5. SW Therapy for Other Ischemic Disorders

Peripheral arterial disease (PAD) is often associated with cardiovascular diseases as a part of systemic atherosclerosis, and its associated morbidity is rapidly increasing worldwide.[43-45] Thus, we examined the effects of our SW therapy on hind-limb ischemia in rabbits.[46] Hind-limb ischemia was induced by surgical excision of the entire unilateral femoral artery. One week after the operation, we started the SW therapy (200 shots/spot at 0.09 mJ/mm^2) to the ischemic region three times a week for 3 weeks. Four weeks after the operation, blood flow, blood pressure, and capillary density were all significantly increased in the SW group compared with the control group.[46]

Based on favorable results in animal studies, we are conducting a clinical study in patients with PAD with intermittent claudication (Fontaine stage II) and those with critical limb ischemia (Fontaine stage III and IV). During the therapeutic procedure, patients lie in a prone position without any anesthesia. SWs are applied to the ischemic calf muscle three times a week for 3 consecutive weeks (200 shots/spot at 0.05 mJ/mm^2 for 40 spots). Walking ability and peripheral blood flow are evaluated at 4, 8, 12, and 24 weeks after the SW therapy.

Recently, the beneficial effects of low-energy SW therapy have also been reported in other ischemic disorders, including the skin flap model in rodents[47,48] and in patients with refractory chronic skin ulcers.[49,50] Also, low to high energy levels of SW are widely used for the treatment of certain orthopedic conditions, such as bone non-unions, tendinosis calcarea, epicondylitis, and calcaneal spur.[51,52] In the orthopedic field, SW therapy is reported to affect the expression of several chemokines and matrix metalloproteinases with resultant anti-inflammatory effects.[24,47,53,54] These findings suggest that multiple signaling pathways are involved in mediating the beneficial effects of the SW therapy.

6. Advantages of Extracorporeal Cardiac SW Therapy

A major advantage of our extracorporeal cardiac SW therapy is its non-invasive nature without any adverse effects. If necessary, we are able to repeatedly treat patients with SW therapy as no surgery or anesthesia is required for the treatment. This is an important factor in determining the clinical usefulness of angiogenic therapies, especially in elderly patients. The combination of cell therapy and SW therapy could be one potential approach. Indeed, enhanced expression of multiple angiogenic factors, such as VEGF and stromal-derived factor 1 (SDF-1), is crucial for the recruitment and incorporation of endothelial progenitor cells (EPCs).[55-61] Also, it was reported that the activation of the SDF-1/CXCR4 axis is essential for the retention of pro-angiogenic stem cells in peripheral organs, although the up-regulation of VEGF is sufficient to mobilize stem or progenitor cells from the bone marrow to the systemic circulation.[56,60] Thus, it is possible that SW therapy enhances the incorporation of circulating EPCs by up-regulating the expression of SDF-1 in ischemic myocardium. This notion has been supported by a recent report showing that the addition of SW therapy enhances the effectiveness of cell-based angiogenic therapy.[62] In this study, low-energy SW therapy was employed to treat hind-limb ischemia in rats in combination with cell-based therapy, where the expression of stromal-derived factor 1 (SDF-1) and recruitment of endothelial progenitor cells by SW therapy were enhanced.[62] In addition, it has been recently reported that the beneficial effects of cell therapy were enhanced by pretreating BMDMNCs with SW before implantation into the infarct area.[63]

7. Conclusions

Extracorporeal low-energy SW therapy appears to be an effective, safe, and non-invasive approach to ischemic heart disease, and its use could be extended to a variety of other ischemic disorders in the near future. The beneficial effects of SW may be mediated by the enhancement of several intrinsic angiogenic systems, although the precise mechanisms remain to be elucidated.

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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

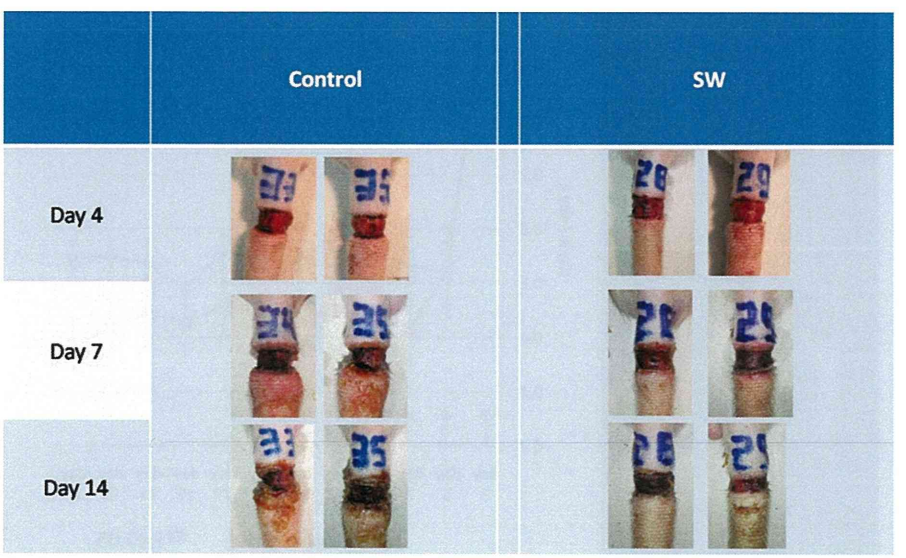

Figure 1 Representative photographs of rat tails. On postoperative day 7, severe oedema and skin redness were observed in the control group whereas the edema in the SW group was modest.

