

● *Original Contribution*

ULTRASOUND-ENHANCED THROMBOLYSIS USING DEFINITY[®] AS A CAVITATION NUCLEATION AGENT

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Abstract—Ultrasound has been shown previously to act synergistically with a thrombolytic agent, such as recombinant tissue plasminogen activator (rt-PA) to accelerate thrombolysis. In this *in vitro* study, a commercial contrast agent, Definity[®], was used to promote and sustain the nucleation of cavitation during pulsed ultrasound exposure at 120 kHz. Ultraharmonic signals, broadband emissions and harmonics of the fundamental were measured acoustically by using a focused hydrophone as a passive cavitation detector and used to quantify the level of cavitation activity. Human whole blood clots suspended in human plasma were exposed to a combination of rt-PA, Definity[®] and ultrasound at a range of ultrasound peak-to-peak pressure amplitudes, which were selected to expose clots to various degrees of cavitation activity. Thrombolytic efficacy was determined by measuring clot mass loss before and after the treatment and correlated with the degree of cavitation activity. The penetration depth of rt-PA and plasminogen was also evaluated in the presence of cavitating microbubbles using a dual-antibody fluorescence imaging technique. The largest mass loss (26.2%) was observed for clots treated with 120-kHz ultrasound (0.32-MPa peak-to-peak pressure amplitude), rt-PA and stable cavitation nucleated by Definity[®]. A significant correlation was observed between mass loss and ultraharmonic signals ($r = 0.85$, $p < 0.0001$, $n = 24$). The largest mean penetration depth of rt-PA (222 μm) and plasminogen (241 μm) was observed in the presence of stable cavitation activity. Stable cavitation activity plays an important role in enhancement of thrombolysis and can be monitored to evaluate the efficacy of thrombolytic treatment. (E-mail: Christy.Holland@uc.edu) © 2008 World Federation for Ultrasound in Medicine & Biology.

Key Words: Ultrasound-assisted thrombolysis, Stroke therapy, Ultraharmonics, Stable cavitation, Therapeutic ultrasound.

INTRODUCTION

The use of ultrasound (US) to enhance thrombolysis has many potential benefits in treatment of diseases like ischemic stroke, myocardial infarction and deep vein thrombosis, which are caused by thrombus formation. Intravenous administration of recombinant tissue plasminogen activator (rt-PA) is the only clinically approved therapy for limited cases of ischemic stroke (NINDS Stroke Study Group 1995; Broderick et al. 2002). In several *in vitro* studies, US has been used to demonstrate thrombolytic enhancement when used as an adjuvant to rt-PA (Lauer et al. 1992; Francis et al. 1992; Blinc et al. 1993). Several studies have also shown that better penetration of US through skull, as well as enhanced fibrin-

olysis can be achieved using frequencies in the 20 to 500-kHz range (Blinc et al. 1993; Suchkova et al. 1998; Akiyama et al. 1998; Behrens et al. 1999; Coussios et al. 2002). Theoretically, Diamond and Anand (1993) demonstrated that the process of thrombolysis is limited by diffusion of fibrinolytic enzymes into the clot. This was experimentally demonstrated by Blinc et al. (1992). In several mechanistic *in vitro* studies, it is speculated that US facilitates transport of fibrinolytic enzymes into the clot (Francis et al. 1995; Pieters et al. 2004; Devcic-Kuhar et al. 2004), along with mechanical effects like microstreaming (Sakharov et al. 2000) and acoustic cavitation (Everbach and Francis 2000; Datta et al. 2005, 2006; Prokop et al. 2007) among other possible mechanisms.

Tachibana (1995) introduced the use of microbubbles to augment thrombolysis using 170-kHz US adjuvant to urokinase in an *in vitro* human blood clot model. They suggested the use of diagnostic contrast

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agents for therapeutic augmentation of thrombolytic drugs. Xie et al. (2005) demonstrated the effectiveness of lipid-encapsulated microbubbles and 1-MHz US in the recanalization of arteriovenous graft thrombi in an animal model. They observed a significantly greater clearing of thrombus using higher intensities (10 W/cm^2) compared with lower intensities (0.4 to 0.6 W/cm^2) and attributed this effect to the presence of cavitation. Molina et al. (2006) administered microbubbles and rt-PA, along with 2-MHz US and accelerated clot lysis in clinical trials. Although this approach appears promising, the mechanisms responsible for this US enhancement are still not well understood. In a previous study, Datta et al. (2006) monitored subharmonic emissions as a result of stable cavitation and correlated this type of bubble behavior with clot mass loss. The detection of subharmonic or ultraharmonic signals provides a possible method to monitor the progress of US enhancement of thrombolysis. Such techniques to monitor and measure cavitation activity during therapy are needed to monitor and perhaps even control thrombolytic progress.

The objective of this investigation was to determine whether 120-kHz pulsed US and an infusion of an echo contrast agent could enhance clot lysis and penetration of rt-PA into human whole blood clots *in vitro*. The hypotheses tested in this study are (a) whether the use of a contrast agent (Definity®) with 120-kHz pulsed US results in a measurable increase in stable cavitation activity over the treatment duration of 30 min and (b) whether the presence of sustained stable cavitation activity results in enhanced thrombolysis and increased penetration of rt-PA into the clots.

MATERIALS AND METHODS

Ultrasound thrombolysis system overview

A prototype 6.14-cm-diameter single circular element, unfocused, 120-kHz transducer (Sonic Concepts Inc., Woodinville, WA, USA) was used for sonication of clots as described previously (Datta et al. 2006). The transducer used in this study has a -3 dB beamwidth of 2.5 cm and a Rayleigh distance of 10.6 cm. An illustration of the experimental set up is shown in Fig. 1. The 120-kHz transducer was driven by a function generator (Agilent 33120A, Palo Alto, CA, USA), power amplifier (75A250, AR Amplifier Inc., Souderton, PA, USA) and a custom-built impedance matching network (Sonic Concepts Inc.). For all experiments described in this study, the 120-kHz transducer was driven in pulsed mode, with an 80% duty cycle and 1,667-Hz pulse repetition frequency consistent with previous studies (Datta et al. 2006; Holland et al. 2002). The sample chamber, consisting of an acoustically transparent latex condom, was placed at the Rayleigh distance of the 120-kHz

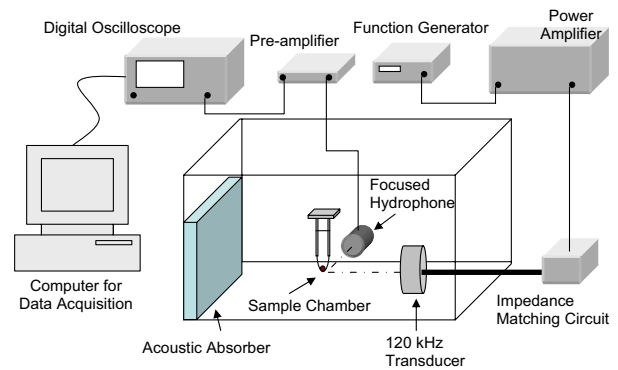


Fig. 1. Experimental setup for US exposure of human blood clots placed in a sample holder containing plasma and Definity®. A focused hydrophone placed 90° to the acoustic axis of the 120-kHz transducer and with its focus coincident with the blood clot was used as a passive cavitation detector.

transducer using a three-axis translation stage (Newport 423, Irvine, CA, USA). Human blood clots suspended in 30 mL of plasma rested in the tip of condom. The sample chamber was placed in a Lucite tank filled with degassed water at 37° C and dissolved oxygen level (PO_2) maintained below 40% saturation as measured by a dissolved oxygen meter (Oakton DO100, Vernon Hills, IL, USA). The tank wall opposite the 120-kHz transducer was lined with acoustically-matched absorbing material (Aptflex F36, Precision Acoustics, Dorchester, UK) to prevent reflection and the formation of standing waves.

