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Extracorporeal Shock Wave Therapy Induces Therapeutic Lymphangiogenesis in a Rat Model of Secondary Lymphoedema

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Abstract *Objective:* Lymphoedema is a common complication after cancer treatment. We have reported that low-energy extracorporeal shock wave (SW) therapy up-regulates vascular endothelial growth factor (VEGF) in ischaemic myocardium. As VEGF plays an important role in lymphangiogenesis, we investigated whether our low-energy SW therapy enhances lymphangiogenesis in rats.
Methods: We created a tail model of lymphoedema in rats. The tail was treated with or without low-energy SW therapy (0.25 mJ mm^{-2}, 500 impulses) four times (days 3, 5, 7, and 9). The tail volume and the fluorescence intensity of indocyanine green (ICG) were measured. The expression of VEGF-C and basic fibroblast growth factor (bFGF) were evaluated by RT-PCR, and the lymphatic vessel density was assessed histochemically.
Results: The tail volume increased significantly in the control group and was significantly improved in the SW group. The lymphatic system function (evaluated with fluorescence intensity of ICG), the lymphatic vessel density, and the expression of VEGF-C and bFGF were all enhanced by the SW therapy (all $P < 0.05$).
Conclusions: The low-energy SW therapy induces therapeutic lymphangiogenesis by up-regulating VEGF-C and bFGF, and improves lymphoedema in a rat-tail model, suggesting that low-energy SW therapy could be a non-invasive and effective strategy for lymphoedema in humans.

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Lymphoedema occurs as a result of an imbalance between the demand for lymphatic flow and the capacity of the lymphatic circulation.[1] It is characterised by the regional accumulation of excess amounts of interstitial protein-rich fluid. Lymphoedema is a slowly progressive, usually painless swelling of the extremities. Primary lymphoedema is caused by developmental abnormalities of the lymphatic vessels. Secondary lymphoedema is the result of acquired obstruction of the lymphatic vessels and lymph nodes. Secondary lymphoedema is a major complication after surgery or radiation treatment for cancer, and the number of patients affected is increasing.[2,3] The standard treatments for lymphoedema are compression and manual drainage, which merely mitigate the symptoms. Therefore, new and effective therapies remain to be developed. Reconstructing the lymphatic circulation system is one promising strategy for lymphoedema.

We have previously demonstrated that low-energy extracorporeal shock wave (SW) therapy effectively induces therapeutic angiogenesis and improves myocardial ischaemia in pigs and humans as well as hindlimb ischaemia in rabbits, through up-regulation of vascular endothelial growth factor (VEGF).[4–8] VEGF-C plays an important and essential role in lymphangiogenesis.[9,10] Basic fibroblast growth factor (bFGF) can also induce lymphangiogenesis,[11,12] and its effect is mediated via VEGF-C and -D.[13] In the present study, we examined whether low-energy SW therapy improves lymphoedema in a rat model of tail lymphoedema and if so, whether VEGF and bFGF are involved.

Materials and Methods

Animals

Male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan) weighing 200–250 g were used ($n = 90$). The animals were cared for in accordance with the principles and guidelines of the Japanese Ministry of the Environment. The protocols of the present study were approved by the ethics committee on animal experiments of Tohoku University (no. 22-303).

