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Two methods of extracorporeal shock-wave therapy in a rat model of secondary lymphedema: a pilot study

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Abstract

Objectives: To compare the effectiveness of two methods of extracorporeal shock-wave therapy (ESWT) in a rat model of forelimb lymphedema, induced by axillary lymph node dissection.
Methods: Sprague–Dawley rats were randomly allocated to a group that received 500 ESWT shocks only in the lymphedematous forelimb (Forelimb/ESWT) and a group that received 300 ESWT shocks in the axilla and 200 shocks in the lymphedematous forelimb (Axilla+Forelimb/ESWT). The circumferences of each limb were then measured. Immunohistochemistry for a pan-endothelial marker (cluster of differentiation [CD]31) and lymphatic vessel endothelial hyaluronan receptor-1, and western blot analysis for vascular endothelial growth factor receptor-3 (VEGFR3) and VEGF-C were performed.
Results: The circumferences of the limbs showed significant effects of group and time following surgery. The circumferences at the carpal joint and 2.5 cm above were smallest in the naïve limbs, larger in the Axilla+Forelimb/ESWT group, and the largest in the control group. VEGFR3 tended to be expressed at a higher level in the Axilla+Forelimb/ESWT group (1.96-fold) than in the Forelimb/ESWT group (1.20-fold) *versus* the opposite non-edematous forelimbs, although this difference was not statistically significant.
Conclusions: These data suggest that ESWT protocols have differential effects on angiogenesis and lymphangiogenesis in lymphedematous limbs.

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Keywords
Extracorporeal shock-wave therapy, secondary lymphedema, rat forelimb lymphedema model, axillary lymph node, vascular endothelial growth factor receptor-3, endothelial hyaluronan receptor-1, breast cancer

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Introduction

Lymphedema is defined as a manifestation of lymphatic system insufficiency and affects up to 120 million people worldwide.[1] It arises from primary lymphatic dysplasia or secondary lymphedema after resection or obstruction of the lymphatic system as a consequence of lymph node dissection or radiotherapy to treat cancer.[2–4] Lymphedema is a chronic problem that manifests as interstitial fluid and protein accumulation, leading to remodeling of the skin and subcutaneous tissue.[5,6] One of the most common treatments for lymphedema is complex decongestive physical therapy (CDPT).[7,8] However, although a reduction in swelling can be achieved using CDPT, lymphedema remains an incurable disease that requires labor-intensive and time-consuming care. Therefore, a number of previous studies have evaluated the use of alternative treatment methods.[9,10]

Recently, extracorporeal shock-wave therapy (ESWT) has been used clinically for the treatment of musculoskeletal diseases, such as calcific tendinitis, epicondylitis of the elbow, and plantar fasciitis.[11] Low-energy shock waves have been shown to cause cavitation (the sudden collapse of micrometer-sized bubbles) and shear stress, which causes an increase in cell permeability, higher expression of growth factors, and activation of intracellular signaling pathways.[12,13] In addition, because low-energy ESWT has been reported to induce neovascularization by stimulating the production of angiogenic growth factors and other cell signaling mechanisms, it has also been used for the treatment of lymphedema. Kubo *et al.*[14] demonstrated that ESWT promotes lymphangiogenesis and ameliorates secondary lymphedema, but the most effective method of utilization has yet to be determined. Therefore, we compared the efficacy of two ESWT protocols for the treatment of axillary lymph node dissection-induced secondary lymphedema in rats.

Methods

Rat model of forelimb lymphedema

The study protocol was approved by the Animal Research Ethics Committee of Daegu Catholic University (DCIAFCR-180416-23-Y) and followed the ARRIVE guidelines.[15] Seventeen male Sprague–Dawley rats weighing 300 to 350 g were used. They were housed in cages at room temperature, with 40% to 60% humidity, under a 12-hour light/dark cycle, with free access to food and water. After adaptation to their environment for 1 week, lymphedema was induced in the rats. They were anesthetized by an intraperitoneal injection of 40 mg/kg tiletamine hydrochloride and zolazepam hydrochloride (Zoletil, Virbac, Carros, France) and 1.0 to 5.0 mg/kg xylazine (Rompun, Bayer AG, Leverkusen, Germany), according to the practices of

the University of California, San Diego (https://blink.ucsd.edu/sponsor/iacuc/links.html#Guidelines). An intradermal injection of 0.1 ml of 0.5% methylene blue was performed in the right footpad, then 10 minutes later, a 10-mm-long incision was made into the dermis across the right axilla and the stained axillary lymph nodes were identified and excised.

