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Effects of low-intensity pulsed ultrasound on clinical parameters in atherosclerotic peripheral artery disease patients with chronic limb-threatening ischemia

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Chronic limb-threatening ischemia (CLTI) is a major public health problem. Low-intensity pulsed ultrasound (LIPUS) has been shown to improve ischemic limb conditions in patients with CLTI. However, the possible mechanisms of these benefits require further understanding. A total of 37 atherosclerotic peripheral artery disease patients with CLTI (Fontaine class III or IV) who were not suitable for standard revascularization therapies were enrolled. Patients were treated with LIPUS daily for 20 min. Clinical parameters were evaluated at baseline and after 4 weeks and 12 weeks of treatment with LIPUS. Rest pain intensity on a visual analog scale (P = 0.018), walking impairment questionnaire score (P < 0.001), skin perfusion pressure (P < 0.001), flow-mediated vasodilation (P < 0.001), nitroglycerine-induced vasodilation (P = 0.002), white blood cell count (P = 0.013), ALT (P = 0.001), AST (P = 0.017), and high-sensitivity C-reactive protein (P = 0.011) were significantly improved after LIPUS treatment. None of the patients withdrew from the study due to adverse effects associated with LIPUS. During a mean follow-up period of 91.4 ± 49.0 months, the rate of survival was 88.9% at 1 year and the rate of limb survival was 88.6% at 1 year. LIPUS exposure may have favorable effects on clinical symptoms, inflammation, perfusion parameters, and vascular function in patients with CLTI and it can be used safely.

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Keywords Chronic limb-threatening ischemia, Atherosclerosis, Endothelial function, Angiogenesis, Inflammation

Abbreviations

ABI	Ankle-brachial index
CLI	Critical limb ischemia
CLTI	Chronic limb-threatening ischemia
FMD	Flow-mediated vasodilation
hs-CRP	High-sensitivity C-reactive protein
LIPUS	Low-intensity pulsed ultrasound

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NID	Nitroglycerine-induced vasodilation
PAD	Peripheral artery disease
SPP	Skin perfusion pressure
VAS	Visual analog scale
WIQ	Walking impairment questionnaire

Peripheral artery disease (PAD) is a systemic atherosclerosis characterized by blood flow restriction in peripheral arteries with a variety of clinical manifestations[1]. Over 200 million persons worldwide have PAD and 1% to 11% of PAD patients have chronic limb-threatening ischemia (CLTI)[1,2]. CLTI, also known as critical limb ischemia (CLI), represents the most advanced stage of the PAD spectrum and is characterized by chronic ischemic rest pain, nonhealing ulcers, and gangrene with a high risk of amputation[1]. Even with optimal medical managements (wound care, medication, and peripheral artery revascularization), the rate of mortality in patients with CLTI ranges from 11.2 to 49.0% at 1 year and the rate of major amputation ranges from 13.2 to 63.2% at 1 year[3–7]. CLTI is a major public health problem due to the high mortality rate, its impact on quality of life, and healthcare costs. In addition, it has been reported that more than 30% of patients with CLTI are considered to be "no-option" at the time of diagnosis[8]. Therefore, the development of a new treatment approach is needed for patients with CLTI to preserve their limbs and to improve their quality of life.

Low-intensity pulsed ultrasound (LIPUS) was shown to be able to induce various responses in different cells and it was developed as a noninvasive therapeutic method for accelerating bone fracture recovery[9,10]. Several clinical studies have shown that LIPUS has favorable effects on lumbar spondylolysis[11], erectile dysfunction[12], and Alzheimer's disease[13]. Experimental studies have demonstrated that LIPUS exposure promotes angiogenesis[14,15]. We previously have reported that LIPUS improves ischemic limb conditions in patients with CLTI compared to the effects of standard treatment[16–19]. However, the possible mechanisms of these benefits have not been elucidated. LIPUS exposure has shown to ameliorate angiotensin II-induced vascular remodeling and inflammation in both in vivo and in vitro studies, suggesting that LIPUS can reduce inflammation and improve vascular function and ischemic limb conditions[20]. The aim of this study was to determine the effects of LIPUS on clinical parameters and vascular function in atherosclerotic PAD patients with CLTI.

Results

Baseline characteristics

The baseline clinical characteristics of the 37 atherosclerotic PAD patients with CLTI are shown in Table 1. The patients included 24 men (65%) and 13 women (35%), and 33 (89%) of the patients had hypertension, 24 (65%) had dyslipidemia, 24 (65%) had diabetes with 75% of those patients taking insulin, 19 (51%) were receiving hemodialysis, 3 (8%) were current smokers, and 24 (65%) had a history of cardiovascular disease. The reasons for disqualification from standard revascularization were as follows: absence of an artery target for endovascular

