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● *Original Contribution*

EFFECTS OF EXTRACORPOREAL SHOCK WAVE THERAPY ON FUNCTIONAL RECOVERY AND NEUROTROPHIN-3 EXPRESSION IN THE SPINAL CORD AFTER CRUSHED SCIATIC NERVE INJURY IN RATS

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Abstract—The study described here investigated the effects of extracorporeal shock wave therapy (ESWT) on functional recovery and neurotrophin-3 expression in the spinal cord after sciatic nerve injury in rats. Forty-five 8-wk-old rats were used and randomly divided into three groups: An experimental group, a control group and a sham group. The experimental group received ESWT after the nerve-crushing damage. The sciatic functional index and Dartfish Software were used to determine the effect of sciatic nerve damage on functional changes. A 1-cm length of spinal cord encompassing the L4–6 level was removed for Western blot analysis. The sciatic functional index significantly changed in both the ESWT and control groups after impairment. In the time course evaluation of the ankle angle in the toe off, the ESWT group had statistically significant increases from day 21 onward. There was a significant difference in neurotrophin-3 expression between the groups on days 1, 7 and 14 after impairment. Early application of ESWT increased the expression of neurotrophin-3 and neurotrophin-3 mRNA, and daily therapy facilitated the activity of macrophages and Schwann cells, which affect the survival and regeneration of neurons. (E-mail: Niceguygil@gmail.com) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Sciatic nerves, Shock waves, Rehabilitation, Peripheral nerves.**

INTRODUCTION

The peripheral nerve is vulnerable to injury resulting from crushing, stretching, compression and avulsion. Such injury results in severe problems at the level of the spinal cord or dorsal root ganglia (Dahlin 2004). Recovery after the impairment of peripheral nerves tends to be slow and incomplete (Rochkind et al. 2001). The disability in sensory and motor capacity after impairment of peripheral nerves decreases not only the partial or overall ability of motor nerves, sensory nerves and autonomic nerves, but also quality of life and functional activities (Rosberg et al. 2005).

Many studies have investigated the impact of neurotrophic factors on the maintenance of neurons and regeneration of axons (Kemp et al. 2011; Markus et al. 2002). After injury, the mature peripheral nerve system exhibits increased production of neurotrophic factors, which facilitate the regeneration of peripheral nerves (Boyd and Gordon 2003; Terenghi 1999). The increase in neurotrophic factors after impairment occurs naturally. An exogenous supply of neurotrophic factors also promotes the regenerative response. The neurotrophic factor neurotrophin-3 (NT-3), which contributes to the survival of neurons, is found in various areas, such as the callosum, hippocampus, cerebral cortex (layer V), primary olfactory cortex, amygdala and spinal cord (Connor and Dragunow 1998). Furthermore, NT-3 controls synaptic functions, neuroplasticity and the organization and development of neurons, not only by stimulating the synthesis of neurotransmitters (Huang and Reichardt 2001), which compensate for the decreased innervation and the decreased number of dendrites after injury, but also by increasing the expression of NT-3 mRNA (Ljungberg et al. 1999; Shimazu et al. 2006; Skaper 2008).

The distal part and the myelin of the nerve fibers around the lesion are degraded by Wallerian degeneration, and the regression of the proximal part is evidenced by the lesion. Schwann cells in the endoneurium of distal

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neurons dedifferentiate into a non-myelinating proliferative phenotype, including growth factors, extracellular matrix and cell adhesion molecules (Hall 2001). The function of the neurons in the injured part then changes from that of a transmitter to a regenerator, which stimulates growth cones (Dahlin and Brandt 2004).

The peripheral nervous system and the central nervous system are functionally integrated. Thus, impairment of the former always leads to long-term modifications and reorganization of the latter. The integration of the two nervous systems has a great impact on the characteristics and form of neurons, the distribution of axons and dendrites, the electrical activity of the neural membrane and the production of transmitters and metabolic molecules (Jaggi and Singh 2011). The plasticity and reorganization of the spinal cord and brain vary, depending on the level of impairment in the peripheral nervous system (Kaas and Collins 2003). Not only can positive changes like functional adaptations occur, but non-adaptive changes, such as pain dysesthesia, hyperreflexia and decreased muscle tone also occur.