Application of ESWT

Ten days after surgery, ultrasonographic gel (Firson Corp., Cheonan, Republic of Korea) was applied and a15-mm applicator for radial-type ESWT (BTL-5000; BTL, Greeneville, TN, USA) was gently applied and used to administer 500 shocks (energy level = 0.05 mJ/mm^2, frequency = 3 pulses/s). ESWT treatment was then repeated under anesthesia every 3 days for 4 weeks.[14,16–18] The rats were randomly allocated to three groups: one that received 500 shocks only in the lymphedematous forelimb (Forelimb/ESWT, n = 5), one that received 300 shocks in the axillary region and 200 shocks in the lymphedematous forelimb (Axilla+Forelimb/ESWT; n = 5), and an untreated control group (n = 7). The treated limbs were compared with the opposite forelimb of each rat.

Assessment of the forelimb circumference

The circumferences of the limb at the carpal joint and 2.5 cm above the carpal joint were measured by passing thread around the limb and measuring its length using a ruler to determine the severity of edema in the rats. These measurements were performed 3, 7, and 10 days post-surgery, every week during the ESWT regimen, and 14 days after the last application of ESWT. Each measurement was made three times on each occasion and a mean value was calculated.

Immunohistochemical analysis

The rats were euthanized by carbon dioxide inhalation 14 days after the last ESWT application. Each forelimb was removed, fixed in 4% paraformaldehyde, and embedded in paraffin, then 4-μm sections were prepared and deparaffinized. Immunohistochemical staining using a BOND-III automated slide stainer (Leica Biosystems, Wetzlar, Germany) was carried out in accordance with the manufacturer's instructions. To characterize angiogenesis and lymphangiogenesis, anti-pan-endothelial marker (cluster of differentiation [CD]31, 1:100, Abcam, Cambridge, UK) and anti-lymphatic endothelial hyaluronan receptor 1 (LYVE-1, 1:300, Abcam) antibodies were applied to the sections, then a Bond Polymer Refine Detection kit (Leica Biosystems) was used, and the sections were counterstained with hematoxylin. Color images were captured using a Nikon Eclipse Ni light microscope (Nikon Corp., Tokyo, Japan) and a Nikon DS-Fi1c digital camera, and the number of stained vessels was counted in five randomly selected ×400 fields per slide in blinded fashion by a pathologist. The measurements were performed in duplicate and the data are expressed as the number of CD31-positive and LYVE-1 positive vessels per high-power field.

Western blotting

Western blot analysis was used to determine the expression of vascular endothelial growth factor receptor-3 (VEGFR3) and VEGF-C in the forelimbs. Skin samples were homogenized in RIPA buffer (Cell Signaling, #9806; Danvers, MA, USA) and then centrifuged at 21,200 × g and 4°C for 10 minutes. Supernatants containing equal amounts (40 μg) of protein were separated by SDS-PAGE and the proteins were transferred to nitrocellulose membranes. After blocking with Tris-buffered saline containing 0.1% Tween 20 and 5%

skim milk, the membranes were incubated with anti-VEGFR3 (1: 1,000; Chemicon, Temecula, CA, USA), anti-VEGF-C (1: 1,000; Santa Cruz Biotechnology, Dallas, TX, USA), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1: 1,000; Santa Cruz Biotechnology) antibodies overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase-linked secondary antibodies for 1 hour at room temperature. The protein bands were visualized using an ECL kit (Thermo Fisher Scientific, Waltham, MA, USA) and densitometric analysis of the band intensity was performed using a Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA, USA). The membranes were then re-probed with anti-GAPDH antibody, which was used as a loading control.

Statistical analysis

Analysis of the changes in limb circumference with time, in each group, and the interaction between the two was conducted using a generalized linear model and *post-hoc* testing was performed using the Bonferroni method. Immunohistochemical and western blot data for the two forelimbs of the Forelimb/ESWT and Axilla+Forelimb/ESWT groups were analyzed using the Kruskal–Wallis test 14 days after the final ESWT application. A medical statistician conducted all the analyses using SPSS ver. 19.0 (IBM Corp., Armonk, NY, USA). All the tests were two-sided and $P < 0.05$ was accepted as indicating statistical significance.

