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Comparison of the effects of 1 MHz and 3 MHz therapeutic ultrasound on endothelium-dependent vasodilation of humans: a randomised clinical trial

M. Hauck [a], C. Noronha Martins [a], M. Borges Moraes [a], P. Aikawa [a], F. da Silva Paulitsch [a], R. Della Méa Plentz [b], S. Teixeira da Costa [c], A.M. Vargas da Silva [c], L.U. Signori [a,c,*]

[a] *Institute of Biological Sciences, Federal University of Rio Grande, RS, Brazil*
[b] *Graduate Programme in Healthy Sciences, Federal University of Health Sciences of Porto Alegre, RS, Brazil*
[c] *Graduate Programme in Physiotherapy and Rehabilitation, Federal University of Santa Maria, RS, Brazil*

Abstract

Objective To compare the effects of different waveforms of 1 MHz and 3 MHz therapeutic ultrasound on endothelial function in healthy subjects.
Design Randomised placebo-controlled, crossover study with concealed allocation and assessor blinding.
Setting Imaging Centre of the University Hospital.
Participants Thirty volunteers aged between 18 and 35 years were divided into two homogeneous groups (1 MHz and 3 MHz).
Interventions Continuous (CUT; 0.4 W/cm^{2SATA}), pulsed (PUT; 20% duty cycle, 0.08 W/cm^{2SATA}) and placebo waveforms (equipment off) of ultrasound (1 MHz and 3 MHz) were randomized and applied over the brachial artery for 5 minutes.
Main outcome measures Endothelial function was evaluated using the flow-mediated dilation (FMD) technique.
Results Both 1 MHz [CUT: mean difference 4%, 95% confidence interval (CI) 2 to 6%, $P < 0.001$; PUT: mean difference 4%, 95% CI 2 to 6%, $P < 0.001$] and 3 MHz (CUT: mean difference 4%, 95% CI 2 to 6%, $P < 0.001$; PUT: mean difference 4%, 95% CI 2 to 6%, $P < 0.001$) of therapeutic ultrasound increased %FMD by approximately 4% compared with the placebo waveforms. The endothelium-dependent vasodilator responses were the same for both types of waves and frequencies. No differences in baseline diameter, hyperaemic flow, and nitroglycerin-mediated diameter and vasodilation were observed between groups.
Conclusion Both CUT and PUT ultrasound waveforms improved endothelial function. The 1 MHz and 3 MHz frequencies of therapeutic ultrasound led to similar improvement in endothelial function in healthy volunteers.

Clinical trial registration number RBR-4z5z3t.

Keywords: Endothelial function; Vascular endothelium; Ultrasonic therapy; Ultrasound; Nitric oxide

Introduction

Therapeutic ultrasound is a form of acoustic energy commonly used in physiotherapy because of its biological effects through thermal and mechanical mechanisms [1]. Thermal and mechanical effects of ultrasound occur simultaneously, so it is inadequate to assume that the thermal effects correspond to exposure to continuous waves and mechanical effects correspond to exposure to pulsed waves [2]. However, these effects are dose-dependent on application parameters that can be manipulated, such as wave type [continuous ultrasound (CUT) or pulsed ultrasound (PUT)], wave frequency

* Corresponding author at: Centro de Ciências da Saúde, Curso de Fisioterapia, Universidade Federal de Santa Maria — UFSM, Av. Roraima no. 1000, Cidade Universitária, Bairro Camobi, 97105-900, Santa Maria, RS, Brazil.
E-mail address: l.signori@hotmail.com (L.U. Signori).

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(1 or 3 MHz), intensity (0.1 to 3 W/cm^{2SATA}), duty cycle (%) and application time (minutes). In addition, the effects on biological tissue are important for better treatment [2,3]. The molecular and cellular action of therapeutic ultrasound is the goal of treatment; however, the mechanisms and parameters are still not fully understood [4]. Clinical trials and experimental studies have revealed that therapeutic ultrasound is able to improve endothelial function [5,6], and increase endothelial NO release [1,7] and vascular angiogenesis [8].

