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Low-energy Shock Wave Therapy Ameliorates Erectile Dysfunction in a Pelvic Neurovascular Injuries Rat Model

Huixi Li,[1,2] Melanie P. Matheu,[3] Fionna Sun,[1] Lin Wang,[1,2] Melissa T. Sanford,[1] Hongxiu Ning,[1] Lia Banie,[1] Yung-chin Lee,[1,4] Zhongcheng Xin,[2] Yinglu Guo,[2] Guiting Lin,[1] and Tom F. Lue[1]

ABSTRACT

Introduction: Erectile dysfunction (ED) caused by pelvic injuries is a common complication of civil and battlefield trauma with multiple neurovascular factors involved, and no effective therapeutic approach is available.

Aims: To test the effect and mechanisms of low-energy shock wave (LESW) therapy in a rat ED model induced by pelvic neurovascular injuries.

Methods: Thirty-two male Sprague-Dawley rats injected with 5-ethynyl-2′-deoxyuridine (EdU) at newborn were divided into 4 groups: sham surgery (Sham), pelvic neurovascular injury by bilateral cavernous nerve injury and internal pudendal bundle injury (PVNI), PVNI treated with LESW at low energy (Low), and PVNI treated with LESW at high energy (High). After LESW treatment, rats underwent erectile function measurement and the tissues were harvested for histologic and molecular study. To examine the effect of LESW on Schwann cells, in vitro studies were conducted.

Main Outcome Measurements: The intracavernous pressure (ICP) measurement, histological examination, and Western blot (WB) were conducted. Cell cycle, Schwann cell activation-related markers were examined in in vitro experiments.

Results: LESW treatment improves erectile function in a rat model of pelvic neurovascular injury by leading to angiogenesis, tissue restoration, and nerve generation with more endogenous EdU^+ progenitor cells recruited to the damaged area and activation of Schwann cells. LESW facilitates more complete re-innervation of penile tissue with regeneration of neuronal nitric oxide synthase (nNOS)-positive nerves from the MPG to the penis. In vitro experiments demonstrated that LESW has a direct effect on Schwann cell proliferation. Schwann cell activation-related markers including p-Erk1/2 and p75 were upregulated after LESW treatment.

Conclusion: LESW-induced endogenous progenitor cell recruitment and Schwann cell activation coincides with angiogenesis, tissue, and nerve generation in a rat model of pelvic neurovascular injuries.

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Key Words: Low Energy Shock Wave; Erectile Dysfunction; Endogenous Progenitor Cells; Schwann Cells; Nerve Regeneration; Angiogenesis

INTRODUCTION

Trauma-related erectile dysfunction (ED) commonly occurs in the setting of pelvic surgery or as a result of local injuries such as improvised explosive device in battlefield, and is most often associated with the damage of cavernous nerves (CN) and/or internal pudendal bundle (IPB).[1,2] After injury, ischemia and neural degeneration lead to both impaired erectile capability and its lack of response to therapy.[3] Current treatments include oral phosphodiesterase V inhibitors, vacuum erection devices, penile injection, transurethral therapy, and penile prosthesis, but none of these can restore normal erectile physiology.[4] In addition, we lack a good animal model to study neurovascular ED. Consequently, both basic and translational researchers are continuing to search for effective strategies.[5]

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[1]Knuppe Molecular Urology Laboratory, Department of Urology, School of Medicine, University of California, San Francisco, CA, USA;

[2]Department of Urology, Peking University First Hospital and the Institute of Urology, Peking University, Beijing, P.R. China;

[3]Diabetes Center, University of California, San Francisco, CA, USA;

[4]Department of Urology, Kaohsiung Medical University Hospital, Department of Urology, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

Low-energy shockwaves (LESW) have been used for years to treat musculoskeletal disorders.[6] Recently, the application of this therapy has been expanded to address ischemic heart disease[7] and vasculogenic ED,[8] but there are few reports concerning the effects shock waves have on nerve fibers or neurovascular ED. In 2001, Ohtori et al reported LESW stimulated reinnervation of sensory fibers,[9] and in 2006 another Japanese group found that shock waves induce the expression of growth-associated protein-43 (GAP-43, a marker for axonal growth cones) in rat dorsal root ganglia (DRG).[10] Shock waves have also been reported to induce DRG cells to express activating transcription factor 3 (ATF3), which promotes neurite outgrowth from the ganglion when the peripheral axon is injured.[10] Also, we have reported that LESW improves diabetic ED in an animal model by promoting nerve regeneration,[11] a finding confirmed by another group.[12] Clinically, LESW therapy also has been proven to be a potential treatment for angiogenesis and penile rehabilitation.[13,14]

Recovery of neurovascular ED is a tough task involving the vascular system and the peripheral nervous system, whereas regeneration of peripheral nerves after pelvic injury is a complex process related to neurons, Schwann cells, basal lamina, and responsiveness of end organs. Among the orchestration of these various cells, Schwann cells are often the "first responders" in this microenvironment[15] and play an important guiding role,[16] which could be promoted by mechanical force.[17] Schwann cells play an important role in axon regeneration after injury, including CN injury that leads to ED.[18] In the penile nerve system, Schwann cells have been found to be functional in Remak bundles/C fibers (mainly composed in the cavernous nerve) and A-δ fibers (mainly composed in the internal pudendal nerve).[15,19] However, the effects of Schwann cells during the penile nerve regeneration have not been well elucidated though indirect evidence claims that treatments aiming to promote the growth of Schwann cells result in better erectile function recovery.[20,21]

In the current study, we developed a new ED rat model of pelvic neurovascular injury (PVNI) by bilateral cavernous nerve injury and internal pudendal bundle injury, and tested the effect of LESW treatment at different energy levels. We hypothesized that LESW might improve function, angiogenesis, and innervations by activating local Schwann cells and increasing progenitor cell recruitment.

