Experimental Neurology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat

Thomas Hausner [a, d, ⁎, 1], Krisztián Pajer [b, 1], Gabriel Halat [a, 2], Rudolf Hopf [a], Robert Schmidhammer [a, c], Heinz Redl [a], Antal Nógrádi [a, b]

[a] Austrian Cluster for Tissue Regeneration and Ludwig Boltzmann Institute for Experimental and Clinical Traumatology at the Research Centre for Traumatology of the Austrian Workers' Compensation Board (AUVA), Donaueschingenstr. 13, A-1200 Vienna, Austria
[b] Department of Ophthalmology, Albert Szent-Györgyi Clinical Centre, University of Szeged, Korányi fasor 10–11, H-6720 Szeged, Hungary
[c] MILLESI Centre for Surgery of Peripheral Nerves at the Vienna Private Clinic, Pelikangasse 15, A-1090 Vienna, Austria
[d] Department for Trauma Surgery and Sports Traumatology, Paracelsus Medical University, A-5020 Salzburg, Austria

arti cle info

Article history:
Received 16 November 2011
Revised 19 March 2012
Accepted 23 April 2012
Available online xxxx

Keywords:
Shock wave treatment
Peripheral nerve
Nerve graft
Axonal regeneration
Axotomy

abstract

De-focused low energy extracorporeal shock wave therapy (ESWT) has been widely used in various clinical and experimental models for the treatment of painful conditions such as epicondylitis and plantar fascitis and also bone and wound healing. There is evidence that ESWT improves the metabolic activity of various cell types, e.g. chondrocytes and endothelial cells but little is known about its effects on nervous tissue. The aim of this study was to investigate whether ESWT improves the regeneration of injured nerves in an experimental rat model.

Sprague–Dawley rats received an 8 mm long homotopic nerve autograft into the right sciatic nerve, fixed with epineurial sutures. Two experimental groups were set up: the group 1 animals received ESWT (300 impulses, 3 Hz) immediately after nerve grafting whereas the group 2 (control) animals received only nerve autografts. Serial CatWalk automated gait analysis, electrophysiological studies and morphological investigations were carried out. The survival time was either 3 weeks or 3 months.

At 6 to 8 weeks of survival the ESWT group of animals exhibited a significantly improved functional recovery relative to the controls. Electrophysiological observations at 3 weeks after surgery revealed marked values of amplitude (3.9± 0.8 mV, S.E.M.) and compound nerve action potential (CNAP, 5.9 ± 1.4 mV·ms, S.E.M.) in the ESWT group, whereas there were no detectable amplitudes in the control group. This finding was accompanied by significantly greater numbers of myelinated nerve fibres in the middle of the graft (4644 ± 170 [S.E.M., ESWT] vs 877 ± 68 [S.E.M.,control]) and in the distal stump (1586 ± 157 [S.E.M., ESWT] vs 308 ± 29 [S.E.M., control]) of ESWT animals relative to the controls 3 weeks after surgery. Three weeks after surgery the nerve grafts of control animals contained great numbers of phagocytes and unmyelinated nerve fibres, while the ESWT nerve grafts were filled with well-myelinated regenerating axons. There was no significant difference between the numbers of endoneural vessels in the ESWT and the control nerves. Three months after surgery, no significant differences were observed in the functional and electrophysiological data. Equally high numbers of myelinated axons distal to the graft could be found in both groups (7693 ± 673 [S.E.M., ESWT] vs 6090±716 [S.E.M., control]).

These results suggest that ESWT induces an improved rate of axonal regeneration, this phenomenon probably involving faster Wallerian degeneration, the improved removal of degenerated axons and a greater capacity of the injured axons to regenerate.

Introduction

An injury to a peripheral nerve often results in large defects in the continuity of the severed nerve. The optimum solution is generally considered to be the bridging of such a defect with an autologous nerve graft, but this procedure does not always provide a satisfactory outcome (Siemionow and Brzezicki, 2009). The causes of unsuccessful regeneration through such grafts are an inflammatory and oedematous microenvironment, scarring within the nerve and the

⁎ Corresponding author at: Austrian Cluster for Tissue Regeneration and Ludwig Boltzmann Institute for Experimental and Clinical Traumatology at the Research Centre for Traumatology of the Austrian Workers' Compensation Board (AUVA), Donaueschingenstrasse 13, A-1200 Vienna, Austria. Fax: + 43 1 33110 460.
E-mail address: thomas.hausner@aon.at (T. Hausner).
[1] Thomas Hausner and Krisztián Pajer contributed equally to this work.
[2] Present address: Department of Traumatology, Medical University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria.

0014-4886/$ – see front matter © 2012 Published by Elsevier Inc.
doi:10.1016/j.expneurol.2012.04.019

host graft interfaces and increased sprouting rather than elongative growth of the axons (Maggi et al., 2003). Numerous experimental strategies are currently applied to facilitate nerve regeneration in cases where nerve stumps have to be bridged, such as the use of artificial tubes, scaffolds, neurotrophic substances, etc. Some of these approaches have gained acceptance and are used in clinical nerve repair, though there is an ongoing debate as concerns their use, effectiveness and side-effects (Arino et al., 2008; Johnson and Soucacos, 2008).

Defocused low-energy extracorporeal shock wave therapy (ESWT) has gained acceptance as a therapeutic tool in different medical disciplines, including urology, orthopaedics and traumatology. The shock wave itself is a longitudinal acoustic wave, travelling at the speed of ultrasound waves in water through the body tissues. It is a single pressure pulse with a short needle-like positive spike~ 1 μs in duration and up to 100 MPa in amplitude, followed by a tensile part of several microseconds at lower amplitude (Mariotto et al., 2009).

Previous studies have shown that shock waves stimulate the metabolic activity of a number of cell types, including osteoblasts (Hausdorf et al., 2011; Martini et al., 2003), tenocytes (Bosch et al., 2007), endothelial cells (Corson et al., 1996; Fleming et al., 1998) and chondrocytes (Murata et al., 2007), and this type of treatment has proved effective in clinical applications relating to bone and wound healing (Moretti et al., 2009a, 2009b; Schaden et al., 2001, 2007) and myocardial ischaemia (Fukumoto et al., 2006; Nishida et al., 2004; Zimpfer et al., 2009). This metabolic activation of cells appears to be at least partially dependent on processes of phosphorylation, including that of nitric oxide synthase (Corson et al., 1996; Fleming et al., 1998).