Extracorporeal shock wave therapy (ESWT) is a non-surgical method used in European countries, including Germany, Switzerland and Austria, to regenerate impaired tissue and destroy calcarea by focusing energy on a single point (McClure and Dorfmüller 2003; Romeo et al. 2011). ESWT can restructure injured tissue by controlling the energy flux density, exposing the impaired area and increasing local growth factors using shock waves (Speed et al. 2002; Wang et al. 2003). ESWT is effective in decreasing muscle tone and increasing muscle power, as well as facilitating pain relief, local vessel remodeling and regeneration of neurons (Loew et al. 1999). ESWT has been reported to have a positive effect on numerous diseases, including tendinitis, tendosynovitis, fasciitis, pelvic pain syndrome, epicondylitis and myofascial pain syndrome (Haake et al. 2002; Zimmermann et al. 2008).

Some studies, however, have reported negative results of this shock wave therapy. Sohn et al. (2011) investigated the effect of ESWT on spasticity in patients with hemiparesis and reported a significant decrease in spasticity. The mechanism underlying this decrease was not clear. Haake et al. (2002) studied the impact of ESWT on reducing neurologic pain and reported that variation in c-fos, acting as a marker of an analgesic agent, was not statistically significant. Most studies of ESWT for nervous system injury have focused on inflammation and neurologic pain after impairment of peripheral nerves (Romeo et al. 2011; Zimmermann et al. 2008, Noguchi et al. 1995). Thus, this study was conducted to investigate the effect of ESWT on the functional activity and expression of NT-3 in rats with crush injury of the sciatic nerve.

METHODS

Experimental animals and surgery

This study used 45 eight-week-old Sprague–Dawley male rats weighing between 250 and 300 g. Forty-five rats were randomly divided into three groups: experimental group, control group and sham group (each n = 15). The experimental and control groups underwent ESWT, but only the ESWT group received ESWT; the control group did not receive any treatment. In the sham group, the sciatic nerve was not damaged and no treatment was administered.

The experimental group received the first EWST immediately after the nerve-crushing damage. From the next day forward, there were five treatments per week for 2 wk (total of 10 treatments). Measurements were obtained on days 1, 7, 14, 21, 28, 35 and 42 after sciatic nerve damage. All surgical procedures and experimental protocols followed Daegu University's guidelines and were approved by the Institutional Animal Care and Use Committee.

For general anesthesia, Zoletil (Virbac, Seoul, South Korea) and Rompun (Bayer, Seoul, South Korea) were mixed in a 1:1 ratio and injected into the abdomen of the rats at a dose of 37 mg/kg. To induce artificial sciatic nerve damage, the region between the right thigh and knee joint was shaved and incised (2 cm long). After the incision, the sciatic nerve was isolated from the surrounding muscles, and a region about 7 cm from the ankle joint, just before the point where the tibial nerve and peroneal nerve separate, was compressed for 30 s under three steps of pressure with hemostatic forceps.

Extracorporeal shock wave therapy

After surgical suture, ESWT was applied to the right crushed area 7 cm above the knee joint. Ultrasound transmission gel (Pharmaceutical Innovations, Newark, NJ, USA) was used as the contact between the shock wave area and the skin. ESWT was applied using an ESWT machine (HAEMIL, Soltar, Seoul, Korea) with low-intensity output, with a PAD5 for the application to a narrow region in the damaged area. We used a frequency of 3 Hz, because application of 15 Hz induced damage (Delius and Brendel 1988), and an energy flux density of 0.09 mJ/mm^2 300 times, because use of 0.49 mJ/mm^2 1000 times induced soft tissue damage, erythema and ischemia (Takahashi et al. 2003; Wu et al. 2008). The left side received no ESWT. Immediately after treatment, the animal's skin was examined for redness, swelling and edema.