MATERIALS AND METHODS

Experimental Design

All procedures were approved by the Institutional Animal Care and Use Committee of University of California, San Francisco. A total 32 newborn male Sprague-Dawley rats were used for this study. Each pup received an intraperitoneal injection of 5-ethynyl-2′-deoxyuridine (EdU, 50 mg/kg, Invitrogen, Carlsbad, CA, USA) as previously reported.[22] At 12 weeks old, they were grouped into 4 (n = 8 each): sham surgery (Sham), pelvic neurovascular injury by bilateral cavernous nerve injury and internal pudendal bundle injury (PVNI), PVNI treated with LESW at low energy (Low), and PVNI treated with LESW at high energy (High). After 4 weeks of LESW treatment and 1 week of washout, all rats underwent erectile function measurement. The rats were then sacrificed and the penis (half for histology and half for Western blot), major pelvic ganglion (MPG), and urethra were harvested for histology and Western blot.

In vitro studies were conducted using primary tissue culture of rat Schwann cells. Four rats (5 weeks old) were sacrificed and the sciatic nerves were harvested for isolation of Schwann cells as previously reported.[23]

Develop Pelvic Neurovascular Injury Rat Model

Bilateral cavernous nerve injury (CNI) was performed as previously described,[24] whereas the IPB injury (IPBI) was conducted as follows: the rat was positioned into lithotomy and a horizontal perineal incision was made. The IPB was identified between the ischiocavernosus muscle (ICM) and the bulbospongiosus muscle (BCM). Suture ligation was performed bilaterally. The sham surgery was performed exactly as the described procedure, except that no CNI or IPBI was induced.

Primary Culture of Rat Schwann Cells

Purified Schwann cells culture was created using methods described by Shen et al.[23]

Low-energy Shockwave Treatment

For the in vivo experiment, LESW therapy was started 48 hours postoperatively. Shockwave was delivered to the pelvic region with a special probe that was attached to a compact electrohydraulic unit with a focused shockwave source (DermaGold, MTS Europe GmbH, Konstanz, Germany). Under anesthesia, each rat was placed in the supine position with its lower abdomen shaved and the preputial skin reduced. Standard commercial ultrasound gel (Aquasonic, Parker Laboratories Inc, Fairfield, NJ, USA) was applied between the probe and the skin of pelvic region for optimal coupling. In the low-energy group, 0.06 mJ/mm^2, 300 pulses at 3 Hz was applied, while 0.09 mJ/mm^2, 1000 pulses at 3 Hz was applied in the high-energy group.

For the in vitro experiment, cell cultures were used for LESW treatment. Schwann cells received LESW treatment (0.02 mJ/mm^2, 200 pulses at 3 Hz) after reaching 70% confluence. The probe was handled under the cell culture dish with standard commercial ultrasound gel applied between dish and probe. The cells were treated once and then harvested or checked at corresponding time points.

Erectile Function Evaluation

An intracavernous pressure (ICP) test was used to evaluate erectile function as previously described.[24] In brief, under

Table 1. Antibodies Used in Immunofluorescence Staining (IF) and Western Blot (WB)

	Name	Abbrev	Dilution	Product information
IF	anti-von Willebrand factor	vWF	1:400	ab6994, abcam, Cambridge, MA, USA
	anti-neurofilament	NF	1:400	MAB5262, Merk Millipore, Billerica, MA, USA
	anti-neuronal nitric oxide synthase	nNOS	1:200	SC-648, Santa Cruz Bio-technology, Santa Cruz, CA, USA
	anti-S100	S100	1:200	Z0311, Dako, Carpinteria, CA, USA
	anti-p-Erk1/2	p-Erk1/2	1:500	9100, Cell Signaling Technology, Framingham, MA, USA
WB	anti-β-actin	β-actin	1:1000	SC-47778, Santa Cruz Bio-technology, Santa Cruz, CA, USA
	anti-neuronal nitric oxide synthase	nNOS	1:200	SC-648, Santa Cruz Bio-technology, Santa Cruz, CA, USA
	anti-SDF-1	SDF-1	1:500	SC-28876, Santa Cruz Bio-technology, Santa Cruz, CA, USA
	anti-p75	p75	1:200	ab3125, Abcam, Cambridge, MA, USA
	anti-p-Erk1/2	p-Erk1/2	1:200	9100, Cell Signaling Technology, Framingham, MA, USA
	anti-Erk1/2	Erk1/2	1:400	9926, Cell Signaling Technology, Framingham, MA, USA

ketamine (100 mg/kg) and midazolam (5 mg/kg) anesthesia, the MPG and CN were exposed via midline laparotomy. The corpus cavernosum was cannulated with a heparinized (200 U/mL) 25-gauge needle and connected to a pressure transducer (Utah Medical Products, Midvale, UT, USA). The stimulus parameters were 1.5 mA, 20 Hz, pulse width of 0.2 ms, and duration of 50 seconds. The maximum increase of the ICP curve of 3 stimuli per side was selected for statistical analysis in each animal. Mean arterial blood pressure (MAP) was recorded using a 25-G needle inserted into the aortic bifurcation after the ICP test. ICP/MAP was calculated as the ratio of maximum change of ICP to MAP. Area under the receiver operating characteristic curve (AUC) was recorded as the proportion under the ICP curve during the stimulation of 50 seconds.

Immunofluorescence Staining

Immunofluorescence (IF) staining of rat tissue was performed according to a previously described protocol.[24] In brief, the tissue was harvested and fixed with 2% formaldehyde and 0.002% picric acid in 0.1 mol/L PBS for 4 hours, followed by immersion in 30% sucrose in PBS overnight at 4°C. Tissues were positioned into the same angle and embedded into an optimal cutting temperature compound (OCT, Sakura Finetek, Torrance, CA, USA). Tissue blocks were stored at −80°C. Before sectioning, the blocks were transferred into −20°C. Frozen sections (−20°C) were cut at a thickness of 5 μm. Schwann cells were cultured on cover slips placed in 6-well plates. Eight hours (for p-Erk) after LESW treatment or mock treatment, the cells were fixed in cold paraformaldehyde (4%) at 4°C for 15 minutes. Then the cover lips were proceeded for IF staining.