Clot formation protocol

Human whole blood was drawn from four volunteers by sterile venipuncture after local Institutional Review Board approval. Clots were prepared by aliquoting 1.5 mL of fresh human blood into 1.3-cm inner diameter Vacutainer tubes, incubating the tubes in a 37° C water bath for 3 h and storing the clots in a 5° C refrigerator for at least 72 h before use in the experiments to allow complete clot retraction. The clot formation and handling protocol has been described previously (Shaw et al. 2006; Datta et al. 2006; Holland et al. 2007). The clots were weighed initially before any treatment and were placed in the sample chamber containing 30 mL of thawed human fresh frozen plasma and held at 37° C . Reconstituted rt-PA ($96 \mu\text{g/mL}$) was added to the plasma for rt-PA-alone treatment and combined US and rt-PA treatments. The treatment times were fixed at 30 min. The rt-PA dose and clinically relevant treatment duration is consistent with previous studies (Datta et al. 2006; Holland et al. 2007). After exposure, each clot was weighed again and the extent of thrombolysis was determined as a percent mass loss.

Contrast agent preparation

Definity[®] vials were activated at room temperature using the Vialmix[™] agent activator (Bristol-Myers Squibb Medical Imaging, MA, USA) according to the manufacturer's instructions. After the 45-s activation cycle, the vials were left for 2 min to return to room temperature. The agent was drawn from the vial using a 20-gauge needle from the base of the vial while venting to the atmosphere with a second 22-gauge needle and a 0.2-micron in-line filter. The volume of agent drawn at a time was 0.1 mL and this aliquot was diluted by a factor of 100 by volume in phosphate-buffered saline (PBS). Subsequently, 100- μ L aliquots of the diluted Definity[®] were infused as a series of bolus injections every 3 min, each at a rate of 80 μ L/s, using a syringe pump (K.D. Scientific, model 230, Holliston, MA, USA). A 4F angiographic catheter (Medi-tech Inc., Watertown, MA, USA) inside an air-filled 9F sheath catheter (Cordis Corp., Miami, FL, USA) was used to deliver contrast agent next to the blood clot inside the sample chamber. The column of air surrounding the inner catheter provided acoustic shielding to the contrast agent within the catheter itself. The number density of microbubbles delivered with each bolus infusion was 1.2×10^8 microbubbles/mL. This microbubble density is two orders of magnitude less than the maximum bolus concentration recommended by the manufacturer for diagnostic applications. The recommended dose is 10 μ L/kg activated Definity (1.2×10^{10} microbubbles/mL) by bolus injection within 30–60 s. In treatments (rt-PA, US, PBS infusions without Definity[®]) without the use of a contrast agent, an equivalent volume of PBS was infused in the sample chamber using the same catheter arrangement.

Cavitation detection system

A cylindrically focused broadband hydrophone (Sonic Concepts Inc.), placed orthogonally to the 120-kHz beam axis, was used to passively detect cavitation activity in the sample chamber as shown in Fig. 1. The cavitation detection hydrophone was mounted on a micrometer-controlled 3-axis translation stage (Newport 423, Irvine, CA, USA) for precise alignment with blood clots. A 5-mm brass ball, positioned at the Rayleigh distance of the 120-kHz transducer, was used as an acoustic target for initial alignment of the broadband cylindrically focused hydrophone. Finally, acoustic scattering from the clot itself was used to finalize the alignment so that a volume encompassing the clot was monitored for cavitation by the focused hydrophone. The cylindrically-focused hydrophone has a -6 dB focal depth-of-field of 3.6 cm along its axis, a focal width of 1.3 cm and thickness of 0.1 cm. Signal acquired by the hydrophone was gated to account for the travel time of the pulse from the 120-kHz transducer and hydrophone.

The signal received by the focused hydrophone was amplified using a preamplifier (Signal Recovery 5185, Oak Ridge, TN, USA) and stored using a digital oscilloscope (Lecroy Waver Surfer 424, Chestnut Ridge, NY, USA). The signal received by the hydrophone was gated to ensure that cavitation was monitored over a region encompassing the clot surface, interior of the clot and surrounding fluid. The start of the gating window was thus set to 102 μ s, the time that corresponds to the travel time of the ultrasound pulse from the 120-kHz transducer to the hydrophone. The duration of the gating window was set to 20 ms to ensure a frequency resolution of 5 kHz. The cavitation detector signal therefore consisted of 500,000 points acquired at a sampling rate of 25 MHz and recorded in binary format twice per second. Each time trace file thus consisted of 33 contiguous pulses recorded in a binary format. The binary files were converted to MATLAB (The MathWorks Inc., Natick, MA, USA) data files and zero padded to 16,384 points. A Hanning window was subsequently applied before performing a fast Fourier transformation for each pulse. The squared frequency spectra for the 33 pulses were averaged in the frequency domain. A schematic of the signal processing is illustrated in Fig. 2.

The appearance of ultraharmonics in the signal received by the hydrophone indicated stable cavitation activity in the vicinity of the blood clot. In this stable cavitation detection scheme, ultraharmonics were frequency components at odd multiples of 60 kHz, half the main excitation frequency. The appearance of broadband noise emissions designated inertial cavitation in that same region. To determine the threshold for stable and inertial cavitation in this system, samples containing a human blood clot, plasma and Definity[®] were exposed to 120-kHz pulsed US, with an 80% duty cycle and a 1667-Hz pulse repetition frequency. The frequency content of the signal received by the focused hydrophone was analyzed for excitation pressure amplitudes in the range 0.06–0.75 MPa. A total of 20 files were recorded over a period of 10 s for each pressure and were analyzed for ultraharmonic and broadband noise emissions. This process was repeated using three different samples at each pressure amplitude. Ten consecutive odd ultraharmonics of the subharmonic (60 kHz) component in the frequency range of 300 kHz–1.5 MHz were extracted from the frequency spectrum using a digital band-pass comb filter. Each frequency band was centered at the corresponding ultraharmonic frequency with a fixed bandwidth of 7.6 kHz, which is comparable to the -6 dB bandwidth of the 120-kHz tone bursts used. Spectral power in 10 bands between the ultraharmonics and harmonics of the fundamental were used to determine the baseline noise floor, as well as changes in the broadband noise energy used as a measure of inertial cavitation