Endothelial function is an important humoral regulator of vessel tone, mainly because of the production of nitric oxide (NO) produced by the conversion of arginine to citruline by NO synthase [1]. This molecule acts together with prostacyclin and endothelium-derived hyperpolarising factor (EDHF) to maintain vascular balance [9–11]. The inhibition of platelet aggregation, suppression of smooth muscle cell proliferation and stimulation of angiogenesis are performed by endothelial cells, which are located in the strategic area between blood and vascular smooth muscle of the cardiovascular system [9–11]. Recent clinical studies have demonstrated that endothelial function evaluated by the flow-mediated dilation (FMD) technique is a predictor of cardiovascular events [9,12,13] and all-cause mortality [13].

By altering various technical therapeutic ultrasound parameters, especially transducer wave frequency, physiotherapists can ensure that the ultrasonic wave reaches deeper tissue layers [14]. Frequencies usually have a depth-dependent effect on tissues, because there is less energy attenuation in surface tissues at lower frequencies, which allows more energy to be available for absorption in deeper tissues [15]. A wave frequency of 3 MHz is absorbed at a depth of approximately 1 to 2 cm and has surface action at skin level [2,3]. Previous studies demonstrating improvement in endothelial function after the application of ultrasound have used low wave frequencies (in kHz) [1,5,7,8] or 1 MHz [6]; however, to the authors' knowledge, no studies to date have evaluated the endothelial response using a wave frequency of 3 MHz. Thus, the aim of this study was to compare the effects of CUT and PUT waveforms of 1 MHz and 3 MHz therapeutic ultrasound on the endothelial function of humans.

Methods

Design

This randomized placebo-controlled crossover study with concealed allocation and assessor blinding was approved by the institutional ethics committee, and was registered in the Brazilian Clinical Trials Registry (Protocol: RBR-4z5z3t).

Initially, a sample of 30 healthy volunteers was divided into two homogeneous groups: the 1 MHz group and the 3 MHz group. Each group was organised into three blocks to perform a crossover study, and the randomised volunteers were submitted to the following treatments: CUT, PUT or placebo waveforms. The randomisation was performed using software (www.random.org) by a blinded researcher (LUS), and the information was sealed in a brown envelope. The volunteers and evaluators (MH, AMVS) were blinded to the type of intervention. %FMD was measured before and immediately after the application of therapeutic ultrasound (Fig. A, see supplementary material in the online version at DOI:10.1016/j.physio.2017.08.010).

Participants

All participants were aged between 18 and 35 years with body mass index (BMI) <30 kg/m^2; non-smokers; free of cutaneous/subcutaneous lesions in the left arm; and free of skeletal muscle, rheumatic, cardiovascular, metabolic, neurological, oncological, immunological, haematological, psychiatric and cognitive disorders. The enrolled participants were not taking any type of medication.

Before and during the interventions, the volunteers were asked not to drink alcohol, coffee or citrus juice, and not to perform intense physical activity 72 h before the evaluations. The exclusion criteria were: arterial diameter <0.25 mm or >5 mm, endothelial dysfunction assessed by FMD (%FMD <8%), inflammatory response (C-reactive protein >3 mg/dL, fibrinogen <200 or >400 mg/dL), and leukocytosis (>11,000 × 10^3/mm^3). Two of the enrolled participants were not included due to the exclusion criteria. Data were collected between January and December 2014 at the Imaging Centre of the University Hospital.

Intervention

The ultrasound equipment (Ultrasound Therapy, Sonopulse III 1 and 3 MHz, IBRAMED, Amparo, SP, Brazil) was calibrated by the Biocare Medical System (Passo Fundo, RS, Brazil) before and after the study [16,17]. The transducer had a diameter of 4.5 cm (No. TR3CCE02), with an effective radiation area of 7 cm^2, and was applied in a stationary mode over the brachial artery above the antecubital fossa of the left arm using a commercially available ultrasound gel as a conduction agent. CUT was applied for 5 minutes at an intensity of 0.4 W/cm^2 spatial averaged-temporal intensity (SATA). PUT was applied with a 20% duty cycle (2 milliseconds on, 8 milliseconds off), which represents 0.08 W/cm^{2SATA} [6,16–18]. The sessions were performed on three consecutive days. The placebo session was performed in the same way but with the equipment turned off.

Clinical and biochemical evaluation

The initial evaluation included anthropometric variables (body weight, height and BMI) and blood samples collected in the fasting state (12 hours). This was undertaken on the first day of intervention.