The tissue sections were incubated with primary antibodies overnight at 4°C. The antibodies used in histology are listed in Table 1.The secondary antibodies used were Alexa-488 and Alexa-594 conjugated antibodies (1:500, Invitrogen, Carlsbad, CA, USA) and the incubation time for secondary antibody is 2 hours at room temperature. Smooth muscle actin (SMA) was stained by Alexa-488—conjugated phalloidin (1:400, Invitrogen) and EdU^+ cells were stained with Click-IT reaction cocktail (Click-IT, Invitrogen) as manual respectively. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen).

The stained slides were examined with a fluorescence microscope (Nikon, Eclipse, 80i). Image analysis was performed by calculating the computerized densitometry or number of positive targets using Image-Pro Plus 5.1(Media Cybernetics, Silver Spring, MD, USA). The following variables were analyzed by number calculating: number of blood vessels and nerve bundles in the dorsal section of the penis, number of EdU positive cells in the cavernosal section of penis, and number of nuclei for Schwann cells. The amount of von Willebrand factor (vWF), neuronal nitric oxide synthase (nNOS), S100, neurofilament (NF), and p-Erk1/2 was analyzed with Image-Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA) based on the integrated optical density of the positively stained area in high-power fields among 4 groups. All the data were calculated in a blinded fashion.

The average number of blood vessels (phalloidin stained green rings) and nerve bundles (the collections of NF stained red dots) within penile dorsal area were counted and calculated (n = 8 for each group).

Western Blot

Protein isolation and Western blot were conducted as previously reported[25] and a total of 20 μg protein were loaded for each sample. The antibodies used in Western blot are listed in Table 1. After the secondary antibody incubation, the resulting images were analyzed with ChemiImager 4000 (Alpha Innotech Corp, San Leandro, CA, USA) to determine the integrated density value of each protein band.

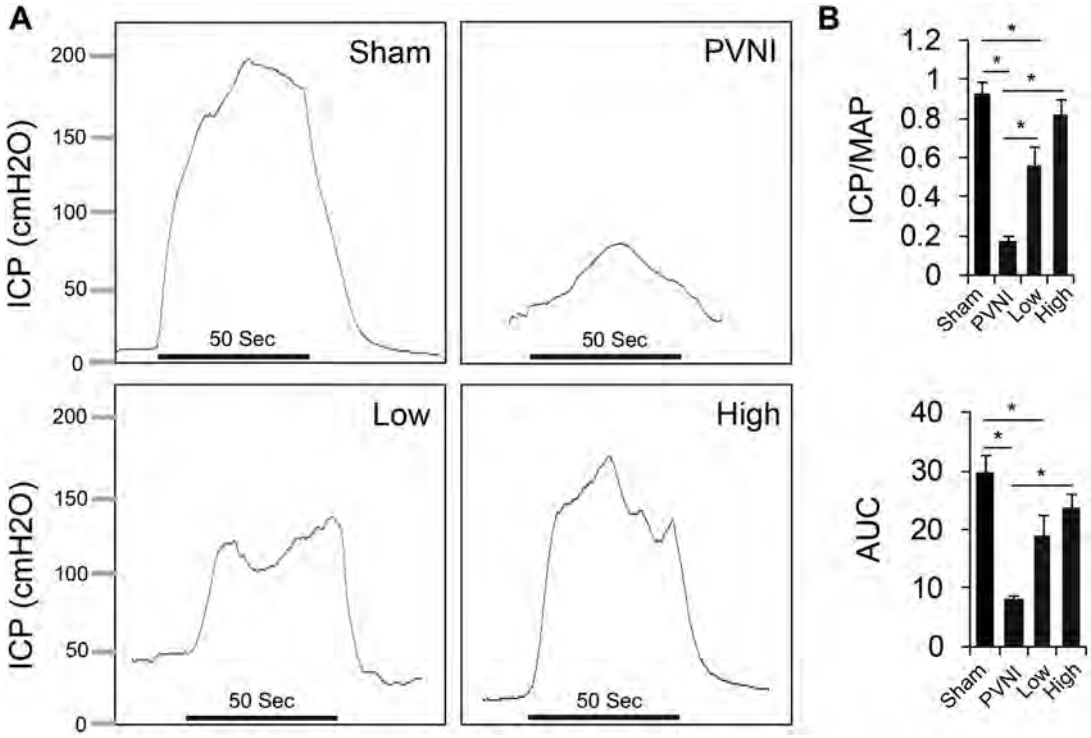

Figure 1. A. Representative intracavernous pressure (ICP) recording of each experimental group. Black curve represents ICP values in response to cavernous nerve (CN) stimulation. The black bar represents 50 seconds electrical stimulation of the CN. B. Data analysis of ICP test. ICP/MAP was calculated as the ratio of maximum change of ICP to MAP. AUC was recorded as the proportion under the ICP curve during the stimulation of 50s. Mean ± standard error of the mean (SEM) for each group. *$P < .05$. MAP = mean arterial pressure; AUC = area under curve.

Cell Cycle Assay

For cell cycle analysis, 10^4 cells were collected for each assay. Cell cycle synchronization was induced by serum starvation. The cells then underwent LESW or mock treatment. The cells were stained with the propidium iodide flow cytometry kit (ab139418, Abcam, Cambridge, MA, USA) according to the manual. The cell cycle was checked at time point 0 hours and 8. 16, and 24 hours after the LESW treatment using flow cytometry (BD, influx, Cell Sorter, Franklin Lakes, NJ, USA) and the results were analyzed with FlowJo (Tree Star, Inc, Ashland, OR, USA).

Statistical Analysis

Results were analyzed using Prism 5 (GraphPad Software, San Diego, CA, USA) and expressed as mean ± standard deviation of the mean (SEM). Multiple groups were compared using *t* test (2 variables) or 1-way analysis of variance followed by the Tukey-Kramer test for post-hoc comparisons (4 variables). Statistical significance was set at $P < .05$.