Despite these well-known effects of ESWT on various cell types and tissues, very little is known as to how it affects either intact or damaged nerve tissue. Several studies have focussed on the analgesic effects of shock waves under circumstances of clinical and experimental applications (Ohtori et al., 2001; Takahashi et al., 2006; Wu et al., 2007). High shock wave doses applied to skin reportedly induced analgesia accompanied by injury of the affected nerves in the exposed area and the corresponding expression of transcription and regeneration-related factors in dorsal root ganglion neurones (Murata et al., 2007).

To the best of our knowledge, no studies have been performed to date as regards whether shock waves influence the regeneration of injured peripheral nerves. The aim of the present study was to investigate whether ESWT improves regeneration within the peripheral nervous system in an experimental model in which the integrity of the injured nerve is restored in a similar manner as in human peripheral nerve injuries.

Materials and methods

Animals and surgery

Experiments were carried out on 49 male Sprague–Dawley rats weighing 300–350 g (Animal Research Laboratories, Himberg, Austria) and lasted for a period of 3 weeks or 3 months. The animals were anaesthetized by the intraperitoneal administration of a combination of ketamine hydrochloride+xylazine (ketamine hydrochloride: 90 mg/kg body weight, Ketavet, Pharmacia & Upjohn Co.; xylazine: 5 mg/kg body weight, Rompun, Bayer Co.). Adequate care was taken in all cases to minimize the levels of pain and discomfort during and after the operation.

The experimental protocol was approved in advance by the Animal Protocol Review Board of the City Government of Vienna (No: MA58-1020/2008/7). All procedures were carried out in full accord with the Helsinki Declaration on Animal Rights and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication NIH 86-23, revised 1985).

Two experimental groups were set up: in the first group of animals (n = 20) the right sciatic nerve received a reversed autograft and consequent ESWT, in group 2 (controls, n = 20) grafting was performed without shock wave treatment. Additionally, 9 intact animals received ESWT and their sciatic nerves were subjected to qualitative histological analysis: animals (3 in each group) received 300, 900 or 1500 shock-wave impulses, featuring the same conditions as described below. The observations on the effects of these doses on intact nerves (see Results section) along with data from the current literature indicated the use of a single dose of 300 impulses throughout the study.

In the operated animals, the right sciatic nerve was exposed at the midthigh level. An 8 mm nerve segment was removed from the sciatic nerve with microsurgical scissors (Fine Surgical Tools). The nerve segment was then rotated through 180°, replaced between the proximal and distal stumps of the transected sciatic nerve and epineurially coapted with two sutures (Ethilon 10-0/BV-2, Ethicon-Johnson & Johnson, Brussels, Belgium) at each end, under an operating microscope (Leica M651, Leica Microsystems, Vienna, Austria). Immediately after wound closure, an ultrasound gel was applied to the skin above the operation area as a protective and conducting layer. In the treatment group (group 1), ESWT was applied with an Orthowave 180 shock wave machine (MTS Europe, Switzerland). Three hundred impulses were applied with a frequency of 3 Hz at energy level 1 (0.1 mJ/mm ^{2}). All animals had access to water and dry chow ad libitum.

The animals that received an autograft were sacrificed after survival times of 3 weeks (n = 10 for controls and treated animals) or 3 months (n=10 in each group). Intact animals (n=9) were sacrificed 1 week after ESWT.

The operated animals had undergone an electrophysiological analysis (see below) before transcardiac perfusion was performed. Following the induction of deep anaesthesia with the ketamine+xylazine combination, the animals that survived for 3 weeks and 3 months were perfused transcardially first with heparinized physiological saline, followed by either 2.5% phosphate-buffered (0.1 M phosphate-buffer, pH 7.4) glutaraldehyde (animals processed for electron microscopy and axon counts), or 4% phosphate-buffered paraformaldehyde (animals processed for immunohistochemistry). The right sciatic nerve with the nerve autograft was removed and immersion-fixed for 12 h. Intact animals were perfused with 4% paraformaldehyde.

Morphological analysis

Intact nerves, removed from perfusion-fixed animals were immersion-fixed for 6 h in 4% paraformaldehyde and then cyroprotected in 30% sucrose in PBS. Parallel cryostat sections were cut on a Leica 1850 cryostat and sections were either stained with cresyl violet or processed for 200 kD neurofilament immunohistochemistry. Section were treated with a 1% milk powder solution and the incubated with an anti-200 kD rabbit neurofilament antibody overnight at 4 °C (Abcam Ltd, UK, Lot. No.: ab8135, rabbit, 1:1000). Then the sections were treated with an anti-rabbit Alexa 546 secondary antibody for 2 h at room, temperature, coverslipped and investigated under an epifluorescence microscope (Olympus FX-50, Olympus Ltd, Tokyo, Japan).

A von Willebrand Factor (vWF) antibody was used to stain endoneural blood vessels in the samples from the animals that survived for 3 weeks (n = 5 in each group). The right sciatic nerves were embedded in paraffin and 5 μm thick sections were cut 1 mm proximal and distal to the proximal suture site, from the middle of the graft, and 1 mm proximal and distal to the distal suture site. These sections were deparaffinized and then blocked in a 1% milk powder solution. An anti-vWF antibody (Invitrogen, Lot No.: 18-0018, 1:100) was applied to the sections for 60 min at room temperature. After several washes in PBS, a biotinylated secondary antibody (goat anti-rabbit, Vector Ltd, 1:200) h was used for 2 h. The antigen–antibody complex was visualized through use of the ABC method, with DAB as chromogen (Vector Ltd). The number of endoneural blood vessels was determined at each location; perineural and epineural vessels were not included in the counts.

After the survival period of 3 weeks and 3 months, semithin sections were cut from peripheral nerves (n = 5 in each group). Remnants of fixative were carefully washed out from the nerve, and the

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019

tissue was next treated in 1% OsO_4 (Agar Scientific, Stansted, UK) (in PBS) for 1 h, dehydrated in a graded ethanol series and propylene oxide and then embedded in Durcupan (Fluka, Switzerland). Semithin sections (0.5 μm) were cut 2 mm proximal and distal to the graft and from the middle of the graft (in case of 3 weeks of survival) on a Leica Ultracut-R ultramicrotome and stained according to Rüdeberg (1967). Nerves taken from animals that survived for 3 months were analysed only 2 mm distal to the graft. Images of the whole cross-sectional area of the nerve were taken with an Olympus DP70 camera attached to an Olympus BX-50 microscope (Olympus Ltd, Tokyo, Japan). Myelinated fibres were counted with the aid of Image J image analysis software (NIH free software).