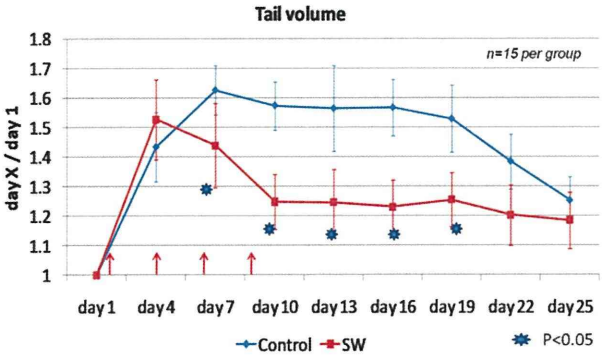

Figure 2 Time course of tail volume. The SW therapy suppressed lymphedema. Up to day 4, lymphedema developed to a similar extent in both groups. On day 7, the tail volume had further increased in the controls, whereas it had started to decrease in the SW group. The difference in tail volume between the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

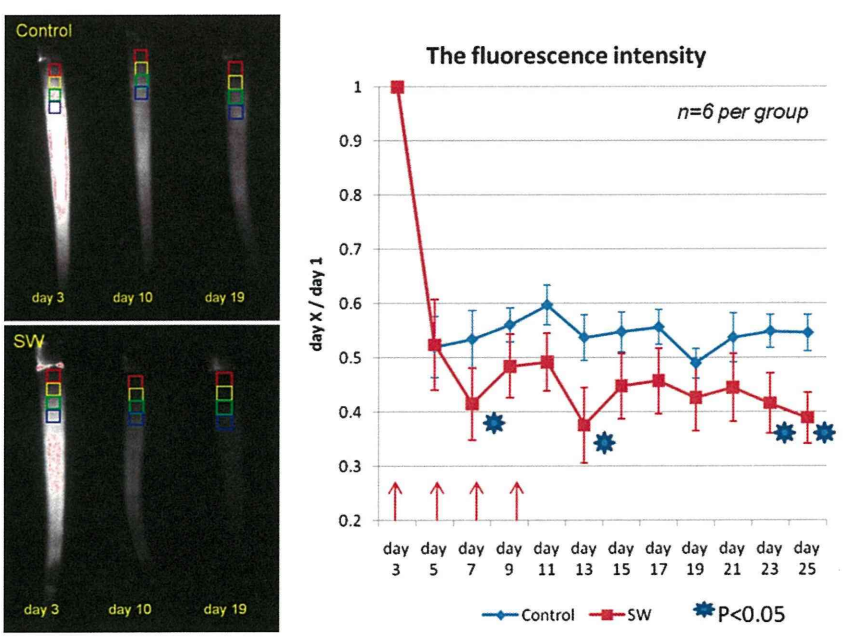

Figure 3 Representative images of fluorescence intensity measurement (right) and the average fluorescence intensity (left). In the SW group, the average fluorescence intensity was lower than in the controls. The red arrows indicate the SW therapy.

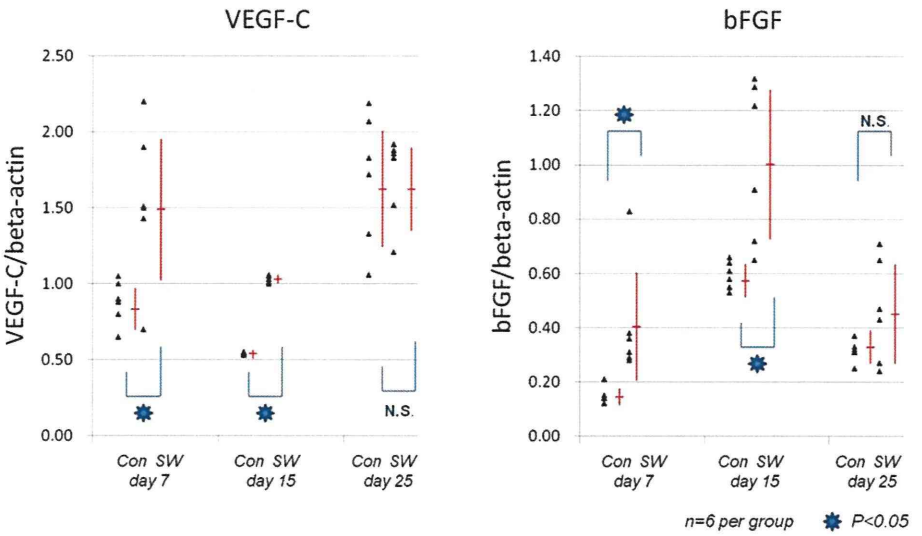

Figure 4 Expression of VEGF-C and bFGF in a surgical site specimen. In the SW group, the expression of VEGF-C and bFGF was significantly enhanced on days 7 and 15, as compared with the control group.

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]

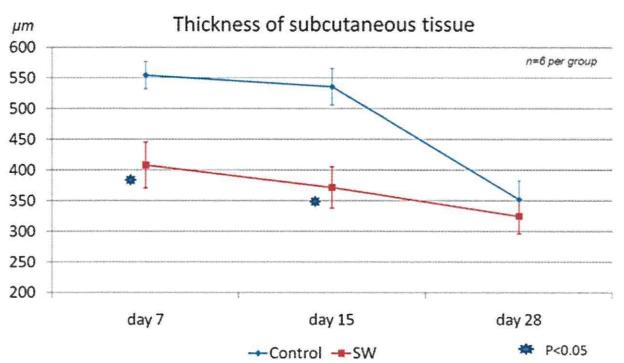

Figure 5 Time course of subcutaneous tissue thickness. The SW therapy reduced the lymphedema compared with the control group.