Functional activity assessment

The sciatic functional index (SFI) was used to determine the effect of sciatic nerve damage on functional changes (Lowdon et al. 1988). Both hindfeet of the rats

were painted with black ink, and the rats were allowed to walk through a dark room (50 × 8 × 10 cm) so that their footprints could be tracked. Analysis of the footprints consisted of measuring the length between the heel and third toe, the length between the first and fifth toes and the length between the second and fourth toes. These measurements were used to calculate the SFI. The value of the SFI of a normal rat is usually around 0, whereas that of a rat with severe injury is close to −100. Experimental (E) and normal (N) data for both the right and left footprints were recorded. The SFI was calculated according to the following equations:

$$\text{print length (PL)} = \text{distance from the heel to the third toe} \quad (1)$$

$$\text{toe spread (TS)} = \text{distance from the first to the fifth toe} \quad (2)$$

$$\text{intermediary toe spread (ITS)} = \text{distance from the second to the fourth toe} \quad (3)$$

$$\text{print length factor (PLF)} = (\text{EPL} - \text{NPL})/\text{NPL} \quad (4)$$

$$\text{toe spread factor (TSF)} = (\text{ETS} - \text{NTS})/\text{NTS} \quad (5)$$

$$\text{intermediary toe spread factor (ITF)} = (\text{EIT} - \text{NIT})/\text{NIT} \quad (6)$$

$$\text{SFI} = (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITF}) - 8.8 \quad (7)$$

Movements of the right hindlimb were measured with Dartfish Software (4.5 ProSuite Version, Dartfish, Fribourg, Switzerland). For sagittal plane kinematic analysis, the joint centers were, after shaving the skin, palpated and marked at the lateral epicondyle, lateral malleolus and head of the fifth metatarsal bone using waterproof black ink (Hayes et al. 2009). Video recordings of hindlimb locomotion were collected in the sagittal plane using a digital video camera at the rate of 60 Hz at 1-m distance. Joint angle trajectories for the right hindlimb were calculated from the joint position at the initial foot contact and the toe off. The ankle angle was defined as the angle between the foot and the shank. In all cases, increasing values indicated extension. Only data from complete walks were included, and the average values of three sets of measurements were used.

Western blot analysis

Western blot analysis was used for quantitative analysis in the time course evaluation of NT-3 expression on days 1, 7 and 14 after impairment. Four rats in each group were measured on each date. Two rats in each group were alive for functional testing.

A 1-cm length of spinal cord encompassing the L4–6 level was removed for Western blot analysis. The harvested tissues were homogenized on ice in a lysis buffer containing 0.05 M Tris–HCL, 0.5 M EDTA, 30% Triton X-100, NaCl, 10% sodium dodecyl sulfate and 1 mM phenylmethanesulfonyl fluoride, followed by centrifugation of 12,000*g* at 4°C for 30 min. Protein concentration was assayed with BCA reagent (Pierce, Rockford, IL, USA). Equal amounts of protein samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinyl difluoride membranes (Thermo Scientific Pierce, Rockford, IL, USA). The membranes were blocked for 2 h in 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20. They were then incubated overnight at 4°C with rabbit anti-NT-3 (1:200) and then with horseradish peroxidase-conjugated secondary antibody (1:4,000) for 1 h at room temperature. After being rinsed with buffer (0.1% Tween-20 in Tris-buffered saline), the immunocomplexes were visualized by chemiluminescence using the Amersham ECL Plus Western Blotting Detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Actin was used as an internal control for sample loading, and each blot was normalized to its corresponding actin value.

Statistical analysis

Groups were compared with a one-way analysis of variance, followed by Tukey's *post hoc* test. All data in this study are given as means ± standard deviations. The data were processed using SPSS for Windows Version 20.0 (IBM, Armonk, NY, USA) and a significance level (α) of 0.05.

RESULTS

Sciatic functional index

The SFI changed significantly in both in the ESWT and control groups in all phases after impairment ($p < 0.05$) (Fig. 1). The SFI of the control and ESWT groups did not differ significantly on day 1, 7, 14 or 21, but the SFI of the ESWT group was significantly higher than that of the control group from days 28 to 42 ($p < 0.05$). However, the SFI score of the ESWT group was significantly lower than that of the sham group until day 42 (Fig. 2).