Variable	Total (n = 37)
Age, year	68 ± 9
Gender, men/women	24/13
Body mass index, kg/m^2	22.6 ± 3.8
Rutherford category, n (%)	
4 (Fontaine III)	7 (19)
5 (Fontaine IV)	26 (70)
6 (Fontaine IV)	4 (11)
Medical history, n (%)	
Hypertension	33 (89)
Dyslipidemia	24 (65)
Diabetes mellitus	24 (65)
Previous cardiovascular disease	24 (65)
Hemodialysis	19 (51)
Current smoker	3 (8)
Former smoker	20 (54)
Medications, n (%)	
Anti-hypertensive therapy	31 (84)
Any lipid modification therapy	21 (57)
Anti-hyperglycemic therapy	22 (59)
Insulin	18 (49)
Anti-platelet therapy	36 (97)
Anti-coagulant therapy	12 (32)

Table 1. Clinical characteristics of the patients. Results are presented as mean ± SD for continuous variables and percentages for categorical variables.

or surgical therapy (n = 21), persistent CLTI for more than 3 months despite repeated endovascular or surgical therapy (n = 14), and refusal to undergo invasive therapy (n = 2).

Effects of LIPUS on parameters

After the start of LIPUS treatment, there were significant improvements in visual analog scale (VAS) (56.2 ± 31.9 at baseline, 49.0 ± 31.9, P = 0.180 at 4 weeks, 37.6 ± 32.8, P = 0.028 at 12 weeks; Fig. 1A), walking impairment questionnaire (WIQ) score (90 ± 89 at baseline, 127 ± 109, P = 0.005 at 4 weeks, 143 ± 106, P < 0.001 at 12 weeks; Fig. 1B), and skin perfusion pressure (SPP) (36.9 ± 17.4 mmHg at baseline, 50.5 ± 17.1 mmHg, P < 0.001 at 4 weeks, 50.6 ± 21.7 mmHg, P < 0.001 at 12 weeks; Fig. 1C), but not in ankle-brachial index (ABI) (0.81 ± 0.45 at baseline, 0.93 ± 0.26, P = 0.159 at 4 weeks, 0.91 ± 0.38, P = 0.166 at 12 weeks; Fig. 1D). Flow-mediated vasodilation (FMD) (3.7 ± 1.2% at baseline, 5.5 ± 2.6%, P = 0.014 at 4 weeks, 7.6 ± 3.6%, P < 0.001 at 12 weeks; Fig. 2A) and nitroglycerine-induced vasodilation (NID) (5.6 ± 1.1% at baseline, 8.3 ± 3.2%, P = 0.005 at 4 weeks, 12.0 ± 7.3%, P = 0.006 at 12 weeks; Fig. 2B) were also significantly improved after the start of LIPUS treatment. There were no significant differences in baseline popliteal artery diameter (4.89 ± 0.86 mm at baseline, 4.80 ± 0.74 mm, P = 0.456 at 4 weeks, 4.58 ± 0.72 mm, P = 0.139 at 12 weeks; Fig. 2C) after the start of LIPUS treatment. Parameters in the 37 patients before and after the start of treatment with LIPUS are summarized in Table 2. There were significant reductions in white blood cell count and levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and high-sensitivity C-reactive protein (hs-CRP) after the start of LIPUS treatment. As a sensitivity analysis, we performed exploratory analysis to evaluate the effects of LIPUS on clinical parameters at 12 weeks and at 48 weeks after the start of LIPUS treatment (Supplementary Table S1). There were significant improvements in VAS, WIQ score, SPP, FMD, NID, and white blood cell count after the start of LIPUS treatment.

Figure 3 shows the changes in ischemic symptoms from baseline to after LIPUS treatment. Of the 30 patients with ulcer/gangrene, 7 patients showed complete healing of the ulcer/gangrene, 22 patients had residual ulcer/gangrene, 2 patients had major amputation, 1 patient had minor amputation, and LIPUS was discontinued in 1 patient due to hospitalization for heart failure during the 12 weeks of LIPUS treatment. Among the 7 patients without ulcer/gangrene at baseline, LIPUS was discontinued in 1 patient due to stroke during the 12 weeks of LIPUS treatment. Among the 22 patients with ulcer/gangrene at 12 weeks of follow-up, 7 patients showed complete healing of the ulcer/gangrene, 13 patients had residual ulcer/gangrene, 1 patient had minor amputation, 1 patient discontinued LIPUS due to death from Fournier's gangrene, and LIPUS was discontinued in 1 patient due to death from pneumonia during the period between 12 and 48 weeks after the start of LIPUS treatment. Among the 13 patients without ulcer/gangrene at 12 weeks of follow-up, 3 patients showed recurrence of ulcer/gangrene.

Correlations between changes in vascular function and changes in inflammatory parameters

Changes in FMD after LIPUS treatment did not correlate with changes in hs-CRP (r = 0.069, P = 0.785) and changes in white blood cell count (r = 0.009, P = 0.972). Changes in NID after LIPUS treatment did not correlate with changes in hs-CRP (r = −0.061, P = 0.805) and changes in white blood cell count (r = −0.207, P = 0.395).

