of treatment is to improve the function and quality of life, in patients with peripheral neuropathy.

Inflammatory reactions occur as macrophages invade the tissue surrounding damaged peripheral nerves. Next, Wallerian degeneration, demyelination and axon regeneration processes are initiated, with various signaling pathways involved. During this process, Schwann cells play a key role in orchestrating repair.^{1–3} Extracorporeal shockwave therapy (ESWT) has long been used in urinary stone lithotripsy treatment. In the musculoskeletal field, it has been widely used and researched in lateral and medial epicondylitis, plantar fasciitis, calcific tendinitis of the shoulder, and bony union after fracture. Recently, it has been suggested that these indications should be expanded to include muscle rigidity, muscular pain, knee degenerative arthritis, and lymphedema.⁴

Peripheral nerves regenerate slowly in the human tissues. Various conservative treatments such as electrical stimulation and steroid injection have been used in an attempt to alleviate symptoms after peripheral nerve injury, but without significant results. Recently, stem cell injection has been proven to be effective in animal studies of peripheral nerve injury, but its safety has not been established for long-term follow-up in clinical practice. Thus, non-invasive ESWT may be a realistic alternative. However, the effects of ESWT on angiogenesis, metabolic activation and inflammation response have been suggested but remain to be established. In addition, stimulation parameters such as optimized stimulation intensity (mJ/mm²), and number and duration of treatment sessions are also different and remain controversial between studies. Although the efficacy and safety of ESWT have been established, studies on the mechanism of effect are essential in order to expand clinical applications.

The ESWT is thought to cause molecular and biological changes, in addition to mechanical and physical stimulation by shockwave, but it is difficult to determine which among various signaling pathways is involved. It has previously been shown that adipose-derived stem cells maintain pluripotency when ESWT is applied and promote differentiation into Schwann-like cells.⁵ It has also been shown that ESWT induces the proliferation of bone marrow stromal cells and their differentiation into osteoprogenitors via induction

of TGF- β 1.⁶ Recent animal studies have also shown that ESWT stimulates peripheral nerve regeneration.^{7–8}

Although ESWT is actively used to treat various diseases other than peripheral nerve damage, its therapeutic effects need to be investigated through animal experiments to validate clinical use and study. We investigated the effects of ESWT on peripheral nerve remyelination and gait function for 5 weeks in a sciatic nerve crush model.

Material and methods

Animal model

Nine-week old male Sprague Dawley rats were anesthetized with isoflurane inhalation, and were subject to right sciatic nerve crush proximal to the bisection area with forceps for 40 s. After the crushed nerve was released, the sciatic nerve remained compressed and became pale (Fig. 1). A single endoneurial suture was conducted using 8-0 nylon in order to identify the site of injury when collecting tissue. After the rat recovered from general anesthesia, acetaminophen syrup was administered orally to relieve postoperative pain. Ninety-seven rats were divided into 5 groups: group 1 – healthy group as a negative control; group 2 – 3 weeks after crush injury with 3 sessions of ESWT; group 3 – 5 weeks after crush injury with 3 sessions of ESWT; group 4 – 3 weeks after crush injury with no ESWT applied; and group 5 – 5 weeks after crush injury with no ESWT. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chungnam National University Hospital, Daejeon, South Korea.

Extracorporeal shockwave therapy

The focused ESWT device (Dornier MedTech, Weßling, Germany), which is widely used in clinical fields, was applied only to the unilateral sciatic nerve injury site. One session consisted of 1,500 stimuli, and the sessions were performed at intervals of 1 week. Peripheral nerves and surrounding tissues were collected from different animals at 1 week and 3 weeks after the 3 sessions of ESWT were completed.

Immunostaining

Immunofluorescence was performed on tissue collected 3 or 5 weeks after crush injury. The rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and perfused transcardially with heparinized phosphate-buffered saline (PBS, pH 7.4), followed by perfusion with 4% paraformaldehyde for 15 min. The sciatic nerve from the injured site was removed immediately. Cryoblocks were sectioned and immunostained with anti-myelin basic protein (MBP) primary antibody (1:400; #MAB386, Merck Millipore, Burlington, USA).