Results

Circumference of the forelimb

Ten days after the axillary lymph node dissection, ESWT was commenced and repeated every 3 days for 4 weeks. The circumference of the limb at the carpal joint and 2.5 cm above the joint showed significant effects of time (days after surgery) ($P < 0.001$) and group (control, Forelimb/ESWT, Axilla+Forelimb/ESWT and naïve) ($P < 0.001$), and a significant group and time interaction ($P < 0.001$). The naïve limb had the smallest circumference, followed by the Forelimb/ESWT and Axilla+Forelimb/ESWT groups, and the control group had the largest circumference at both locations on the limb ($P < 0.001$) (Figure 1).

Immunohistochemistry and quantification

A representative image of immunohistochemically stained lymphatic vessels is shown in Figure 2. Immunohistochemistry of the rat forelimbs showed that the mean (±SD) numbers of CD31-positive vessels were 5.7 (±0.61) in the Axilla+Forelimb/ESWT group, 4.6 (±0.91) in the Forelimb/ESWT group, and 4.02 (±1.06) in the control contralateral forelimbs. These did not significantly differ among the groups. The mean (±SD) numbers of LYVE-1-positive vessels were 9.77 (±2.02) in the Axilla+Forelimb/ESWT group, 9.52 (±0.87) in the Forelimb/ESWT group, and 8.86 (±0.88) in the contralateral control forelimbs. There were also no significant differences among these groups (Figure 2).

Expression of VEGFR3 and VEGF-C

Western blot analysis was performed 14 days after the last ESWT application to measure the protein expression of VEGTR3 and VEGF-C in developing lymphatic vessels in the skin. The expression of VEGFR3 was 1.20- and 1.96-fold higher in the Forelimb/ESWT and Axilla+Forelimb/ESWT groups, respectively, *versus* the contralateral control limbs. The expression in the Axilla+Forelimb/ESWT group tended to be higher than in the other groups, but this difference did not achieve statistical

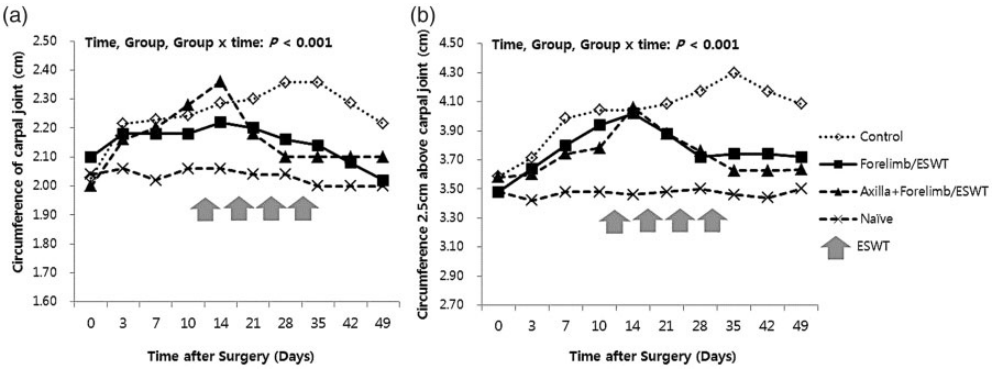

Figure 1. Circumferences of the forelimb at the level of the carpal joint (a) and 2.5 cm above the carpal joint (b) in the Forelimb/ESWT, Axilla+Forelimb/ESWT, control (lymphedematous limb) groups, and contralateral control (naïve) forelimbs. There were significant effects of time ($P < 0.001$) and group ($P < 0.001$), and a group × time interaction ($P < 0.001$) between the Forelimb/ESWT, Axilla+Forelimb/ESWT, and control (lymphedematous limb) groups, according to a generalized linear model.