Effects of LIPUS on clinical outcomes

During a mean follow-up period of 91.4 ± 49.0 months, the rates of survival were 88.9% at 1 year and 48.1% at 5 years and the rates of limb survival were 88.6% at 1 year and 71.7% at 5 years. Twenty-one patients died (11 from cardiovascular causes) and two patients had acute myocardial infarction, three had stroke, six had coronary revascularization, and four were hospitalized for heart failure. There were eight major amputations and seven minor amputations (Table 3). The causes of deaths are shown in Table 4. None of the patients withdrew from the study due to adverse effects associated with LIPUS. Next, we divided the patients into two groups according to the changes in SPP from baseline to after 12 weeks of LIPUS treatment: an improved SPP group with changes in SPP of 1 mmHg or more and a no change or reduced SPP group with changes in SPP of less than 1 mmHg. Clinical characteristics of the two groups are summarized in Supplementary Table S2. The Kaplan–Meier curves for survival were not significantly different in the two groups (Fig. 4A). The Kaplan–Meier curves for major amputation were significantly different in the two groups (Fig. 4B).

Discussion

This study was a single-arm, prospective, and interventional study to evaluate the effects of LIPUS on symptoms, clinical parameters, and vascular function in 37 atherosclerotic PAD patients with CLTI (Fontaine class III or IV) who were not suitable for standard revascularization therapies. Our study results suggested that LIPUS treatment had favorable effects in white blood cell count, ALT, AST, hs-CRP, VAS, WIQ score, SPP, FMD, and NID.

It is well known that atherosclerotic PAD patients have many complications such as hypertension, diabetes, hyperlipidemia, and chronic renal failure[1,2]. In the present study, the prevalence of cardiovascular risk factors was high and nearly half of the participants were receiving hemodialysis and insulin therapy. Several investigators have reported that the rates of amputation and death are extremely high in patients with CLTI on hemodialysis and/or insulin treatment[21–24]. Our data suggested that the study patients were consistent with severe cases of CLTI.

It is well known that cardiovascular drugs such as statins and renin-angiotensin system inhibitors improve vascular function and inflammation[25–27]. Although the patients enrolled in this study were receiving optimal medical management, treatment with LIPUS further improved FMD and NID in the PAD patients with CLTI. Inflammation is associated with endothelial dysfunction by reducing the bioavailability of nitric oxide[27,28]. In the present study, SPP was improved after LIPUS treatment, suggesting that LIPUS induced angiogenesis in ischemic limbs. LIPUS-induced angiogenesis accelerates wound healing, leading to a reduction in wound inflammation,