RESULTS

Low-energy Shockwave Treatment Improves Erectile Function

PVNI-impaired erectile function compared with sham procedure, whereas significant improvement was evident after LESW treatment (Figure 1A). To evaluate recovery of erectile function, we analyzed the ratio of maximum change of ICP to MAP (ICP/MAP) and AUC of the ICP results. Both treated groups showed a significant increase in ICP/MAP compared with control group (0.56 ± 0.10 and 0.82 ± 0.08 vs 0.17 ± 0.03, $P <$ .05). Rats in the higher LESW group showed significant recovery ($P < .05$) compared with the control group with larger AUC (23.83 ± 2.42 vs 8.13 ± 0.55). Overall, these measures suggest partial recovery of erectile function in both treated groups, with rats in the high group demonstrating better recovery relative to the low group (Figure 1B).

LESW Treatment Enhances Penile Angiogenesis and Regains Blood Circulation in PVNI Group of Rats

To determine if the improvement in erectile function correlated to changes in tissue vascularization in the dorsal sections we counted the number of blood vessels. Post the PVNI, the penile dorsal artery collapsed and endothelium were significantly atrophied (Figures 2A and 2B). Impressively, collapsed penile dorsal arteries regain a normal-looking structure with many small blood vessels around it (Figure 2A). Also, LESW promoted the expression of vWF in the penile tissue in both the lower- and higher-energy groups (Figures 2B and 2D). In line with improvements in erectile function, the higher LESW treated group had improved outcome of tissue regeneration. These results

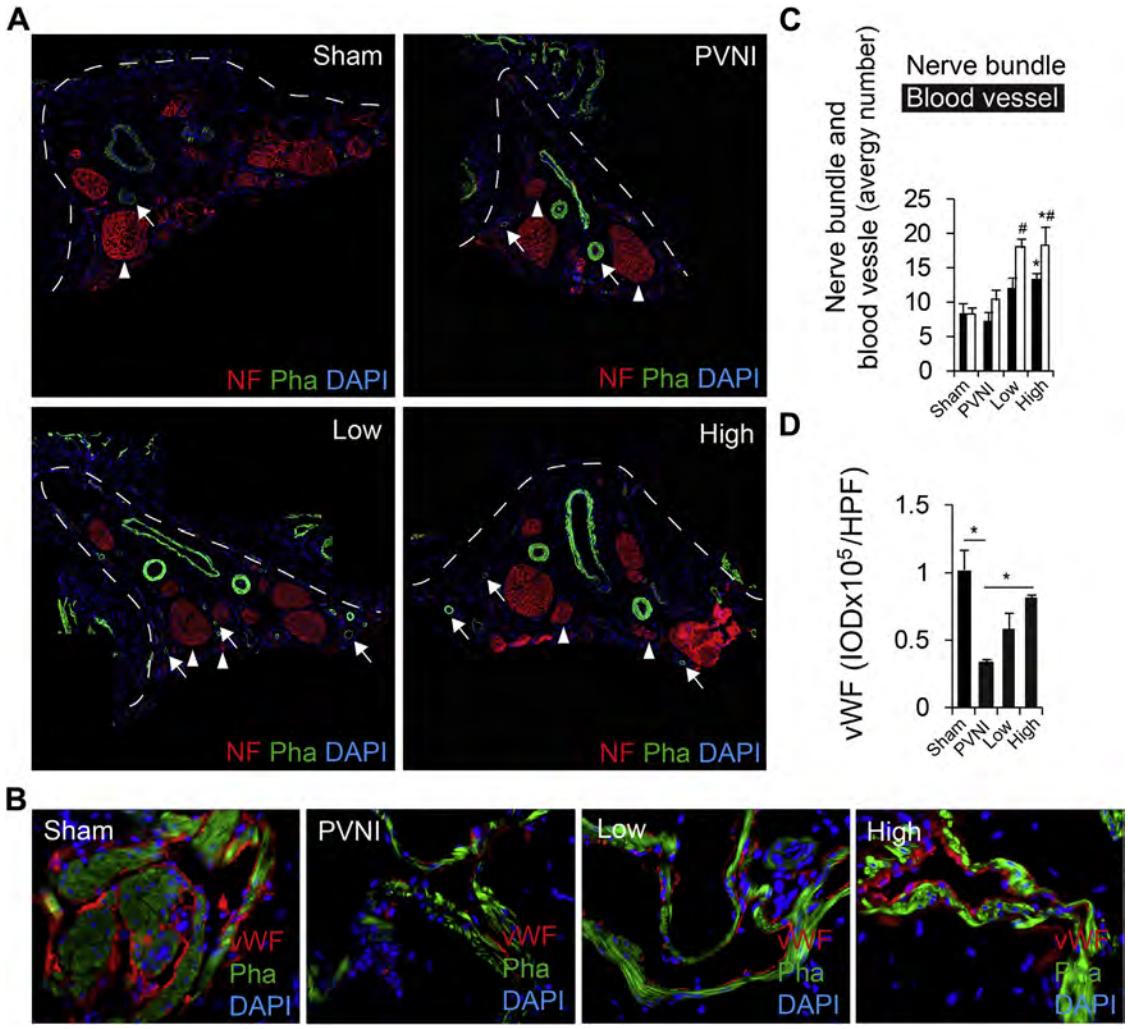

Figure 2. General neurovascular changes in penile tissue. A. Representative images of immunofluorescence staining for vascular smooth muscle (Pha- Alexa 488 conjugated phalloidin-green) and dorsal nerve (NF-red, original magnification is ×4) in 4 groups. Dotted lines surround the dorsal parts. Typical blood vessels were indicated with white arrows and typical nerve bundles were indicated with white triangles. B. Representative images of immunofluorescence staining for endothelium (vWF-red, original magnification is ×400) in sinusoid. C. Average number (± SEM) of nerve bundles (NF dots collections) and blood vessels (smooth muscle rings) in the dorsal neurovascular area of each sample, $^{*}P < .05$ compared with control group, $^{\#}P < .05$ compared with sham group; D. Average IOD amount (± SEM) of vWF staining in sinusoid, $^{*}P < .05$. NF = neurofilament; wWF = von Willebrand factor.

indicate that while at 5 weeks after pelvic injury, the control rats could regenerate the number of blood vessels to a nearly normal level, but the erectile function was not restored in these animals. Therefore, after pelvic injury LESW treatment stimulates increased generation of blood vessel that is strongly correlated with improved function. These studies indicate that LESW is beneficial in cavernous tissue rehabilitation.