Secondary lymphoedema model in the rat

On day 1, we performed surgery to cause secondary lymphoedema in rat tails. The rat-tail model of secondary lymphoedema has been described previously.[14,15] Anaesthesia was induced with diethyl ether and maintained with the intra-peritoneal injection of pentobarbital (30 mg kg^{-1}) during surgery. Two parallel circumferential incisions were made 5 mm apart through the dermis, close to the tail base. The skin band and subcutaneous tissues, including lymphatic vessels, were removed completely. Lymphatic vessels were identified with the subcutaneous injection of 0.5% Evans blue dye. The major underlying blood vessels and tendon were preserved to prevent the distal tail from becoming necrotic. Both skin edges were cauterised with a radio knife for haemostasis and to delay wound closure. Postoperatively, the animals were randomly divided into the control and the SW groups. First, we treated 30 rats to measure tail volume; 15 for the control group and the remaining 15 for the SW group. On day 25, all animals were euthanised. Among them, 12 (six from each group) were used for RT-PCR analysis and another 12 for histochemical analysis; the remaining six were used for blood test. Second, we treated 48 rats (24 in each group) and euthanised 24 rats (12 from each group) on day 7 and 15, respectively. Among them, 12 (six from each group) were used for RT-PCR analysis and another 12 for histochemical analysis. Third, we treated additional 12 (six from each group) to evaluate lymphatic system function using indocyanine green (ICG) and an infrared camera. Thus, we used a total of 90 rats in this study.

Extracorporeal SW therapy

One SW treatment consisted of 0.25 mJ mm^{-2} (total energy flux density), 500 impulses, using a SW generator (DUOLITH® SD1; Storz Medical, Switzerland) based on our previous studies, in which maximal up-regulation of VEGF expression was achieved at ~0.1 mJ mm^{-2} (positive energy flux density).[4–8] According to the manufacturer, 0.1 mJ mm^{-2} (positive energy flux density) is equivalent to 0.25 mJ mm^{-2} (total energy flux density). Animals in the SW group received low-energy SW therapy to the surgical site four times (postoperative days 3, 5, 7 and 9), whereas those in the control group received the same procedures but without the SW treatment.

Tail volume measurement

Tail volume was measured using water displacement volumetry every 3 days ($n = 15$ per group).[16]

Evaluation of lymphatic system function

Lymphatic system function was evaluated by the ICG method ($n = 6$ per group). Briefly, we injected 0.1 mg of ICG (Daiichi Sankyo, Japan) subcutaneously in the end of the tail on day 1. As the injected ICG was absorbed in the lymphatic ducts and transported from the tail to the body, the fluorescence intensity gradually decreased. We evaluated the drainage function of lymphatic fluid by measuring the average fluorescence intensity with an infrared camera (PDE System® C9830; Hamamatsu Photonics, Japan) at the distal area of the surgical site every 2 days. The camera was fixed at 20 cm from the tail, and the measured area was 4.4 × 20 mm.

RT-PCR analysis

All tissues except bone were harvested from the surgical site on days 7, 15 or 25 ($n = 6$ per group). The samples were homogenised and used for total RNA extraction with a TRIzol® Plus RNA purification kit (Life Technologies Japan, Japan). RNA concentrations were determined using GeneQuant Pro® (Biochrome, UK). Reverse transcriptase M-MLV® (2640A, Takara Bio, Japan), three gene-specific primer pairs (Sigma–Aldrich Japan), and a LightCycler 2.0® (Roche Diagnostics, Tokyo, Japan) were used for PCR. The primers were as follows (5′ → 3′): for VEGF-C (286-bp fragment), GCCAATCACACT TCCTGCCG (sense) and CTGGCAGGTGTCTTCATCCAAC (antisense); for bFGF (225-bp fragment), CCAGTTGGTATGT GGCACTG (sense) and CAGGGAAGGGTTTGACAAGA (antisense); and for β-actin (612-bp fragment), ATATCGCTGC

GCTCGTC (sense) and TTTCCCTCTCAGCTGTGGT (anti-sense). The PCR conditions for VEGF-C were 40 cycles of 1 min at 94 °C, 90 s at 55 °C, and 90 s at 72 °C. The PCR conditions for bFGF were 40 cycles of 30 s at 94 °C, 60 s at 56 °C, and 105 s at 72 °C. The expression levels of the two genes were compared between the SW and control groups. Values are reported as the quotients of the copy number of the gene of interest relative to that of b-actin, as a housekeeping gene (VEGF-C/b-actin or bFGF/b-actin). The PCR reaction mixtures (20 μl) were separated electrophoretically in 2% agarose gels containing ethidium bromide, observed, and photographed under ultraviolet light.

