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Therapeutic Ultrasound Promotes Reperfusion and Angiogenesis in a Rat Model of Peripheral Arterial Disease

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Background: Shock wave therapy (SWT) is an acoustic technology clinically used for the non-invasive treatment of ischemic heart disease (IHD). Therapeutic ultrasound (TUS) has more recently been developed for the same indication, although its effects on reperfusion and angiogenesis have yet to be directly compared to those of SWT.

Methods and Results: TUS and SWT acoustic parameters were matched, and their ability to promote angiogenesis and reperfusion in a rat hindlimb ischemia model was compared. After left femoral artery excision, 3-weekly TUS, SWT or sham treatments (n=10 rats each) of the left hindlimb were performed for 2 weeks. Laser Doppler perfusion imaging demonstrated improved perfusion with TUS (66±4% L:R hindlimb perfusion, mean±SEM, P=0.02), but not with SWT (59±4%, P=0.13) compared with sham (50±4%). Immunohistochemistry of CD31 demonstrated increased microvascular density with TUS (222.6 vessels/high-power field, P=0.001) and SWT (216.9, P=0.01) compared to sham-treated rats (196.0). Tissue vascular endothelial growth factor mRNA levels were elevated in the left hindlimb of TUS-, but not SWT- or sham-treated rats.

Conclusions: Direct comparison demonstrates that TUS is more effective than SWT at promoting reperfusion, whereas both therapies promote angiogenesis in ischemic gastrocnemius muscle. These results suggest that TUS may be more effective than SWT for the treatment of IHD and peripheral arterial disease.

Key Words: Angiogenesis; Ischemic heart disease; Peripheral arterial disease; Shock wave therapy; Ultrasound

Global burdens of ischemic heart disease (IHD) and peripheral arterial disease (PAD) have been increasing.[1] IHD leads to approximately 7.2 million deaths per year,[2] and PAD affects over 200 million patients globally.[3] While modern revascularization methods such as bypass graft surgery and percutaneous interventions have decreased morbidity and mortality associated with these diseases, these treatments remain limited by small vessel disease and the risks of reoperation.[4] Thus, there is a strong need for novel, non-invasive and effective therapies for the promotion of angiogenesis for the treatment of these diseases.

Extracorporeal shock wave therapy (SWT) is an acoustic treatment modality that sends individual acoustic shock waves in a beam focused onto the area of interest. Each shock wave consists of an approximately 1 μs-long, positive (compressional) pulse followed by a longer (~5 μs), lower amplitude negative (rarefactional) pressure pulse (**Figure 1A**). In a landmark study, Nishida et al demonstrated the potential of SWT as a treatment for IHD; in a porcine model of chronic IHD, SWT improved ejection fraction, wall thickening, myocardial blood flow and capillary density compared with sham therapy.[5] Similar results were demonstrated in porcine models of acute myocardial infarction[6] and ischemia-reperfusion injury.[7] Human studies soon followed, with Fukumoto et al first describing decreased angina and nitroglycerine use, and improved nuclear myocardial perfusion among patients with IHD who underwent SWT 3 times a week for 3 weeks.[8] Following small randomized, double-blinded trials,[9–11] clinical use of SWT for IHD has spread through Japan, Israel, and parts of Europe, where it received CE Mark approval in 2010.[12] However, clinical use has yet to spread globally, potentially due to the small subject size and efficacy of clinical trials.

Unlike SWT, therapeutic ultrasound (TUS) is an acoustic technique in which multiple cycles of a single-frequency (usually 1–10 MHz) sinusoidal ultrasound wave are transmitted from an extracorporeal-focused source into the tissue of interest (**Figure 1B**). The frequency, amplitude and duration of the TUS pulses can be varied in a wide range, depending on the desired effect on the target tissue. When used at high intensities (high-intensity-focused ultrasound; HIFU), TUS is clinically