Ultrathin sections were cut from the graft and 2 mm distal to the graft (animals surviving for 3 weeks only) and mounted on copper grids. Sections, contrasted in uranyl acetate and stained with lead citrate were investigated in a JEOL JEM 1010 electron microscope (JEOL Ltd, Tokyo, Japan).

Electrophysiological analysis

At the end of the survival period, electrophysiological analysis (NeuroMax-XLTEK, Oakville, ON, Canada) was carried out during the terminal operations in all animals in order to assess the extent of reinnervation in the various groups. Stimulation electrodes were placed 2 mm proximal and 2 mm distal to the graft for calculation of the nerve conduction velocity. A needle electrode was placed as a recording electrode into the tibialis anterior muscle, and the sciatic nerve was stimulated for 0.05 ms first proximally and then distally to the graft, so as to achieve the supramaximal stimulation amplitude. The compound action potential, the amplitude and the nerve conduction velocity were determined. All measurements were carried out at a body temperature between 38 and 39 °C.

Functional analysis

Functional analysis was performed with the aid of the CatWalk (version 7.1) automated gait analysis system (Noldus Information Technology, Wageningen, The Netherlands), which makes dynamic measurements of the footprints of a rat, via which locomotor deficits and pain syndromes can be assessed. The apparatus comprises an enclosed walkway with a glass floor, located in a darkened room. A rat or mouse traverses the walkway from one side to the other, while light enters the long edge of the glass floor. Light is able to escape only at those areas where a paw makes contact with the floor, and hence illuminates the animal's paws.

The following parameters were assessed:

1. Footprint intensity (the mean pressure exerted by one paw, expressed in arbitrary units, a.u.)
2. Footprint width (the mean width of each footprint of the affected hind limb, in mm)
3. Footprint length (the mean length of each footprint of the affected hind limb, in mm)
4. Stride length (the total length of the step cycle, in mm)
5. Stance duration (the duration of the stance phase of the hind limb, in s)
6. Swing duration (the duration of the swing phase of the hind limbs, in s).

Statistical analysis

The statistical analysis was carried out with the Graph Pad Prism statistical software (Graph Pad Software Inc., San Diego, CA, USA). Groups were compared by the use of ANOVA, followed by Tukey's post hoc test. Functional evaluations were compared with the Mann–Whitney U test. All data in this study are given as means ± standard error (S.E.M.).

Results

Observations of movement pattern of operated animals

All of the animals survived the surgery and the subsequent ESWT. No side-effects were seen.

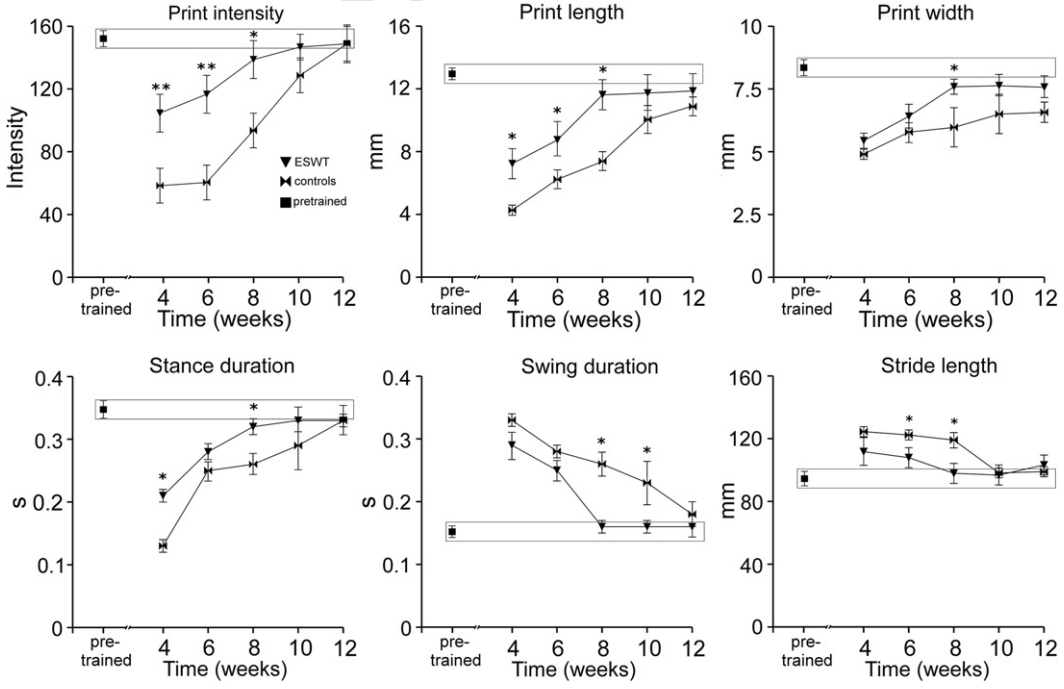

Fig. 1. CatWalk automated gait analysis data 4 to 12 weeks postoperatively. Significant differences were observed in various parameters, indicating earlier restoration of the hind limb motor function in the shock wave-treated (ESWT) animals. Averaged values of pretraining are shown in framed boxes. *Significant difference between the control and ESWT groups, $p < 0.05$, **$p < 0.01$, by the Mann–Whitney U test. Values are expressed as means ± S.E.M.