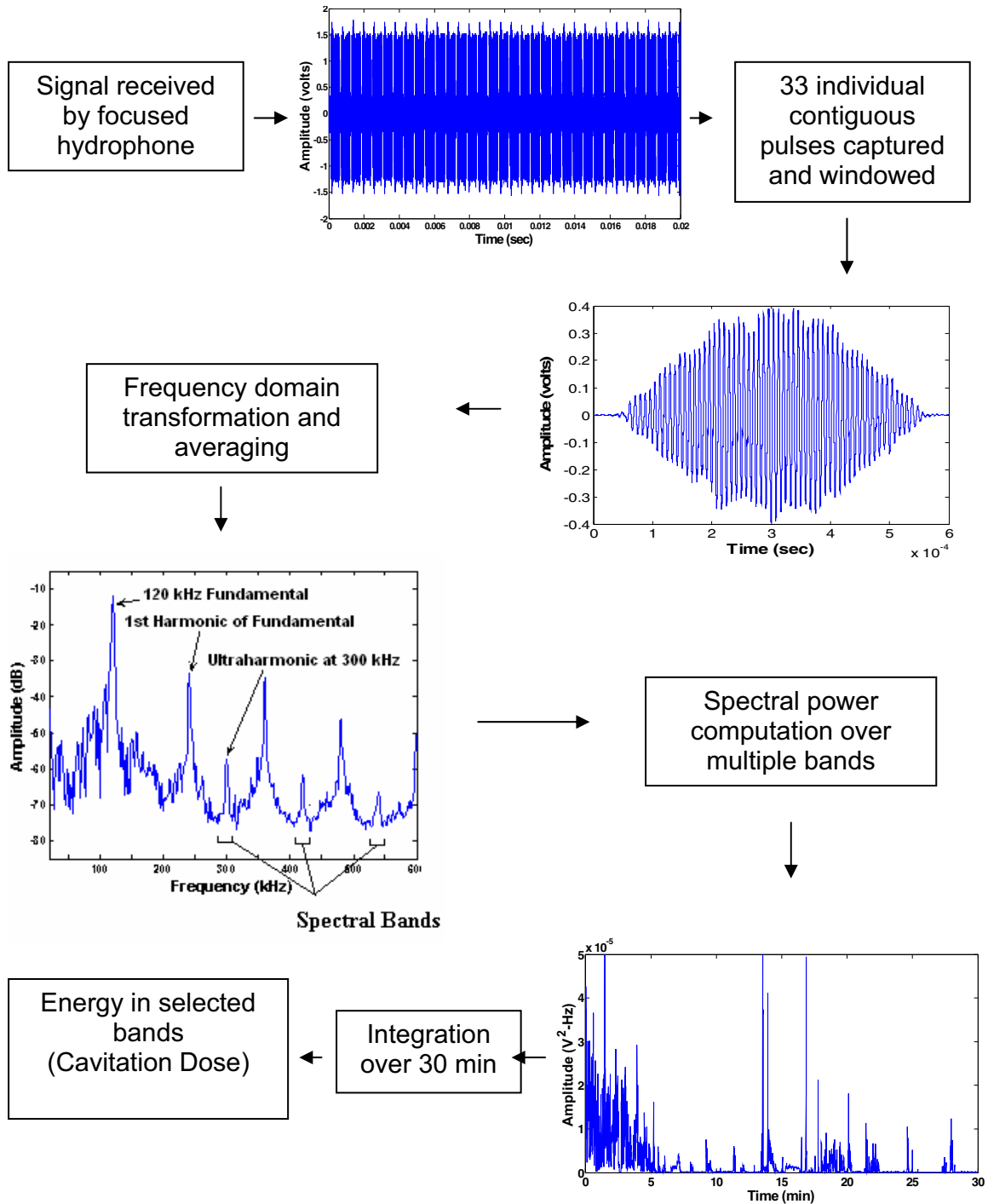


Fig. 2. Schematic of the signal processing algorithm to determine cavitation dose from scattered acoustic signals received by the broadband-focused hydrophone.

activity. The levels of ultraharmonic and broadband noise power as a function of excitation pressure are shown in Fig. 3.

During the clot mass loss experiments, a total of 3,600 files were recorded over a period of 30 min. Spectral power in the ultraharmonic bands was inte-

grated over the 30-min duration and used as a measure of the extent or dose of stable cavitation as illustrated conceptually in Fig. 2. The fraction of the exposure time during which the energy in ultraharmonic bands was higher than the baseline spectral components because of scattering from the contents of the sample holder was

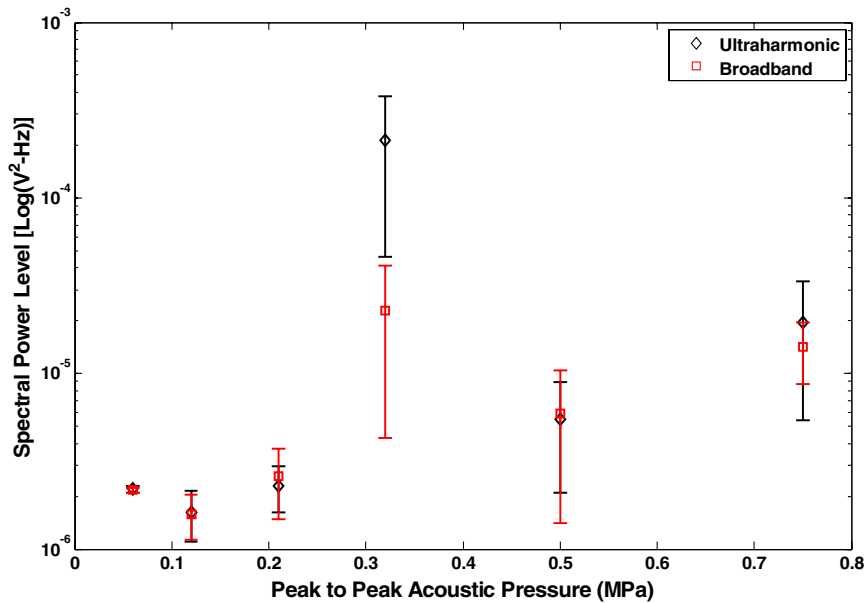


Fig. 3. Spectral power levels in ultraharmonic and broadband bands as a function of increasing peak-to-peak acoustic pressure amplitude. The scattered signal is received by a hydrophone placed 90° to the source transducer axis with its focus coincident with the region of the sample holder containing a human clot in plasma and Definity®. Three different samples were exposed at each pressure, and error bars represent the standard deviation of 60 measurements.

used as a measure of the duration of stable cavitation activity. Spectral power in the bands between the ultraharmonics and harmonics of the fundamental were used as a measure of inertial cavitation activity. In this approach, the selected spectral bands were sufficiently close to enable meaningful comparison without the need to correct for the frequency response of the hydrophone.

To evaluate the effect of Definity® on scattered energy, the harmonics of the fundamental detected from the contents of the sample holder including clot, plasma and Definity® were also measured using a similar approach. To estimate the fractional stable cavitation duration, the mean and standard deviation measurements of spectral power in bands representing broadband or baseline emissions were used. The fractional stable cavitation duration was defined as the percentage of time the combined spectral power in the ultraharmonic bands was greater than two standard deviations above the mean of broadband or baseline spectral bands for that treatment.