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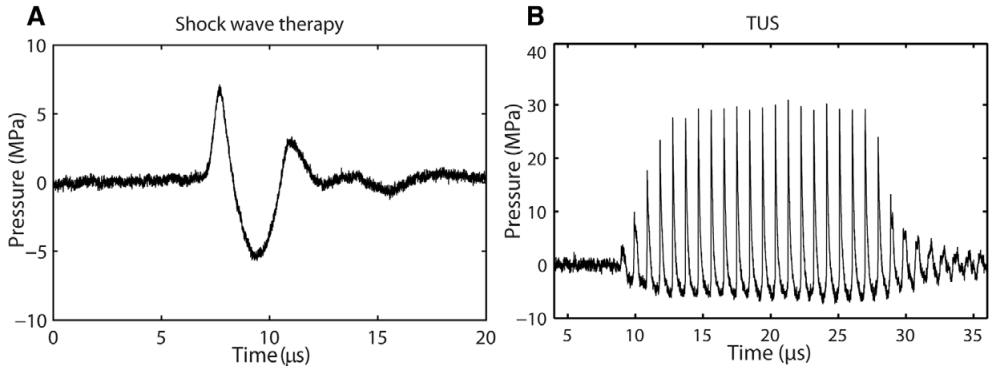

Figure 1. Representative acoustic/pressure waveforms of shock wave therapy (**A**; SWT) and therapeutic ultrasound (**B**; TUS). Acoustic parameters were matched in terms of p^- (–5.8 MPa). Displayed TUS pulse waveform (**B**) truncated to 30 cycles (1,054 cycles used for treatment).

Table. Comparison of SWT and TUS Acoustic and Treatment Parameters

	SWT	TUS
p^+ (MPa)	7.1	30.5
p^- (MPa)	–5.8	–5.8
Frequency (MHz)	NA	1.054
Pulse duration	Single shockwave	1 ms (1,054 waves)
PRF (Hz)	5	5
Shocks/pulses per spot	200	200
Duty cycle	NA	0.5%

NA, not applicable; p^-, peak negative pressure; p^+, peak positive pressure; PRF, pulse repetition frequency; SWT, shockwave therapy; TUS, therapeutic ultrasound.

and experimentally used for thermal ablation of solid tumors.[13] At lower intensities, TUS is used to promote acoustic cavitation (formation and oscillation of gas bubbles in liquid when exposed to ultrasound) in target tissue, for a variety of clinical applications, including chemotherapeutic drug delivery[14] and opening of the blood-brain barrier.[15] Only recently, TUS has been investigated as an acoustic treatment for IHD and PAD. TUS has been demonstrated to have pro-angiogenic effects in cultured endothelial cells,[16] chick chorioallantoic membrane,[17] and a porcine chronic IHD model.[18] Unlike SWT, which only allows control of pulse repetition frequency and amplitude, TUS is a more flexible technology, as it allows adjustment of all acoustic treatment parameters to optimize non-thermal bioeffects while limiting thermal damage. Thus, TUS may be a more effective method of acoustic energy delivery for angiogenesis applications, in comparison with SWT. However, no direct comparison between TUS and SWT has been made previously.

In this study, we aimed to match TUS and SWT by their peak negative pressures (p^-), as p^- is most relevant to the dominant ultrasound-mediated, non-thermal effects[19,20] such as cavitation. We then compared TUS and SWT with sham therapy to assess their effects on angiogenesis and reperfusion in a rat model of PAD.

Methods

Hindlimb Ischemia Rat Model

Thirty 8-week-old, female Sprague-Dawley rats were used for a femoral artery excision hindlimb ischemia model. Briefly, rats were anesthetized using inhaled isoflurane. After shaving and depilation, a 1.5 cm incision was made longitudinally along the ventral surface of the left hindlimb. The left femoral artery and all side branches were ligated with 7-0 silk suture between the inguinal ligament proximally and the popliteal fossa distally, and then excised. The skin was closed with 4-0 absorbable suture. Rats were allowed to recover for 3 days prior to initiation of treatments. All animal protocols were approved by the University of California Institutional Animal Care and Use Committee (IACUC).