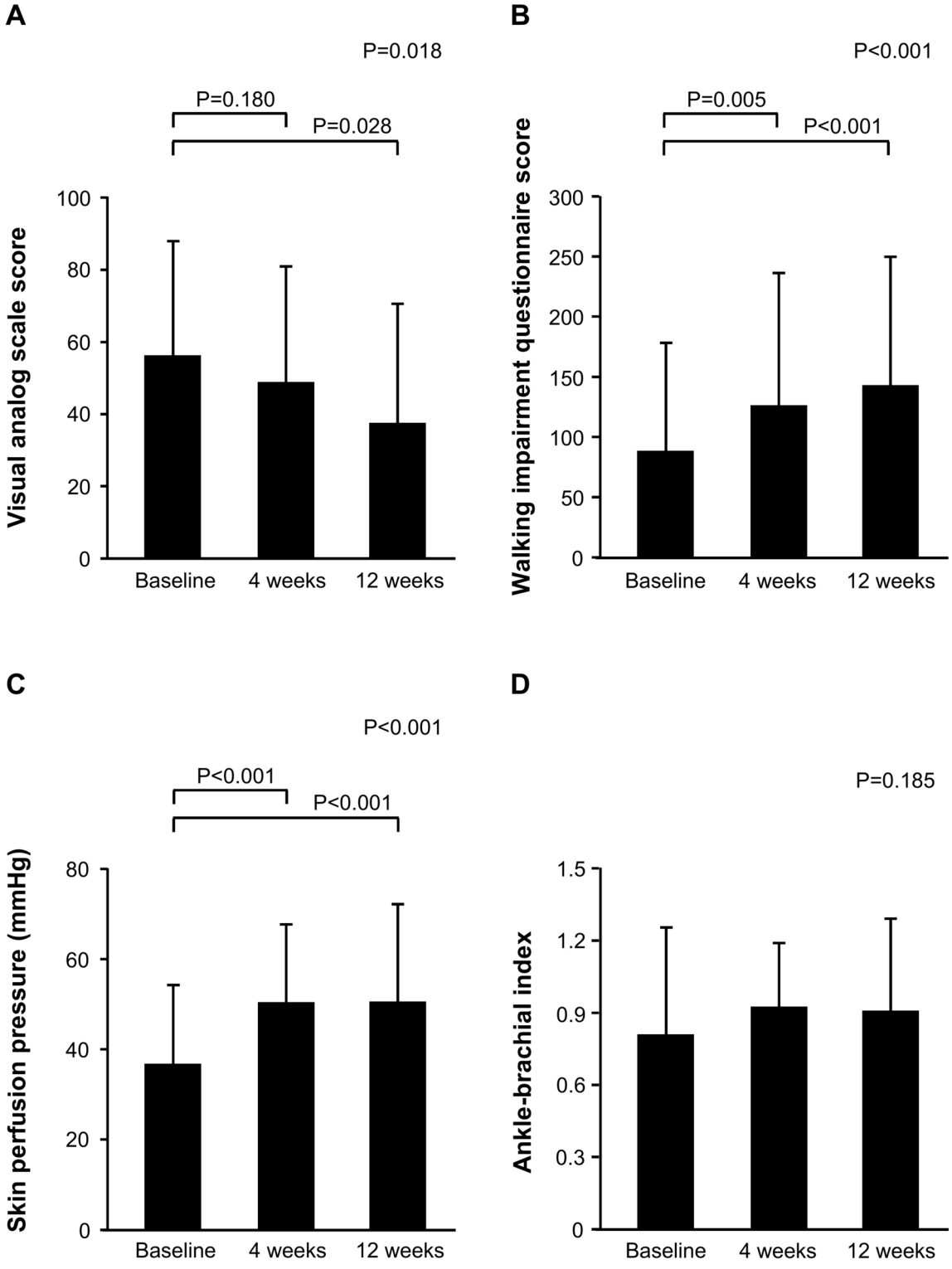

Fig. 1. Bar graphs show visual analog scale score (**A**), walking impairment questionnaire score (**B**), skin perfusion pressure (**C**), and ankle-brachial index (**D**) at baseline and after 4 weeks and 12 weeks of treatment with LIPUS.

which is reflected in the reductions of white blood cell count and hs-CRP in our study. In addition, experimental studies have demonstrated that LIPUS exposure per se has anti-inflammatory effects[20,29,30]. LIPUS stimulation has been shown to increase the expression levels of anti-inflammatory cytokines (arginase-1, peroxisome proliferator-activated receptor gamma, interleukin-4) and reduce the expression levels of pro-inflammatory cytokines (transforming growth factor beta 1, interleukin-1β, tumor necrosis factor -alpha) and induce a change

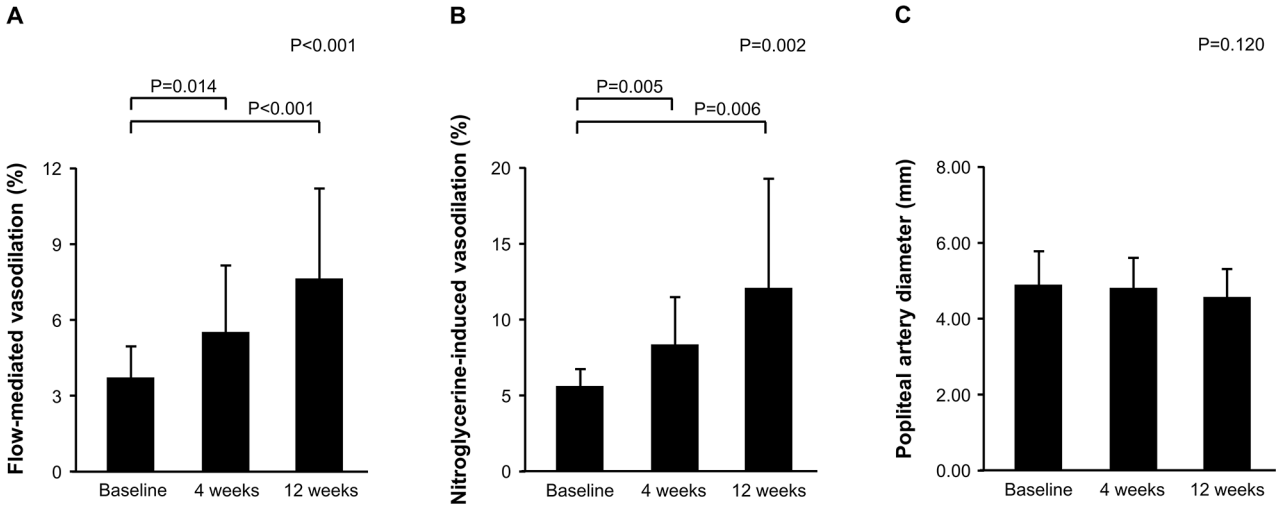

Fig. 2. Bar graphs show flow-mediated vasodilation (**A**), nitroglycerine-induced vasodilation (**B**), and popliteal artery diameter (**C**) at baseline and after 4 weeks and 12 weeks of treatment with LIPUS.