Three rt-PA concentrations of 1.4 $\mu\text{g}/\text{mL}$, 11.0 $\mu\text{g}/\text{mL}$ and 96.0 $\mu\text{g}/\text{mL}$ were used in this work to study the effect of Definity® infusions on US enhancement of thrombolysis. The lowest concentration chosen was approximately eight times lower than the maximum allowable human clinical dose (NINDS Stroke Study Group 1995; Broderick *et al.* 2002). Similarly, the highest concentration used was approximately eight times higher than human dose as was used in previous study (Datta *et al.* 2006). The approach of sustaining stable cavitation

throughout the treatment period was explored at the lower rt-PA concentrations (1.4 $\mu\text{g}/\text{mL}$ and 11.0 $\mu\text{g}/\text{mL}$) using Definity® infusions every 3 min and US exposures with a peak-to-peak pressure amplitude of 0.32 MPa. Both rt-PA alone and rt-PA, US, PBS infusions without Definity® data were also acquired for the lower rt-PA concentrations.

Fluorescence dual-antibody staining

After treatment, the clots were immersed in optimal cutting temperature gel (Tissue Tek, Redding, CA, USA) and snap-frozen in liquid nitrogen and stored at -80°C . The clots were thawed to room temperature for pathologic analysis and fixed by immersion in 3% paraformaldehyde solution for 48 h and 30% sucrose solution for 24 h. The clots were cut in 25- μm -thick sections using a microtome for fluorescence dual-antibody staining. The primary antibodies used were monoclonal antibody (mouse immunoglobulin, IgG) against human plasminogen (1:1000 dilution; AB10178; Abcam Inc., Cambridge, MA, USA) and polyclonal antibody (goat IgG) against human tissue plasminogen activator (1:1000 dilution; 387; American Diagnostics Inc., Stamford, CT, USA). Dilutions were done in blocking buffer (2% horse serum, 0.3% Triton and 0.1% bovine serum albumin in 0.1 mol/L phosphate buffer). The two secondary antibodies that were used were donkey anti-goat IgG (dilution 1:500; A11055; Molecular Probes Inc., OR, USA) and donkey

antimouse IgG (dilution 1:500; A31570; Molecular Probes Inc.).

Clot sections were incubated in the primary antibodies for 1 h, washed three times with saline solution and blocked before incubating in secondary antibodies for 1 h. After incubation with secondary antibodies, clot sections were washed again three times with saline solution, mounted on glass slides (Fisher Scientific, Pittsburgh, PA, USA), and sealed with glass cover slips. Clot sections were imaged using a fluorescent microscope BX51 (Olympus America Inc., Melville, NY, USA) with a 10x objective, and images were acquired using Spot Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and stored on a desktop computer. The exposure parameters (time and gain) were adjusted to calibrate images of each clot section, and the same fixed exposures parameters and filter were used to analyze all the images for that particular section. A total of five images were taken for each clot section, one at the center and one each at randomly selected 3, 6, 9 and 12 o'clock positions encompassing clot edge and a portion of interior of the clot section. In the images, the area of rt-PA localization appears bright green and the area of plasminogen localization appears red. For each clot section, the image of the interior of the clot served as its own control. The brightness level for the image obtained for clot center was adjusted using the window/level function available in ImageJ software (National Institutes of Health, Bethesda, MD, USA) such that the whole image appeared dark. The same values of the window/level function were then applied to the remaining images of the same section. This ensured that only signal sufficiently stronger than the background brightness level appears in the images. The measurements for penetration depth were performed on the processed images. The penetration depths of rt-PA and plasminogen were measured using a line scan tool available in the software. An average of six measurements per image per section was used to evaluate penetration depth. Overlapping region of rt-PA and plasminogen in the images represents the clot lysis zone, and the depth of this zone was measured from the surface of the clot. Images of the clot sections obtained for the following treatments were analyzed to evaluate the difference in penetration depths of rt-PA and plasminogen: (i) no rt-PA, Definity[®] or US treatment; (ii) rt-PA alone treatment (no US or Definity[®]); (iii) rt-PA, US (0.32 MPa), PBS infusions without Definity[®] treatment; (iv) rt-PA, Definity[®] infusions; and (v) US (0.32 MPa) treatment.

Statistics

Correlation between mass loss, the energy in ultraharmonic bands, the energy in broadband bands, the energy in harmonic bands and fractional stable cavitation

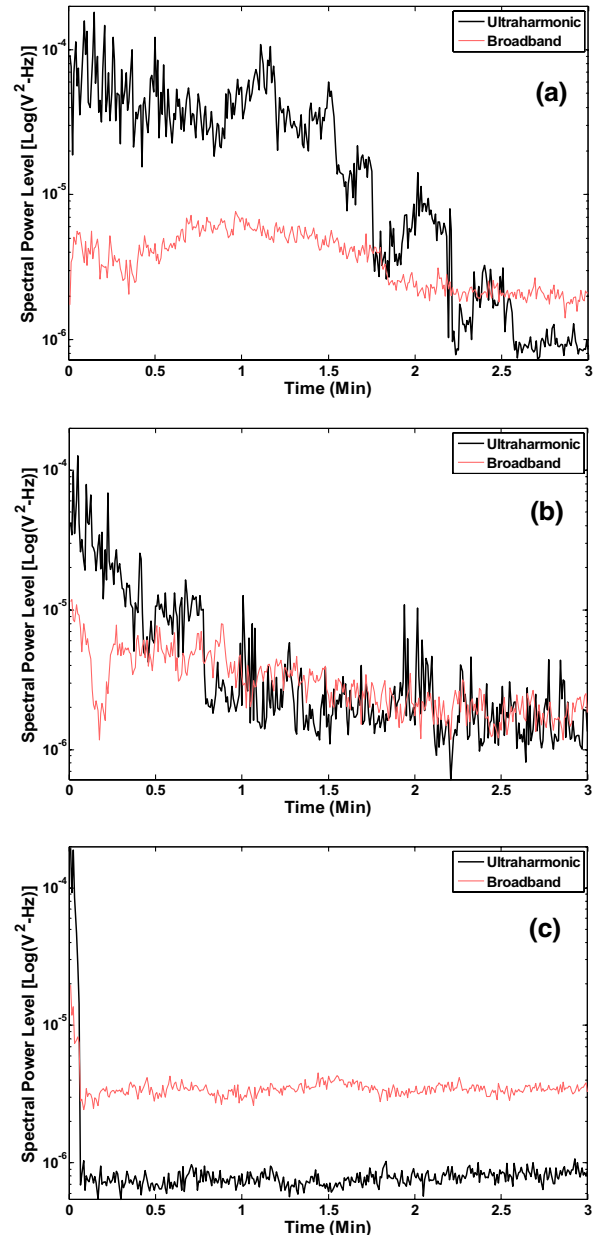


Fig. 4. Representative ultraharmonic and broadband signal levels as function of time after a single infusion of Definity[®] with peak-to-peak US exposure of (a) 0.32 MPa, (b) 0.50 MPa and (c) 0.75 MPa.

duration was evaluated statistically using the “corr” function in MATLAB (The MathWorks, Inc.). The comparison of mass loss for exposures to different combinations of rt-PA, US and Definity[®] was accomplished using a one-way ANOVA and two sample Student’s *t*-tests using MINITAB (MINITAB Inc., State College, PA, USA) statistical analysis software. A two-sample Student’s *t*-test was performed on penetration depth measurements from the images of clot sections.