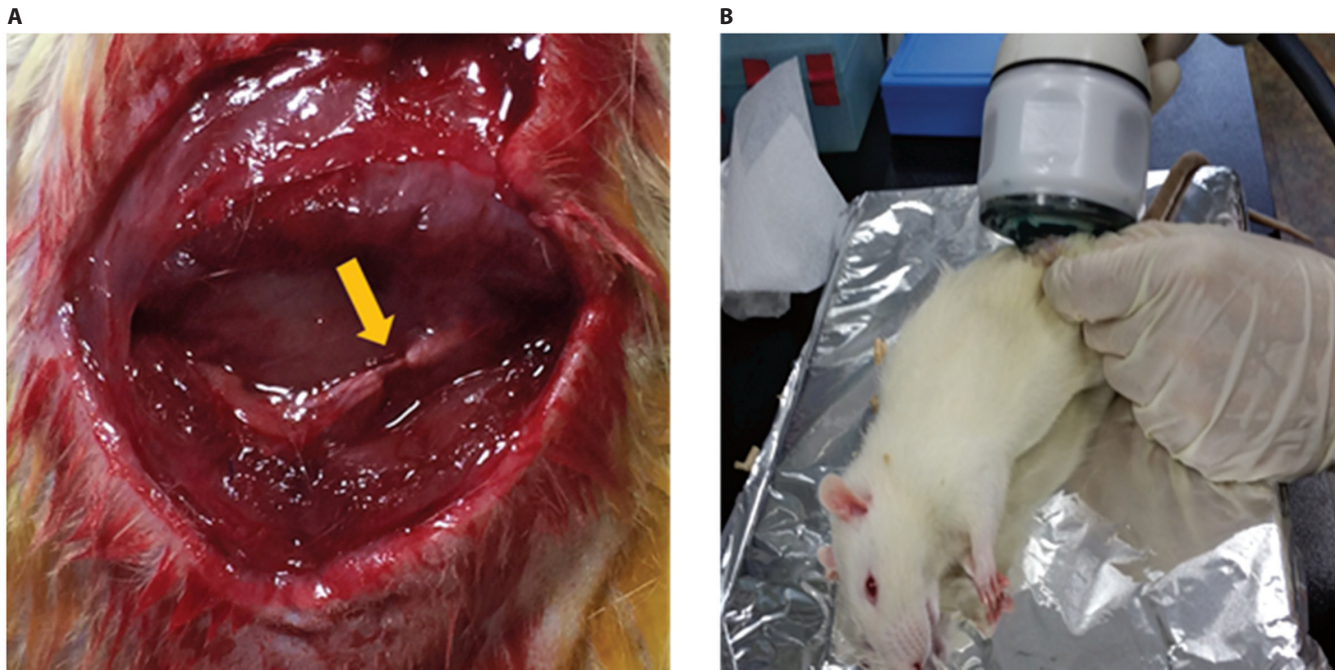


Fig. 1. Sciatic nerve crush and application of ESWT. A. When sciatic nerve crush was applied to the proximal portion of the sciatic nerve for 40 s, the nerve became compressed and pale (arrow). B. ESWT was applied to the sciatic nerve injury site

Electron microscopy

The sciatic nerve was removed and stored in the fresh fixative solution overnight at 4°C. Tissues were washed in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide for 2 h and dehydrated through an ascending series of ethanol and propylene oxide, and then embedded in Epon812 mixture (Oken Shoji, Tokyo, Japan). Semi-thin sections (×200 magnification) were made using Leica EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany), mounted on #200 mesh copper grids, stained with 2% uranyl acetate and Reynold's lead citrate for 5 min each, and observed under a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at the accelerating voltage of 80 kV.