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

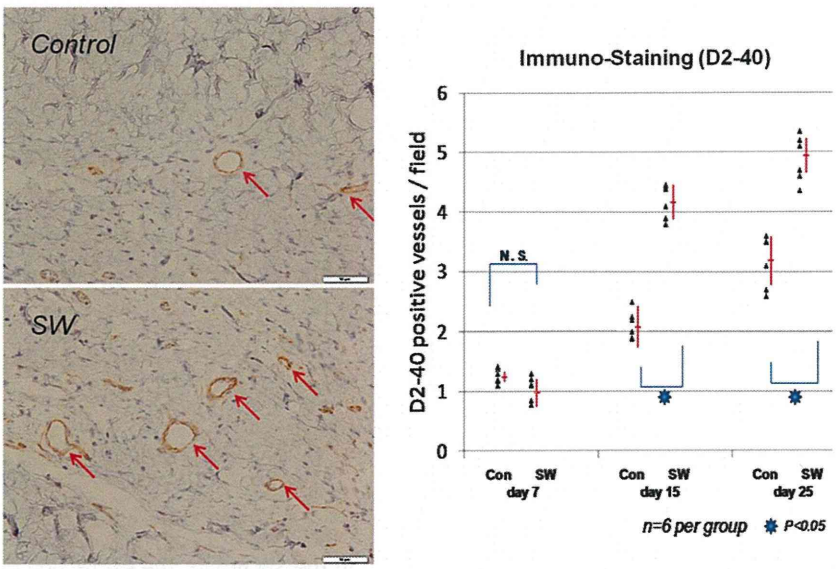

Figure 6 Time course of lymphatic vessel density. The number of D2-40-positive vessels (red arrow) per field was increased significantly in the SW group as compared with the control group on days 15 and 25. Bar, 50 μm.

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.

Acknowledgements

The authors are grateful to Dr. Ernest H. Marlinghaus, Storz Medical AG, Switzerland, for invaluable comments on our study.

Conflict of Interest

None.

Funding

None.

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Extracorporeal Shock Wave Therapy Improves the Walking Ability of Patients With Peripheral Artery Disease and Intermittent Claudication

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Background: Despite the recent advances in bypass surgery and catheter interventional therapy for peripheral artery disease (PAD), the long-term outcome of revascularization therapy for infrapopliteal lesions remains unsatisfactory. We have previously demonstrated that low-energy extracorporeal shock wave (SW) therapy effectively induces neovascularization through upregulation of angiogenic factors and improves myocardial ischemia in pigs and humans and in hindlimb ischemia in rabbits. In this study, we thus examined whether our SW therapy also improves the walking ability of patients with PAD and intermittent claudication.

Methods and Results: We treated 12 patients (19 limbs) in Fontaine II stage (males/females, 10/2; 60–86 years old) with low-energy SW therapy to their ischemic calf muscle 3 times/week for 3 consecutive weeks. After 24 weeks, the pain and distance subscale scores of the walking impairment questionnaire were significantly improved (33±25 vs. 64±26, 27±16 vs. 64±23, respectively, both $P<0.01$). Maximum walking distance was also significantly improved at 4 weeks (151±37% from baseline, $P<0.01$) and was maintained at 24 weeks (180±74% from baseline, $P<0.01$). Moreover, the recovery time of the tissue oxygenation index in the calf muscle during a treadmill test, which reflects local O_2 supply, was significantly shortened (295±222 s vs. 146±137 s, $P<0.01$). Importantly, no adverse effects were noted.

Conclusions: Non-invasive SW therapy improves the walking ability of PAD patients.

Key Words: Angiogenesis; Ischemia; Peripheral artery disease; Shock wave therapy

Peripheral artery disease (PAD) is caused by arterial stenosis and/or occlusion in the lower extremities, mainly because of atherosclerosis, and is associated with poor prognosis.[1] The number of patients with PAD has been recently increasing worldwide. Reduced blood supply causes tissue ischemia and subsequent various symptoms depending on its severity, including intermittent claudication, limb coldness, rest pain, and tissue necrosis. These ischemic symptoms impair exercise capacity and quality of life, together with increased risk of cardiovascular disorders.[1,2] Therapeutic strategies for PAD are several, including medication, exercise, bypass surgery, and catheter intervention. Although long-term outcomes of bypass surgery and endovascular intervention for ilio-femoral artery are acceptable,[3-6] the long-term patency rate for infrapopliteal lesions remain low,[7,8] which often requires repeated invasive procedures for these patients. New, non-invasive therapeutic strategies remain to be developed.

Editorial p????

We have previously demonstrated that low-energy extracorporeal shock wave (SW) therapy effectively induces therapeutic angiogenesis and improves myocardial ischemia in pigs and humans, and in hindlimb ischemia in rabbits, through upregu-

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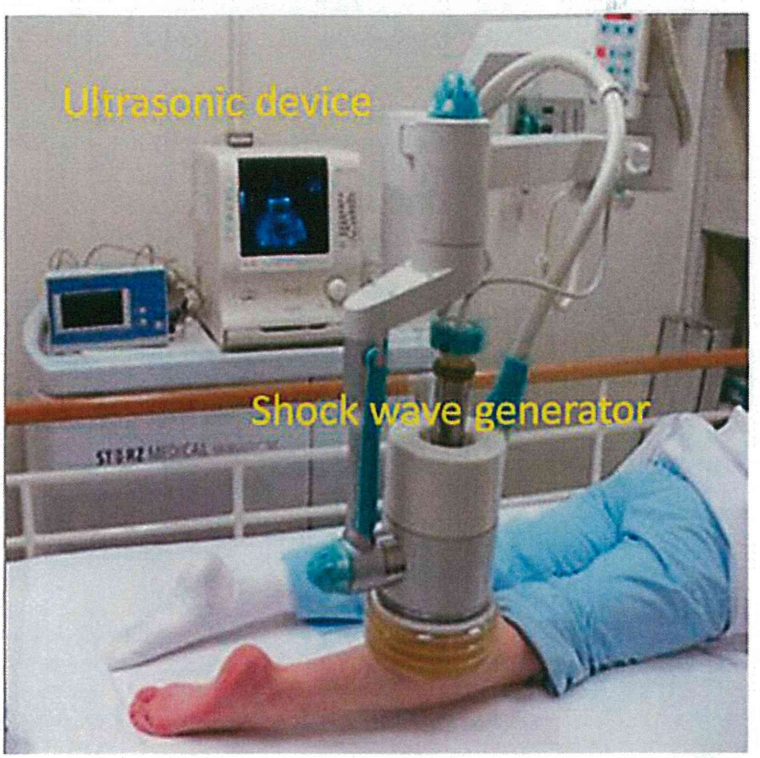


Figure 1. Shock wave therapy was performed with the guidance of an inline ultrasound device. Patients were awake and no invasive procedures were needed for this therapy.