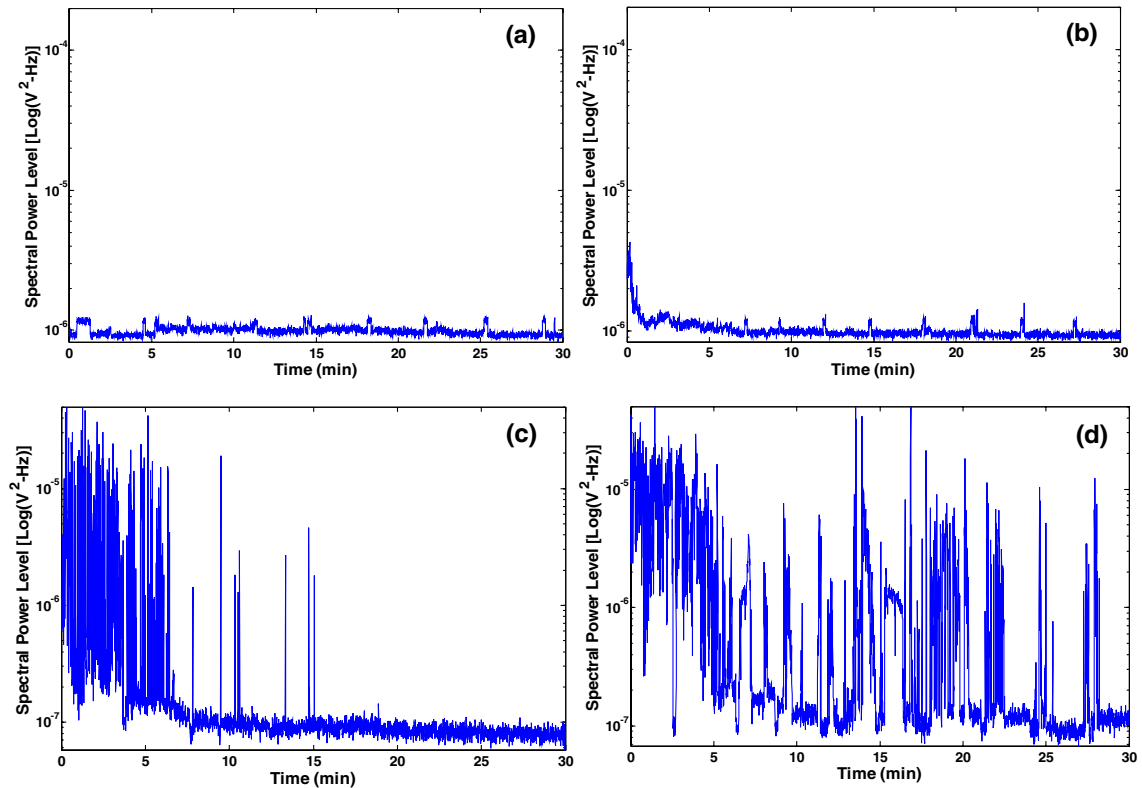


Fig. 5. A representative example of the spectral power in the ultraharmonic band over the treatment duration (a) with Definity[®] infusion but no cavitation (0.12 MPa peak-to-peak), (b) with Definity[®] infusion but no cavitation (0.21 MPa peak-to-peak), (c) with infusion of PBS alone at a peak-to-peak pressure of 0.32 MPa (note only initial cavitation activity is observed) and (d) with infusion of Definity[®], resulting in relatively sustained cavitation at the same peak-to-peak pressure (0.32 MPa).

RESULTS

The change in the level of spectral power in ultraharmonic bands as function of increasing peak-to-peak pressure amplitude and their relative magnitude compared with spectral power in broadband bands is shown in Fig. 3. Each data point is an average of 60 measurements over three sample preparations, and the error bars represent the standard deviation of the measurements. No significant difference between the levels of signal in ultraharmonic or broadband signals was observed for the pressure steps up to 0.21 MPa. As the acoustic pressure

is increased to 0.32 MPa peak-to-peak in the next step, the amplitude of both broadband and ultraharmonic power bands increases, but the increase in the ultraharmonic bands is significantly higher than the broadband energy. Thus, 0.32 MPa peak-to-peak pressure amplitude is interpreted as the onset of stable cavitation for this experimental set up. At pressure steps greater than 0.32 MPa, the level of ultraharmonic energy decreases.

An example of the effect of infusion of Definity[®] can be observed in Fig. 4. When Definity[®] was infused initially, the spectral power in ultraharmonic bands in-

Table 1. Effect of contrast agent Definity[®] on enhancement of stable cavitation activity as measured by energy in the ultraharmonic signals and fractional stable cavitation duration

	PBS, rt-PA and US at 0.32 MPa	Definity [®] , rt-PA and US at 0.32 MPa	<i>p</i> -value
Ultraharmonic energy (V ²)	0.0162 ± 0.0145	0.0482 ± 0.031	0.046
Fractional stable cavitation Duration (%)	10.1 ± 4.4	20.8 ± 6.8	0.003

The *p*-value shown is for two sample Student's *t*-test for a sample size of six for each treatment.

Table 2. Correlation coefficient (*r*) for measured acoustic emissions and mass loss

	Mass Loss	Ultraharmonic Emissions	Broadband Emissions	Harmonic of Fundamental	Fractional Stable Cavitation Duration
Mass Loss	1	0.85 (<i>p</i> < 0.0001)	0.00 (<i>p</i> = 1.0)	0.51 (<i>p</i> = 0.01)	0.83 (<i>p</i> < 0.0001)
Ultraharmonic Emissions		1	0.01 (<i>p</i> = 0.97)	0.34 (<i>p</i> = 0.10)	0.84 (<i>p</i> < 0.0001)
Broadband Emissions			1	0.06 (<i>p</i> = 0.77)	-0.19 (<i>p</i> = 0.38)
Harmonic of Fundamental				1	0.29 (<i>p</i> = 0.17)
Fractional Stable Cavitation Duration					1

The *p*-value for correlation is shown in parentheses and the statistically significant correlations are shown in bold. A combined sample size of 24 was used to evaluate correlation coefficients.

creased for approximately 2.5 min in the presence of pulsed US at peak-to-peak pressure amplitude of 0.32 MPa (Fig. 4a). At higher pressures, initial infusion of Definity[®] enhanced the level of ultraharmonic emissions but for shorter durations (Fig. 4b and c). This information was used to select the frequency of intermittent infusions of Definity[®] (one infusion every 3 min) and pressure amplitude of 120 kHz US (0.32 MPa peak-to-peak) to sustain stable cavitation during the 30-min exposures. The effect of Definity[®] infusions on sustaining cavitation over a period of 30 min can be observed in Fig. 5. The peak-to-peak pressure amplitudes used for data plotted in Fig. 5 were 0.12 MPa, 0.21 MPa and 0.32 MPa. In Fig. 5a, ultraharmonic spectral power level in the presence of Definity[®] but without any detectable stable cavitation activity (0.12 MPa) is shown. Note that no enhancement in the ultraharmonic spectral power bands is observed. Fig. 5b shows ultraharmonic spectral power level at a higher pressure (0.21 MPa). Fig. 5c shows the cavitation activity without Definity[®] at a higher-pressure amplitude (0.32 MPa), which is closer to the previously reported stable cavitation threshold of 0.40 MPa (Datta et al. 2006). Note that PBS was infused in this treatment every 3 min. An increase in ultraharmonic signal can be observed for the first 5 min, after which subsequent stable cavitation activity subsided. However, in Fig. 5d, when Definity[®] was infused intermittently every 3 min, the cavitation activity is sustained throughout the 30-min exposure duration.