Haemogram blood tests (erythrogram and leukogram) were processed automatically using ABX kits (Horiba Diag-

nóstica, Curitiba, PR, Brazil) and microscopy. Cholesterol, triglycerides, high-density lipoproteins and urea were analysed using LAB MAX 240 (Tokyo, Japan) equipment. Concentrations of low-density lipoproteins were calculated using Friedewald's formula. Glucose levels were measured using the Trinder assay (calorimetry), and glycosylated haemoglobin was determined by enzymatic methods, both with LAB MAX 240. Insulin was assessed by the chemiluminescence method using Immulite (Diagnostic Products Corporation, Los Angeles, CA, USA). Fibrinogen was analysed using START equipment (Diagnóstica Stago, Asnieres, France) using LAB TEST commercial tests (Lagoa Santa, MG, Brazil). Ultra-sensitive C-reactive protein was evaluated by nephelometry (Nephelometer Beckman Coulter, IMMAGE, Fullerton, CA, USA).

Evaluation of endothelial function

Arterial endothelium-dependent vasodilation was evaluated by FMD in the same place where the ultrasound was applied, with high-resolution vascular ultrasound and a 5 to 12 MHz linear transducer (Logiq P6, GE Healthcare, GE Ultrasound Korea Ltd, Seoul, Korea) according to the American Heart Association Guidelines and adjustments [5,6,12,19]. Briefly, after adjustments, the baseline image was acquired, and changes in the brachial artery diameter until 60 seconds of reactive hyperaemia after deflation of the cuff positioned around the upper arm and inflated to 50 mmHg above systolic blood pressure for 5 minutes were compared with baseline measures. Mid-artery pulsed Doppler velocity signals were used to evaluate basal blood flow and flow immediately after cuff release (obtained no later than 15 seconds after cuff deflation). Brachial artery diameter was measured at the same time in the cardiac cycle by electrocardiographic monitoring during image acquisition, and data were calculated as an average of three consecutive heart cycles (three measurements per cycle) at basal evaluation and after the intervention. Onset of the R-wave was used to identify end-diastole, which corresponds to the minimum diameter of the artery. The increased diameter after a sublingual nitroglycerin spray (0.4 mg) was used as a measurement of endothelium-independent vasodilation. Vessel diameter responses to reactive hyperaemia and nitroglycerin are expressed as percentage changes in relation to diameter before cuff inflation and before drug administration [5,6,12,20]. Measurements of brachial artery diameter were accomplished offline by two evaluators (blind measures) after interventions. Differences >0.01 mm between the assessors (mean vessel diameter) were repeated.

Outcome measures

Primary outcome

The primary outcome measure was endothelial function, as measured by %FMD before and after application of therapeutic ultrasound.

Secondary outcomes

Secondary outcome measures were endothelium-dependent vasodilation (as evaluated by vascular response to nitroglycerin after FMD), baseline arterial diameter, hyperaemic arterial diameter, baseline flow and hyperaemic flow, as measured by FMD.

Data analysis

Sample size

On the basis of a previous study [6], it was estimated that a sample size of 15 volunteers in each study group (ultrasound and placebo) would have a power of 80% to detect a 2% difference between means of %FMD with 2% standard deviation (SD) for $\alpha = 0.05$.

Results are presented as mean (SD). Variations between interventions are reported as mean differences and 95% confidence intervals (95% CI). The distribution of variables was tested by the Kolmogorov–Smirnov normality test. In the statistical analysis, Student's *t*-test was applied in order to compare the means or two repeated measures. Two-way analysis of variance with repeated measures (waveforms, time and interaction) was applied, followed by the Bonferroni post hoc test. Categorical variables were evaluated by Fisher's exact test and expressed as proportions. Pearson's correlation coefficient was calculated to show the relationship between ultrasound effect and brachial artery %FMD, and the reproducibility of the ultrasound effect. Differences were considered to be significant when $P < 0.05$.