Acoustic Devices

A Duolith SD1 (Storz Medical, Tägerwilen, Switzerland) SWT device was used. These SWT waveforms, including p^+ and p^-, were previously characterized at various energy settings in a degassed waterbath using a fiber optic hydrophone (FOPH 2000; RP Acoustics, Germany), as previously reported by our group.[21] The handheld transducer for this SWT device has a 50 mm focal depth, which was used with a 30 mm standoff coupling cone.

A focused US transducer was designed similarly to the Duolith SD1 SWT. This consisted of a lead-zirconate-titanate (PZT) 1.054 MHz transducer spherically focused to 63 mm. A 35-mm standoff coupling cone was applied, and filled with degassed water prior to treatments. The transducer was powered by a function generator (Agilent 33120A; Hewlett-Packard, Santa Clara, CA, USA) and a power amplifier (ENI 400B; E&I., Rochester, NY, USA). The peak focal acoustic pressures generated by this transducer at various applied voltages were measured in a similar manner to the SWT device. The voltage setting for TUS treatment was then chosen to match the corresponding peak negative pressure to that produced by SWT (–5.8 MPa) at its typical clinically used[9] energy level of 0.10 mJ/mm^2 (**Table**).

Acoustic Treatments

Rats were allocated to 3 groups of 10 each: TUS, SWT, sham. After 3 days of recovery, rats underwent treatment 3 times a

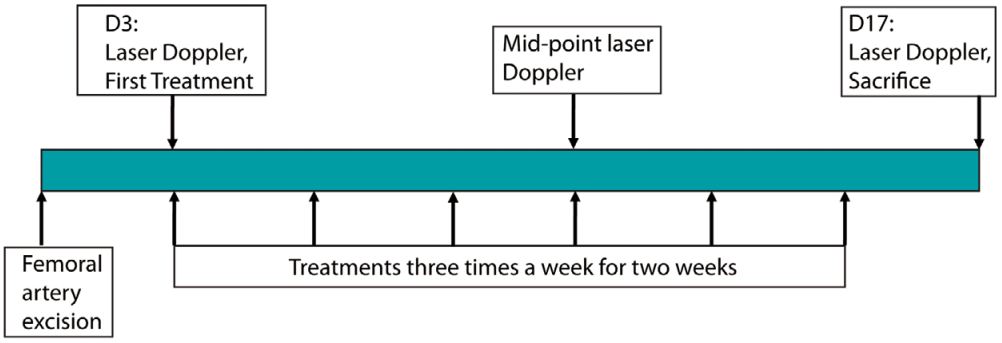

Figure 2. Experimental protocol timeline of left femoral artery excision, acoustic treatments, and laser Doppler imaging.

week for 2 weeks (**Figure 2**). At each treatment point, rats were anesthetized with inhaled isoflurane on a 37°C warming pad, the left hindlimb was depilated with depilatory cream, and they were then treated with either TUS, SWT or sham (in which rats were only anesthetized and depilated). The TUS and SWT transducers were coupled to the skin using Aquasonics 100 Ultrasound Transmission Gel (Parker Laboratories, Franklin, NJ, USA). Treatments were applied at 3 spots along the area of femoral artery excision, and 3 spots longitudinally along the left gastrocnemius muscle. Rats were monitored regularly for signs of acute or chronic limb ischemia, as well as skin changes at the site of treatments.

Laser Doppler Perfusion Imaging

Three days after left femoral artery excision (prior to first treatment), left hindlimb ischemia was verified using a MoorLDI Laser Doppler scanner (Moor Instruments, Devon, United Kingdom). Under a stable dose of isoflurane anesthesia, rats were positioned prone on a 37°C warming pad. Three consecutive laser Doppler scans of the plantar surfaces of both hindlimbs were performed. Laser Doppler flux units (directly proportional to the volume and velocity of red blood cells in tissue[22]) were averaged for each hindlimb, for all points distal to the ankle, across all 3 scans (**Figure 3**). Perfusion of the left ischemic hindlimb was reported as ratio of left:right mean laser Doppler flux. Scanning was repeated at the study midpoint (immediately prior to the fourth treatment), and at end of the study (immediately prior to sacrifice).