Dartfish software

In the time course evaluation, the ankle angle in the toe off of the control and ESWT groups increased

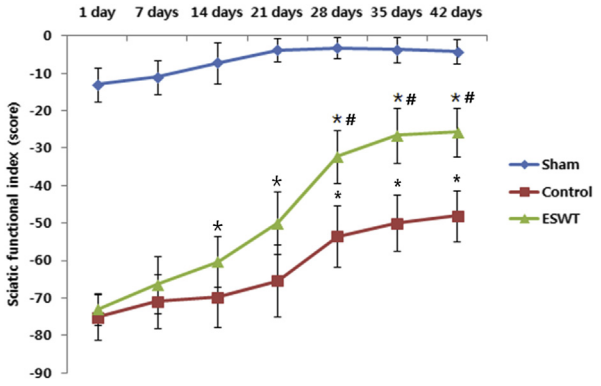

Fig. 1. Time course of the sciatic functional index (SFI) in the sham, control and extracorporeal shock wave therapy (ESWT) groups. Data are presented as means ± standard deviations. n = 15 per group. $^{*}p < 0.05$, significantly different from value 1 d after sciatic nerve injury. $^{\#}p < 0.05$, significantly different from control group.

statistically significantly from day 21 onward ($p < 0.05$) (Fig. 3). However, from day 28, there was no significant change in ankle angle between the ESWT and sham groups.

In the time course evaluation of the ankle angle in foot contact, the ESWT group had a statistically significant increase compared with the control group from day 21, but there was no significant difference in ankle angle compared with the sham group from day 21 (Fig. 4). Thus, compared with the control group, functional gait was increased by ESWT from days 21 to 28.

Western blot analysis

Western blot analysis was used for quantitative analysis in the time course evaluation of NT-3 expression (Fig. 5). There was a significant difference in NT-3 expression between the groups on days 1, 7 and 14 after impairment ($p < 0.05$). After impairment, the nervous system immediately releases large quantities of neurotrophic factors to facilitate survival and regeneration of nerves. Although this process lasts only a short time, it provides the neurotrophic foundation. AT 1 d, NT-3 expression is upregulated in response to peripheral nerve impairment. With time, NT-3 expression in the control group rapidly declined and was reduced on days 7 and 14 after impairment compared with the sham and ESWT groups. Seven days after impairment, the early upregulation of NT-3 continued in the ESWT group. Fourteen days after impairment, the ESWT group expressed much greater levels of NT-3 compared with the sham and control groups.

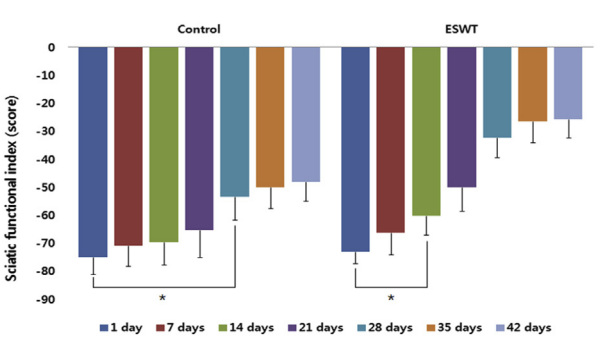

Fig. 2. Comparison of sciatic functional index in the control and extracorporeal shock wave therapy (ESWT) groups. Data are presented as means ± standard deviations. n = 15 per group. $^{*}p < 0.05$.

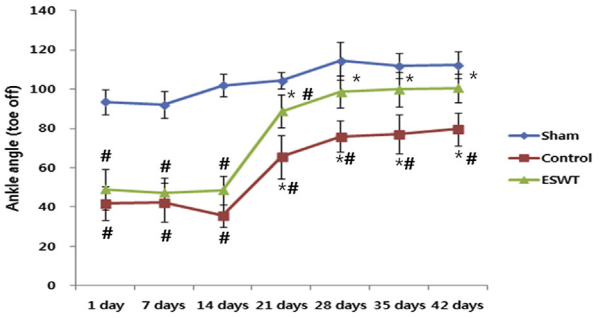

Fig. 3. Time course of ankle angle in toe off in the sham, control and extracorporeal shock wave therapy (ESWT) groups. Data are presented as means ± standard deviations. n = 15 per group. $^{*}p < 0.05$, significantly different from value at 1 d. $^{\#}p < 0.05$, significantly different from sham group.

