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Ultrasound-enhanced tissue plasminogen activator thrombolysis in an *in vitro* porcine clot model

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Abstract

Introduction—Thrombolytics such as recombinant tissue plasminogen activator (rt-PA) have advanced the treatment of ischemic stroke, myocardial infarction, deep vein thrombosis and pulmonary embolism.

Objective—To improve the efficacy of this thrombolytic therapy, the synergistic effect of rt-PA and 120 kHz or 1.0 MHz ultrasound was assessed *in vitro* using a porcine clot model.

Materials and methods—Fully retracted whole blood clots prepared from fresh porcine blood were employed to compare rt-PA thrombolytic treatment with and without exposure to 120-kHz or 1-MHz ultrasound. For sham studies (without ultrasound), clot mass loss was measured as a function of rt-PA concentration from 0.003 to 0.107 mg/ml. For combined ultrasound and rt-PA treatments, peak-to-peak pressure amplitudes of 0.35, 0.70 or 1.0 MPa were employed. The range of duty cycles varied from 10% to 100% (continuous wave) and the pulse repetition frequency was fixed at 1.7 KHz.

Results—For rt-PA alone, the mass loss increased monotonically as a function of rt-PA concentration up to approximately 0.050 mg/ml. With ultrasound and rt-PA exposure, clot mass loss increased by as much as 104% over rt-PA alone. Ultrasound without the presence of rt-PA did not significantly enhance thrombolysis compared to control treatment. The ultrasound-mediated clot mass loss enhancement increased with the square root of the overall treatment duration.

Conclusions—Both 120-kHz and 1-MHz pulsed and CW ultrasound enhanced rt-PA thrombolysis in a porcine whole blood clot model *in vitro*. No clear dependence of the observed thrombolytic enhancement on ultrasound duty cycle was evident. The lack of duty cycle dependence suggests a more complex mechanism that could not be sustained by merely increasing the pulse duration.

Keywords

Ultrasound-enhanced thrombolysis; Stroke therapy; Fibrinolysis; Therapeutic ultrasound; Stable cavitation

Introduction

Stroke is the third leading cause of death and the leading cause of disability in the US. The cost of treatment, care, and lost productivity associated with stroke is \$62.7 billion [1]. Despite

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significant improvements in mortality rates from stroke during the past two decades, stroke still affects over 700,000 Americans each year [1,2]. Ischemic stroke, which accounts for about 87% of all strokes, is caused by sudden loss of blood flow to a region of the brain due to a thrombus or embolus. Investigational therapies have focused on limiting the severity of ischemic injury (neuronal protection) and reducing the duration of ischemia (restoring blood flow). However, at present, the only therapy for ischemic stroke that is approved by the FDA is the thrombolytic agent, recombinant tissue plasminogen activator (rt-PA). In 1995, the National Institute of Neurological Disorders and Stroke Tissue Plasminogen Activator Stroke Study Group [3] reported that rt-PA reduced disability in patients with acute ischemic stroke. Recombinant t-PA is moderately effective in lysing thrombi in ischemic stroke patients and improves neurologic deficits if given within 3 h after the onset of stroke symptoms [4]. Unfortunately, thrombolytics also can cause intracerebral hemorrhage. Potent thrombolytic agents, such as rt-PA, have permitted effective thrombolysis in occluded arteries, particularly for myocardial salvage in acute myocardial infarction [5]. However, a hemorrhagic event, such as cerebral or gastrointestinal bleeding, can occur during or after thrombolysis, causing substantial complications [6-9]. Adjuvant therapies that lower the dose of rt-PA or increase its efficacy would represent a significant breakthrough. Improved effectiveness or greater safety would provide a powerful impetus for physicians to administer rt-PA to a larger portion of patients with ischemic stroke.

Effective methods of enhancing thrombolysis have been examined in an attempt to reduce the dosage of the thrombolytic agent and reduce the risk of hemorrhagic events. A higher rate of intracranial hemorrhagic complications is associated with higher rt-PA dose in clinical rt-PA thrombolytic treatment [10]. In the absence of rt-PA, kilohertz-frequency ultrasound has been found to disrupt peripheral arterial and venous thrombi in animal models [11-14]. However, the use of ultrasound without a thrombolytic drug has had limited clinical success to date.