Muscle Histology and Immunohistochemistry (IHC)

At the end of the study, rats were euthanized in a flow-regulated CO_2 chamber. Bilateral gastrocnemius muscles were dissected and weighed. Muscles were suspended from cork paper by 10% Tragancanth (Sigma-Aldrich, St. Louis, MO, USA) and snap-frozen in 100% isopropanol cooled in a bath of liquid nitrogen. The muscles were sectioned on a cryostat to a thickness of 8 μm (100 μm apart, n=20 sections per muscle) and fixed in 4% paraformaldehyde. Hematoxylin and eosin (H&E) staining was performed to assess myocyte morphology. IHC was performed labeling endothelial cells with mouse anti-rat CD31 antibody and anti-mouse Ig horseradish peroxidase detection kit (both Beckton-Dickinson, East Rutherford, NJ, USA) on an eosin background stain. Random sections (6 per section) were imaged using light microscopy at 20× magnification, and myocytes and vessels were counted using an ImageJ software (National Institutes of Health, Bethesda, MD, USA) particle counting algorithm standardized across all 3 groups.

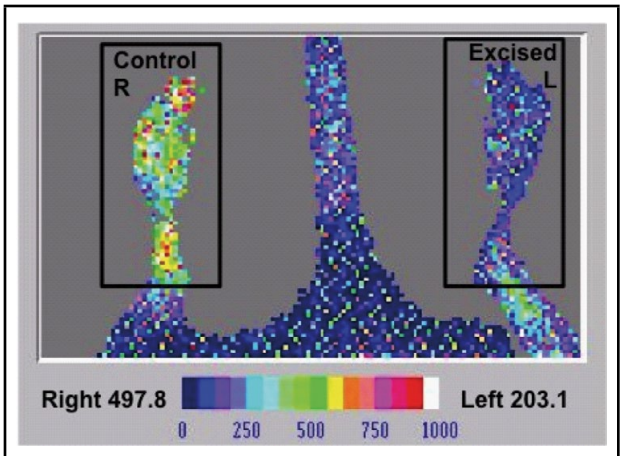

Figure 3. Laser Doppler scan of prone rat hindlimbs 3 days after left femoral artery excision. Flux units of all pixels above heel (in boxes) averaged for each hindlimb. Left hindlimb relative perfusion quantified as ratio of L:R mean flux (40.8%).

Quantitative Polymerase Chain Reaction

Mid-anterior gastrocnemius fragments of approximately 50 μg were homogenized in 500 μl Trizol reagent, and mRNA was isolated. RT-PCR was performed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) on a Bio-Rad S1000 Thermal Cycler. QPCR was performed for vascular endothelial growth factor (VEGF) and VEGF receptor 1 (FLT-1) using a Bio-Rad iQ SYBR Green Supermix kit on a Bio-Rad CFX Connect Real-Time PCR Detection System. mRNA expression of target genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the following primers:

VEGF	5'-GTCGGAGAGCAACGTCACTA-3',
	3'-TCACAGTGAACGCTCCAGGAT-5';
FLT-1	5'-GTGGCTGCGACACTCTTTTG-3',
	3'-CAAATGCCGAAGCCTGAACC-5';
GAPDH	5'-TGTGAACGGATTTGGCCGTA-3',
	3'-GATGGTGATGGGTTTCCCGT-5'.