DISCUSSION

Impairment of the peripheral system after trauma or accidents is more common than impairment of the central nervous system. This peripheral injury can result in severe physiologic, morphologic, cytologic and functional disability (Campbell 2008). The action of muscles and the integration of senses are also limited after impairment of peripheral nerves, resulting in a decrease in functional

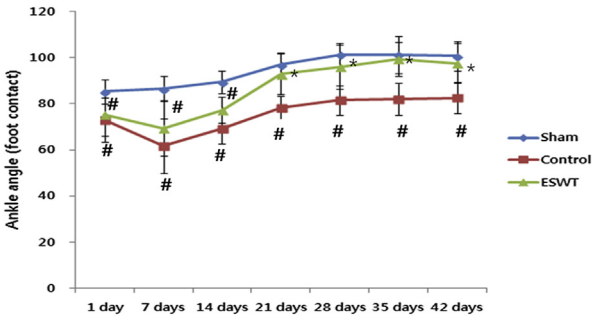

Fig. 4. Time course of ankle angle in foot contact in the sham, control and extracorporeal shock wave therapy (ESWT) groups. Data are presented as means ± standard deviations. n = 15 per group. $^{*}p < 0.05$, significantly different from the control group. $^{\#}p < 0.05$, significantly different from sham group.

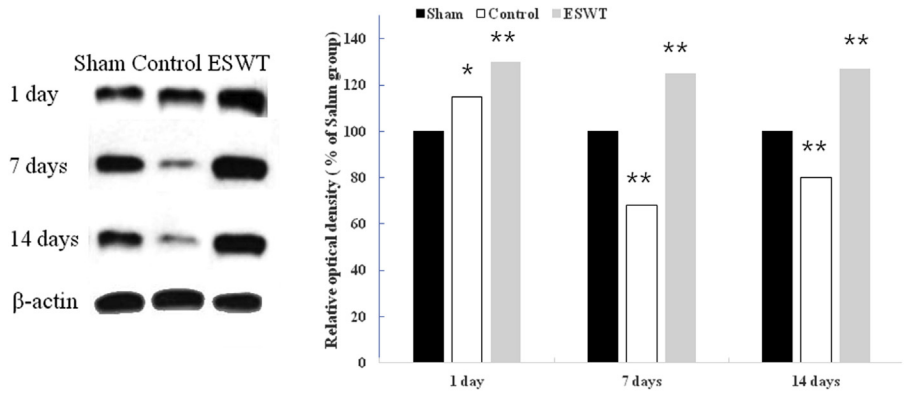

Fig. 5. Western blot analysis of neurotrophin-3 proteins in the lumbar spinal cord derived from the sham, control and extracorporeal shock wave therapy (ESWT) groups. Data are presented as means ± standard deviations. Relative optical density values expressed as percentages of immunoblot band are also illustrated. Data are presented as means ± standard deviations. *$p < 0.05$, **$p < 0.01$, significantly different from sham group.

body activity. Unless appropriate therapeutic interventions are provided in the early phase of impairment, many problems resulting in functional disability can occur after injury (Wu et al. 2008). The functional defects associated with nerve injury can be repaired by three mechanisms: reinnervation through remyelination of paralyzed axons, collateral sprouting around the impaired part and remodeling of nervous system circuitry (Zochodne and Levy 2005).

Diverse factors, such as age, region of impairment, shape of the lesion and delay of surgical therapy, affect the regeneration of axons and recovery of function after peripheral nerve impairment (Valero-Cabré et al. 2004). If the impairment is moderate, regeneration and restoration occur immediately after impairment. After the impairment of peripheral nerves, the distal end of the injured axon is disconnected from the nerve cell body, resulting in degeneration. In this phase, the neurons undergo phenotypic changes known as neuronal reactions and chromatolysis (Raivich et al. 2004).

It is possible for impaired parts of axons to be regenerated, but the redistribution of neurons does not always result in the recovery of sensory function. There is significant important regulation in the reconnection between the proximal and distal parts after injury (Valero-Cabré et al. 2004). The various aspects of the reconnection depend on the shape of the end organ connected to the injured neuron and the character of the distal axon with the sprouting of the proximal axon. Schwann cells between the motor and sensory nerves control the regeneration of axons by regulating the secretion of cell adhesion molecules (Eberhardt et al. 2006).

Factors that have negative effects on the functional recovery of impaired peripheral nerves include retrograde degeneration, impairment of the cell body by axotomy, poor axonal growth caused by neuropathy and disease and the appearance of wrong integration by the regenerated axons during neural redistribution (Navarro et al. 2007). Composite actions, such as the construction of the micro-environment, the extension and sprouting of axons, the activity of neurotrophic factors and the activity of Schwann and mast cells, influence the recovery of peripheral nerves after impairment. In addition, continuous therapy is necessary for the effective regeneration and redistribution of neurons.