Other researchers have investigated the ability of ultrasound to enhance the efficacy of thrombolytic agents. Lauer et al. [15] demonstrated that 1-MHz intermittent ultrasound, with an “on” interval of 2 s followed by a rest interval of 2 s, increased the percent mass loss in a whole human blood clot model exposed to rt-PA *in vitro*. They proposed that acoustic streaming alone, without cavitation effects, was responsible for the increased thrombolysis. Careful investigations by Francis et al. suggest that ultrasound accelerates enzymatic fibrinolysis by increasing transport of reactants through a cavitation-related mechanism [16-18]. However, experiments employing ultrasound exposure of clots in a hyperbaric chamber revealed that other mechanisms in addition to inertial cavitation were present [19]. Several investigators have utilized low-frequency, low-intensity ultrasound to accelerate rt-PA thrombolysis *in vitro* [20,21]. In addition, mechanistic studies *in vitro* have revealed that stable cavitation is correlated with enhanced rt-PA thrombolysis [22], yet strategies to optimize the occurrence of such bubble activity and avoid potential harmful bioeffects have yet to be identified. Stable cavitation is characterized by bubbles pulsating gently in response to the time-varying acoustic pressure in an ultrasound field. Siegel et al. found that noninvasive transthoracic application of low-frequency (27 kHz) ultrasound augmented the efficacy of t-PA-mediated thrombolysis in a canine model, and improved coronary patency without increasing the risk of bleeding [23].

In a clinical study Alexandrov et al. used 2-MHz transcranial Doppler ultrasound to expose and monitor the middle cerebral artery in acute ischemic stroke patients [24]. These authors observed an increase in the rate of sustained complete recanalization within 2 h after the administration of rt-PA in those patients receiving the combined therapy. Building on this strategy for therapeutic success, Molina et al. administered galactose-based microbubbles (Levovist™) in combination with 2.0-MHz transcranial Doppler ultrasound during rt-PA therapy and observed a further increase in the rate of complete recanalization [25]. However,

another study carried out in patients using 300-kHz transcranial ultrasound with a spatial peak temporal average intensity of 0.7 W/cm^2 in combination with rt-PA demonstrated an increased rate of cerebral hemorrhage and the study was prematurely stopped [26]. More research in animal models is necessary to uncover the role of ultrasound in such injury, including the understanding of the potential for reperfusion injury.

Given the wide range of ultrasound frequencies employed by researchers to enhance rt-PA thrombolysis to date, (20-kHz–2-MHz), unresolved questions include the optimization of ultrasound parameters such as frequency, the use of pulsed vs. continuous wave, and the choice of pressure amplitude to enhance thrombolysis yet avoid potentially harmful bioeffects. In this study, two simple designs for ultrasound application at 120 kHz and 1 MHz were tested in an *in vitro* porcine clot model. The synergistic thrombolytic effect of recombinant tissue plasminogen activator (rt-PA) and either 120-kHz or 1-MHz pulsed or continuous wave ultrasound was assessed *in vitro* [27–29]. The objective of this study was to determine the thrombolytic efficacy of pulsed ultrasound over a range of duty cycles at moderate pressure amplitudes. In addition, the effect of ultrasound exposure time was explored over the time period of 0.25 to 2.0 h. This work is an initial step in identifying optimal acoustic parameters for ultrasound-enhanced thrombolysis.

Materials and methods

Experimental apparatus

All experiments were conducted in a Lucite tank (42 cm×24 cm×23 cm) filled with 0.2- μm filtered, deionized water. The water in the tank was maintained at $37\pm0.5^\circ\text{C}$ at all times with a temperature-controlled circulator (Neslab D4571, Newington, New Hampshire, USA).

The overall experimental arrangement is shown in Fig. 1. One of two large, single-element, unfocused narrowband transducers (120 kHz and 1 MHz), described in greater detail hereafter, was placed inside the tank facing the sample holder. The tank wall opposite the source transducer was lined with absorbing rubber (Aptflex F36, Precision acoustics, Dorchester, UK) to minimize reflections and prevent the formation of standing waves. The sample holder consisted of an acoustically transparent latex condom (with reservoir tip) stretched over a plastic frame, which was filled with 30 cc of thawed, porcine fresh frozen plasma (Animal Biotech Industries, Danboro, PA, USA) maintained at 37°C in a temperature-controlled bath for at least 10 min before use.