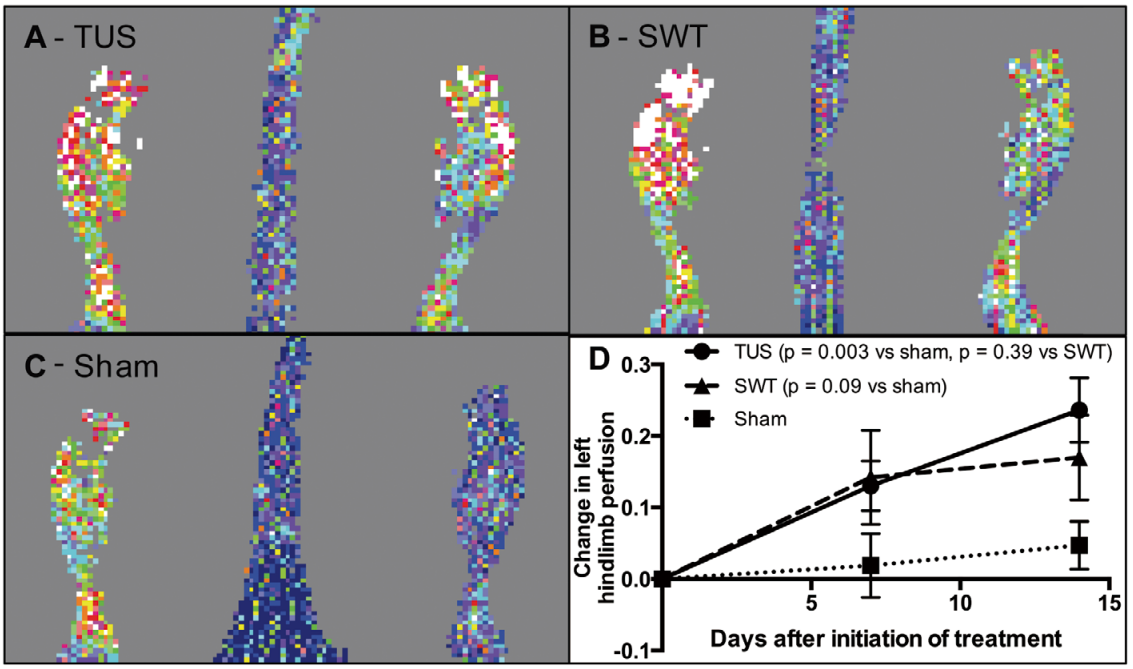

Figure 4. Laser Doppler scan after 2 weeks of therapeutic ultrasound (TUS) (**A**), shock wave therapy (SWT) (**B**) and sham (**C**) therapy. Reperfusion quantitated as change in L:R perfusion (**D**) over the course of 2 weeks' therapy.

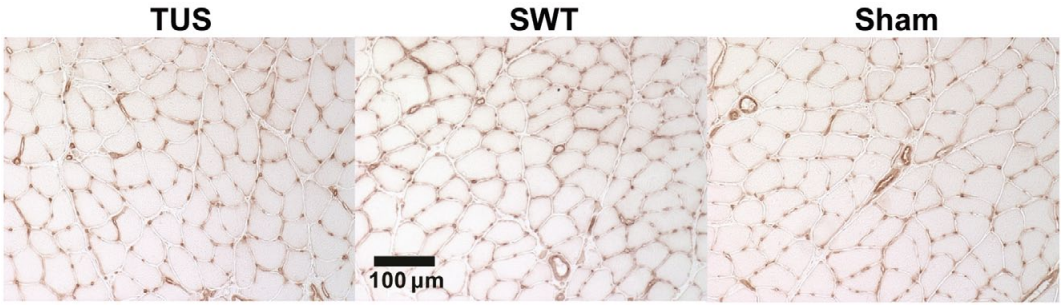

Figure 5. Immunohistochemistry of left (ischemic) gastrocnemius muscle cross-sections labeling endothelial cells with mouse anti-rat CD31 antibody and horseradish peroxidase on an eosin background stain. Representative images of gastrocnemius treated with therapeutic ultrasound (TUS) (**Left**), shock wave therapy (SWT) (**Middle**), and sham (**Right**). Microvascular density was quantified using ImageJ software (**Figure 6**).