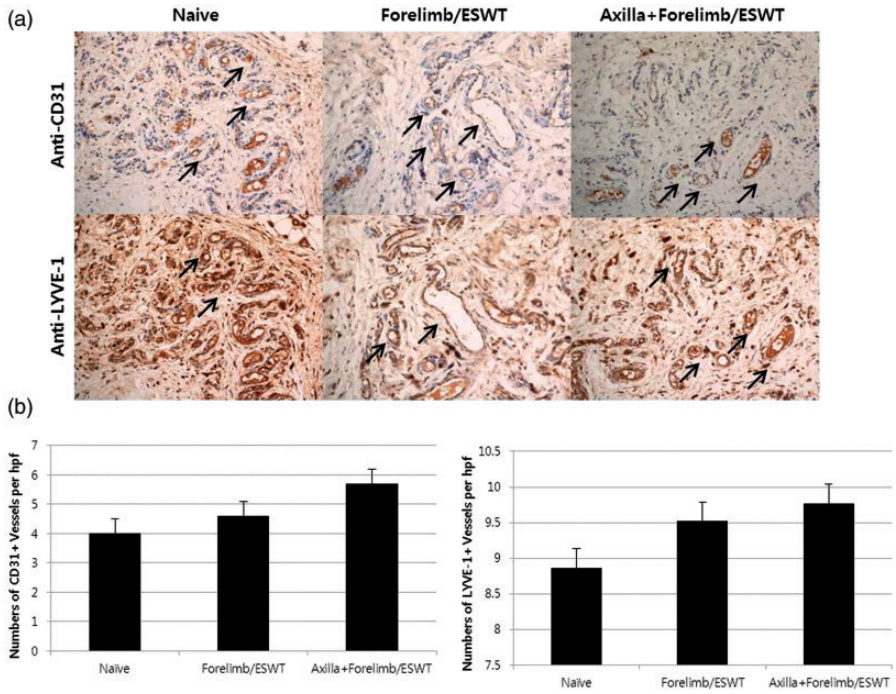

Figure 2. Immunohistochemical analysis of CD31 and LYVE-1 expression in the forelimbs of the rats 14 days after the final ESWT application. (a) Immunohistochemical staining (brown) for CD 31 and LYVE-1 in rats with lymphedema that underwent ESWT only in lymphedematous forelimb (Forelimb/ESWT) or in both the axillary area and forelimb (Axilla+Forelimb/ESWT), in the non-edematous contralateral forelimbs (naïve). The vessels are marked by arrows. (b) Quantification of the numbers of CD31-positive and LYVE-1-positive vessels in each group. The numbers of each were recorded in 10 consecutive high-power fields (hpf) at ×400 magnification. Data are mean ± SEM. The Kruskal–Wallis test was used to compare the groups. CD31, cluster of differentiation 31; LYVE-1, lymphatic endothelial hyaluronan receptor 1; ESWT, extracorporeal shock-wave therapy.

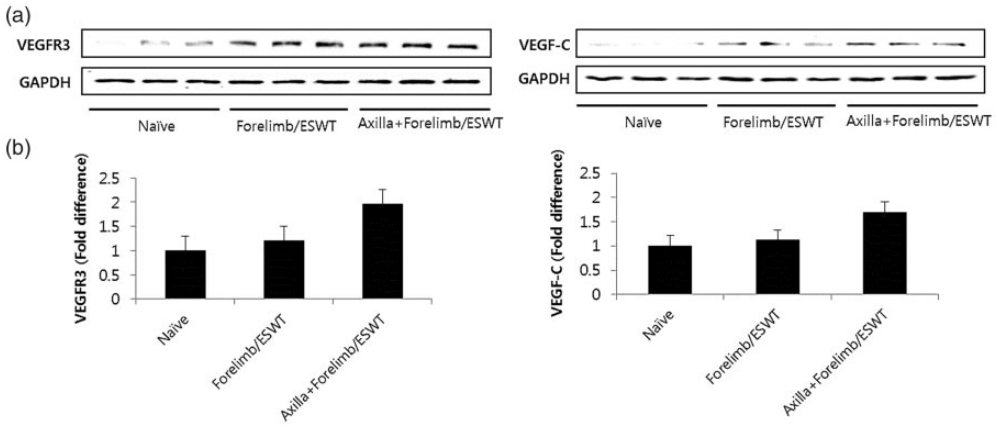

Figure 3. Expression of VEGFR3 and VEGF-C proteins in the forelimbs of the rats 14 days after the last ESWT application. Rats were subjected to ESWT in their lymphedematous forelimb (Forelimb/ESWT) or in the axillary area as well as the lymphedematous forelimb (Axilla+Forelimb/ESWT) and the contralateral forelimbs were non-edematous and untreated (Naïve). (a) Protein levels of VEGFR3 and VEGF-C in the skin of each forelimb were measured by western blotting. A representative blot is shown. (b) Relative fold differences are presented (mean ± SEM) and the groups were compared using the Kruskal–Wallis test. VEGFR3, vascular endothelial growth factor receptor-3; VEGF-C, vascular endothelial growth factor C; ESWT, extracorporeal shock-wave therapy.

significance. VEGF-C expression was 1.12-fold higher in the Forelimb/ESWT and 1.69-fold higher in the Axilla+Forelimb/ESWT group than in the contralateral control forelimbs, but the difference between the two was not significant (Figure 3).