Quantitative polymerase chain reaction

Since the tissue volume of the rat sciatic nerve is low, right sciatic nerves of 3 rats were harvested and pooled. Total RNA was extracted from the sciatic nerve tissue using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. After purification with a RNA isolation kit (Hybrid-R; GeneAll Biotechnology, Seoul, South Korea; 305-101) the concentration and purity of RNA were assessed using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Total RNA was used for cDNA synthesis with a TOPscript cDNA synthesis kit (Enzymomics, Daejeon, South Korea; RT 220) in a 20 µL reaction. Subsequently,

quantitative polymerase chain reaction (qPCR) was performed in duplicate in a total volume of 10 µL, including each 5 pM primer, cDNA and TOPreal qPCR SYBR mix (Enzymomics; RT 500). The qPCR conditions were 95°C for 10 min, then 40 amplification cycles of 95°C for 15 s and 60°C for 1 min (AriaMx Realtime PCR System; Agilent Technologies Santa Clara, USA). The primer sequences (CosmoGenetech, Daejeon, South Korea) used for qPCR were as follows: rat *GAPDH*, forward: 5'- CTC ATG ACC ACA GTC CAT GC -3', reverse: 5'- TTC AGC TCT GGG ATG ACC TT -3'; rat *MBP* forward 5'-agagaccctcacagcgacac-3', reverse 5'-agggagccgtagtgggtagt-3'. The mRNA expression level was normalized to *GAPDH*, and the relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, as previously described.⁹

CatWalk-automated gait analysis

We performed gait analysis using the CatWalk XT system (Noldus Information Technology, Wageningen, the Netherlands) as a functional evaluation after 1 week of adaptation. Print width (cm) and area (cm²) of the right leg were divided by those of the left leg at 3 and 5 weeks respectively after crush injury to derive a ratio of print width and area between both legs.

Statistics

Statistical analyses were carried out with PASW Statistics v. 18 software (SPSS Inc., Chicago, USA). The Mann-Whitney U test was used to compare differences in qPCR

results. The independent t-test and analysis of variance (ANOVA) were used to compare differences in CatWalk data at 3 and 5 weeks each. Differences were considered significant at a p-value <0.05.

Results

Degree of myelination

The distal part of the injured sciatic nerve was prepared and observed. In transmission electron microscopy ($\times 3,000$ magnification) and immunofluorescence staining, the degree of myelination tended to increase in the ESWT stimulation groups compared to the no-ESWT groups 3 and 5 weeks after crush injury (Fig. 2A,B). In qPCR, expression of MBP tended to be higher in the ESWT groups ($n = 18$) than no-ESWT groups ($n = 18$) when shown using the pooled average method (Fig. 2C).

Functional gait recovery

In the right sciatic nerve crush model, we performed gait analysis using the CatWalk apparatus as a functional evaluation. Three weeks after crush injury, the print width

and print area (%) of the ESWT groups were significantly larger than in the no-ESWT groups ($p = 0.030$ for width and $p = 0.003$ for area). This improvement was maintained 5 weeks after crush injury ($p = 0.009$ for width and $p = 0.006$ for area) (Fig. 3). This indicates that gait recovery was achieved when ESWT was applied after sciatic nerve injury.

Discussion

Microsurgical repair has been performed in cases of traumatic or postoperative peripheral nerve transection, but is not applicable to partial neuronal injury. Electrical stimulation has also been used to prevent muscle atrophy caused by peripheral nerve injury, but is not effective on promoting neural recovery in severe injury.

Stem cell application has recently been suggested as a potential innovative treatment.¹⁰ Stem cells are being tested for amyotrophic lateral sclerosis and serious central nervous system disorders such as stroke and spinal cord injury, and are not commercially available for peripheral neuropathy due to possible long-term adverse effects. The PTEN blockers and microRNA-222 have been studied in animals,^{11,12} but they have not been used in the clinical