Histochemical examination

The surgical site was excised, including 1 cm on each side on days 7, 15 or 25 ($n = 6$ per group). The samples were fixed with formalin, embedded in paraffin, and divided into two parts for staining: one with haematoxylin and eosin (HE) and the other with D2-40 (code: 413451, mouse monoclonal; Nichirei, Japan), an antibody against a lymphatic-specific marker.[17] We measured the thickness of the dermis and subcutaneous tissue just distal to the surgical site in the HE-stained samples (original magnification, ×200), and used the D2-40-stained samples to assess lymphatic vessel density (original magnification, ×400). The number of D2-40-positive vessels was counted in randomly selected microscopic fields, and the results are expressed as the number of D2-40-positive vessels/field. The observer counting the lymphatic vessels was blinded to treatment allocation of the rats. All histochemical examinations were performed with a BX51® microscope (Olympus, Japan).

Statistical analyses

Statistical analyses were performed with the unpaired *t*-test using StatMate 4. The results are expressed as means ± standard deviations (SDs). Differences were considered statistically significant at $P < 0.05$.

Results

Tail volume

Until day 4, the tail volume increased similarly in both groups. After day 4, the tail volume further increased in the control group, whereas it decreased in the SW group (Figs. 1 and 2). A significant difference in the tail volume was observed between the two groups from days 7–19 (day 10: 7.0 ± 0.8 vs. 8.4 ± 0.4 ml; day 19: 7.0 ± 0.6 vs. 8.2 ± 0.2 ml, both $P < 0.05$).

Lymphatic system function

The average fluorescence intensity value was significantly lower in the SW group than in the control group ($P < 0.05$) on days 7, 13, 23, and 25 (Fig. 3). These data indicate that the drainage of lymphatic fluid was enhanced by the SW therapy.

RT-PCR analysis

VEGF-C expression was significantly up-regulated in the SW group compared with the control group on days 7 and 15 (day 7: 1.52 ± 0.47 vs. 0.83 ± 0.14; day 15: 1.03 ± 0.02 vs. 0.54 ± 0.01, both $P < 0.05$), as was bFGF expression (day 7: 0.41 ± 0.21 vs. 0.15 ± 0.03; day 15: 1.01 ± 0.27 vs. 0.59 ± 0.05, both $P < 0.05$) (Fig. 4).

Histochemical examination

In the HE-stained histological specimens, the dermis and subcutaneous tissue of the control group were significantly swollen, as compared with the SW group, on days 7 and 15 (day 7: 408 ± 74 vs. 555 ± 45 mm; day 15: 371 ± 67 vs. 536 ± 60 mm, both $P < 0.05$) (Fig. 5). Upon immunostaining with D2-40, a specific marker of lymphatic vessels, newly formed lymphatic vessels were readily visualised in the subcutaneous tissue in the SW group. On days 15 and 25, the number of lymphatic vessels was significantly higher in the SW group than