The effect of Definity[®] on the enhancement of cavitation activity as measured by ultraharmonic energy and fractional stable cavitation duration is shown in Table 1. Infusion of Definity[®] increased the ultraharmonic energy from a mean value of 0.0162–0.0482 V² (*p* = 0.046). The stable cavitation duration was also signif-

icantly increased from a mean of 10.1–20.8% (*p* = 0.003). The effect of combinations of rt-PA at a concentration of 96 μg/mL, US and Definity[®] infusions with or without cavitation activity on clot mass loss is presented in Fig. 6. The greatest clot mass loss of 26.2% was observed for the combination of rt-PA, Definity[®] infusions and US treatment at the peak-to-peak pressure amplitude of 0.32 MPa (*p* = 0.001). A significantly lower clot mass loss of 16.0% was observed for the same peak-to-peak pressure amplitude of 0.32 MPa in rt-PA, US, PBS infusions without Definity[®] treatment. The other rt-PA, Definity[®] infusions and US treatments at lower pressures (0.12 MPa and 0.21 MPa) resulted in a

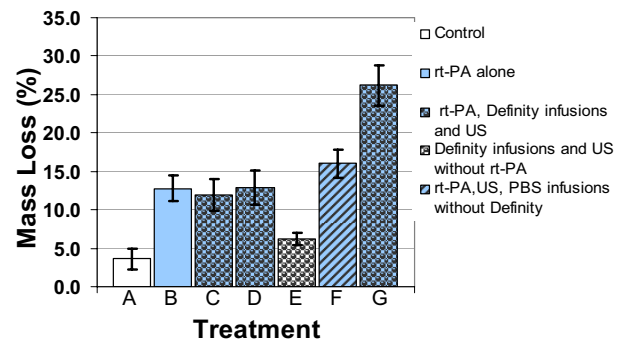


Fig. 6. Mass loss (%) as a function of peak-to-peak acoustic pressure for different treatments: (a) No rt-PA, Definity[®] or US, which is the control treatment; (b) rt-PA alone; (c) rt-PA, Definity[®] infusions and US (0.12 MPa peak-to-peak pressure amplitude); (d) rt-PA, Definity[®] infusions and US (0.21 MPa peak-to-peak pressure amplitude); (e) Definity[®] infusions and US (0.32 MPa peak-to-peak pressure amplitude) without rt-PA treatment; (f) rt-PA, PBS infusions without Definity[®] and US (0.32 MPa peak-to-peak pressure amplitude); and (g) rt-PA, Definity[®] infusions, and US (0.32 MPa peak-to-peak pressure amplitude). A sample size of six was used for each treatment.

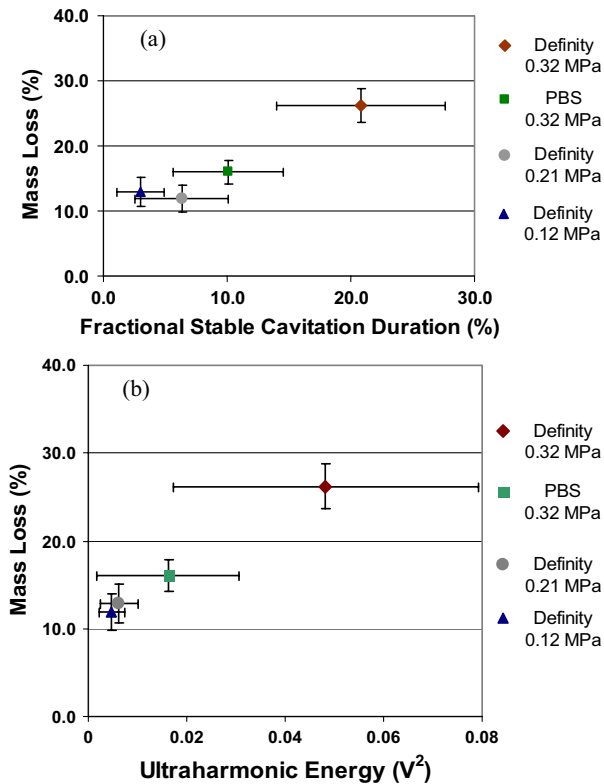


Fig. 7. Mass loss (%) and cavitation activity relationship as a function of (a) fractional stable cavitation duration, which is a fraction of the total time for which the ultraharmonic energy level was above the noise floor; and (b) ultraharmonic energy integrated over a 30-min exposure. A sample size of six was used for each treatment, and error bars represent standard deviation of the measurements.

lower clot mass loss, which was not significantly different from the rt-PA alone treatment (without US). Treatment with Definity[®] infusions and US without rt-PA at a pressure amplitude of 0.32 MPa resulted in a mean clot mass loss of 6.3%, which is higher than control (no US, no Definity[®], no rt-PA) but lower than rt-PA alone treatment.

The correlation between the fractional stable cavitation duration and clot mass loss is shown in Fig. 7a and the correlation between ultraharmonic energy and clot mass loss is shown in Fig. 7b. The clot mass loss increased with both the fractional stable cavitation duration and ultraharmonic energy. Table 2 shows the values of correlation coefficients between the measured mass loss, ultraharmonic energy, broadband emissions, harmonic energy and stable cavitation duration, for a sample size of 24. A statistically significant correlation of $r = 0.85$ is observed between clot mass loss and ultraharmonic energy ($p < 0.0001$). Also, there was a significant correlation of $r = 0.83$ between stable cavitation duration and mass loss ($p < 0.0001$). A relatively weaker correlation

of $r = 0.51$ with harmonic emissions was also observed ($p = 0.01$).

Results of the dual antibody fluorescence imaging are shown in Fig. 8. The images in the left column show rt-PA localization in green and the right column show localization of plasminogen in red. The scale bar in the images is 0.3 mm. Images in Fig. 8a show the control treatment, Fig. 8b shows results for rt-PA alone treated clots, Fig. 8c shows US, rt-PA and saline treated clots and Fig. 8d shows US, rt-PA and Definity[®]-treated clots. It can be qualitatively observed that rt-PA penetration is largest for the combined US, rt-PA and Definity[®] treatment. Width of the clot lysis zone can be observed by penetration depth of rt-PA in the images and accumulation of plasminogen in the same region, as measured from the surface of the clot using the line scan tool in the Spot Advanced software. The penetration depth measurements are shown in Table 3 for three clots processed for each treatment. Treatments with rt-PA, Definity[®] infusions and US exhibited the greatest mean rt-PA penetration of almost 222 μm , which is statistically significantly greater than any other treatment ($p = 0.001$). There was a greater variation of plasminogen penetration depth, and the mean value for combined US, rt-PA and Definity[®] treatment was 241 μm , but the mean was not statistically significantly greater than the rt-PA, US, PBS infusions without Definity[®] ($p > 0.05$).

The effect of infusions of Definity[®] at lower rt-PA concentrations on clot mass loss can be observed in Fig. 9. At the lowest rt-PA concentration of 1.4 $\mu\text{g/mL}$, thrombolysis by rt-PA alone dropped to a mean value of 7.9%. The use of Definity[®] at this concentration still enhanced thrombolysis significantly ($p < 0.001$).