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019

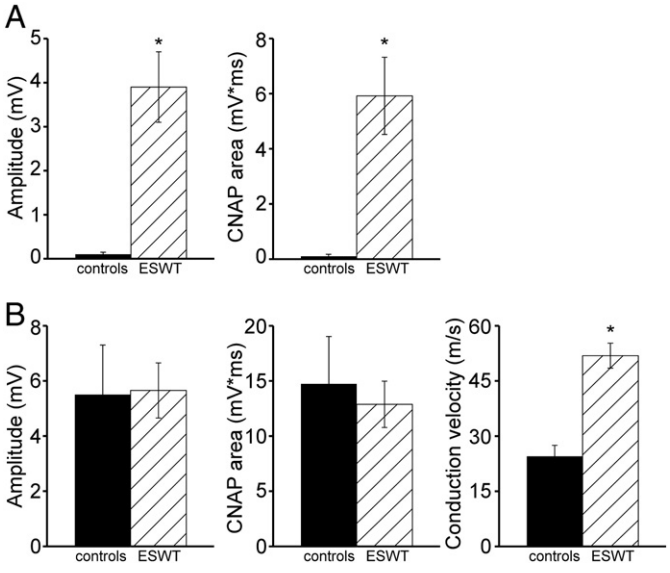

Fig. 2. Electrophysiology stimulation data 3 weeks (A) and 3 months (B) postoperatively. A: The amplitude and the compound nerve action potential area (CNAP) values are negligible in control animals as compared with the shock wave-treated ones (ESWT). B: At the end of the survival period the amplitude and CNAP values do not significantly differ whereas the conduction velocity in the controls is still considerably lower than that in the ESWT group. *Significant difference between the control and ESWT groups, p b 0.05, by ANOVA, computed by using Tukey's all pairwise multiple comparison procedures. Values are expressed as means ± S.E.M.

The functional observations were carried out on freely-moving animals. Immediately after surgery, all the animals developed paralysis in the operated right hind limb corresponding to the musculature supplied by the sciatic nerve. The animals that received ESWT started to produce movements detectable by naked eye using the affected musculature in their operated hind limbs as early as 21 days following surgery. However, the control animals showed the first signs of improvement only 28 days postoperatively. The functional deficit was more pronounced in the control animals than in the ESWT group up to a survival time of 10 weeks, but from this time on the animals in the two groups did not display any detectable movement pattern differences.

CatWalk automated gait analysis system

CatWalk analysis was performed biweekly on the animals in both experimental groups from week 4 to week 12. The results of the automated gait analysis confirmed our functional observations on freely-moving animals. Parameters such as print intensity, print width and length, swing duration, stance duration and stride length were evaluated. These parameters revealed that the functional recovery proceeded much more rapidly in the ESWT group, the differences proving statistically significant typically at early survival times (4 to 8 weeks after surgery, Fig. 1). However, by 10 weeks of survival, the differences between the control and treatment groups had disappeared, except for swing duration. The improved regeneration in ESWT animals resulted in a more pronounced rate of increase of stance duration and decrease of swing duration and stride length as the sole of these animals developed a more stable contact with the ground during the fast functional recovery. These data indicate that the sciatic nerves of the control animals underwent regeneration with a delay of ~ 4 weeks.

Electrophysiology

Electrophysiological recordings were made from the tibialis anterior muscle at 3 weeks and 3 months after surgery. Stimulating electrodes were placed either proximal or distal to the nerve graft, and the conduction velocity within the grafted nerve segment could therefore be calculated. At survival time of 3 weeks, considerable amplitude (3.9 ± 0.8 mV, S.E.M.) and compound nerve action potential area values (CNAP, 5.9 ± 1.4 mV·ms, S.E.M., Fig. 2A) could be observed in the ESWT animals, while these parameters were typically not detected in the control animals. The conduction velocity was not evaluated because of the poor innervation pattern in the control animals. The animals that survived for 3 months displayed amplitudes and compound nerve action potential areas that did not significantly differ between the two experimental groups. However, there was a significant difference in nerve conduction velocity between the ESWT and the control animals (54.9 ± 3.4 m/s vs 24.5 ± 3 m/s, S.E.M., respectively; Fig. 2B).

Morphometry and morphological analysis of intact and grafted nerves after ESWT

Intact nerves that received 300 impulses of ESWT and were stained with cresyl violet and processed for 200 kD neurofilament immunohistochemistry displayed no signs of degeneration or interruption of axons. However, animals that received 900 impulses of ESWT showed interrupted axons, while ESWT of 1500 impulses induced complete degeneration of the axons (Fig. 3). The connective tissue structures (endo-, peri- and epineurium) appeared intact, except for nerves treated with 1500 impulses, where these connective tissue structures appeared damaged, too.

To detect the early differences in nerve regeneration between the ESWT and the control animals, we determined the numbers of myelinated fibres in the graft and proximal and distal to it in the sciatic nerve at 3 weeks after injury. In the sciatic nerve 2 mm proximal to the graft we found 7868 ±171 (S.E.M.) and 7508± 192 (S.E.M.) myelinated axons in the control and the ESWT animals, respectively (Fig. 4). In contrast with this, in the middle of the graft and 2 mm distal to the graft there were pronounced differences in the numbers of myelinated fibres between the ESWT and the control groups (4644 ± 170 [S.E.M.] vs 877±68 [S.E.M.] for the graft and 1586 ± 157 [S.E.M.] vs 308 ± 29 [S.E.M.] for the sciatic nerve distal to the graft, Fig. 4A). These data suggest that axons may regenerate faster in the ESWT animals than in the control nerves. In the sciatic nerve of animals that survived for 3 months, we

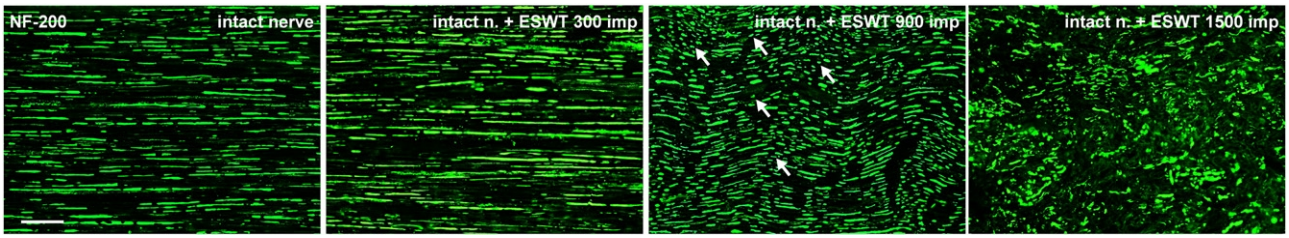

Fig. 3. Intact nerves receiving various doses of ESWT and processed for 200 kD neurofilament immunohistochemistry. Longitudinal sections show that a single shock wave treatment of 300 impulses did not induce axonal degeneration 1 week after ESWT, while treatment with 900 or 1500 impulses resulted in moderate and severe degeneration, respectively. Scale bar = 50 μm.