Results

Flow of participants

There was no significant difference in mean age [1 MHz group 26.6 (SD 5.4) years, 3 MHz group 27.1 (SD 5.2) years, $P = 0.785$] or BMI [1 MHz group 24.3 (SD 3.4) kg/m^2, 3 MHz group 23.7 (SD 3.1) kg/m^2, $P = 0.591$] between the two groups. Systolic blood pressure [1 MHz group 108 (SD 9.6) mmHg, 3 MHz group 111.3 (SD 8.9) mmHg, $P = 0.333$] and diastolic blood pressure [1 MHz group 74.3 (SD 6.3) mmHg, 3 MHz group 75.3 (SD 5.5) mmHg, $P = 0.647$] were within normal limits in both groups. The basic attributes of the clinical and metabolic characteristics of the fasting participants are presented in Table A (see supplementary material in the online version at DOI:10.1016/j.physio.2017.08.010).

Ultrasound measurements in the brachial artery

Measurements of the brachial artery in response to different wave frequencies of therapeutic ultrasound are shown in Table 1. Nitroglycerin resulted in no modifications in baseline diameter ($P = 0.746$), hyperaemic flow ($P = 0.798$), hyperaemic diameter ($P = 0.834$) or %FMD ($P = 0.730$) for either of the ultrasound frequencies used. CUT and PUT waveforms

Table 1
Results of ultrasound measurements in the brachial artery.

Endothelial function	Frequency	Basal	Placebo	PUT	CUT	Group	Time	Interaction
Baseline diameter (mm)	1 MHz	3.51 (0.7)	3.52 (0.7)	3.51 (0.7)	3.54 (0.7)	0.746	0.773	0.401
	3 MHz	3.36 (0.6)	3.45 (0.6)	3.43 (0.6)	3.42 (0.6)			
Hyperaemic diameter (mm)	1 MHz	3.89 (0.7)	3.89 (0.7)	3.99 (0.7)[a,b]	4.05 (0.7)[a,b]	0.789	<0.001	0.583
	3 MHz	3.85 (0.6)	3.85 (0.6)	3.94 (0.6)[a,b]	3.95 (0.6)[a,b]			
Hyperaemic flow (ml/min)	1 MHz	194 (116)	244 (177)	234 (117)	239 (128)	0.798	0.504	0.908
	3 MHz	222 (128)	233 (125)	247 (120)	245 (139)			
NMD diameter (mm)	1 MHz	4.06 (0.8)	4.08 (0.8)	4.22 (0.8)	4.11 (0.8)	0.834	0.712	0.501
	3 MHz	4.10 (0.8)	4.24 (4.7)	4.20 (0.8)	4.16 (0.8)			
NMD (%)	1 MHz	25.0 (4.1)	26.1 (5)	24.9 (5.1)	26.8 (4.2)	0.730	0.291	0.459
	3 MHz	25.1 (3.9)	25.2 (3.7)	26.2 (4.7)	26.2 (4.7)			

Basal, average of three baseline measurements; PUT, pulsed ultrasound therapy; CUT, continuous ultrasound therapy; NMD, nitroglycerin-mediated vasodilation. All values are presented as mean (standard deviation).
[a] Compared with baseline measures.
[b] Compared with placebo.

of the 1 MHz frequency increased the diameter of the brachial artery after reactive hyperaemia compared with the baseline evaluation ($P < 0.001$ for CUT, mean difference 0.16 mm, 95% CI 0.05 to 0.26 mm; $P < 0.001$ for PUT, mean difference 0.09 mm, 95% CI 0.00 to 2.03 mm), and when compared with the placebo group ($P < 0.001$ for CUT, mean difference 0.16 mm, 95% CI 0.05 to 0.26 mm; $P < 0.001$ for PUT, mean difference 0.10 mm, 95% CI 0.05 to 2.0 mm). Similar results were reported for the 3 MHz frequency for both waveforms compared with the baseline evaluation ($P < 0.001$ for CUT, mean difference 0.10 mm, 95% CI 0.00 to 0.21 mm; $P < 0.001$ for PUT, mean difference 0.09 mm, 95% CI 0.00 to 2.00 mm) and the placebo group ($P < 0.001$ for CUT, mean difference 0.1 mm, 95% CI 0.00 to 0.21 mm; $P < 0.001$ for PUT, mean difference 0.09 mm, 95% CI 0.00 to 2.00 mm). There were no significant differences in diameter and other variables after reactive hyperaemia between the CUT and PUT waveforms of the 1 MHz and 3 MHz frequencies (mean difference 0.06 mm, 95% CI -0.04 to 0.16 mm; mean difference 0.01 mm, 95% CI -0.10 to 0.11 mm, respectively).