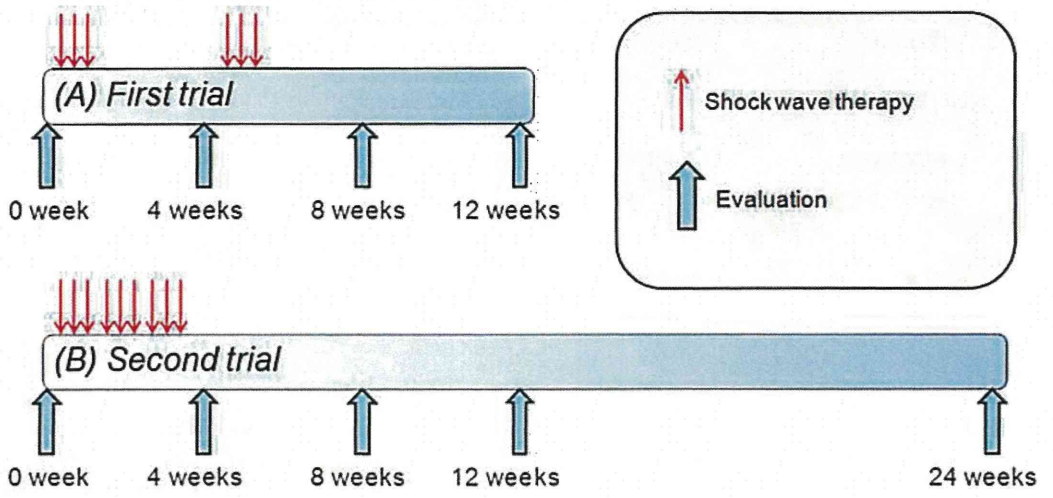


Figure 2. Treatment protocols. (**A**) First trial (pilot study) in which patients were treated in the 1st and 5th weeks. (**B**) Second trial in which patients were treated in 3 consecutive weeks.

lation of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS).[9–14] Because of its non-invasive nature, our low-energy SW therapy is applicable for both patients with complicating disorders and elderly patients, and can be repeated if needed.

In the present study, we thus examined whether our low-energy SW therapy also improves the walking ability of PAD patients with intermittent claudication.

Methods

Patients

We enrolled PAD patients who were classified as Fontaine II. In the first trial (a pilot study), we treated 6 patients from September 2007 to July 2008. After modifying the treatment protocol based on the results of the pilot study, we then treated another 12 patients in the second trial from September 2008 to

Table. Basic Characteristics of the Patients (Second Trial)

Age (years)	Sex	Primary disease	Site of lesion (CT findings)	Hypertension	Hyperlipidemia	Diabetes mellitus	Smoking history	Cilostazol	Sarpogrelate
77	M	PAD	F, IP	+	+	+	+	+	+
60	M	PAD	F, IP	+	+	+	+	−	+
75	M	BD	F, IP	−	−	−	+	−	+
86	M	PAD	I, F, IP	+	−	−	+	−	−
67	M	PAD	I, F, IP	+	+	+	+	+	−
75	M	PAD	F, IP	+	+	+	+	+	+
60	F	PN	I, IP	−	−	−	−	−	+
67	M	PAD	IP	+	−	−	+	+	−
67	M	PAD	I	−	+	−	+	+	−
70	F	PAD	I, F, IP	+	−	−	+	−	−
68	M	PAD	IP	+	−	−	+	+	−
84	M	PAD	F, IP	+	−	−	+	−	+

PAD, peripheral artery disease; Buerger, Buerger's disease; PN, polyarteritis nodosa; I, iliac region (from aortic bifurcation to external iliac artery); F, femoral region (common/superficial/deep femoral artery); IP, infra-popliteal region.

May 2011. Exclusion criteria were as follows: absence of PAD (ankle-brachial pressure index [ABI] >0.90 at rest), asymptomatic PAD, unstable coronary artery disease, current smoking, inability to perform treadmill test, active cancer, and dementia. Smoking history was obtained from the patient's self-report and non-smoking for more than 6 months was required to participate in the present study. Antiplatelet agents were administered for at least 1 year before enrolment and all antiplatelet agents were continued during the follow-up period. Both trials were approved by the ethical committees of Tohoku University, and written informed consent was given by each patient.

Low-Energy SW Therapy

Low-energy SW therapy was performed with a SW generator (Modulith® SLC, Storz Medical AG, Switzerland) (Figure 1). Based on our previous work, 1 SW session consisted of 200 shots in each of 40 sites on the ischemic calf muscle at 0.1 mJ/mm^2, approximately 10% of the energy level that is used for lithotripsy.[9–17] If the patient felt discomfort in the legs during the SW therapy, the energy level was reduced to a tolerable level and then the energy level was gradually increased.

First Trial (Pilot Study)

We treated 6 limbs in 6 patients (5 males/1 female, 67–82 years old; all patients had arteriosclerosis obliterans) with the low-energy SW therapy 3 times/week in the first (days 1, 3, 5) and fifth weeks (days 29, 31, 33) (Figure 2).

Second Trial

Based on the results of the pilot study, we modified the treatment protocol. In the second trial, the low-energy SW therapy was performed 3 times/week for 3 consecutive weeks (Figure 2). We treated 19 limbs in another 12 PAD patients (10 males/2 females, 60–86 years old), comprising arteriosclerosis obliterans in 10, Buerger's disease in 1 and polyarteritis nodosa in 1 (Table).