LESW Promotes Penile Nerve Regeneration

Within the penis, recovery of the dorsal nerve was examined with IF staining of NF. The number of nerve bundles was significantly improved in LESW-treated groups (Figures 2A and 2C). The majority of nNOS^{+} nerve fibers originate from DCR-MPG. They form Remak bundles and join other nerve fibers to become the cavernous nerves. To tracing the regeneration process of nNOS^{+} Remak nerve fibers, 4 levels of tissue sections were performed: at the MPG, the cavernous nerve at distal site from nerve crush along urethra (CN); dorsal penile nerve (DPN); and the penile sinusoid (Figure 3A).

Nerve injury significantly decreased nNOS^{+} nerve fibers at all 3 nerve levels (CN, DPN, and sinusoid) except in the MPG (Figures 3A and 3B). After LESW treatment, the numbers of nNOS containing fibers increased (Figure 3B). Increased nNOS expression in the treatment groups was confirmed with Western blot using the protein lysates from penile tissue (Figures 3C and 3D). High-energy level LESW treatment might lead to enhanced regeneration of nNOS^{+} nerve fibers compared with low energy levels of LESW; however, these differences were not significant between the 2 groups.

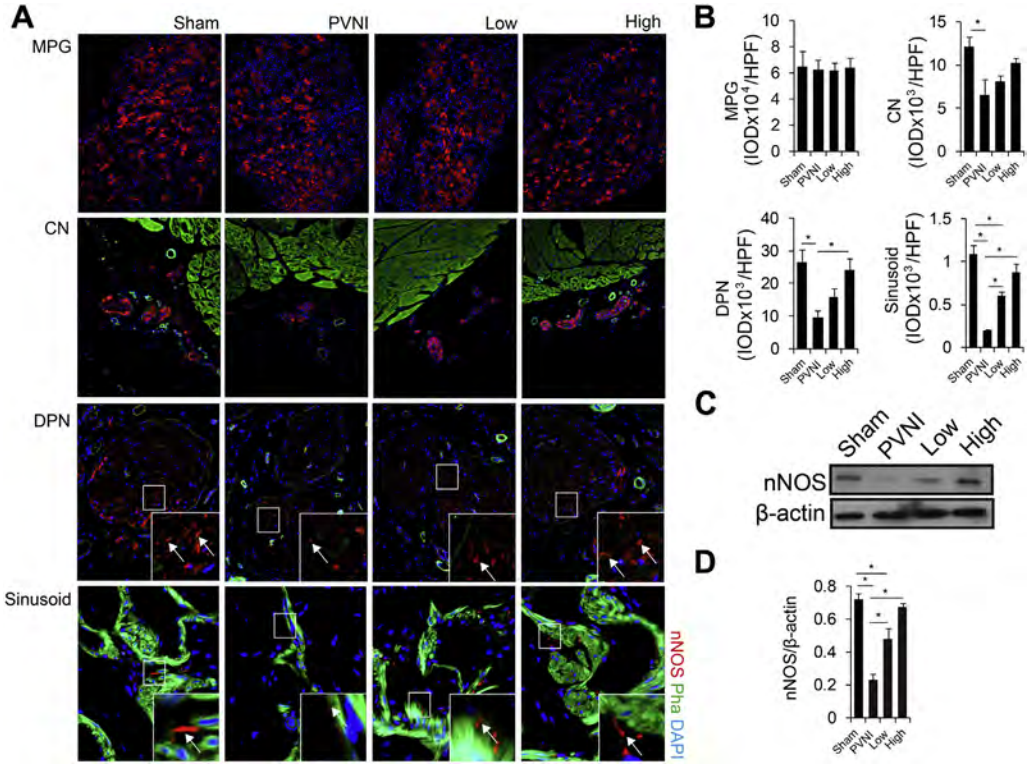

Figure 3. LESW treatment promotes the regeneration of neuronal nitric oxide synthase (nNOS) positive nerves from the major pelvic ganglion (MPG), cavernous nerve (CN) to the penis (dorsal penile nerve or DPN and sinusoid). A. Representative images of immunofluorescence staining for nNOS (red). Original magnification is ×100 (MPG, CN), ×200 (DPN) and ×400 (sinusoid).The white rectangles denote the area selected for amplification. Typical nNOS$^+$ nerves were indicated with white arrows. B. Average IOD amount (± SEM) of nNOS by calculating the densitometry IF staining. C. Representative image of Western blot for nNOS in penile tissue. D. Western blot analysis of nNOS. *$P < .05$.

LESW Treatment Enhances Recruitment of EdU Positive Cells

Because progenitor cells or cells with stem properties are recognized by their ability to retain thymidine analog EdU for an extended period of time, we examined recruitment of EdU$^+$ cells in animals given a single EdU injection at birth.[22] An energy-level–dependent increase between treatment groups ($P < .05$) in EdU$^+$ cells in the cavernosal tissues was evident (Figures 4A and 4B). Using lysates from penile tissue, we measured the expression level of chemokine stromal derived factor 1 (SDF-1; Figures 4C and 4D), a classic chemoattractant for progenitor cell recruitment.[26] After pelvic injury, higher expression of SDF-1 correlated with increased number of EdU$^+$ cells, and this effect was significantly enhanced by LESW treatment, especially in the higher LESW group ($P < .05$).