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019

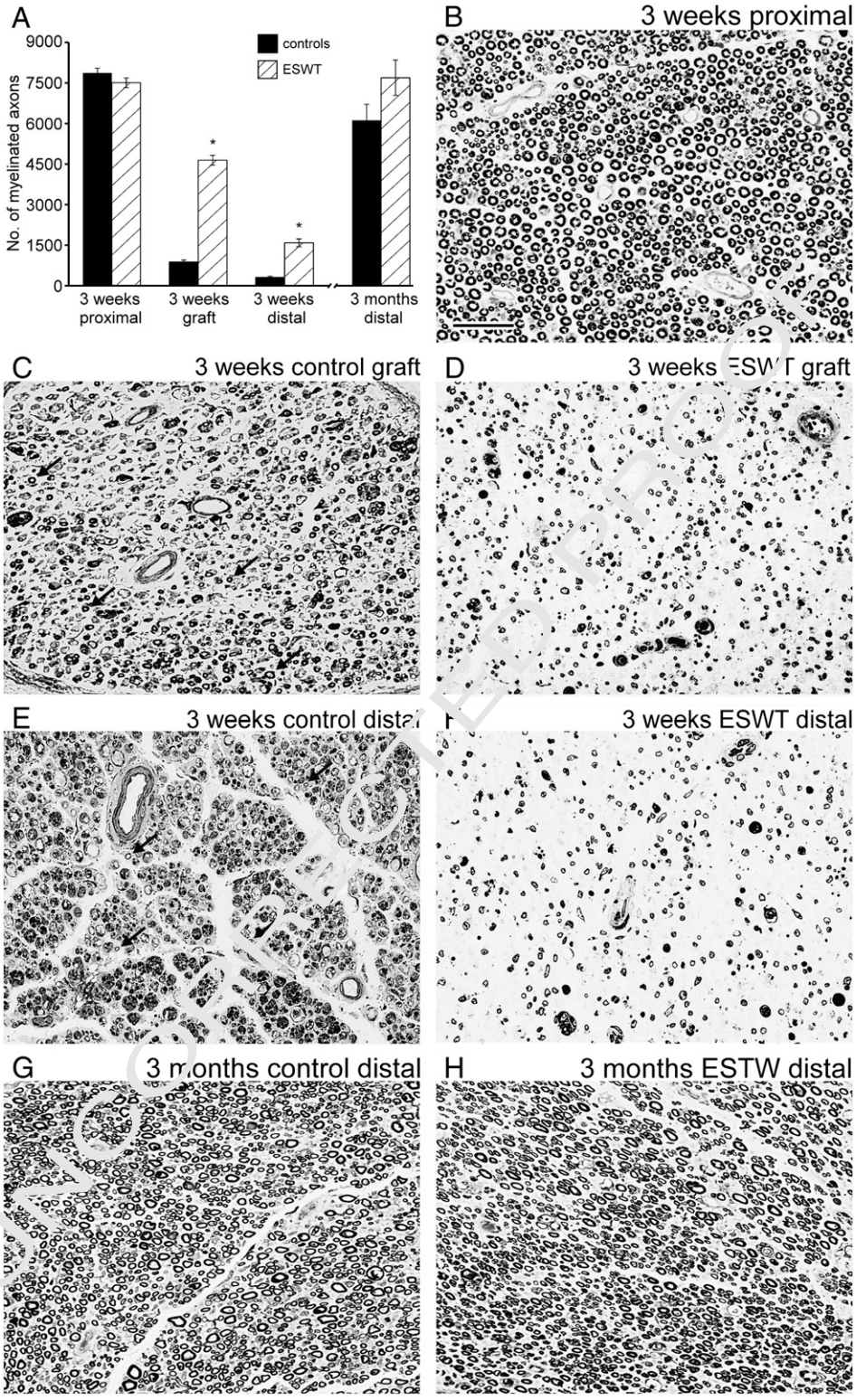

Fig. 4. Axonal regeneration in control and shock wave-treated (ESWT) peripheral nerves 3 weeks and 3 months after surgery. A: The chart shows the numbers of myelinated fibres found in the middle of the graft and 2 mm proximal and distal to the graft in ESWT and control animals 3 weeks after axotomy (left). The numbers of myelinated axons in the graft and distal to the grafting site are much higher in the ESWT animals than in the controls. There was no significant difference between controls and ESWT animals in the numbers of myelinated axons distal to the graft 3 months after axotomy and grafting (right). *Significant difference between the control and ESWT groups, p b 0.05,by ANOVA, computed by using Tukey's all pairwise multiple comparison procedures. B–F: Photographs of semithin cross-sections from the proximal stump (B), the middle of the graft (C, D) and the distal stump (E, F) 3 weeks after axotomy. The shock wave-treated peripheral nerves (ESWT) contain more myelinated axons, while the control nerves display far fewer regenerated axons (arrows) and are full of degenerated myelin sheaths and reactive cells. G–H: Photographs of semithin cross-sections from the distal stump 3 months after axotomy. There is no striking difference between the ESWT and control nerves, although the myelin sheaths of the regenerated axons appear thinner compared with those seen in the intact proximal stump (B). Methyleneblue-thionin staining according to Rüdeberg, scale bar = 25 m.

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019

found equally high numbers of myelinated axons distal to the graft, although nerves treated with ESWT had non-significantly more axons compared to controls (7693 ± 673 [S.E.M.] vs 6090 ± 716 [S.E.M.], respectively). No structural or visible morphological differences were observed at this stage between treated and control nerves (Figs. 4G–H), however, the regenerated axons in both cases appeared less well myelinated yet, than in the proximal stump seen in Fig. 4B.

Angiogenesis

The analysis of the sections taken from various levels of the investigated nerves and processed for vWF immunohistochemisry did not reveal any significant difference between the control and the ESWT nerves at 3 weeks of survival (Fig. 4). It could be observed, however, that the number of vWF-positive vessels gradually increased towards the distal stump of the nerves in both groups.