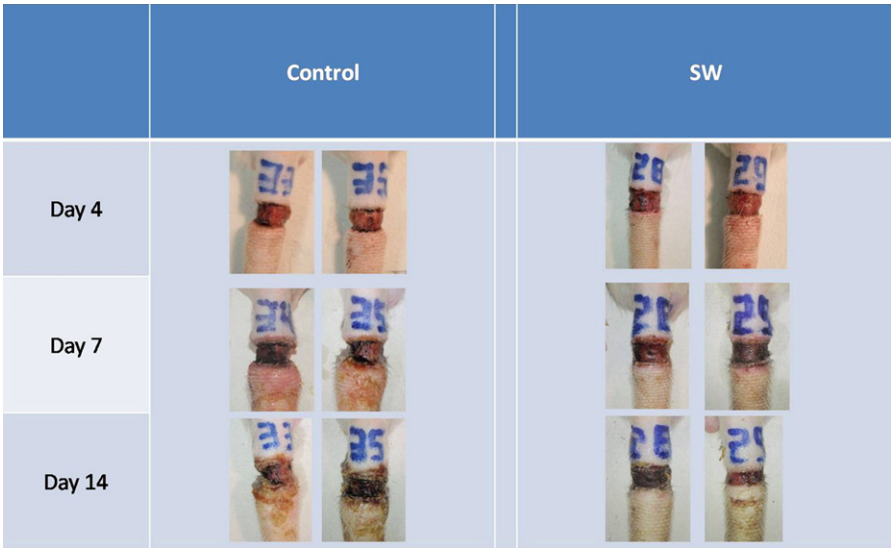

Figure 1 Representative photographs of rat tails. On postoperative day 7, severe oedema and skin redness were observed in the control group whereas the edema in the SW group was modest.

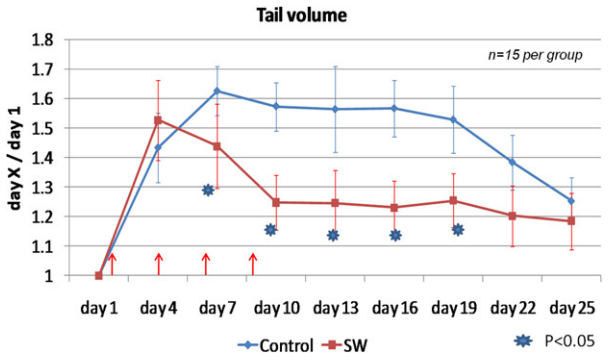

Figure 2 Time course of tail volume. The SW therapy suppressed lymphedema. Up to day 4, lymphedema developed to a similar extent in both groups. On day 7, the tail volume had further increased in the controls, whereas it had started to decrease in the SW group. The difference in tail volume between the two groups was statistically significant from days 7–19.

in the control group (day 15: 4.2 ± 0.5 vs. 2.1 ± 0.7 per field; day 25: 4.8 ± 0.7 vs. 3.2 ± 0.8 per field, both $P < 0.05$) (Fig. 6).

Biochemical analysis

No significant differences were observed between two groups (total protein: 5.8 ± 0.12 vs. 5.8 ± 0.16; albumin 2.4 ± 0.04 vs. 2.3 ± 0.08). All results were shown in Table 1.

Discussion

The novel finding of the present study was that low-energy SW therapy induced effective therapeutic lymphangiogenesis in a rat model of secondary lymphoedema, and which was accompanied by the up-regulation of VEGF-C and bFGF.

Beneficial effects of SW therapy on lymphoedema

In the present study, the increased tail volume decreased significantly in the SW group with a significant increase in the number of D2-40-positive vessels at the site of surgery as compared with the control group. These results indicate that low-energy SW therapy enhanced lymphangiogenesis, thus reducing the lymphoedema. We also examined the removal rate of injected ICG from the distal part of the surgical site by measuring its fluorescence intensity with an infrared camera. Until day 5, the fluorescence intensity decreased similarly in both groups. The fluorescence intensity was significantly lower in the SW group than in the control group during the experimental period following SW therapy. These results indicate that low-energy SW therapy enhanced the drainage of lymphatic fluid. The fluorescence intensity in the control group also decreased between days 3 and 5 without any treatment, even though most of the superficial lymphatic system was removed by the surgery. Thus, it is possible that some ICG drained through the deep lymphatic vessels or diffused through deep tissues.