Schwann Cells During Penile Nerve Regeneration and Effects From LESW Treatment

Schwann cells are critical for nerve fiber growth and regeneration. To further examine the mechanism of nerve regeneration, we explored the process of Schwann cell activation, which is characterized by dedifferentiation, redifferentiation, proliferation, and maturation. Two kinds of Schwann cells were distributed in penile nerves: myelinated Schwann cells (mSC) and nonmyelinated Schwann cells (nmSC). In the tissue section of dorsal nerve, we quantified the relative number of DAPI$^+$ dots in the dorsal nerve fiber (primarily Schwann cell nuclei of both mSC and nmSC),[27] as well as mature Schwann cell marker S100 (Figures 5A and 5B). The number of Schwann cells within dorsal nerves increased in the higher LESW group compared with the other 3 groups, whereas S100 was highly expressed in both sham and higher LESW-treated groups (Figure 5B). Based on the DAPI results it appears that 5 weeks after pelvic injury, the number of cells (including Schwann and other cells) increased to normal levels spontaneously (i.e. the PVNI group) whereas LESW treatment increased the number further. The decreased expression of S100 in the PVNI group compared with the sham group possibly indicates degeneration of some Schwann cells after nerve injury. S100 staining was increased in the LESW treatment groups, suggesting that LESW may enhance proliferation of Schwann cells. Moreover, we examined 2 markers for Schwann cell dedifferentiation and proliferation after pelvic injury: p75 and p-Erk1/2. Western blot of the penile tissue indicated that both p75 and p-Erk1/2 were significantly increased after LESW therapy (Figures 5C and 5D).

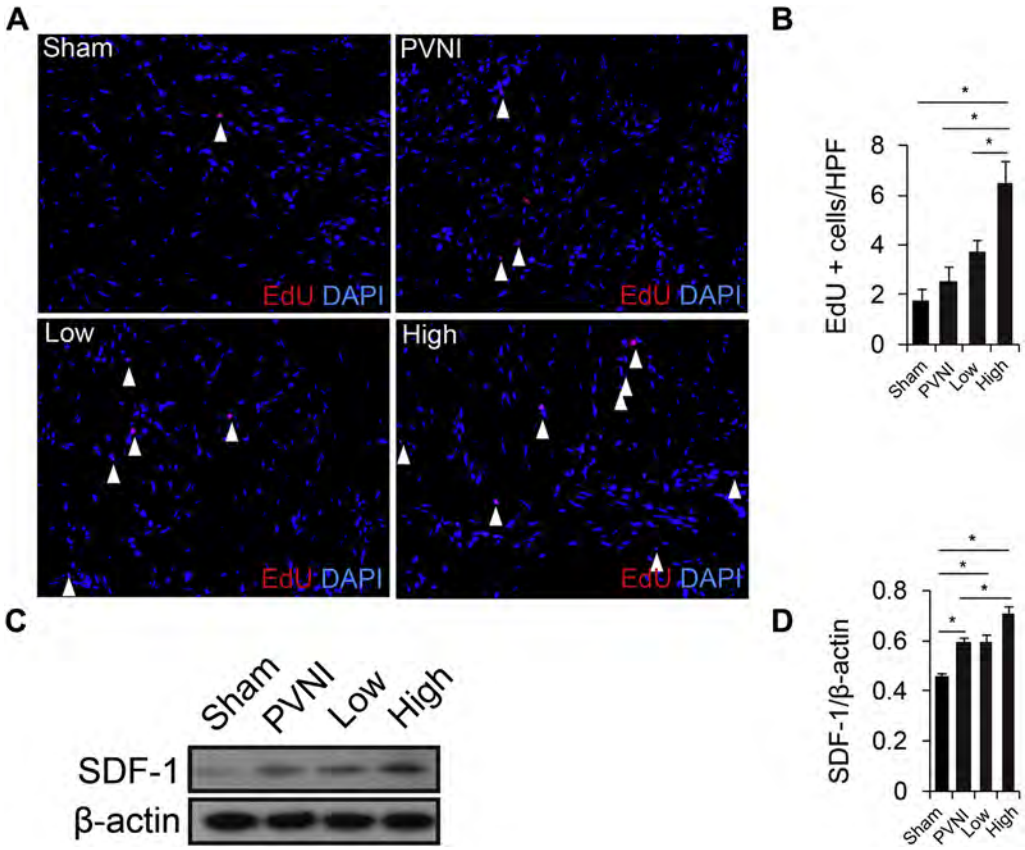

Figure 4. Recruiting of endogenous progenitor cells by LESW in vivo. A. Representative of 5-ethynyl-2′-deoxyuridine (EdU, red, indicated with white triangles) positive cells in cavernosum. Original magnification is ×200. B. Average number (± SEM) of EdU-positive cells per high power field. C. Representative image of Western blot for SDF-1 in penile tissue. D. Western blot analysis of SDF-1. *$P < .05$. SDF-1 = stromal derived factor 1.

Effect of LESW on Cultured Rat Schwann Cells In Vitro

Using LESW on cultured adherent Schwann cells, similar to the in vivo results, the expression of p-Erk1/2and p75 also were significantly elevated after LESW treatment (Figures 6A and 6B). In IF staining, we observed that p-Erk1/2 tended to accumulate in Schwann cell nuclei after LESW treatment, possibly indicating that LESW therapy triggers the initiation of p-ERK1/2—mediated downstream pathways in Schwann cells (Figure 6C). Additionally, we quantified the number of cells in particular cell cycle phases. G1/G0 phase typically indicates dormancy or resting phase before proliferation, S phase indicates the DNA replication, and G2/M phase is when cell division occurs. Within 8 hours after LESW treatment, a higher percent of Schwann cells entered the S phase and the G2/M relative to untreated cells, and this increase in the percentage of cycling Schwann cells remained for 24 hours (Figures 6D and 6E). Together, these data demonstrate the growth-promoting effect of LESW on Schwann cells.