The source transducer was mounted on a 3-axis translation stage (Newport 423, Irvine, CA, USA) to enable alignment of the ultrasound beam relative to the clot located in the reservoir tip of the sample holder. Alignment was achieved for each experiment by placing a piezoelectric hydrophone (8103, Brüel and Kjaer, Norcross, GA, USA or Reson, TC 4038, Goleta, CA, USA) inside the reservoir tip and by moving the source transducer in both the axial and transverse directions until the amplitude of the signal recorded by the hydrophone was maximal. The B&K 8103 was utilized for the 120 kHz transducer alignment and the Reson TC4038 was employed for the 1-MHz alignment. Thereafter, the hydrophone was removed and a blood clot was placed into the tip of the sample holder. The lyophilized powder form of rt-PA (Activase, Genetech, South San Francisco, CA, USA) was reconstituted in sterile water following the manufacturer's instructions, aliquoted and stored at -80°C . In experiments where blood clots were exposed to rt-PA, the reconstituted aliquots were thawed in a 37°C water bath and added to the porcine plasma.

Two single-element, unfocused sources were employed: a 120-kHz transducer with a diameter of 6.14 cm (Sonic Concepts Inc., Bothell, WA, USA), made of 1–3 piezocomposite material, and a 1-MHz transducer with a diameter of 2.5 cm (Applied Science, Inc, Cedarville, OH).

The aperture of each transducer was chosen such that, at each frequency, the depth of field and beam width would be sufficient to encompass a clot in the human middle cerebral artery [30].

The 120-kHz transducer was driven by a function generator (Agilent, 33120A, Santa Clara, CA, USA), power amplifier (T&C Power Conversions Inc., Ultra 2021, Rochester, NY, USA), and custom-built impedance matching network (Sonic Concepts Inc., Bothell, WA, USA) intended to maximize the electroacoustic power conversion efficiency. The 1-MHz transducer was driven with a custom-built waveform generator (Applied Sciences, Inc., Cedarville, OH, USA) and low-noise power amplifier (Amplifier Research, Model 50 A15, Souderton, PA). Both transducers were driven in either pulsed or continuous wave (CW) modes. The porcine clots were always placed at the natural focus (Rayleigh distance) of each transducer.

***In vitro* clot preparation**

Whole blood clots were prepared by aliquoting 1.5 ml samples of fresh porcine blood into 8-mm inner diameter glass tubes, immersing the tubes in a 37 °C water bath for 3 h and storing the clots at 5 °C, which ensured complete clot retraction. The blood collection protocol was approved by the local institutional animal care and use committee. Additional aliquots of blood from each pig were used to obtain a complete coagulation panel from Antech Diagnostics (Chicago, IL, USA), including D-Dimer, activated partial thromboplastin time, fibrinogen and prothrombin time testing, as well as a complete blood count. A decision to use or reject the resulting clots was made based both on the test results and the clot appearance. The pigs used in this study were found to be slightly anemic, with hematocrits in the range 25–35%. Only donors with values in the range 10–900 ng/ml for the D-Dimer test, 10–25 s for A-PTT, 250–700 mg/dl for the fibrinogen concentration and 9–13 s for prothrombin time were used. The resulting clots are normally dark red in color, roughly cylindrical in shape with an average diameter of 1 cm, and have an initial mass of 0.44 ± 0.06 g.

Calibration of 120-kHz and 1-MHz transducers

The acoustic output of the 120-kHz and 1-MHz transducers was calibrated in a water-filled (97 cm × 57 cm × 54 cm) Lucite tank using a 0.5 mm hydrophone (Reson, TC 4038, Goleta, CA, USA) mounted on a computer-controlled three-axis positioner (Velmex, NF-90 series, Bloomfield, NY, USA) and the beam patterns were carefully mapped. The Rayleigh distance of the 120-kHz transducer was 8.5 cm, the 3-dB depth of field was 7.6 cm and the 3-dB transverse beamwidth was 2.4 cm. Thus the focal region of the 120-kHz transducer is significantly larger than the mean clot diameter (1 cm). The Rayleigh distance of the 1-MHz transducer was 8.3 cm, the 3-dB depth of field was 6.0 cm, and the 3-dB transverse beamwidth was 1 cm. Thus the clot volume is contained within the 3-dB beam width of the 1-MHz transducer. The calibration and alignment protocol ensured uniform sonication of the clots.