Evaluation of Walking Ability

Walking Impairment Questionnaire (WIQ) Subjective walking ability was evaluated in the second trial with a WIQ (Japanese version).[18] The patients answered the WIQ before and 4, 8, 12 and 24 weeks after the SW therapy.

ABI ABI was examined before and 4, 8, and 12 weeks after the SW therapy in the first trial, and before and 4, 8, 12, and 24 weeks after the SW therapy in the second trial. All measurements were performed with an ABI measurement device (VaSera VS-1500A®; Fukuda Denshi, Tokyo, Japan) at rest.

Maximum Walking Distance The treadmill test was performed under the condition of 2.4 km/h, 12% degrees in incline with treadmill device, and the maximum walking distance was measured (up to 400 m in 10 min). The maximum walking distance was evaluated before and 4, 8, and 12 weeks after the SW therapy in the first trial, and before and 4, 8, 12, and 24 weeks after the SW therapy in the second trial. Seven patients enrolled in the second trial had bilateral lesions and their maximum walking distance was measured in the more severely diseased leg.

Recovery Time of Tissue Oxygenation Index (TOI) TOI was measured with near-infrared spectroscopy (NIRO-200®; Hamamatsu Photonics, Japan) during the treadmill test in the second trial before and 4, 8, 12 and 24 weeks after the SW therapy (Figure 3).

TOI = oxygenated hemoglobin (O_2Hb)/concentration of hemoglobin (cHb)

The near-infrared spectroscopy probes were attached to the calf muscle, and the recovery time of TOI, which reflects local O_2 supply, was obtained.

CT Angiography CT angiography was performed to evaluate collateral vascular growth before and 12 weeks after the SW therapy in the first trial, and before and 24 weeks after the SW therapy in the second trial. The angiographic images were independently evaluated by 2 radiologists in a blinded manner.

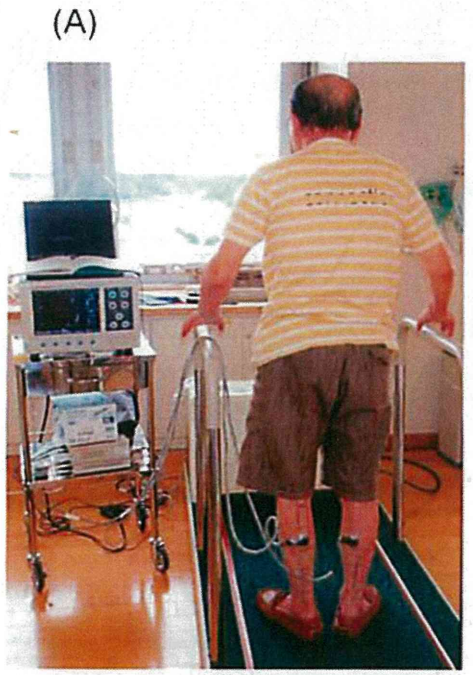
Statistical Analysis

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

Results

First Trial (Pilot Study)

There were no significant changes in ABI during the follow-up period in the first trial (data not shown). After the SW therapy, the maximum walking distance was significantly increased at 4 weeks (130±27% from baseline, $P<0.05$) and 8 weeks



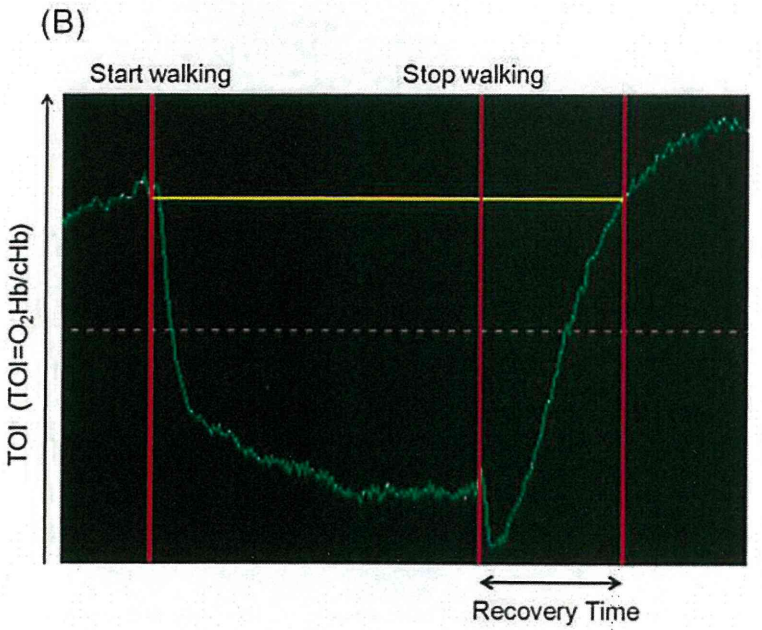


Figure 3. Treadmill test and near-infrared spectroscopy. (**A**) Treadmill test in a patient with peripheral artery disease. (**B**) The tissue oxygenation index (TOI) decreased after starting exercise and recovered to the pre-exercise level after stopping exercise.

(162±30% from baseline, P<0.05). However, the increased maximum walking distance was not sustained at 12 weeks in 3 of the 6 patients (146±63% from baseline, P=0.14). Thus, we modified the treatment protocol following our previous study of hindlimb ischemia in a rabbit model.[11] No detectable change in collateral vessels was observed with CT angiography at 12 weeks after the SW therapy.

Second Trial

WIQ The pain and distance subscale scores were significantly increased at 8, 12 and 24 weeks (Figure 4). The stairs subscale score was increased only at 8 weeks and no significant change was observed in the speed subscale score (Figure 4).

ABI There were no significant changes in ABI during the follow-up period (baseline: 0.57±0.15, 4 weeks: 0.58±0.13, 8 weeks: 0.58±0.13, 12 weeks: 0.57±0.14, 24 weeks: 0.59±0.12, all P=NS).