DISCUSSION

Though the standard CNI-induced ED model is consistently used to study ED,[28] there is currently no good animal model to mimic neurovascular ED. In this study, we combined CNI with IPBI to establish an ED model in the rat that closely replicates human pelvic injury during surgery and trauma. This combined injuries model successfully impaired erectile function for a long duration, allowing for extended duration studies of ED therapies.[29]

CNI and IPBI lead to ischemia, neurodegeneration, and impaired erectile capability. We tried to establish a new clinical approach to fix this condition. Recently, low energy shock wave (LESW) treatment is proved to be a promising therapeutic strategy for ED that has been tested in clinical trials (NCT01317693, NCT01811797, NCT01274156, NCT00901056, and others at http://clinicaltrials.gov). The mechanisms of the precise therapeutic and biological effects in LESW treatment are still not completely understood. Prior experiments found that LESW induces neovascularization by upregulating the expression of VEGF and its receptor[30] and mobilization of progenitor cells.[31] The biologic responses of LESW appear to be time dependent and according to a previous report, the peak response occurs 4 weeks after treatment.[32]

In this project, rats in the treated groups showed improved functional and histologic recovery after 4 weeks of LESW treatment. We noted that angiogenesis and recirculation were

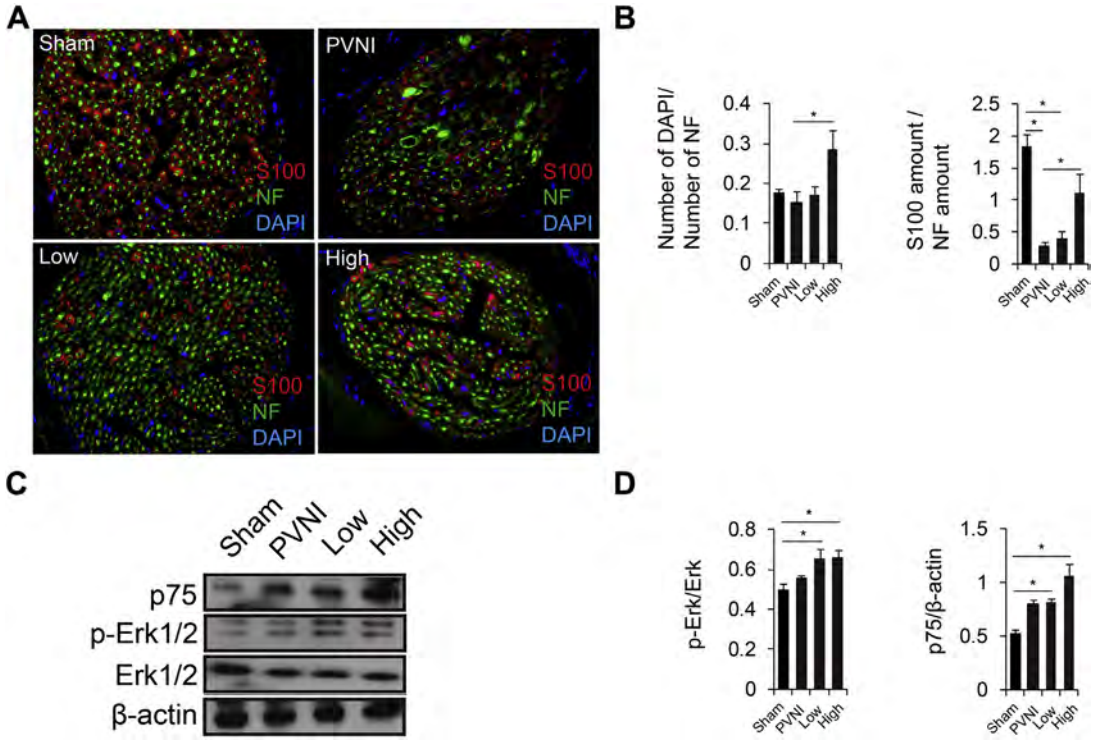

Figure 5. Activation of Schwann cells by LESW in vivo. A. Representative images of immunofluorescence staining for NF (green) and S100 (red) in dorsal nerve. Original magnification is ×200. B. The number of Schwann cells by calculating the number of DAPI dots (standardized to the number of nerve fiber dots which was stained with NF) and expression of S100, which is a marker for mature Schwann cells (standardized to the densitometry of NF). C. Representative image of Western blot for p75 neurotrophin receptor (p75) and Phosphor Erk1/2 (p-Erk1/2) in penile tissue. D. Western blot analysis of p-Erk and p75. *$P < .05$. NF = neurofilament.

significantly promoted after LESW treatment. The major nerve components in the penile nerve system demonstrated significantly more regeneration after LESW treatment when compared with the spontaneous regeneration in the untreated control group rats. The accelerated regeneration of nerve fibers, including nNOS$^+$ nerve fibers, is especially exciting as neural injury and lack of functional nerve recovery are believed to be the crux of why neurovascular ED is refractory to therapy.

Recruitment of sufficient progenitor cells through the vascular network and interstitial tissues is usually the first step of tissue regeneration, and is required for tissue maintenance and injury repair.[33] However, in most studies exogenous progenitor cell application has not been highly successful for various reasons, including inefficient migration to target organs.[33,34] It has been suggested that direct recruitment of endogenous progenitor cells to the target organ of interest might improve the outcome of progenitor cell treatment.[33,35] In our present work we used label retaining cell technique with EdU[36,37] and found more endogenous progenitor cells in penile tissue after pelvic injury, consistent with the process of tissue repair and a previous report.[38] Additionally, we found that LESW therapy led to a significant increase in local progenitor cell numbers relative to untreated animals, in line with our previous report using a diabetic ED model,[11] and that the expression of SDF-1, which plays a primary role in progenitor cell recruitment,[39] is correlated with the number of EdU$^+$ cells in penile tissue. LESW might act to increase and maintain the concentration of SDF-1 in penile tissue post injury, thus potentiating and prolonging the recruitment of endogenous progenitor cells and amplifying in situ tissue regeneration. Many different types of cells can secrete SDF-1, including endothelial cells and smooth muscle cells during injury or in response to hypoxia, and progenitor cells that express CXCR4 could be recruited through SDF-1/CXCR4 axis.[39,40] Progenitor cells predominantly contribute to tissue regeneration through their paracrine ability.[41] It is currently unclear if progenitor cells in LESW-treated tissues are local cells induced to divide, recruited from other sites, or both. In addition, the biological effects of either progenitor cell division or recruitment to penile tissue in this ED model remain open questions.