Ultrastructural analysis

Semithin sections taken from control and ESWT sciatic nerve grafts at 3 weeks of survival demonstrated a striking difference: not only did the nerves from the ESWT animals have more myelinated fibres, but the endoneurium was free of cells other than Schwann cells related to the newly formed myelin (Figs. 4D,F) . In contrast, the control nerve grafts exhibited far fewer regenerated axons with new myelin sheaths, and the endoneurium contained large numbers of reactive cells (Figs. 4C,E). Electron microscopic investigations revealed that the regeneration of severed axons was more advanced in the nerve grafts of the ESWT animals: degenerating myelin sheaths could hardly be seen and the endoneurium around the regenerated axons contained only normal amounts of collagen fibres and few fibrocytes. On the other hand, the axon regeneration appeared to be less advanced in the control nerve grafts, where large numbers of degenerated myelin sheath fragments, phagocytes and reactive fibroblasts could still be observed (Fig. 5).

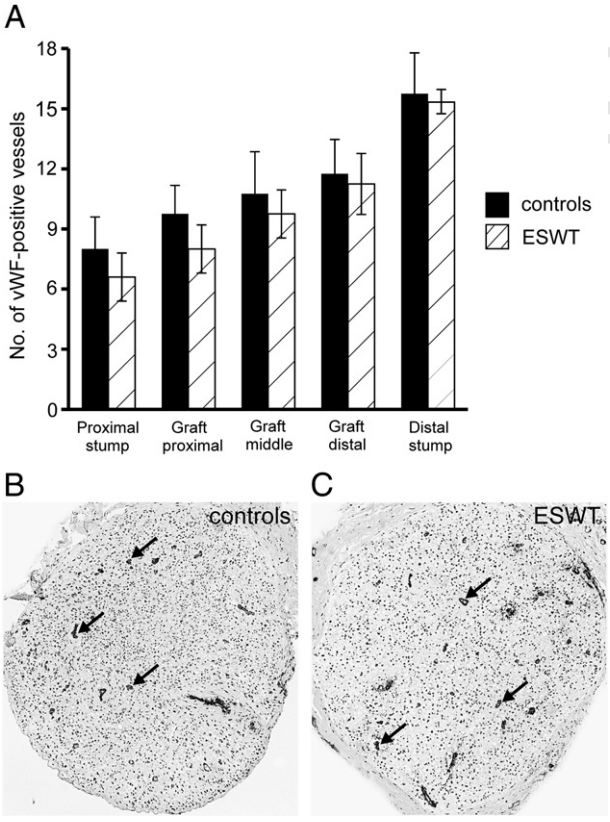

Fig. 5. Vascularization of nerve grafts 3 months postoperatively. A: The number of von Willebrand factor-immunoreactive vessels is shown. There is no significant difference between the control and shock wave-treated (ESWT) samples. B,C: Representative cross-sections of nerve grafts of ESWT and control animals, showing von Willebrand factor-immunoreactive vessels (arrowheads). Scale bar = 250 μm.

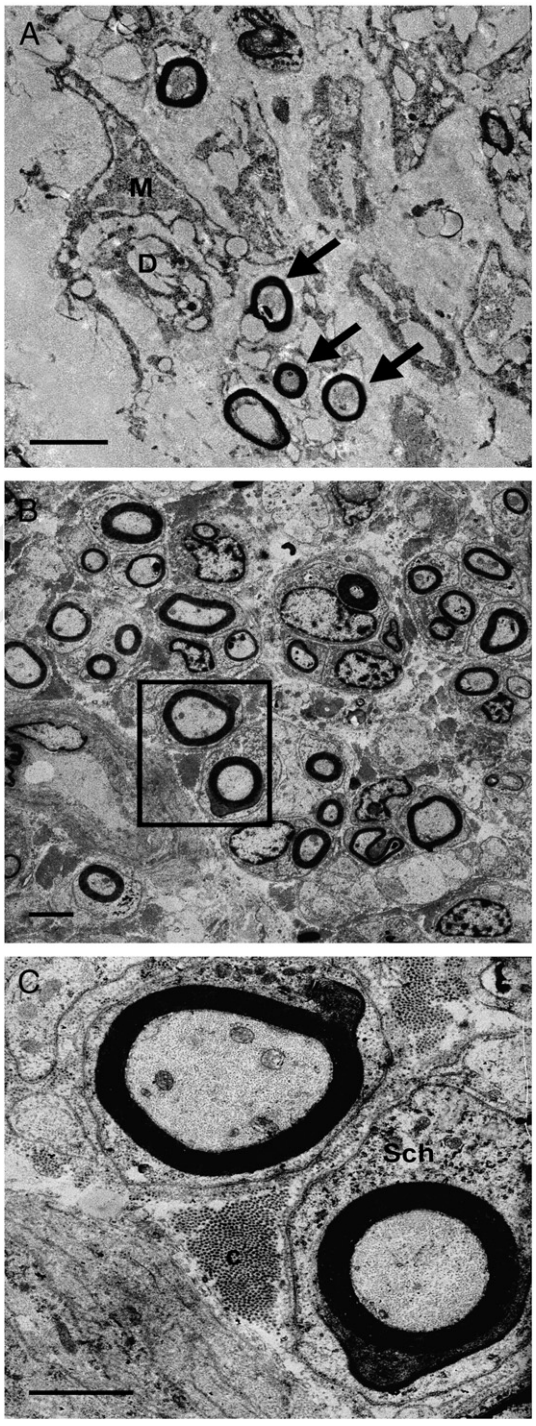

Fig. 6. Electron microscopic photographs of control (A) and shock wave-treated peripheral nerves 3 weeks after surgery. Panel A shows several degenerated myelin sheaths (D) engulfed by macrophages (M). A few myelinated regenerated axons (arrows) too can be seen. In panel B a high number of myelinated axons are present without reactive cells, but surrounded by Schwann cells. Panel C shows a higher magnification of the framed area in B. Note the remyelinating Schwann cells (Sch) and some collagen bundles (C) in the endoneurium. Scale bar in A and B = 2 μm, in C = 1 μm.

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019

Discussion

This study has first provided evidence that defocused low-energy ESWT induces an improved rate of functional recovery in the initial phase of regeneration following injury to the rat sciatic nerve. The functional and morphological data presented here suggest that this improved functional recovery is achieved through faster elongation of the myelinated axons within the nerves following ESWT.