Variables	Baseline	4 weeks	12 weeks	P value
Systolic blood pressure, mmHg	135 ± 22	132 ± 22	135 ± 24	0.746
Diastolic blood pressure, mmHg	75 ± 13	73 ± 12	76 ± 14	0.454
Heart rate, bpm	77 ± 13	75 ± 12	75 ± 11	0.316
Total cholesterol, mg/dL	170 ± 42	161 ± 44	167 ± 41	0.145
Triglycerides, mg/dL	125 ± 51	119 ± 52	115 ± 53	0.562
HDL-C, mg/dL	52 ± 20	49 ± 19	54 ± 23	0.105
LDL-C, mg/dL	93 ± 31	83 ± 32	86 ± 32	0.095
Glucose, mg/dL	169 ± 108	162 ± 64	172 ± 80	0.859
White blood cells, $\times 10^3/\mu L$	6748 ± 1632	6127 ± 1638*	6100 ± 1644*	0.013
Red blood cells, $\times 10^6/\mu L$	393 ± 60	389 ± 63	394 ± 66	0.720
Platelets, $\times 10^3/\mu L$	18.6 ± 4.4	18.7 ± 6.0	18.4 ± 4.4	0.869
ALT, U/L	15 ± 9	12 ± 8*	12 ± 8*	0.001
AST, U/L	19 ± 7	17 ± 8*	17 ± 7*	0.017
Blood urea nitrogen, mg/dL	29.5 ± 15.6	28.8 ± 15.2	26.2 ± 13.3	0.064
Creatinine, mg/dL	3.08 ± 2.97	3.13 ± 3.03	2.99 ± 2.91	0.326
eGFR, mL/min/1.73 m^2	38.5 ± 28.6	38.7 ± 29.0	38.3 ± 28.0	0.942
Uric acid, mg/dL	6.0 ± 2.2	5.7 ± 1.9	5.6 ± 1.6	0.133
Sodium, mmol/L	139 ± 3	138 ± 3	139 ± 3	0.933
Potassium, mmol/L	4.6 ± 0.6	4.6 ± 0.7	4.5 ± 0.6	0.552
Chloride, mmol/L	102 ± 3	103 ± 3	103 ± 3	0.728
hs-CRP, mg/dL	1.32 ± 1.81	1.11 ± 1.90	0.43 ± 0.52*	0.011

Table 2. Clinical parameters at baseline and during follow-up. Results are presented as mean ± SD. *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *WBC* white blood cell, *RBC* red blood cell, *ALT* alanine aminotransferase, *AST* asparate aminotransferase, *eGFR* estimated glomerular filtration rate, *hs-CRP* high-sensitivity C-reactive protein. *P < 0.05 vs. baseline.

in the phenotype of macrophages to immunosuppressive M2[30,31]. Although these findings suggest that LIPUS has favorable effects on vascular function by reducing inflammation, there were no significant correlations between changes in vascular function and changes in inflammatory markers in our study. Other potential mechanisms by which LIPUS improves vascular function have been postulated. LIPUS has been shown to exert protective effects on endothelial cells against oxidative stress through suppressing the oxidative stress-induced endothelial-mesenchymal transition, activating the phosphatidylinositol 3-kinase/protein kinase B pathway under oxidative stress, and limiting cell migration and excessive extracellular matrix deposition[31]. Indeed, randomized placebo-controlled clinical studies have shown that LIPUS exposure for 5 min improves FMD in healthy volunteers[32,33]. The improvement in endothelial function induced by LIPUS may reduce the incidence of cardiovascular events.

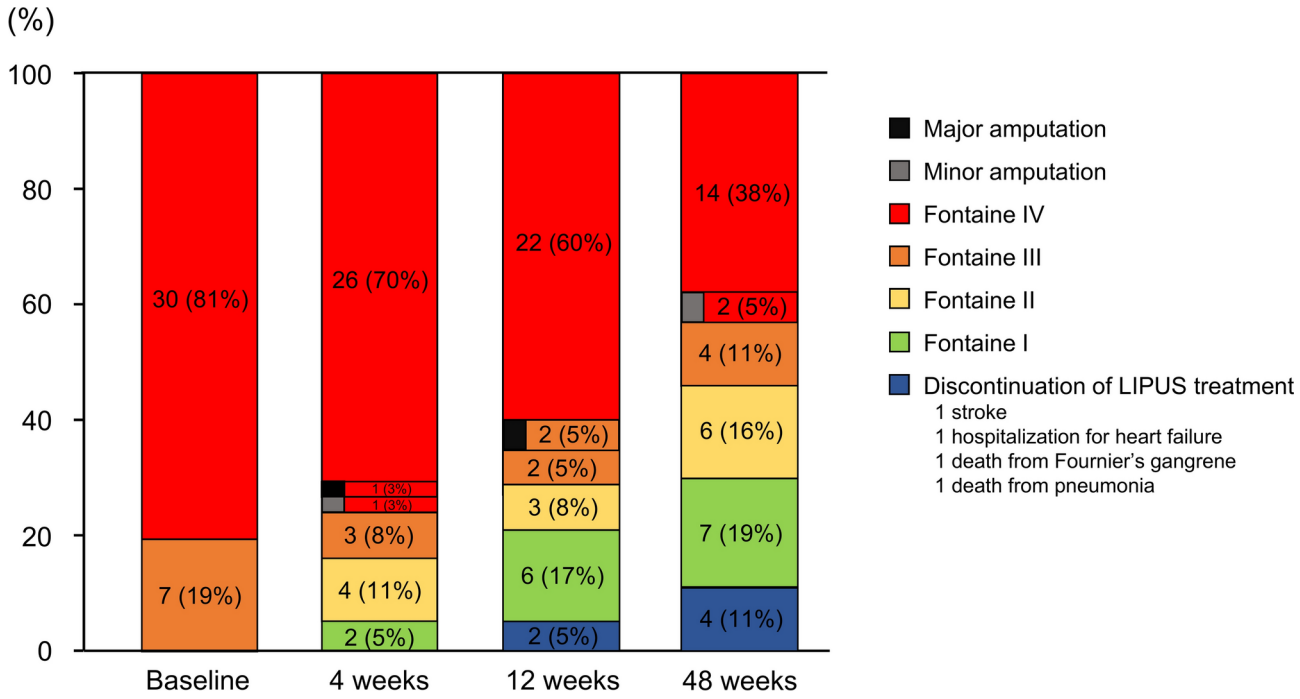

Fig. 3. Changes in ischemic symptoms from baseline to after 48 weeks of LIPUS treatment.