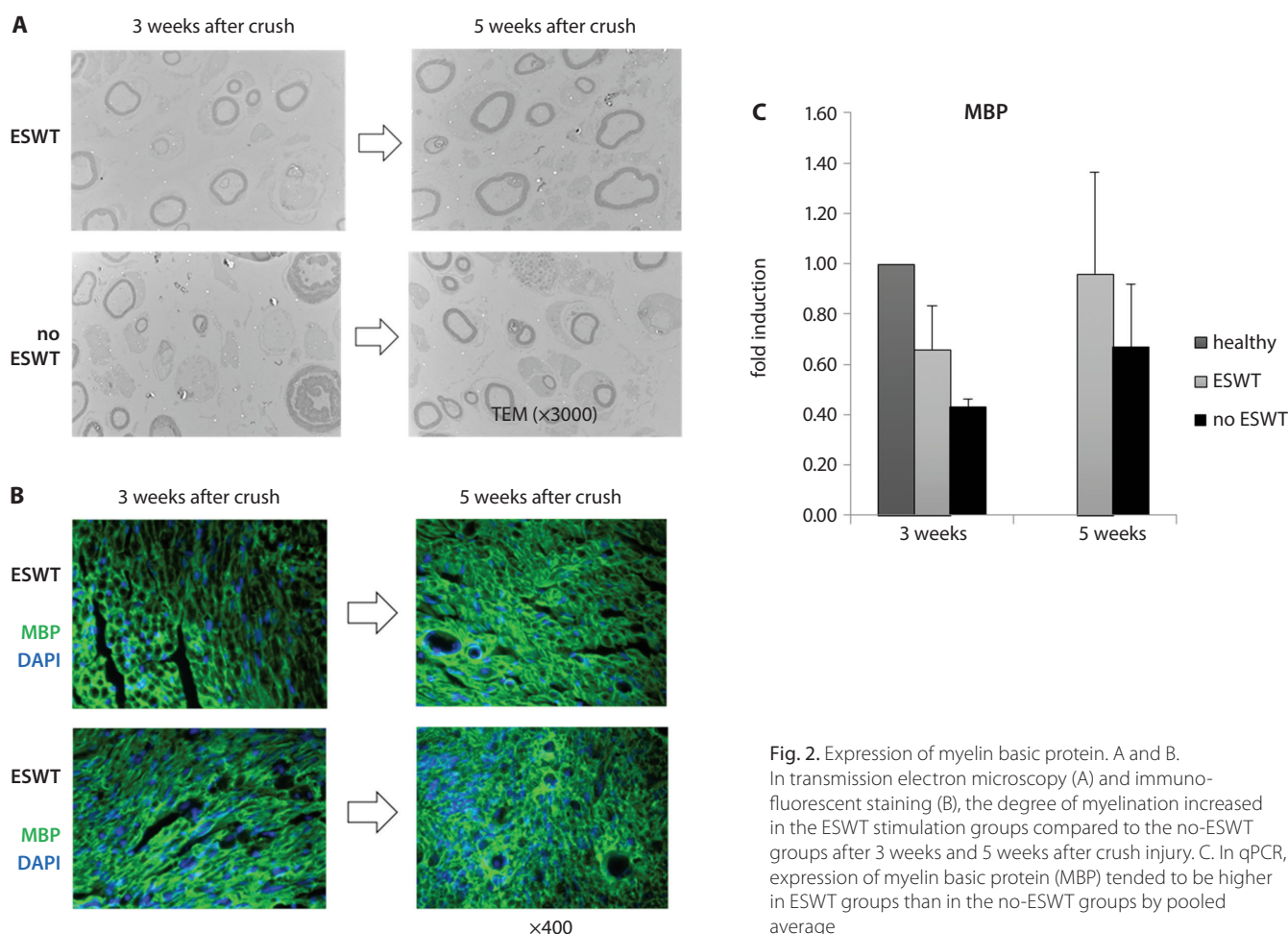


Fig. 2. Expression of myelin basic protein. A and B. In transmission electron microscopy (A) and immunofluorescent staining (B), the degree of myelination increased in the ESWT stimulation groups compared to the no-ESWT groups after 3 weeks and 5 weeks after crush injury. C. In qPCR, expression of myelin basic protein (MBP) tended to be higher in ESWT groups than in the no-ESWT groups by pooled average