Functional analysis of the movement patterns of the ESWT rats indicated a faster initial functional improvement relative to control animals. This improvement was most impressive at 8 weeks after the nerve injury, but the difference between the ESWT and control groups had become non-significant or had disappeared by 12 weeks of survival. In contrast with the early morphological findings of far fewer regenerating myelinated fibres in the untreated peripheral nerves than in the ESWT nerves at 3 weeks postoperatively, a functional improvement was first observed only at 6 to 8 weeks of survival (Fig. 6). The reason for this difference is that the regenerating fibres must first reach their peripheral targets (skeletal muscles), reinnervate them and then produce a functional reinnervation. As concerns the apparently faster regeneration in the ESWT animals, it seems that the regenerating fibres in this group attain a measurable functional reinnervation by 6 weeks postoperatively.

This accords with our electrophysiological observation that nerve action potentials with considerable amplitudes could be evoked at 3 weeks of survival by stimulation of the intact segment of the sciatic nerve in the ESWT animals, whereas no response was generated in the control nerves, but by 3 months of survival the responses detected in the two groups had become identical.

As regards the rate of regeneration in normally regenerating rodent nerves (4 mm/day) (Forman and Berenberg, 1978; McQuarrie and Grafstein, 1973), it can be argued that all the regenerating motor axons were likely to reach their target muscles by the end of the survival period in both experimental groups. This suggestion is justified by the electrophysiological and functional test data. However, the non-significant differences between the two experimental groups in some of the movement pattern analysis (CatWalk) data indicate that minor differences between the ESWT and control animals may still exist 3 months after surgery. Indeed, the significant difference in nerve conduction velocity that we observed, may suggest that the faster-regenerating axons in the ESWT animals may achieve characteristics closer to those of intact axons more quickly than without ESWT.

ESWT is a well-established method for the therapy of various disorders, such as soft and hard tissue defects, skin ulcerations and plantar fasciitis (Loew and Jurgowski, 1993; Rompe et al., 1996a, 1996b). However, despite the observed effectiveness of this method in such clinical applications, the mechanism of action is poorly understood, but is mainly considered to involve improved angiogenesis in the repaired tissues (Stojadinovic et al., 2008). Other mechanisms have also been suggested, such as the release of various growth factors, the activation of innate stem cells and changes in mechanotransduction, e.g. integrin-mediated cytoskeletal and other cellular changes (Thiel, 2001). Our study did not indicate an improved vascular supply in the treated nerves, and it can therefore be argued that angiogenesis is not responsible for the improved rate of regeneration induced by ESWT.

However, improved axonal regeneration may be supported by other mechanisms acting in the nerves of the ESWT animals. Our electron microscopic analysis revealed the faster clearance in the regenerating nerves as another morphological change in the ESWT animals, which also displayed fewer fibroblasts and less endoneural collagen in the nerves. This may be interpreted as a lower degree of endoneural scarring in consequence of the different fibrocytic activity, and therefore improved reorganization of the injured nerves.

This study has focused on the locomotor recovery induced by regenerating motor axons. However, ESWT reportedly induces injury of the small-diameter unmyelinated nerve fibres (C-fibres) in the rat skin (Murata et al., 2006; Ohtori et al., 2001; Takahashi et al., 2006), accompanied by the loss of immunoreactivity for calcitonin gene-related peptide in the dorsal root ganglia and in the free nerve endings in rats (Takahashi et al., 2003). Moreover, there are significant increases in the number of neurons immunoreactive for activating transcription factor 3 (ATF3) and growth-associated phosphoprotein (GAP-43) as markers of nerve injury and axonal regeneration (Murata et al., 2006). We did not investigate the analgesic effect of ESWT, but in contrast with the deleterious effects of ESWT on unmyelinated fibres, we observed a growth-promoting effect on myelinated motor axons.

The results presented here also raise the question of whether ESWT will find a place in the arsenal of treatment strategies related to peripheral nerve injuries. ESWT has been demonstrated to be a non-invasive method, regardless of field of application. Moreover, it is widely accepted that the spinal cord and peripheral nervous system structures in rats and humans exhibit considerable similarities. It therefore appears likely that ESWT would bring about the same beneficial effects in humans as it does in rodent experiments, provided that conditions for uninterrupted axonal growth are established.

Acknowledgments

The authors are indebted to the Lorenz Böhler Fonds for financial support. The excellent technical assistance of Mrs I. Kovács is gratefully acknowledged. We thank Dr. David Durham for critical reading of the manuscript.

References

Arino, H., Brandt, J., Dahlin, L.B., 2008. Implantation of Schwann cells in rat tendon autografts as a model for peripheral nerve repair: Long term effects on functional recovery. Scand. J. Plast.Reconstr. Surg. Hand Surg. 42, 281–285.

Bosch, G., Lin, Y.L., van Schie, H.T.M., van de Lest, C.H.A., Barneveld, A., Weeren, P.R., 2007. Effect of extracorporeal shock wave treatment on the biochemical composition and metabolic activity of tenocytes in normal tendinous structures in ponies. Equine Vet. J. 39 (3), 226–231.

Corson, M.A., James, N.L., Latta, S.E., Nerem, R.M., Berk, B.C., Harrison, D.G., 1996. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ. Res. 79 (5), 984–991.

Fleming, I., Bauersachs, J., Fisslthaler, B., Busse, R., 1998. Ca2 +-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. Circ. Res. 82 (6), 686–695.

Forman, D.S., Berenberg, R.A., 1978. Regeneration of motor axons in the rat sciatic nerve studied by labeling with axonally transported radioactive proteins. Brain Res. 156 (2), 213–225.

Fukumoto, Y., Ito, A., Uwatoku, T., Matoba, T., Kishi, T., Tanaka, H., Takeshita, A., Sunagawa, K., Shimokawa, H., 2006. Extracoporeal cardiac shock wave therapy ameliorates myocardial ischemia in patients with severe coronary artery disease. Coron. Artery Dis. 17, 63.

Hausdorf, J., Sievers, B., Schmitt-Sody, M., Jansson, V., Maier, M., Mayer-Wagner, S., 2011. Stimulation of bone growth factor synthesis in human osteoblasts and fibroblasts after extracorporeal shock wave application. Arch. Orthop. Trauma Surg. 131 (3), 303–309.

Johnson, E.O., Soucacos, P.N., 2008. Nerve repair: experimental and clinical evaluation of biodegradable artificial nerve guides. Injury 39S, S30–S36.