Discussion

In the present study, we compared the efficacy of two ESWT protocols for the treatment of forelimb lymphedema in rats, induced by axillary lymph node dissection. We found that ESWT application in the axillary area as well as in the edematous forelimb tended to lead to higher expression of VEGFR3 in this rat model of secondary lymphedema.

Lymphedema is a manifestation of lymphatic system insufficiency that is characterized by the accumulation of protein-rich interstitial fluid in tissues. Secondary lymphedema commonly develops as a complication of cancer surgery or radiotherapy,[5,6] but the options for the management of lymphedema remain limited, despite recent technical advances in surgery and radiotherapy.

Interestingly, ESWT has been shown to be an effective means of treating lymphedema in some previous studies.[19,20] Bae and Kim assessed the efficacy of ESWT in seven patients with breast cancer-related lymphedema, and found that it reduced the circumference of the arms with lymphedema.[20] Cebicci *et al.*[19] found that ESWT caused a significant reduction in lymphedema in all the patients assessed, and this reduction was maintained for 6 months. Furthermore, animal studies have shown the effectiveness of low-energy ESWT with respect to angiogenesis and lymphangiogenesis, and the expression of related genes.[14,16,17,21] It has been demonstrated that the biologic effects of low-energy ESWT are mediated through mechanical forces, such as cavitation (sudden μm-sized collapse of bubbles) and

shear stress.[12,13] These mechanical forces increase the permeability of cell membranes and lead to the induction of growth factor expression. Human and animal studies have shown that low-energy ESWT promotes angiogenesis, reduces neutrophil count and inflammation, and also reduces the number of adipocytes in the affected region.[14,17,19,20] These findings are consistent with those of the present study, in which significant decreases in the circumferences of the lymphedematous forelimb at the level of the carpal joint and 2.5 cm proximal were induced by ESWT, and these may be the results of lymphangiogenesis and an improvement in lymphatic drainage. Furthermore, we have also shown a tendency for the expression of a regulator of lymphangiogenesis, VEGFR3, to increase after ESWT application.

Many ESWT protocols have been suggested and many animal models of lymphedema have been used in previous studies. Several ESWT protocols aimed at reducing lymphedema are currently being tested in animal models, including rabbit ear and rat or mouse tail models.[14,22–26] However, unlike in these previous studies, we used a rat model of axillary lymph node dissection-induced secondary lymphedema.[26] One of the most clinically important types of secondary lymphedema occurs in the upper extremity as a complication of breast cancer. Patients with breast cancer are at a higher risk of lymphedema after axillary lymph node dissection and radiotherapy, and the present model of secondary lymphedema, which involves an obstruction of lymphatic drainage, mirrors this. We used this model to identify an effective ESWT protocol by comparing the therapeutic efficacy of ESWT targeting the lymphedematous area and ESWT targeting both this area and the axillary lymph node dissection area. To this end, we applied 500 shocks only to the lymphedematous forelimb or 200 shocks to the lymphedematous forelimb and 300 shocks to the axillary area. We found that both protocols led to a reduction in swelling of the forelimb, but the numbers of LYVE-1-positive vessels and CD31-positive vessels did not significantly differ between the two groups. The expression of VEGFR3 tended to be slightly higher in the Axilla+Forelimb/ESWT group, but this difference was not statistically significant. Several previous studies have demonstrated that overexpression of VEGFR3 and its ligand VEGF-C causes an increase in the density of lymphatic capillaries, which improves lymphatic function.[28–30] Although we had hypothesized that there would be a synergistic effect of ESWT applied to both the axillary lymph node dissection area and the lymphedematous area, our findings did not support this. However, given the importance of VEGF-C/VEGFR3 signaling in the lymphangiogenesis pathway, even the slightly higher VEGFR3 expression in the Axilla+Forelimb/ESWT group may imply that differing ESWT protocols have differential effects on lymphangiogenesis.

The present study had several limitations. First, it was conducted without performing a sample size calculation, and should therefore be regarded as a pilot study. The sample size may have been insufficient to obtain statistically significant results. Second, we used immunohistochemistry and western blot analysis to analyze protein levels in the Axilla+Forelimb/ESWT and Forelimb/ESWT groups, but did not compare the differences found in these two groups with the control group. Third, we did not evaluate the fibrosis associated with lymphedema and ESWT in the rats.

Conclusion

Although we did not identify a synergistic effect of ESWT when it was applied to both the lymphedematous and axillary areas, the results of this pilot study suggest that

angiogenesis and lymphangiogenesis may differ according to the ESWT protocol used for the treatment of lymphedema. Further larger studies are warranted to corroborate and extend these findings.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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