Variable	Total (n = 37)
Death from any cause, n (%)	21 (56.8)
Death from cardiovascular disease, n (%)	11 (29.7)
Acute myocardial infarction, n (%)	2 (5.4)
Stroke, n (%)	3 (8.1)
Coronary revascularization, n (%)	6 (16.2)
Hospitalization for heart failure, n (%)	4 (10.8)
Major amputation, n (%)	8 (21.6)
Minor amputation, n (%)	7 (18.9)

Table 3. Clinical outcomes of all subjects. All results are presented as number (%).

Variable	Total (n = 37)
Sudden death, n (%)	6 (16.2)
Heart failure, n (%)	2 (5.4)
Sepsis, n (%)	4 (10.8)
Acute myocardial infarction, n (%)	1 (2.7)
Stroke, n (%)	1 (2.7)
Gastrointestinal bleeding, n (%)	1 (2.7)
Renal failure, n (%)	1 (2.7)
Fournier's gangrene, n (%)	1 (2.7)
Pneumonia, n (%)	2 (5.4)
Caducity, n (%)	2 (5.4)

Table 4. Cause of death. All results are presented as number (%).

None of the patients withdrew from the study due to adverse effects associated with LIPUS. However, it remains unknown whether daily LIPUS exposure has other biological effects. We evaluated blood samples to investigate potential effects of LIPUS exposure, and our data showed that there were significant improvements in liver function and inflammation marker levels. A recent experimental study has demonstrated that LIPUS significantly decreased liver injury and fibrosis through increasing anti-inflammatory factors and reducing pro-

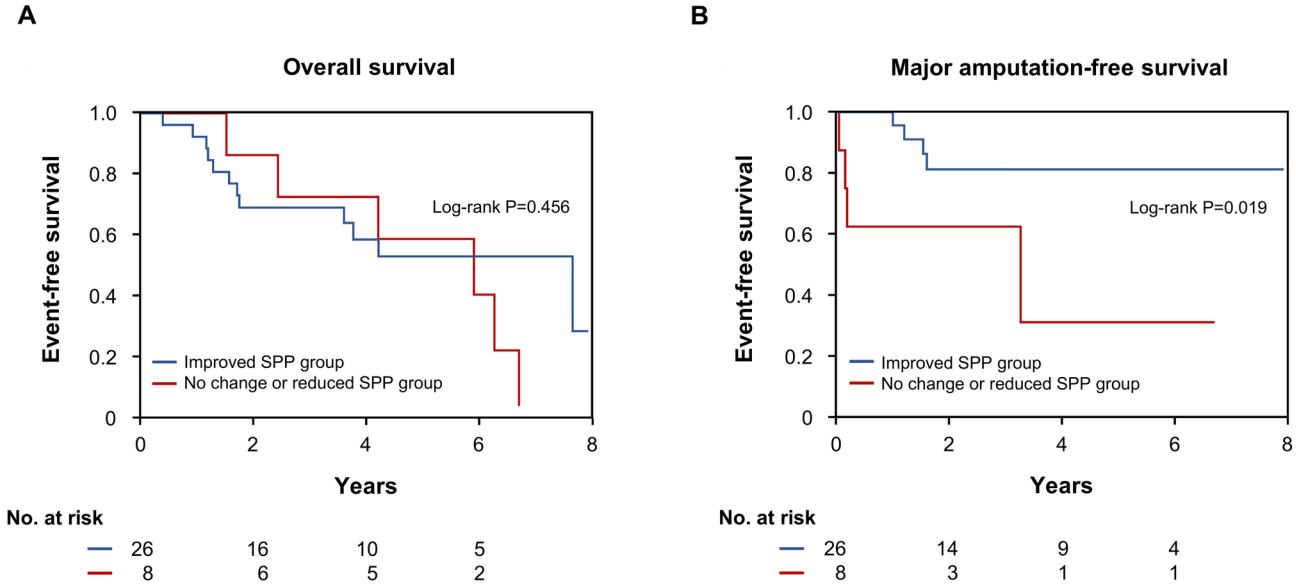

Fig. 4. Kaplan–Meier curves of overall survival (**A**) and major amputation-free survival (**B**) according to changes in SPP. "Improved SPP group" indicates the changes in SPP from baseline to after LIPUS treatment ≥ 1 mmHg and "No change or reduced SPP group" indicates changes in SPP < 1 mmHg.

inflammatory factors in a rat model[34]. These findings suggest that LIPUS can be used safely for treatment of CLTI.