Results

Hindlimb Perfusion

Within 3 days of femoral artery excision, rats were noted to have mild pallor of the left hindlimb compared to the sham-operated right hindlimb, as well as small (<3 mm), superficial skin ulceration on the plantar surface of the left paw with no evidence of critical limb ischemia, infection or gangrene. Pallor and ulceration qualitatively improved over the course of the study, with no clear difference between groups. There was no significant difference in the weight of the left gastrocnemius muscle, which was approximately 90% the weight of the right at the end of the study in all 3 treatment groups (**Figure S1**). Mean left hindlimb laser Doppler perfusion was 42±2% (mean±SEM; **Figure 3**), with small but non-significant differences between treatment groups prior to treatment initiation (TUS 39%, SWT 42%, Sham 45%, P>0.05 by ANOVA). By 2 weeks, left hindlimb perfusion in the TUS group had improved to 66±4% (P=0.02 vs. sham, P=0.33 vs. SWT), in the SWT group to 60±4% (P=0.13 vs. sham), and in the sham group to 50±4% (**Figures 4A–C**). To account for baseline differences in baseline perfusion differences, the change in hindlimb perfusion from post-excision/pre-treatment baseline was compared, and similar improvements in the

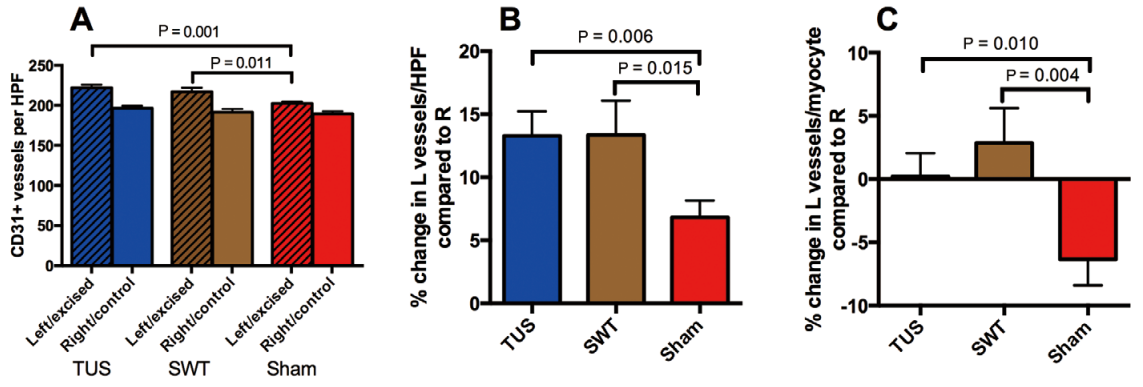

Figure 6. Quantitation of gastrocnemius microvascular density after 2 weeks of treatment. (**A**) Vessels/high power field (hpf) calculated for both limbs in all 3 treatment groups demonstrating increased microvascular density with therapeutic ultrasound (TUS) and shock wave therapy (SWT) compared with sham (P=0.37 comparing TUS and SWT). (**B**) Percent increase in vessels/hpf of left gastrocnemius over right gastrocnemius confirming increased angiogenesis with TUS and SWT treatment compared with sham (P=0.98 comparing TUS and SWT). (**C**) Percent change in left:right vessels/myocyte, normalizing for ischemic atrophy. Decrease of 6.4% in the sham group suggests increased left gastrocnemius vessels/hpf, as noted in (**A**) and (**B**), is due to ischemic atrophy. Both TUS and SWT led to slight increases in vessels/myocyte (P=0.40 comparing TUS and SWT).