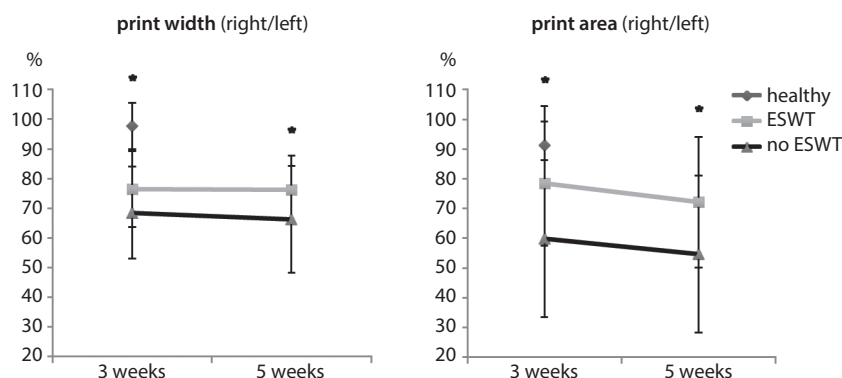


Fig. 3. Gait analysis using CatWalk. Three weeks after crush injury, the ESWT group had significantly larger print width and print area (%) than the no-ESWT group ($p = 0.030$ for width and $p = 0.003$ for area). This improvement was maintained 5 weeks after crush injury ($p = 0.009$ for width and $p = 0.006$ for area).

field. Therefore, there is a need to develop a realistic and non-adverse treatment modality that can be immediately applied to patients with peripheral nerve damage causing functional decline and poor quality of life.

Recent studies have shown that Schwann cells are the major expression cells of *GDF15*, and may be involved in the regeneration of peripheral nerves through increases in the myelination of injured peripheral nerves or through improving nerve conduction velocity.^{13–15} However, in vivo experiments focused on the mechanisms have not been performed. Schwann cells are an important cell type involved in the myelination of peripheral nerves, and Schwann cell plasticity and de-differentiation are newly suggested restoration mechanisms after injury. In normal conditions, Schwann cells are in a resting state that has no other function except for its basic role of nerve conduction. However, when the peripheral nerve is damaged, Schwann cells maintain the function of neuronal cells. This is a substantial phenotypic transformation of Schwann cells, referred to as plasticity or de-differentiation.¹⁶ Schwann cell de-differentiation is largely divided into 2 mechanisms. Firstly, it participates in the demyelination process by activating lysosome or protease proteins. Secondly, it secretes neurotrophic factors involved in peripheral nerve survival or axonal regeneration.

It is also known that the neurotrophic factors secreted during Schwann cell de-differentiation are glial cell-derived neurotrophic factor (GDNF) and extracellular matrix proteins. These factors influence the survival of motor neurons and dorsal root ganglion in the spinal cord, and signal transduction through c-jun is involved in axonal regeneration. These factors and the relationship between *GDF15* and *Krox20* in the peripheral nerves have not been elucidated. In this study, induction of Schwann cell plasticity could be suggested as a mechanism of peripheral nerve remyelination and functional recovery.

We demonstrated that degree of myelination and expression of MBP tended to increase in the ESWT groups compared to the no-ESWT groups 3 and 5 weeks after crush injury. Regarding functional gait recovery, the print width and areas of the injured leg in the ESWT groups were significantly larger than those in the no-ESWT groups

measured 5 weeks after crush injury. Considering these findings, ESWT may enhance gait function through peripheral nerve remyelination in a crush model.

There could be several limitations in this study. First, toxin-induced demyelination would be more suitable than nerve crush for pure demyelination without axonal loss. However, we selected the crush model that is similar to clinical situation for peripheral nerve injury. Second, the ESWT applicator was relatively large for the lower limb of adult rats. Thus, we tried to focus the center of the applicator on the sciatic nerve crush site. Third, different individuals were analyzed at 3 and 5 weeks after crush injury because rats were sacrificed and tissues harvested at 3 and 5 weeks, respectively. Although longitudinal follow-up in the same individuals would be more reliable to interpret the results, it is not possible in animal studies. Long-term follow-up after ESWT with a small applicator suitable for rats will be needed to confirm the therapeutic effects. Furthermore, molecular mechanisms regarding various signaling pathways are yet to be investigated.

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