DISCUSSION

A strategy for the use of contrast agent microbubbles along with US and thrombolytics was explored in this work. A simultaneous monitoring of cavitation activity could help optimize therapeutic benefits of US-enhanced thrombolysis. The results suggest that an increase in stable cavitation activity resulted in a corresponding increase in clot mass loss. In the present work, simultaneous measurement of microbubble-enhanced cavitation activity showed a statistically significant correlation between ultraharmonic signals and mass loss. Earlier, Porter *et al.* (1996) observed thrombolytic enhancement *in vitro* using perfluorocarbon-exposed sonicated dextrose albumin microbubbles together with 20-kHz US (0.845 MPa peak negative pressure amplitude) and urokinase. Along the same lines, Mizushige *et al.* (1999) compared different contrast agents used with rt-PA and catheter-based 10-MHz US. They reported a correlation between the persistence of microbubbles dur-

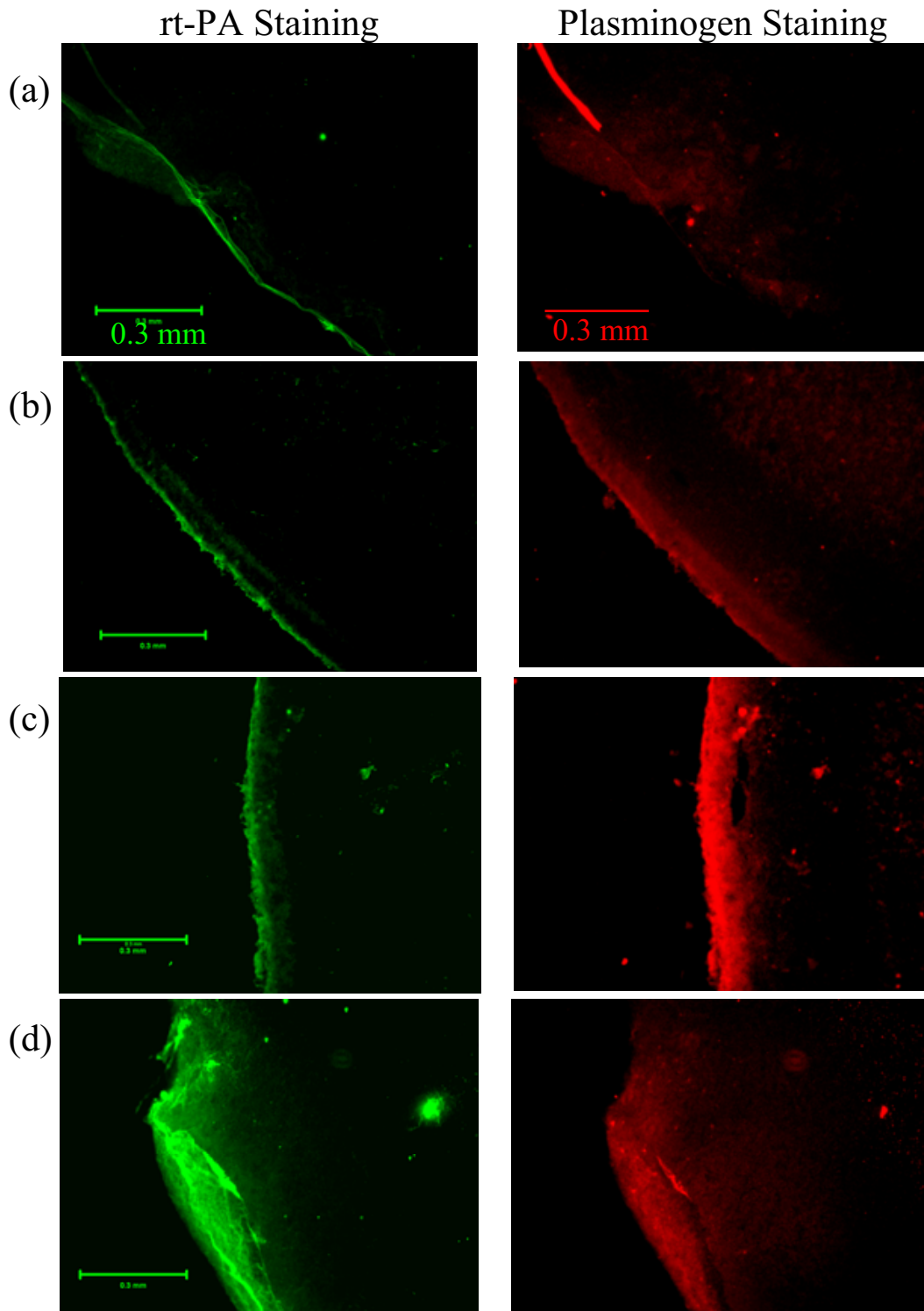


Fig. 8. Representative examples of fluorescent dual-antibody-stained images of clot sections. The left column images are stained green for rt-PA by fluorescein isothiocyanate (FITC)-conjugated secondary antibody. For the same section, the right column images show accumulation of plasminogen stained red by Texas red (TRITC)-conjugated secondary antibody. Representative clot section images are shown for (a) no rt-PA, Definity[®] or US (the control treatment); (b) rt-PA alone treatment; (c) rt-PA, PBS infusions and US (0.32 MPa peak-to-peak amplitude) treatment; and (d) rt-PA, Definity[®] infusions and US (0.32 MPa peak-to-peak amplitude) treatment.

Table 3. Penetration depth of rt-PA and plasminogen measured from the surface of the clot

Treatment	rt-PA penetration depth (μm)	Plasminogen penetration depth (μm)
Control	15.2 \pm 7.5	64.2 \pm 51.5
rt-PA alone	22.0 \pm 7.8	65.2 \pm 36.2
rt-PA, US, PBS infusions without Definity®	52.5 \pm 21.5	149.9 \pm 56.9
rt-PA, Definity® infusions and US	221.8 \pm 54.9	240.9 \pm 102.8

Measurements were made on dual antibody-stained clot sections for each case. The data shown here is mean \pm standard deviation for measurements done on sections obtained from three clots for each treatment and 18 measurements of penetration depth made for each section. The peak-to-peak pressure amplitude for US was 0.32 MPa.

ing the US exposure and thrombolysis in their *in vitro* experiments. Several other *in vitro* studies have since shown US-assisted thrombolysis with and without rt-PA in the presence of microbubbles and have suggested cavitation-related phenomena are responsible for this effect (Porter *et al.* 2001; Nedelmann *et al.* 2002; Cintas *et al.* 2004). Nedelmann *et al.* (2005) further demonstrated that US-induced blood clot dissolution without a thrombolytic drug is more effective at 20 kHz when compared with 40 or 60 kHz using the same acoustic intensity (0.2 W/cm^2). These studies have already demonstrated a potential therapeutic use of microbubbles in breaking up blood clots mediated possibly by cavitation activity. In an important contrast to the present study, no direct or indirect measurement of cavitation activity was reported in any of the studies mentioned above. The results of the present study demonstrate that a microbubble infusion was effective in sustaining stable cavitation activity and this treatment protocol resulted in the highest mass loss. It is important to note that fully retracted clots (3 to 14 d old) were used in this study. These clots tend to be more resistant to lysis than newly formed clots (Holland *et al.* 2002, 2007).