In humans, peripheral nerve regeneration after injury is known to be a slow process, and may be an underlying factor in the loss of innervated tissue function. Therefore, a potential avenue for ED treatment would be a therapy that enhances the kinetics of nerve regeneration after injury. Successful peripheral nerve regeneration is promoted by Schwann cell activation. After injury, it is thought that some Schwann cells dedifferentiate into a progenitor-like state, proliferate, and then repopulate the damaged nerve.[42] This is critical to navigate the growth of new nerve fibers, especially within the first week or 2 after injury.[43,44]

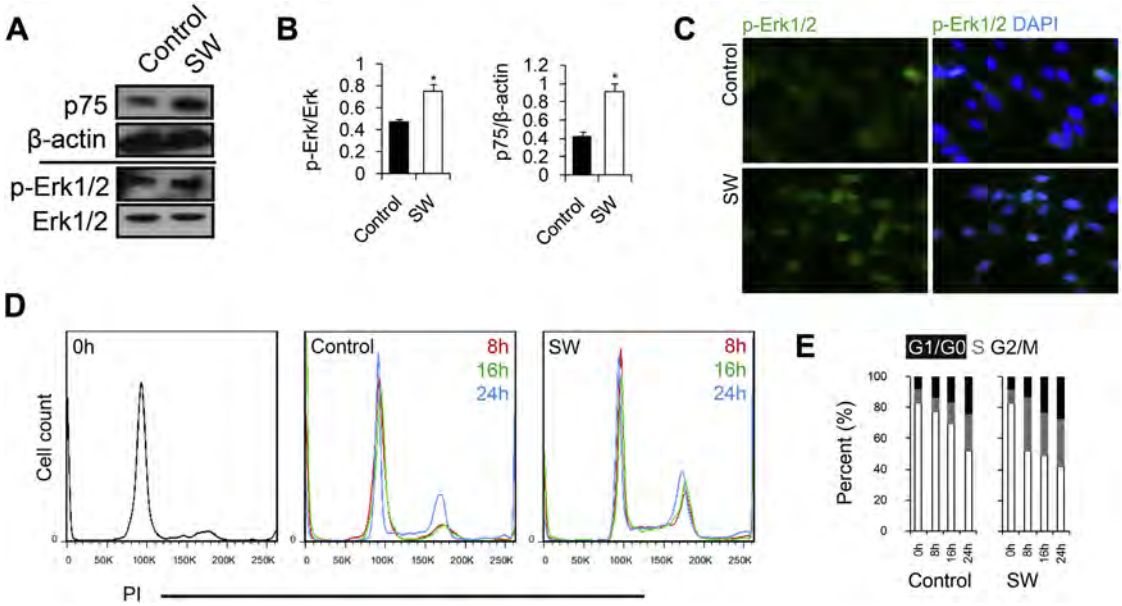

Figure 6. LESW promotes the activation of Schwann cells in vitro. A. Representative image of Western blot for p75 neurotrophin receptor (p75), Phosphor Erk1/2 (p-Erk1/2) in Schwann cells 24h (p75) or 8h (p-Erk1/2) after LESW treatment (SW) or mock treatment (Control). B. Western blot analysis of p-Erk (8h) and p75 (24h). C. Representative images of immunofluorescence staining for p-Erk1/2 (green) in Schwann cells 8 hours after LESW treatment (SW) or mock treatment (Control). Original magnification is ×400. D. Representative of cell cycle image before treatment (0h) and 8, 16, 24 hours after LESW treatment (SW) or mock treatment (Control). E. Percent of cells in different cell cycle periods. *$P < .05$.

In our experiment, upregulation of the crucial activation signaling pathway mediator phosphorylated ERK (p-Erk) is significant after treatment. ERK/MAPK signaling is a classic pathway for induction of cell proliferation[45] and high levels of p-Erk are also a crucial trigger of dedifferentiation of Schwann cells.[42] p75 neurotrophin receptor (p75) expression is a hallmark of Schwann cell dedifferentiation. Currently, the kinetics of p75 expression after injury is unknown, but we also found that expression of p75 is significantly upregulated after LESW treatment both in vitro and in vivo. Together, increased expression of these markers indicates that LESW induces both dedifferentiation and proliferation (also verified by cell cycle analysis) of Schwann cells. Though S100 has been widely used as a cell marker for Schwann cells in vitro and in vivo, it is worth noting that S100 is not a specific marker. Downstream of dedifferentiation and proliferation, S100 is recognized as a maturation gene and represents the amount of mature Schwann cells.[46] In our experiment, the amount of mature Schwann cells decreased after pelvic injury because of nerve degeneration. Dedifferentiation and proliferation of Schwann cells distal to the site of injury along with activation of ERK/MAPK and p75 result in more mature Schwann cells and creating and maintaining an environment amenable to nerve regrowth by LESW.

CONCLUSION

LESW treatment improves erectile function in a rat model of pelvic neurovascular injuries. Penile tissue components, especially vascular and neuronal tissue, demonstrated improved recovery after LESW therapy. The mechanism of these beneficial effects appears to be through the recruitment of endogenous progenitor cells and activation of Schwann cells.

Corresponding Author: Tom F. Lue, MD, Department of Urology, University of California, San Francisco, 400 Parnassus Ave., Ste A-633, San Francisco, CA 94143-0738, USA. Tel: 415-476 1611; Fax: 415-476 8849; E-mail: tlue@urology.ucsf.edu

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STATEMENT OF AUTHORSHIP

Category 1

(a) Conception and Design
Guiting Lin, Tom F. Lue

(b) Acquisition of Data
Huixi Li, Melanie P. Matheu, Fionna Sun, Lin Wang, Melissa T. Sanford, Lia Banie

(c) **Analysis and Interpretation of Data**
Huixi Li, Melanie P. Matheu, Hongxiu Ning, Yung-chin Lee, Zhongcheng Xin, Yinglu Guo, Guiting Lin, Tom F. Lue

Category 2

(a) **Drafting the Article**
Huixi Li, Melanie P. Matheu, Melissa T. Sanford, Guiting Lin, Tom F. Lue

(b) **Revising It for Intellectual Content**
Melanie P. Matheu, Guiting Lin, Tom F. Lue

Category 3

(a) **Final Approval of the Completed Article**
Tom F. Lue

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