Mechanisms for the beneficial effects of SW therapy on lymphoedema

VEGF-C and bFGF are important factors for lymphangiogenesis.[9–12,18] VEGF-C gene transfer reduces lymphoedema in several animal models.[9,10,16] In the present study, the expression of VEGF-C and bFGF was enhanced significantly at the surgical site, where low-energy SW therapy was applied, suggesting that VEGF-C and bFGF were up-regulated by the low-energy SW therapy with a resultant therapeutic lymphangiogenesis. Recently, Kubo et al. also reported that low-energy SW therapy induced lymphangiogenesis and ameliorated secondary lymphoedema in rabbit-ear model.[19] They

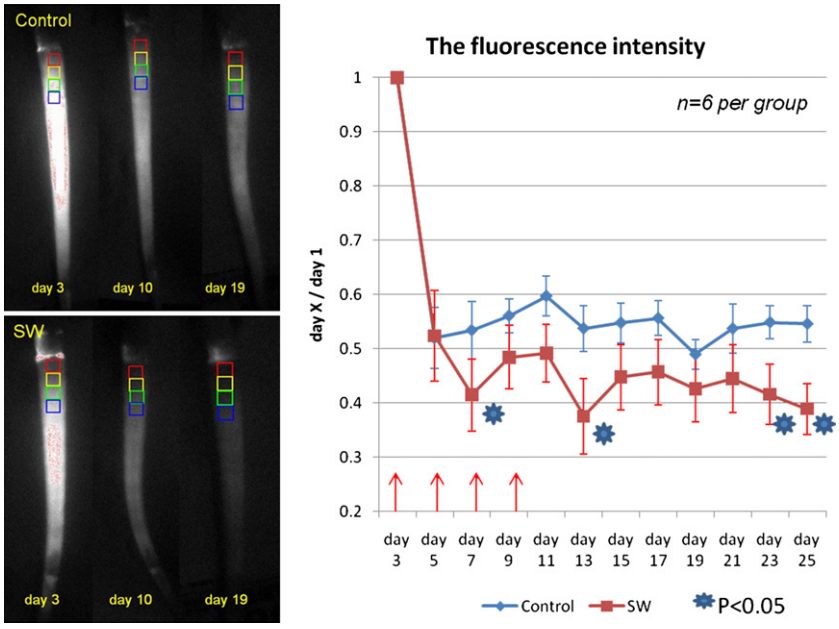

Figure 3 Representative images of fluorescence intensity measurement (right) and the average fluorescence intensity (left). In the SW group, the average fluorescence intensity was lower than in the controls. The red arrows indicate the SW therapy.

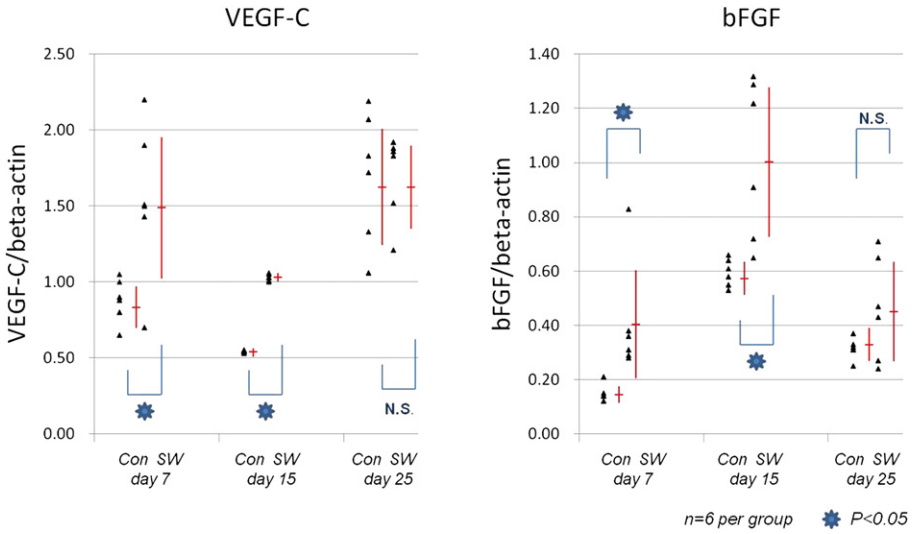

Figure 4 Expression of VEGF-C and bFGF in a surgical site specimen. In the SW group, the expression of VEGF-C and bFGF was significantly enhanced on days 7 and 15, as compared with the control group.