Microbubbles and US have already been successfully used to augment the recanalization of vessels oc-

cluded by small clots in an *in vivo* rabbit iliofemoral artery clot model without the use of lytic agents such as rt-PA (Birnbaum *et al.* 1998). In a canine coronary artery thrombus model, transcutaneous 27-kHz US along with rt-PA demonstrated significant recanalization (Siegel *et al.* 2000). Microbubble potentiation of thrombolysis in intracranial arteries was demonstrated in a porcine model by Culp *et al.* (2003) using 1-MHz US. These results were repeated in other arteriovenous graft thrombi models in canines (Xie *et al.* 2005) and in a porcine ascending pharyngeal artery model (Culp *et al.* 2004) using platelet-targeted microbubbles. The authors in these studies attributed this enhancement to cavitation activity and suggested a possible additive effect when used with a thrombolytic such as rt-PA. In contrast to these *in vivo* studies, a significant observation in our *in vitro* study is that Definity® infusions in the presence of US but without rt-PA did not result in significantly higher mass loss. An explanation of this enhancement *in vivo* could be attributed to the presence of a sufficient quantity of endogenous tissue plasminogen activator and its synergistic effect in the presence of US. We propose that this endogenous tissue plasminogen activator, together with the cavitation activity nucleated by a contrast agent, may have been sufficient to cause enough thrombolysis to result in recanalization.

In human clinical trials Alexandrov *et al.* 2004a, 2004b) demonstrated improved arterial recanalization and improved clinical outcomes with the use of 2-MHz transcranial Doppler and rt-PA. Molina *et al.* (2006) added Levovist™ microbubbles to the transcranial Doppler and rt-PA treatment and reported further thrombolytic enhancement. However, Daffertshofer *et al.* (2005) employed a low-frequency broad-beam 300-kHz therapeutic device in clinical trials and observed a significant increase in intracranial hemorrhage, and the study was stopped prematurely. The authors pursued the study of bioeffects in a rat model and reported no deleterious effects as evaluated by histopathological evaluation of brain and temperature measurements (Fatar *et al.* 2006). These studies highlight the importance of *in vitro* and *in vivo* studies aimed at understanding the potential

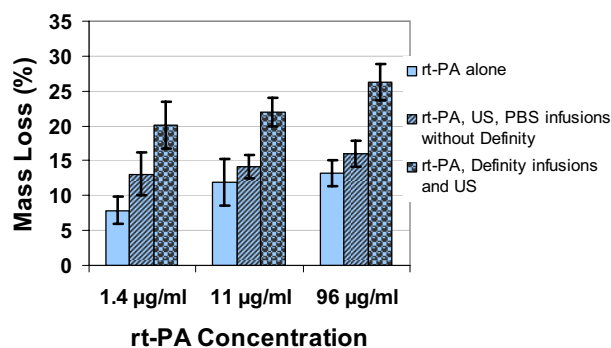


Fig. 9. Effect of Definity® on clot mass loss as a function of rt-PA concentration. The peak-to-peak pressure amplitude used in all US treatments was 0.32 MPa. A significant enhancement over rt-PA alone treatment can be observed at lowest concentration.

adverse bioeffects of US-assisted thrombolysis while optimizing US parameters to maximize thrombolysis. Monitoring the stable cavitation activity as demonstrated in this study could possibly be extended to an *in vivo* setting to have better control on therapy.

Several mechanistic *in vitro* studies have been conducted to investigate the interaction of US with thrombolytic drugs (Francis et al. 1992; Blinc et al. 1993; Francis 1995; Suchkova et al. 1998; Akiyama et al. 1998; Behrens et al. 1999; Everbach et al. 2000; Holland et al. 2007). Some of these studies have shown that US potentiation of fibrinolysis is related to increased transport of reactants through cavitation-related mechanisms. Our dual antibody staining results provide experimental evidence of a significantly broader lysis zone observed in the presence of sustained stable cavitation. A similar result was reported in an earlier study using a similar immunohistochemistry technique but without any detection of cavitation (Devic-Kuhar et al. 2004). The rt-PA penetration depth reported in the study ($21.2 \pm 7.2 \mu\text{m}$) is comparable to our rt-PA, US, PBS infusions without Definity[®] treatment ($52.5 \pm 21.5 \mu\text{m}$). In our study, the rt-PA penetration depth in the presence of stable cavitation activity increased to almost $222 \pm 55 \mu\text{m}$.

Onundarson et al. (1992) reported saturation in thrombolysis for rt-PA concentrations $>5 \mu\text{g/mL}$ in human whole blood clots. A similar value of $3 \mu\text{g/mL}$ was reported by Trusen et al. (1998). In our study, we observed a significant thrombolytic enhancement at an rt-PA concentration as low as $1.4 \mu\text{g/mL}$ in the presence of stable cavitation activity. The use of microbubbles at such low concentration of rt-PA was sufficient to enhance thrombolysis significantly compared with treatment with rt-PA alone. This demonstrates a possibility of lowering the rt-PA dose and thus lowering the hemorrhagic risk associated with the use of thrombolytics.

In our previous study, Datta et al. (2006) demonstrated that the largest increase in clot mass loss was achieved in the presence of stable cavitation and showed that inertial cavitation activity decreased thrombolytic efficacy. The presence of inertial cavitation could have served as a limiter of sustained stable cavitation. In the present study cavitation activity was measured and correlated significantly with the enhancement of thrombolysis. It is also important to note that, for treatments with rt-PA, Definity[®] infusions at lower US peak to peak pressures (0.12 and 0.21 MPa) did not cause any enhancement of thrombolysis over rt-PA alone treatment in the absence of ultraharmonic signals.

This study demonstrates an approach to nucleate stable cavitation with a contrast agent for therapeutic benefit. The serial bolus injection protocol used in this study does not allow for detailed knowledge of the concentration of acoustically-activated microbubbles

around the clot. Thus, a limitation of the study is that the relationship between density of cavitation nuclei (*i.e.*, the contrast agent) and the number of activated microbubbles is unknown. In addition, the specific effects of microbubble aggregation as a result of secondary Bjerknes forces, acoustic radiation forces, microbubble coalescence and fluid mixing as a result of streaming were not investigated and could all contribute to the enhanced thrombolytic efficacy.

CONCLUSIONS

Stable cavitation activity can be sustained using an intermittent infusion of a commercial contrast agent, Definity[®]. Ultraharmonic emissions can be used to monitor and measure cavitation activity, and such signals are significantly correlated with increased clot mass loss. Treatments with Definity[®] infusion along with rt-PA and US below any detectable cavitation activity did not result in any increase in mass loss compared with rt-PA alone treatment. An increased penetration of rt-PA and plasminogen was also observed in clots exposed to stable cavitation nucleated by Definity[®]. The synergistic effect of sustained cavitation activity was also observed at a significantly lower concentration of rt-PA. Ultraharmonic emissions could potentially be used to monitor thrombolytic efficacy during treatment, and further studies are still warranted to develop *in vivo* cavitation detection techniques.

Acknowledgements—This research was supported by the National Institutes of Health, grant number NIH/NINDS R01-NS047603. We gratefully acknowledge the assistance of Drs. George Shaw and Jason Meunier for the human whole blood clot model and valuable discussions. We also acknowledge the assistance of Stella Nicolaou and Sampada Vaidya for developing the fluorescent imaging technique.

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