Endothelium-dependent vasodilation

The %FMD measurements in response to different waveforms (CUT and PUT) and different wave frequencies (1 MHz and 3 MHz) of therapeutic ultrasound are shown in Fig. 1. For 1 MHz frequency, %FMD increased for both CUT (mean difference 4%, 95% CI 2 to 6%, $P < 0.001$) and PUT (mean difference 3%, 95% CI 1 to 5%, $P < 0.001$) waveforms compared with the baseline evaluation. When compared with the placebo group, %FMD increased by 4% for CUT (95% CI 2 to 6%, $P < 0.001$) and by 4% for PUT (95% CI 2 to 6%, $P < 0.001$). For 3 MHz frequency of therapeutic ultrasound, CUT (mean difference 4%, 95% CI 2 to 6%, $P < 0.001$) and PUT (mean difference 4%, 95% CI 2 to 6%, $P < 0.001$) waveforms caused an increase in %FMD compared with the baseline evaluation. In relation to the placebo group, there

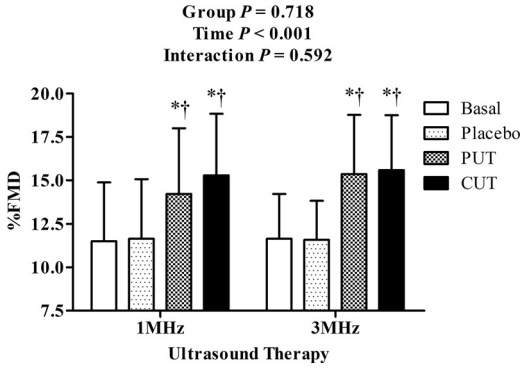

Fig. 1. Therapeutic ultrasound effects on endothelial function. FMD, flow-mediated vasodilation; PUT, pulsed ultrasound therapy; CUT, continuous ultrasound therapy.

was an increase of 4% for CUT (95% CI 2 to 6%, $P < 0.001$) and 4% for PUT (95% CI 2 to 6%, $P < 0.001$).

The type of wave (mean difference 0%, 95% CI -3 to 4% for CUT; mean difference 1%, 95% CI -3 to 5% for PUT; $P = 0.712$) and different ultrasound frequencies (mean difference 1%, 95% CI -1 to 3% for 1 MHz; mean difference 0%, 95% CI -2 to 2% for 3 MHz; $P = 0.718$) did not modify %FMD (group: $P = 0.537$; time: $P = 0.271$; interaction: $P = 0.473$). The reproducibility of the ultrasound effects is shown in Fig. B (see supplementary material in the online version at DOI:10.1016/j.physio.2017.08.010). The results show strong correlation (superior results to $r > 0.7$) between brachial artery %FMD basal and vasodilation in response to different wave frequencies (1 MHz and 3 MHz) and different waveforms (CUT and PUT) of therapeutic ultrasound Fig. B.

Discussion

This study found that both CUT and PUT waveforms of 1 and 3 MHz therapeutic ultrasound increased the brachial artery diameter and improved the

endothelium-dependent vasodilator function in healthy volunteers. Anti-inflammatory, proliferative and remodelling actions of therapeutic ultrasound are dependent on the application parameters that can be modified. In the present study, the parameters were manipulated by the type of wave and frequency [2,3].

The appropriate wave frequency is normally between 0.8 and 3 MHz, and is important for the production of a penetration pattern and generation of thermal and acoustic effects in tissues [15]. A frequency of 3 MHz causes therapeutic effects in superficial tissues, and is absorbed at a depth of approximately 1 to 2 cm [2,3]. The ultrasonic energy penetration depth is inversely proportional to frequency, and this energy is absorbed rapidly by tissues, causing superficial therapeutic effects [2,3]. However, the present study showed that even with lower penetration frequency, CUT and PUT waveforms of 3 MHz ultrasound were able to improve endothelial function.