The therapeutic effect of intervention can be increased in the early phase of impairment by stimulation of an exogenous supply during the connection between distal and proximal axons. In a study that investigated the effect of the early application of ESWT on nerve regeneration, nerve conduction velocity and amplitude increased more in the group that received ESWT immediately after sciatic nerve impairment than in the control group (Hausner et al. 2012). In another study of the effect of ATF3 and GAP-43 on the regeneration of neurons, their expression was significantly increased in the ESWT group compared with a control group (Murata et al. 2006). The authors concluded that the early application of ESWT has a positive effect on the regeneration and redistribution of neurons after impairment of peripheral nerves.

In the present study, expression of NT-3 and the subsequent regeneration of neurons were increased in the ESWT group compared with the control group from the 1st to the 14th days after impairment. The continued expression of this neurotrophic factor likely aided the regeneration and redistribution of neurons. This resulted in a continuous and statistically significant increase in functional activity in the ESWT group from days 21 and 28 through day 42 compared with the control group.

Extracorporeal shock wave therapy is known to have a positive effect on the survival and maintenance of soft

tissue. It not only stimulates after impairment caused by trauma, but inhibits the conduction of pain by changing the permeability of the cell membrane and blocking the gate control mechanism through hyperstimulation of the peripheral nervous system (Speed 2004). In addition, ESWT inhibits the production of free radicals, which lead to neural deformation (Munver et al. 2002). In this study, ESWT was applied to the crush injury region. The ESWT group exhibited increased functional activity from day 21 onward compared with the control group. ESWT may exert its positive effect on the regeneration of impaired axons by preventing oxidative damage caused by free radicals generated via axonotmesis and by stimulating a change in metabolism. That is, the stimulation of cell recovery by ESWT may enable effective regeneration and redistribution of sensory and motor fibers, thereby facilitating an increase in functional activity.

After peripheral nerve injury, the deficiency in neurotrophic factors causes neuronal death and pathologic changes and impairment. NT-3 facilitates axonal regeneration and the degree of histomorphologic or electrophysiologic limitation according to the nervous injury (Chen et al. 2010). It has an important role in axonal extension, survival and maintenance of neurons, myelination and regeneration of neural fibers (Li et al. 2006).

Expression of NT-3 and NT-3 mRNA rapidly increases immediately after impairment of peripheral nerves, because macrophages stimulate the production of NT-3 by releasing interleukin-1β. Macrophages affect phagocytosis and regeneration, and Schwann cells maintain the survival of the neurons through the production of NT-3, region, as well as other neurotrophic factors, around the injured. The concentration of NT-3 receptor in Schwann cells in which a Büngner band has already formed is increased after peripheral nerve impairment, and NT-3, combined with the receptor, leads to the sprouting of re-growing axons. NT-3 ensures continuous stimulation for the growth of axons and for retrogressive development from growth cones through axons to the nerve cell body (Mark and Eric 2004).

The expression of NT-3 occurs within a week and lasts 2 to 3 wk. Early application of therapy not only increases the expression NT-3, but also protects against the decreased expression of NT-3 as time passes (Kemp et al. 2011; Shakhbazau et al. 2012).

In this study, therapy was initiated immediately after nerve impairment, and the expression of NT-3 1 d after impairment was significantly increased in the ESWT group compared with the sham and control groups. Expression of NT-3 was also greater in the ESWT group on days 7 and 14 compared with the control group. This result suggests that early application of ESWT increases the expression of NT-3 and NT-3 mRNA and that daily therapy facilitates the activity of macrophages and Schwann cells, which affect the survival and regeneration of neurons.

CONCLUSIONS

Early application of shock wave therapy is effective in increasing functional activity and gait by improving neuro-regeneration and the range of motion of the ankle joint. Expression of NT-3, which has an effect on neuro-reorganization and redistribution, was also increased after shock wave therapy. Thus, it is believed that extracorporeal shock wave treatment is therapeutic for the regeneration of peripheral nerves and the rehabilitation of peripheral nerve injury.

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