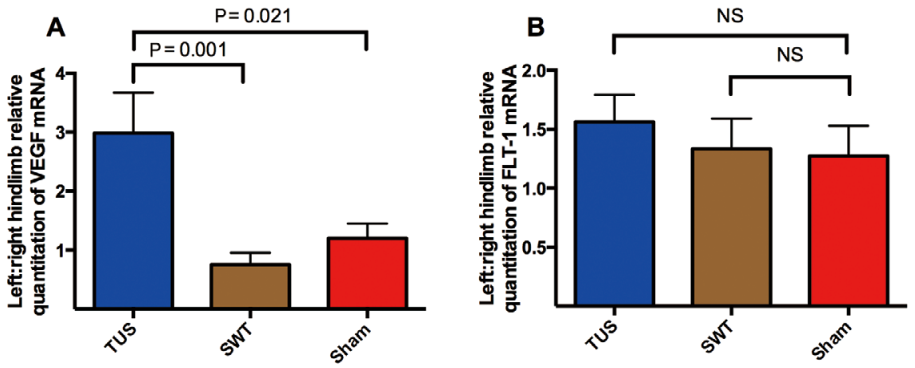

Figure 7. Quantitative polymerase chain reaction (QPCR) of homogenized gastrocnemius tissue after 2 weeks of treatment. (**A**,**B**) QPCR was performed in triplicate, mRNA levels were normalized to GAPDH, and expressed as a ratio of left:right gastrocnemius expression. FLT-1, vascular endothelial growth factor receptor 1; VEGF, vascular endothelial growth factor.

TUS and SWT groups compared with the sham group were demonstrated (**Figure 4D**).

Histologic and Microvascular Analyses

H&E sections demonstrated normal myocyte morphology, with no myocyte rounding, centralized nuclei, adipocyte or inflammatory cell infiltrate (**Figure S2**). Mild atrophy was noted, with decreased myocyte cross-sectional area in the left compared with right gastrocnemius (2,659±33 vs. 2,951±44 μm^2, P<0.001) resulting in an 11% decrease with no significant difference between treatment groups. Anti-CD31 IHC labeling of endothelial cells (**Figure 5**) demonstrated an increase in vessel count of the left ischemic hindlimb of both TUS-(222.6±3.8 vessels/high power field (hpf), P=0.001) and SWT-(216.9±5.2vessels/hpf, P=0.011) treated animals compared to sham-treated animals (196.0±3.8vessels/hpf). There was no statistically significant difference between TUS and SWT (P=0.37) (**Figure 6A**). When normalizing vascular density of the left to right hindlimb, TUS treatment resulted in a 13.3% increase in vessels per hpf in the left hindlimb, compared with 13.4% in the SWT group (P=0.98 vs. TUS), and 6.6% in the sham group (P=0.006 vs. TUS, P=0.015 vs. SWT) (**Figure 6B**). This increase in vascular density of the left ischemic hindlimb is largely abrogated when controlling for myocyte atrophy. Indeed, when comparing vessels per muscle fiber between groups, TUS resulted in a 0.2% increase in left hindlimb vessels/myocyte compared with a 2.9% increase with SWT (P=0.40 vs. TUS), and a 6.4% decrease for sham (P=0.01 vs. TUS, P=0.004 vs. SWT) (**Figure 6C**).

Molecular Mechanism of Pro-Angiogenic Effect

The cellular mechanism of angiogenesis induced by TUS or SWT has yet to be clarified. To assess effects of the 2 treatments on the VEGF pathway, QPCR analysis of mRNA levels

of *VEGF* and *FLT-1* normalized to *GAPDH* were performed in homogenates of gastrocnemius muscle from left and right hindlimbs. TUS treatment resulted in an ~3-fold increase in left hindlimb VEGF mRNA (P=0.001 compared with SWT, P=0.021 compared with sham; **Figure 7A**). There was no significant increase in VEGF mRNA with SWT or sham therapy (**Figure 7A**). All 3 groups demonstrated an upregulation of *FLT-1* mRNA (TUS 56%, SWT 26%, SWT 33%, P=0.64 by ANOVA) in the left ischemic hindlimb, with no significant differences between treatments (**Figure 7B**).

Discussion

In large animal and human studies, SWT has demonstrated the capacity to promote angiogenesis, tissue perfusion, and improvement in angina endpoints, leading to clinical use in several countries. TUS has more recently emerged as a comparable acoustic technology that may offer more effective treatment than SWT by its ability to deliver longer pulses of acoustic energy rather than single shocks. The opportunity to adjust a wide array of TUS acoustic parameters (frequency, pulse repetition frequency, pulse duration/duty cycle, p^+, p^-) may also allow optimization of its angiogenic, sub-lethal bioeffects while avoiding potentially harmful, thermal damage. However, this wide range of parameters also makes it difficult to directly compare the therapeutic effects of SWT and TUS. As non-thermal ultrasound bioeffects are known to be mediated by p^-, designing TUS pulses to match the p^- generated by clinically used SWT allowed us to make a direct comparison between the 2 therapies.