Maximum Walking Distance After the SW therapy, the maximum walking distance was significantly increased at 4 weeks (151±37% from baseline, P<0.01), and was maintained at 8 weeks (161±56%, P<0.01), 12 weeks (171±75%, P<0.01) and 24 weeks (180±74%, P<0.01) (Figure 5). One patient

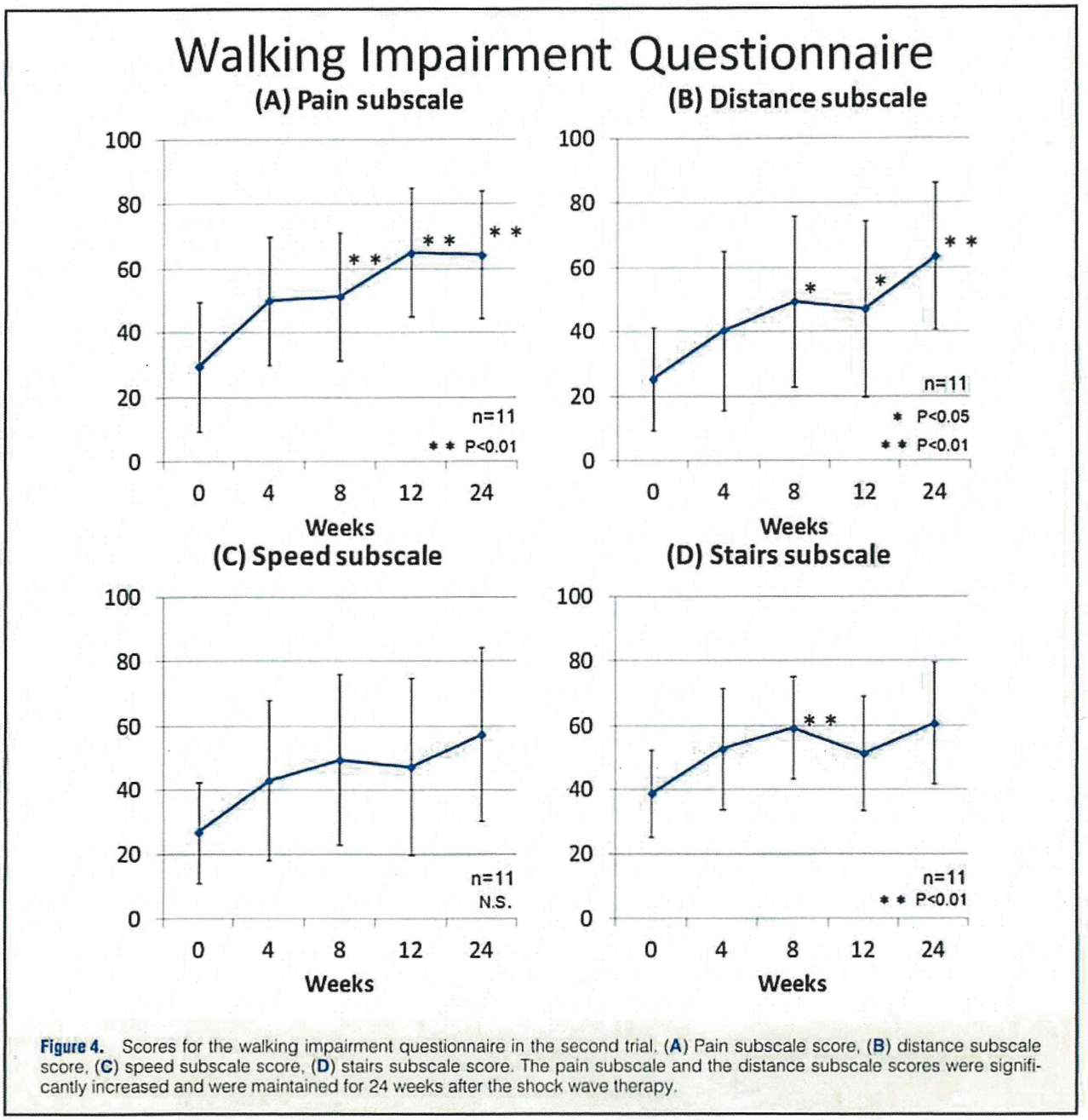


Figure 4. Scores for the walking impairment questionnaire in the second trial. (**A**) Pain subscale score, (**B**) distance subscale score, (**C**) speed subscale score, (**D**) stairs subscale score. The pain subscale and the distance subscale scores were significantly increased and were maintained for 24 weeks after the shock wave therapy.

failed to undergo the treadmill test at 4 weeks because of knee joint pain, and another patient at 24 weeks because of a respiratory disorder.

Recovery Time of TOI The recovery time of TOI was significantly shortened at 4, 8, 12 and 24 weeks compared with baseline (all P<0.01) (Figure 6).

CT Angiography No increase in visible collateral vessels was noted on the CT angiograms at 24 weeks after the SW therapy.

Discussion

In the present study, the low-energy SW therapy significantly improved symptoms, walking ability and peripheral perfusion without any adverse effects in PAD patients with intermittent claudication. To our knowledge, this is the first report to demonstrate the beneficial effects of low-energy SW therapy for PAD patients.