evaluated the effects of SW therapy on skin thickness, expression of VEGF-C and VEGFR3, and lymphatic duct count. In both their and our studies, SW therapy reduced the increased skin thickness, up-regulated the expression of VEGF-C, and increased the lymphatic duct count. These results suggest that the SW therapy enhances up-regulation of VEGF-C leading to lymphangiogenesis. It is possible that the mechanical stress caused by the low-energy SW, such as cavitation (the formation of vapour bubbles in a flowing liquid) and shear stress,[20,21] induced VEGF-C and bFGF up-regulation. Further investigation is needed to clarify the detailed mechanisms of the beneficial effects of low-energy SW therapy.

Animal models of secondary lymphoedema

Several animal models of secondary lymphoedema have been reported, including rabbit-ear- and rat- or mouse-tail models.[14–16,19,22,23] In the present study, we used the rat-tail model because it allows precise measurements of the compartment volume. Pathologically, secondary lymphoedema results from a decreased transport capacity of the lymphatic system due to acquired lymphatic vessel or lymph node obstruction. The result of this condition is the stagnation of lymphatic fluid in the affected compartment, which is directly reflected in the compartment volume. Therefore, tail volume measurement would be superior to assessing skin thickness in evaluating the severity of lymphoedema. In fact, a heterogeneous distribution of lymphoedema in the same compartment is often observed in humans.[24]

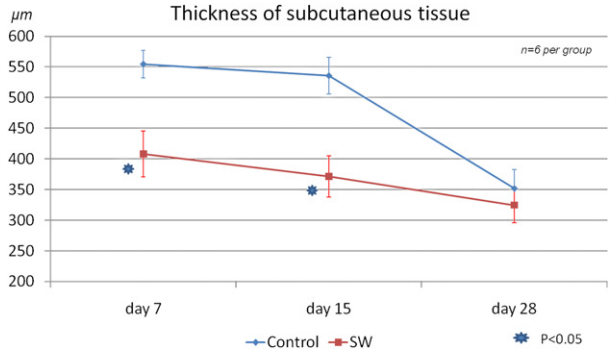

Figure 5 Time course of subcutaneous tissue thickness. The SW therapy reduced the lymphedema compared with the control group.

Limitations

Several limitations of the present study should be mentioned. First, lymphoedema in the present rat-tail model might be different from that in humans. Rats have a powerful healing ability, and lymphoedema in the rat tail heals almost completely even with no treatment. By contrast, secondary lymphoedema in humans is always slowly progressive and does not recover naturally. No exact animal model of chronic secondary lymphoedema in humans is available. Nevertheless, the results of this study suggest that low-energy SW therapy may be effective, at least, in the acute phase of secondary lymphoedema in humans. Second, the optimal therapeutic condition is not clear. Low-energy SW therapy has been used to treat a variety of diseases, such as ischaemic heart disease, hindlimb ischaemia, and orthopaedic disorders; however, the doses and number of applications vary among studies (0.037–0.62 mJ mm^{-2}, 100–12 000 impulses).[4–8,19,25–29] Regarding the SW level, we have previously demonstrated that VEGF expression peaks at ~0.1 mJ mm^{-2} (positive energy flux density),[4,6] and according to the manufacturer, 0.1 mJ mm^{-2} (positive energy flux density) is equivalent to 0.25 mJ mm^{-2} (total energy flux density). Thus, in the present study, we employed 0.25 mJ mm^{-2}. Regarding the number of SW impulses, the most effective number of impulses is unknown for lymphoedema. However, in the clinical trial of the SW therapy for severe angina pectoris, satisfactory outcome was achieved with 4000–8000 impulses.[5,8] As the human heart (200–300 g) is about 15 times as heavy as the rat tail (15–20 g), we