After left femoral artery excision and 2 weeks of treatment, TUS-, but not SWT-treated rats demonstrated improvements in hindlimb perfusion, as measured by laser Doppler and compared with sham-treated rats. Interestingly, SWT-treated rats demonstrated a trend toward improvement; however, the effect was not statistically significant compared with sham-treated rats. Histologic assessment of ischemic gastrocnemius muscle with IHC of CD31 demonstrated increased microvascular density with both TUS and SWT treatment of the left ischemic hindlimb. This result remained significant when quantifying absolute vessels (vessels/hpf) or when controlling for mild myocyte atrophy noted after femoral artery excision (vessels/myocyte).

The mechanism of angiogenesis mediated by TUS or SWT remains an active area of research. Our results demonstrate an elevation of VEGF expression after 2 weeks of treatment with TUS, but not with SWT or sham treatments. This finding may reflect inherent differences in tissue and molecular interactions of TUS and SWT, or differences in the time-courses of rise and fall of VEGF levels after these treatments. Levels of *FLT-1* mRNA were increased in the left hindlimb compared to the right hindlimb (control) in all 3 experimental groups, suggesting that the increase in *FLT-1* may be related more to the ischemia induced by femoral artery excision than to treatment. Further investigation of the mechanism of angiogenesis and reperfusion by TUS is warranted. Measurement of tissue VEGF and FLT-1 at various time points after excision/treatment may be helpful to distinguish different time-courses of the angiogenic response to TUS or SWT. Other cellular and molecular mechanisms that have been described in acoustic-mediated regeneration (anti-inflammatory effects,[23] nitric oxide pathway,[24,25] stem cell differentiation[25,26]) or repair response to PAD (satellite cell differentiation,[27] prostacyclin pathways[28]) should be compared between TUS and SWT.

In this study, TUS and SWT treatments were performed over a wide area of the rat hindlimb, encompassing the area around the femoral artery excision and the gastrocnemius muscle. Therefore, it is difficult to distinguish whether the findings are due to "collateralogenesis" around the area of femoral artery excision or direct angiogenesis in the distal ischemic tissue. However, our finding of increased microvascular density in gastrocnemius muscle confirms a direct angiogenic effect. Furthermore, an increase in collateral vessels is likely to be involved, as laser Doppler assesses distal perfusion to the paw, which was not treated by TUS or SWT. Additionally, while p^- was matched for TUS and SWT treatment, it is possible that differences in p^+ or other acoustic parameters, which were not able to be perfectly matched, may have contributed to observed differences.

In summary, our study is the first side-by-side comparison of TUS and SWT treatment modalities, and demonstrates that with 2 weeks of treatment with matched p^-, TUS but not SWT was able to promote significant reperfusion in a rat model of PAD. Both technologies similarly increased microvascular density in ischemic gastrocnemius muscle, while only TUS was associated with an increase in tissue VEGF levels. Future comparison studies at various p^- or other acoustic parameters are necessary for TUS to progress from animal studies to human trials to clinical care, and may reveal: (1) further differences in the therapeutic effects of SWT and TUS; and (2) the molecular mechanisms in mediating angiogenesis.

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Disclosures

There are no conflicts of interest.

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Supplementary Files

Supplementary File 1

Figure S1. Weight of left (ischemic) gastrocnemius muscle normalized to right, 17 days after left femoral artery excision.

Figure S2. Hematoxylin and eosin-stained cross-sections of left (ischemic) gastrocnemius muscle treated with TUS (**Left**), SWT (**Middle**), and sham (**Right**).

Please find supplementary file(s);
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