Treatment Protocol

In the first trial, although the maximum walking distance was increased at 4 weeks after the SW therapy, the beneficial effect was not sustained for a longer period. Thus, we modified the protocol by increasing the duration of the SW therapy in the second protocol, and we were able to confirm sustained beneficial effects of the therapy on the walking ability of PAD patients. In our previous studies, 1-week treatment (total 3 times) was enough for the treatment of myocardial ischemia in pigs and humans,[9,10,12-16] whereas the 3-week treatment (total 9 times) was required for the treatment of hindlimb ischemia

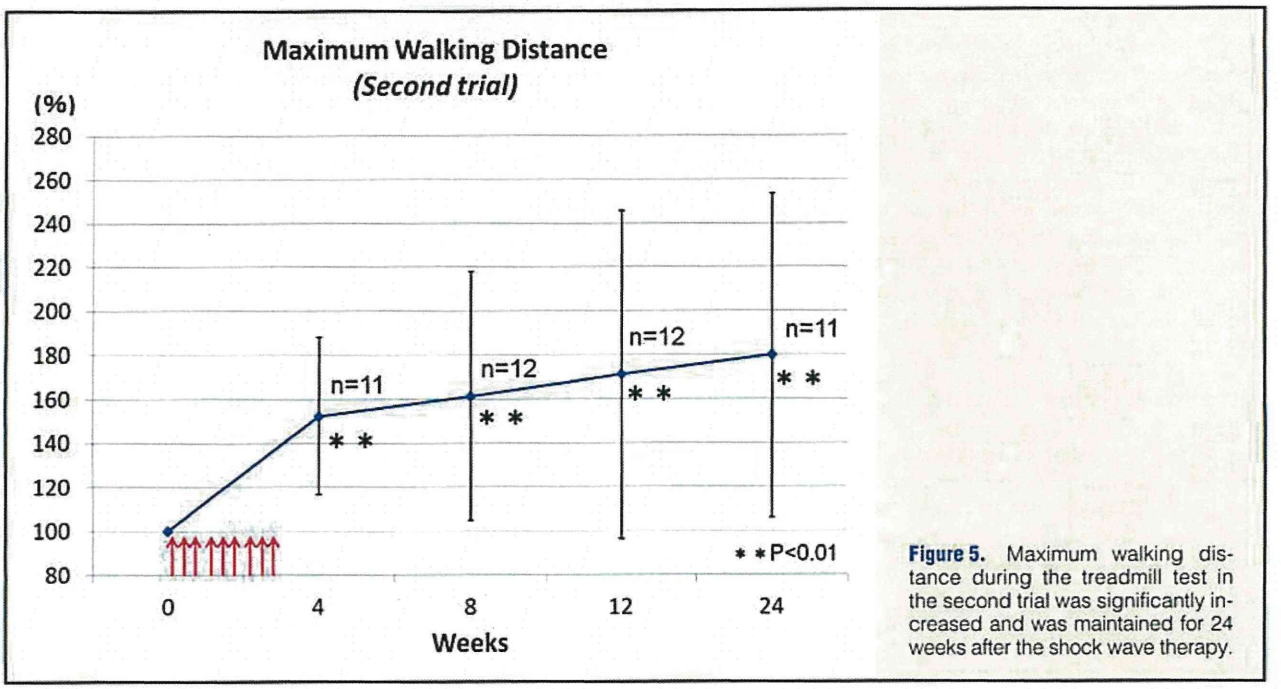


Figure 5. Maximum walking distance during the treadmill test in the second trial was significantly increased and was maintained for 24 weeks after the shock wave therapy.

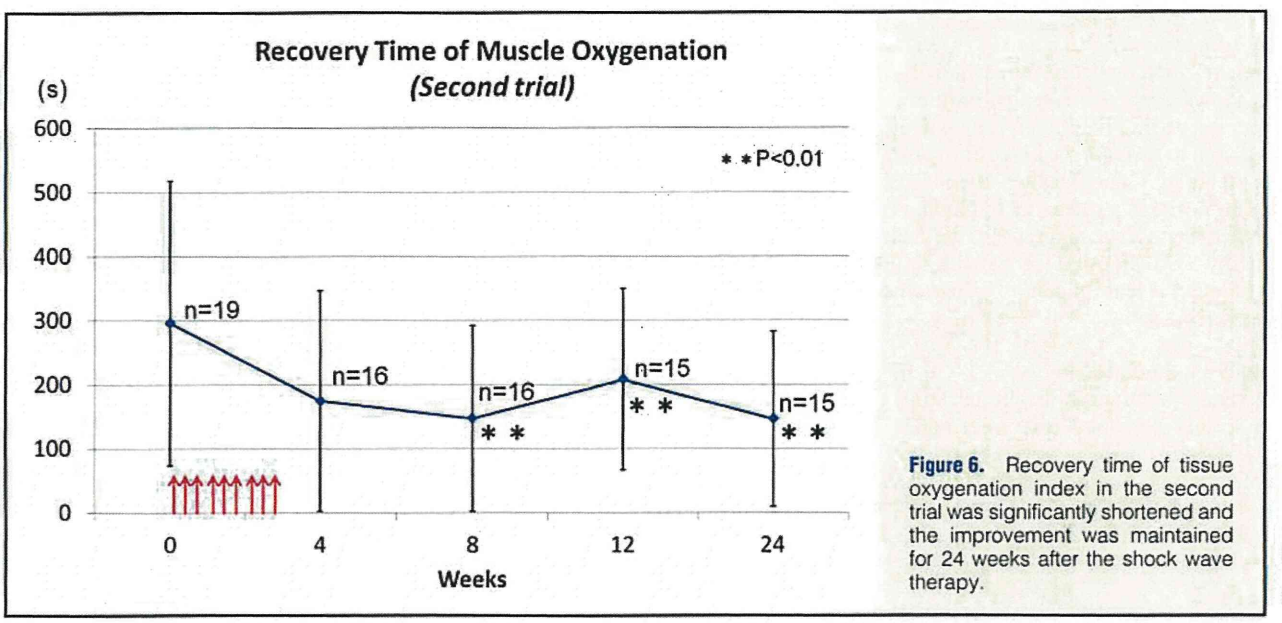


Figure 6. Recovery time of tissue oxygenation index in the second trial was significantly shortened and the improvement was maintained for 24 weeks after the shock wave therapy.

in rabbits.[11] Although the mechanisms for the different optimal SW conditions between the heart and the legs remain unclear, it is conceivable that therapeutic angiogenesis may be more effectively induced in the heart than in the legs. Further studies are needed to address this point.