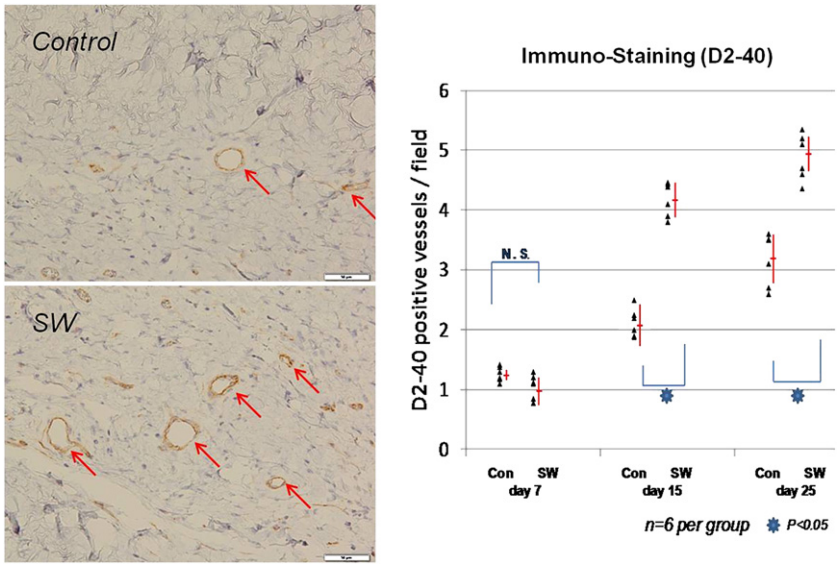

Figure 6 Time course of lymphatic vessel density. The number of D2-40-positive vessels (red arrow) per field was increased significantly in the SW group as compared with the control group on days 15 and 25. Bar, 50 μm.

expected that 500 impulses may be enough to induce lymphangiogenesis. Regarding the number of treatment series, when we started the present study, we were not sure how many series of SW therapy were most effective. However, on day 7 (after two series of SW therapy), the tail volume began to decrease and the difference between the SW and the control groups became significant. On day 10 (after four series of SW therapy), significant difference became more clear. Thus, we considered that four series of SW therapy are sufficient for the present study. Further studies are required to find the optimal condition for each disease. Third, the number of rats used in this study might have been small for a well-grounded conclusion. Fourth, we must consider the anti-inflammatory effects of low-energy SW therapy. Because low-energy SW therapy suppresses inflammation,[30] it is possible that low-energy SW therapy could reduce lymphoedema through an anti-inflammatory effect as well.

Clinical implications

Although patients with lymphoedema suffer from physical and psychological impairments,[31,32] the available treatment options are limited. Traditional compression treatments with a bandage or manual lymph drainage are not curative. Lymphatico-venous anastomosis is effective for lymphoedema,[33,34] but is invasive and requires a surgeon skilled in microsurgery. Thus, it is desirable to develop a safe, non-invasive treatment for lymphoedema. In this study, we demonstrated that low-energy SW therapy ameliorated lymphoedema by enhancing lymphangiogenesis in rats. Furthermore, no side effects were observed. Because of no need for anaesthesia or invasive procedures, low-energy SW therapy might be suitable even for patients with severe lymphoedema that required repeated therapy. However, we need further consideration before clinical use because the rat-tail model is not exactly fit for the lymphoedema in humans.

Table 1 No significant differences were observed between two groups.

	Control ($n = 3$)	SW group ($n = 3$)
Total protein (g/dl)	5.8 ± 0.12	5.8 ± 0.16
Albumin (g/dl)	2.4 ± 0.04	2.3 ± 0.08
AST (I.U/l)	82 ± 10.6	84 ± 9.2
ALT (I.U/l)	31 ± 3.1	28 ± 3.3
HGB (g/dl)	15.4 ± 0.66	15.8 ± 0.71
Ht (%)	44.2 ± 1.00	44.4 ± 1.06

Conclusions

We demonstrated that low-energy SW therapy enhanced lymphangiogenesis and improved secondary lymphoedema in rats, suggesting that the low-energy SW therapy may have a potential to be a safe and non-invasive strategy for treating lymphoedema in humans.

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Conflict of Interest

None.

Funding

None.

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