The aim of treatment for patients with CLTI is to preserve limbs and improve the quality of life. Currently, there are limited therapeutic options for patients with severe CLTI[1,2,8]. In the present study, the rates of survival were 88.9% at 1 year and 48.1% at 5 years and the rates of limb survival were 88.6% at 1 year and 71.7% at 5 years after treatment with LIPUS. Although our patients presented with severe complications at the time of enrollment, treatment with LIPUS showed favorable effects on survival and amputation-free survival compared with those in previous reports[3–7]. In addition, we confirmed that VAS, WIQ score, and SPP were significantly improved after treatment with LIPUS, suggesting that treatment with LIPUS may improve quality of life and ischemic limb conditions. We also found that patients in the improved SPP group had better limb salvage than did patients in the no change or reduced SPP group. Therefore, LIPUS has the possibility of being a therapeutic option for atherosclerotic PAD patients with CLTI who are not suitable for current standard revascularization options. Further studies are needed to assess the effects of LIPUS on clinical outcomes in a multicenter study with a longer follow-up period.

Our study has several limitations. First, this study was a single-arm study. We enrolled patients with CLTI who had no other option for treatment of chronic ischemic rest pain and nonhealing ulcers and who were at high risk of major amputation. Therefore, it is ethically unacceptable to enroll patients in a random assignment. Second, in the present study, LIPUS was applied to the calf region of the symptomatic limb. If there is sufficient blood flow in the leg arteries, local application of LIPUS at the site of ulcers may be effective. CLTI is characterized by diffuse arterial stenosis or occlusion and is caused by compromise of blood supply to the lower limbs. Improvement in leg blood supply is necessary for patients with CLTI to promote blood flow for pre-existing arterioles in the foot. Therefore, the LIPUS device was used on the calf region. Third, although we confirmed that symptoms, severity of CLTI, and vascular function were improved after LIPUS treatment, the number of patients was small and we did not evaluate the changes in ulcer area and depth. Fourth, we do not deny the possibility that other factors such as medication and rehabilitation may have affected outcomes. Fifth, the effects of LIPUS on clinical parameters after more than one year are unknown. Future studies are needed to confirm the effect of LIPUS on CLTI including ulcer area, ulcer depth, clinical parameters, and outcomes in a large number of patients.

In conclusion, LIPUS exposure may have favorable effects on clinical symptoms, inflammation, perfusion parameters, and vascular function in patients with CLTI and can be used safely for a mean follow-up period of 91.4 months. Further studies are needed to determine the overall effects in a larger number of patients.

Methods

Subjects

Between October 2010 and March 2022, we enrolled 37 atherosclerotic PAD patients with CLTI (Fontaine class III or IV) who had no other option for standard revascularization therapies for this study. Rheumatoid factor, lupus anticoagulants, and results of serological investigations were assessed to rule out other vasculitis and hypercoagulable states. The diagnosis of arterial occlusion leading to ischemia was confirmed by angiography. CLTI/CLI was classified according to the guidelines of the TransAtlantic Inter-Society Consensus II (TASC II)[35]. The exclusion criteria were as follows: (1) a history of malignant disease within five years prior to the study, (2) a history of myocardial infarction or cerebrovascular disease within three months prior to the study, and (3)

pregnancy or possible pregnancy. Cardiovascular disease was defined as a history of angina pectoris, myocardial infarction, unstable angina, ischemic stroke, hemorrhagic stroke, or transient ischemic attack.

The study protocol was approved by Hiroshima University ethical committee. The study was executed in accordance with the Good Clinical Practice guidelines and the tenets of the Declaration of Helsinki. Informed consent for participation in the study was obtained from all patients. The protocol was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN000004901: first registration data 07/09/2010, UMIN000014757: first registration data 01/04/2014) and Japan Registry of Clinical Trials (jRCTs062200008: first registration data 02/07/2020).

Study protocol

This study was a single-arm, prospective, and interventional study. Patients were treated with LIPUS daily for 20 min. Clinical parameters were evaluated at baseline and after 4 weeks and 12 weeks of treatment with LIPUS. We evaluated changes in clinical parameters from baseline to 4 weeks and 12 weeks. Clinical parameters included blood pressure, heart rate, blood sampling results, rest pain intensity on a VAS, WIQ score, SPP, ABI, FMD, and NID. The patients were instructed to abstain from eating, drinking alcohol, smoking and taking caffeine for at least 12 h prior to the measurements. Measurement of SPP was performed while each patient was in the supine position. Venous blood samples were obtained from the left antecubital vein. We measured FMD and NID in the popliteal artery without stenosis and in occluded arteries in the patients[36]. The patients were kept in the prone position in a quiet, dark, air-conditioned room (constant temperature of 22–25 °C) throughout the study. Thirty minutes after maintaining the prone position, basal popliteal artery diameter was measured. Then FMD was measured in the popliteal artery. After completion, we next measured NID with confirmation that the popliteal artery diameter had recovered to the baseline value. The observers were blind to the protocol of this study.

Next, we investigated the effects of LIPUS on clinical outcomes. From January to March 2024, we collected information on potential outcomes or adverse events from medical records and a telephone survey. Major amputation was defined as above-the-ankle amputation. We obtained complete follow-up data for all patients.