Effects of Low-Energy SW Therapy on Symptoms

In the second trial, the low-energy SW therapy significantly improved the pain and distance subscale scores of the WIQ, probably because of improved peripheral perfusion and local O_2 supply as evidenced by the improved recovery time of TOI. In contrast, the SW therapy did not sufficiently improve the speed or stairs subscale scores. Most PAD patients with intermittent claudication walk slowly and subconsciously avoid the use of stairs in their daily life, which could be one of the reasons why the SW therapy did not improve the speed and stairs subscale scores. Although cilostazol has also been reported to improve the WIQ scores,[19] the beneficial effect of the SW therapy on the WIQ scores in the present study is superior to that of cilostazol.

Effects of the SW Therapy on Walking Ability

In the second trial, the SW therapy significantly improved the walking ability of PAD patients with intermittent claudication. This beneficial effect was associated with a significant reduction in the recovery time of TOI, reflecting improved calf

muscle blood flow and oxygenation. These results indicate that the SW therapy ameliorates the walking disability by improving peripheral perfusion in ischemic limbs.

Recently, Norgren et al demonstrated that sarpogrelate ameliorated the walking disability and increased the maximum walking distance at 24 weeks by 40% in patients classified as Fontaine II.[20] Regensteiner et al[19] and Pande et al[21] also demonstrated that cilostazol prolonged the maximum walking distance at 24 weeks by 50–76%. Exercise rehabilitation has also been reported to improve walking capacity by 25–65%.[22–24] The present study demonstrates that the SW therapy improves walking ability to the same extent as cilostazol without any adverse effects.

Mechanism of the Low-Energy SW Therapy

We have previously reported that low-energy SW therapy ameliorates myocardial ischemia in patients with severe angina pectoris[10,13] and increases capillary density in ischemic myocardium and ischemic limbs in animal models.[9,11,15,16] There are also several animal studies and case reports in humans showing that SW therapy accelerates wound healing in skin graft and chronic ulcers.[25–28] We and others have demonstrated in cultured human umbilical vein endothelial cells that low-energy SW therapy enhances NO production and expression of VEGF and its receptor, fms-related tyrosine kinase 1 (Flt-1), in vitro[9,29] and that the upregulation of VEGF and eNOS was involved in the SW-induced angiogenesis in vivo.[9,16] In addition, it was reported that low-energy SW applied to bone-marrow-derived mononuclear cells enhances VEGF production from the cells and their differentiation into endothelial phenotype cells[30] and that low-energy SW activates proliferation and differentiation in cardiac primitive cells.[31] Low-energy SW therapy was also reported to increase the expression of stromal-derived factor 1 in ischemic tissue, leading to enhanced recruitment of progenitor cells in a rat model of hindlimb ischemia.[32] Taken together, these data suggest that the beneficial effects of low-energy SW therapy on the walking ability of PAD patients are attributed, at least in part, to enhancement of several intrinsic angiogenic pathways.

Study Limitations

Several limitations of the present study should be mentioned. First, this was not a randomized controlled study and there was a small number of patients. We created the present protocol because patients can easily feel the SW-induced compression sensation (but not pain) when SW is applied to the calf muscle. Furthermore, we found a statistically significant beneficial effect of the SW therapy in the present patients (80% increase in the walking distance at 24 weeks) and the effect was greater than in a previous study in which some placebo effects were noted (35% increase at 6 months in a phase III clinical trial of oral beraprost sodium in PAD patients with intermittent claudication).[33] Thus, we consider that our SW therapy is superior to the placebo effect. However, this point needs to be confirmed in future studies with a large number of patients. Second, SW therapy failed to significantly improve the ABI and no increase in visible collateral vessels was noted by CT angiography although the SW therapy improved not only symptoms and walking ability but also local O_2 supply. Although the effect of the SW therapy appears to be mediated primarily by angiogenesis, the ABI and CT may not be sensitive enough to detect the angiogenesis in the ischemic calf muscle. A similar phenomenon was often observed in excercise rehabilitation studies of PAD patients and a recent meta-analysis reported that exercise rehabilitation could ameliorate the walking disability but did not improve ABI.[23] In PAD patients' calf muscles, increased muscle cell apoptosis and decreased capillary density are noted at the cellular and tissue levels, and muscle energetics are associated with mitochondrial dysfunction.[34,35] Bauer et al reported that mitochondrial dysfunction may affect the muscle oxygen utilization rate and accelerate endothelial cell damage.[36,37] We consider that our SW therapy may contribute, at least in part, to an improvement in mitochondrial dysfunction and muscle oxygen utilization. Although muscle biopsy may be useful to examine the histological changes, biopsy of the calf muscle of PAD patients is not usually recommended because of its invasive nature. Third, although supervised exercise training has been reported to improve walking ability by 25–65% in PAD patients,[22–24] it was not performed in the present study because the patients did not have an access to daily supervised exercise training. The possible effects of low-energy SW therapy combined with conventional therapeutic strategies, such as supervised exercise training, bypass surgery and endovascular intervention, remain to be examined. Fourth, the maximum walking distance at 24 weeks was increased in all patients. However, in only 1 patient wasthe maximum walking distance increased by less than 35% at 24 weeks. This patient had a single lesion caused by an occluded external iliac stent and the vascular tree below the femoral artery was intact. Considering potential mechanisms such as angiogenesis, SW therapy applied to the ischemic calf muscle may be more effective in patients with infrapopliteal lesions. Finally, in the present study, only PAD patients in Fontaine stage II were enrolled. In order to examine whether low-energy SW therapy is also effective in PAD patients with critical limb ischemia, we are now conducting a third clinical trial with PAD patients in Fontaine stages III and IV.

Conclusions

The present study demonstrates for the first time that our noninvasive low-energy SW therapy ameliorates the walking disability of PAD patients without any adverse effects. Further studies are needed to elucidate the detailed mechanisms of the beneficial effects of SW therapy.

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