The improvement in endothelial function evaluated by FMD in this study has been reported in a previous clinical study that used frequencies of 29 kHz [5] and 1 MHz [6]. Furthermore, Iida *et al.* [5] showed that the application of PUT wave (30% duty cycle) of low-frequency therapeutic ultrasound (0.12 W/cm^{2SATA}) improved the endothelial function of healthy volunteers. These clinical results are predominantly caused by NO, because prostacyclin was inhibited [6] and EDHF acts mainly in small blood vessels [21,22]. Additionally, the low-frequency ultrasound (27 kHz, 0.25 W/cm^{2SATA}, 10 minutes) enhanced NO production in endothelial cells from 10 seconds, and for 30 minutes after exposure [1]. In humans, this effect lasts for approximately 20 minutes after the application of 29 kHz [5] and 1 MHz [6] therapeutic ultrasound. The abovementioned studies suggest that 29 kHz, 1 MHz and 3 MHz frequencies have similar effects on the endothelial function of the brachial artery.

Established biological effects of therapeutic ultrasound are the result of pressure waves and an increase in temperature [23–25]. The mechanical effects occur due to the propagation of ultrasonic pressure waves through tissues that stimulate the formation of stable microbubbles (acoustic streaming and microstreaming) [7,24] and induce shear stress over the endothelial cells [23,25]. These mechanisms are transformed into specific cellular signals which enhance NO bioavailability [11,23] and, consequently, the endothelium-dependent vasodilation [11]. The application of therapeutic ultrasound in stationary mode (especially with CUT waves) can induce the risk of endothelial damage, especially in in-vitro experiments, but they are much less likely to occur *in vivo* [2], as shown in a previous study [6]. In the present study, stationary mode ultrasound was used to attenuate the mechanical effect of the ultrasound head on the brachial artery and to generate shear stress.

Thermal effects are frequency dependent, although the heating depth of the 0.86 MHz, 1 MHz, 2 MHz and 3 MHz wave frequencies remained the same [15], which suggests that a lower frequency does not cause a deep heating pattern. In previous clinical studies using low [5] and high [6] wave frequencies to investigate endothelial function, it was not possible to observe any increase in skin temperature. The authors suggest that other application parameters must be considered [2], particularly an effective low intensity (such as used in the present study) and other parameters [5,6] that do not cause heat generation [26]. The presence of thermal effects cannot be disregarded, especially for CUT waves [29], as the thermal gradient at the skin activates the vasodilator nerves which mediate a decrease in smooth muscle tone, leading to arteriolar vasodilation and an increase in skin blood flow [27,28]. However, this study found no difference in the improvement of endothelial function between the two waveforms, suggesting that the main effects are due to the mechanical effects of waves on vascular reactivity.

The 3 MHz therapeutic ultrasound has superficial and local vascular effects that act by inhibiting platelet aggregation, suppressing smooth muscle cell proliferation, and stimulating angiogenesis as endothelial cells are responsible for these actions [9–11]. An increase in endothelium-dependent vasodilation is important because systemic vascular homeostasis is dependent of NO vasodilator action [11,30] for musculoskeletal tissue repair [30] and angiogenesis [8], which justifies the clinical importance of these results. This way, these essential actions on blood vessels justify and support the use of 3 MHz ultrasound for the treatment of acute cutaneous and subcutaneous lesions, such as in the dermatological area, especially burns and scars as they do not cause tissue heating. The lack of evaluation of the depth of the brachial artery and tissue temperature are limitations of this study, that contributed to clarify the impact of ultrasound effects on vascular reactivity.

Conclusion

This study showed that CUT and PUT waveforms of therapeutic ultrasound at frequencies of 1 and 3 MHz cause vasodilation in the brachial artery, and improve endothelial function. Although the 3 MHz wave frequency reaches more superficial regions in the cutaneous and subcutaneous levels, its ultrasonic wave has the same effect on endothelial function as the 1 MHz wave frequency

Ethical approval: This study was approved by the Health Research Ethics Committee (CEP/FURG No. 88/2012) of the Federal University of Rio Grande, and was registered in the Brazilian Clinical Trials Registry (Protocol RBR-4z5z3t).

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Conflict of interest: None declared.

Key messages

- Continuous and pulsed therapeutic ultrasound waveforms are able to improve endothelial function.
- This improvement in endothelial function is independent of the wave frequency (1 MHz and 3 MHz) of therapeutic ultrasound.
- The vascular effects of therapeutic ultrasound act by inhibiting platelet aggregation and leukocyte adhesion, suppressing smooth muscle cell proliferation, and stimulating angiogenesis and tissue repair.

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