LIPUS treatment

The LIPUS device (SX-1001, Nippon Sigmax Co. Ltd., Tokyo, Japan) with a transducer element (Nippon Sigmax Co. Ltd.) emits ultrasound frequency of 2 MHz ± 10%, ultrasound output power of 30 mW/cm^2, beam nonuniformity ratio of 5 ± 2, pulse duration of 200 μs ± 5%, pulse repetition frequency of 1 kHz ± 5%, pulse duty of 20% ± 5%, area of ultrasound exposure of 6.85cm^2 ± 10%, and output duration of 20 min per probe[16,17]. Ultrasound exposure was applied over the skin of the calf region of the symptomatic limb for 20 min per day and the patient could be either in a supine or seated position (Supplementary Fig. S1). The gap between the transducer and the skin was filled with ultrasonic gel. Eight transducer probes were attached to the symptomatic lower limb. The LIPUS device is portable and was used by patients at home with recorded log data[16,17].

Measurement of vascular function

A high-resolution linear artery transducer was coupled to computer-assisted analysis software (Aloka-α7, ALOKA Co., Tokyo, Japan) that used an automated edge detection system for measurement of artery diameter (Supplementary Fig. S2). A blood pressure cuff was placed around the thigh of the treated limb. The target artery was scanned longitudinally. When the clearest B-mode image of the intimal interfaces between the lumen and vessel wall was obtained, the transducer was held at the same point throughout the scan by a special probe holder (MP-PH0001, ALOKA Co.) to ensure consistency of the image. A baseline image was acquired and blood flow was estimated by time averaging the pulsed Doppler velocity signal obtained from a sample volume. Then the blood pressure cuff was inflated to 50 mmHg above systolic pressure for 5 min. Depth and gain setting were set to optimize the images of the arterial lumen wall interface. When the tracking gate was placed on the intima, the artery diameter was automatically tracked and the waveform of diameter changes over the cardiac cycle was displayed in real time using the FMD mode of the tracking system. This allowed the ultrasound images to be optimized at the start of the scan and the transducer position to be adjusted immediately for optimal tracking performance throughout the scan. Pulsed Doppler flow was assessed at baseline and during peak hyperemic flow, which was confirmed to occur within 15 s after cuff deflation. Blood flow velocity was calculated from the color Doppler data and was displayed as a waveform in real time. The baseline longitudinal image of the artery was acquired for 30 s and then the blood pressure cuff was inflated to 50 mmHg above systolic pressure for 5 min. The longitudinal image of the artery was recorded continuously until 5 min after cuff deflation. Pulsed Doppler velocity signals were obtained for 20 s at baseline and for 10 s immediately after cuff deflation. Changes in popliteal artery diameter were immediately expressed as percent change relative to the vessel diameter before cuff inflation. FMD was calculated as the percent change in peak vessel diameter from the baseline value. %FMD [(peak diameter – baseline diameter)/baseline diameter] was used for analysis. Blood flow volume was calculated by multiplying the Doppler flow velocity (corrected for the angle) by heart rate and vessel cross-sectional area (-r^2). Reactive hyperemia was calculated as the maximum percentage increase in flow after cuff deflation compared with baseline flow. After a 10-min period to allow baseline conditions of the artery to be reestablished, another baseline scan was performed.

The response to nitroglycerine was used for assessment of endothelium-independent vasodilation. NID was measured as described previously[37]. In brief, after acquiring baseline rest images for 30 s, a sublingual tablet (75 μg nitroglycerine) was given, and images of the artery were recorded continuously until the dilation reached a plateau after administration of nitroglycerine. Patients who received nitrate treatment and patients in whom the sublingually administered nitroglycerine tablet did not dissolve during the measurement, were excluded from this study. NID was automatically calculated as a percent change in peak vessel diameter from the baseline value. Percentage of NID [(peak diameter – baseline diameter)/baseline diameter] was used for analysis.

The coefficient of variation for the baseline diameter was 2.9% in our laboratory.

Statistical analysis

Baseline characteristics are presented as means ± SD for continuous variables and as percentages for categorical variables. Differences between mean values of continuous variables at baseline and at 4 weeks and 12 weeks were compared by multivariate analysis of variance (MANOVA). We divided the patients into two groups according to changes in SPP from baseline to after 12 weeks of LIPUS treatment: an improved SPP group with changes in SPP of 1 mmHg or more and a no change or reduced SPP group with changes in SPP of less than 1 mmHg. Comparison of continuous variables between the two groups was performed by using Student's t test or the chi-squared test for categorical data. Time-to-event end-point analyses were performed by using the Kaplan–Meier method. A log-rank test was used to compare outcomes between the two groups. All analyses were conducted using JMP version 16.0 software (SAS Institute, Cary, NC, USA).

Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Author contributions

M.K. and Y.H., drafting the article and conception of this study; M.K., F.M.Y., T.Y., T.M., S.K., T.H., A.M., S.T., C.G., and A.N. acquiring subjects and/or data; Y.N. and K.C. revising the article critically for important intellectual content. Y.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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