Loew, M., Jurgowski, W., 1993. Initial experiences with extracorporeal shockwave lithotripsy (ESWL) in treatment of tendinosis calcarea of the shoulder [in German]. Z. Orthop. Ihre Grenzgeb. 131, 470–473.

Maggi, S.P., Lowe III, J.B., MacKinnon, S.E., 2003. Pathophysiology of nerve injury. Clin. Plast. Surg. 30, 109–126.

Mariotto, S., Carcereri de Prati, A., Cavalieri, E., Amelio, E., Marlinghaus, E., Suzuki, H., 2009. Extracorporeal shock wave therapy in inflammatory diseases: molecular mechanism that triggers anti-inflammatory action. Curr. Med. Chem. 16, 2366–2372.

Martini, L., Giavaresi, G., Fini, M., Torricelli, P., de Pretto, M., Schaden, W., Giardino, R., 2003. Effect of extracorporeal shock wave therapy on osteoblast like cells. Clin. Orthop. Relat. Res. 413, 269–280.

McQuarrie, I.G., Grafstein, B., 1973. Axon outgrowth enhanced by a previous nerve injury. Arch. Neurol. 29 (1), 53–55.

Moretti, B., Notarnicola, A., Garofalo, R., Moretti, L., Patella, S., Marlinghaus, E., Patella, V., 2009a. Shock waves in the treatment of stress fractures. Ultrasound Med. Biol. 35 (6), 1042–1049.

Moretti, B., Notarnicola, A., Maggio, G., Moretti, L., Pascone, M., Tafuri, S., Patella, V., 2009b. The management of neuropathic ulcers of the foot in diabetes by shock wave therapy. BMC Musculoskelet. Disord. 27 (10), 54.

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019

Murata, R., Ohtori, S., Ochiai, N., Takahashi, N., Saisu, T., Moriya, H., Takahashi, K., Wada, Y., 2006. Extracorporeal shockwaves induce the expression of ATF3 and GAP-43 in rat dorsal root ganglion neurons. Auton. Neurosci. 128 (1–2), 96–100.

Murata, R., Nakagawa, K., Ohtori, S., Ochiai, N., Arai, M., Saisu, T., Sasho, T., Takahashi, K., Moriya, H., 2007. The effects of radial shock waves on gene transfer in rabbit chondrocytes in vitro. Osteoarthritis Cartilage 15 (11), 1275–1282.

Nishida, T., Shimokawa, H., Oi, K., Tatewaki, H., Uwatoku, T., Abe, K., Matsumoto, Y., Kajihara, N., Eto, M., Matsuda, T., Yasui, H., Takeshita, A., Sunagawa, K., 2004. Extracoporeal cardiac shock wave therapy ameliorates markedly ameliorates ischemia-induced myocardial dysfunction pigs in vivo. Circulation 110, 3055.

Ohtori, S., Inoue, G., Mannoji, C., Saisu, T., Takahashi, K., Mitsuhashi, S., Wada, Y., Takahashi, K., Yamagata, M., Moriya, H., 2001. Shock wave application to rat skin induces degeneration and reinnervation of sensory nerve fibres. Neurosci. Lett. 315 (1–2), 57–60 (23).

Rompe, J.D., Hopf, C., Küllmer, K., Heine, J., Burger, R., 1996a. Analgesic effect of extracorporeal shock-wave therapy on chronic tennis elbow. J. Bone Joint Surg. Br. 78, 233–237.

Rompe, J.D., Hopf, C., Nafe, B., Burger, R., 1996b. Low-energy extracorporeal shock wave therapy for painful heel: a prospective controlled single-blind study. Arch. Orthop. Trauma Surg. 115, 75–79.

Rüdeberg, C., 1967. A rapid method for staining thin sections of Vestopal W-embedded tissue for light microscopy. Experientia 23 (9), 792 (Sep 15).

Schaden, W., Fischer, A., Sailler, A., 2001. Extracorporeal shock wave therapy of non-union or delayed osseous union. Clin. Orthop. Relat. Res. 387, 90–94.

Schaden, W., Thiele, R., Kolpl, C., Pusch, M., Nissan, A., Attinger, C.E., Maniscalco-Theberge, M.E., Peoples, G.E., Elster, E.A., Stojadinovic, A., 2007. Shock wave therapy for acute and chronic soft tissue wounds: a feasibility study. J. Surg. Res. 143, 1–12.

Siemionow, M., Brzezicki, G., 2009. Chapter 8: current techniques and concepts in peripheral nerve repair. Int. Rev. Neurobiol., 87, pp. 141–172.

Stojadinovic, A., Elster, E.A., Anam, K., Tadaki, D., Amare, M., Zins, S., Davis, T.A., 2008. Angiogenic response to extracorporeal shock wave treatment in murine skin isografts. Angiogenesis 11, 69–380.

Takahashi, N., Wada, Y., Ohtori, S., Saisu, T., Moriya, H., 2003. Application of shock waves to rat skin decreases calcitonin gene-related peptide immunoreactivity in dorsal root ganglion neurons. Auton. Neurosci. 107, 81–84.

Takahashi, N., Ohtori, S., Saisu, T., Moriya, H., Wada, Y., 2006. Second application of low-energy shock waves has a cumulative effect on free nerve endings. Clin. Orthop. Relat. Res. 443, 315–319.

Thiel, M., 2001. Application of shock waves in medicine. Clin. Orthop. Relat. Res. 387, 18–21.

Wu, Y.H., Lun, J.J., Chen, W.S., Chong, F.C., 2007. The electrophysiological and functional effect of shock wave on peripheral nerves. Conf. Proc. IEEE Eng. Med. Biol. Soc. 2369–2372.

Zimpfer, D., Aharinejad, S., Holfeld, J., Thomas, A., Dumfarth, J., Rosenhek, R., Czerny, M., Schaden, W., Gmeiner, M., Wolner, E., Grimm, M., 2009. Direct epicardial shock wave therapy improves ventricular function and induces angiogenesis in ischemic heart failure. J. Thorac. Cardiovasc. Surg. 137, 963–970.

Please cite this article as: Hausner, T., et al., Improved rate of peripheral nerve regeneration induced by extracorporeal shock wave treatment in the rat, Exp. Neurol. (2012), doi:10.1016/j.expneurol.2012.04.019