Experimental protocol

The whole blood porcine clots were blotted and weighed before and after treatment to determine the percent mass loss. Each clot was placed in an acoustically transparent latex condom filled with thawed porcine fresh frozen plasma (PFFP) and maintained in a 37 °C temperature-controlled water bath (Fig. 1). Reconstituted rt-PA was added to the PFFP for sham (rt-PA only, no ultrasound) and ultrasound-treated clots. Clots treated as controls were exposed to PFFP alone in order to quantify the effect of exposure to plasma and handling alone. To evaluate the effect of clot age on rt-PA thrombolysis (no ultrasound), mass loss data was collected for clots formed from blood taken from a single pig and stored at 5 °C from 1 to 16 days after clot formation ($N=5$ each). Shown in Fig. 2 is the effect of clot age on sham thrombolysis studies with an rt-PA concentration of 0.05 mg/ml. Clots that were one day old exhibited significantly higher clot mass loss ($p<0.05$) compared to aged clots (2–16 day old). Because aged clots

exhibit less thrombolysis when exposed to rt-PA alone, probably due to clot retraction over the first 48 h, only clots aged 3 to 14 days were used in subsequent treatment protocols.

To study the effect of rt-PA concentration on thrombolysis efficacy, mass loss data were collected for clots exposed to rt-PA concentrations varying between 0.003 and 0.107 mg/ml ($N=5$ each). The rt-PA concentration was doubled for each incremental step and the exposure duration was fixed at 30 min. Shown in Fig. 3 is the clot mass loss measured as a function of rt-PA concentration (without ultrasound exposure). The mass loss increased monotonically as a function of rt-PA concentration up to 0.05 mg/ml. Based on this data an rt-PA concentration of 0.107 mg/ml was chosen for all the subsequent ultrasound exposure protocols so that small errors in the rt-PA concentration would not yield a large effect on the clot mass loss.

To evaluate pig-to-pig variability, sham mass loss data were gathered for a fixed exposure time of 30 min using an rt-PA concentration of 0.107 mg/ml. Both control and sham treatments for blood clots obtained from 5 different pigs ($N=5$ each) were performed and mass loss data are shown in Table 1. The pig-to-pig variability in the sham and control mass loss data was not statistically significant ($p=0.065$), as determined using single factor fixed effect ANOVA (Minitab, State College, PA, USA).

To explore the effect of ultrasound duty cycle on rt-PA thrombolytic efficacy at an rt-PA concentration of 0.107 mg/ml, each transducer was driven either in pulsed or CW mode with a duty cycle of 10, 20, 50, 80 and 100% (CW) and pulse repetition frequency (PRF) of 1.7 kHz. A peak-to-peak pressure amplitude of either 0.35 or 0.7 MPa was employed for the 120-kHz treatment protocol and a peak-to-peak pressure amplitude of either 0.35 or 1.0 MPa was used for the 1.0-MHz treatment protocol. The treatment period was fixed at 30 min for the duty cycle experiments. The clot mass loss was assessed for four experimental groups: 1) control (PFFP alone) ($N=34$), 2) sham (rt-PA-treated) ($N=35$), 3) combined ultrasound and rt-PA for all duty cycles and both pressure amplitudes ($N=5$ each) and 4) ultrasound alone for 50% duty cycle and CW at both pressure amplitudes ($N=5$ each). All treatment protocols were compared statistically using single factor, fixed effect ANOVA analysis and Student's t -test with Bonferroni correction for multiple comparisons (Minitab). A p -value of 0.05 was considered significant. Relative thrombolytic enhancement, TE, for each ultrasound and rt-PA combined treatment protocol was compared to rt-PA treatment alone using the following formula,

$$TE\% = \frac{\Delta M_{US+rt-PA} - \Delta M_{rt-PA}}{\Delta M_{rt-PA}} 100\% \quad (1)$$

where ΔM_{rt-PA} is the sham clot mass loss and $\Delta M_{US+rt-PA}$ is the mass loss for clots exposed to ultrasound and rt-PA. The standard error of the mean for each combination of measurements defined in Eq. (1) was calculated using the standard expression for a function of several variables [31].

The ultrasound peak-to-peak pressure amplitude dependence was further investigated at five pressures (0.30 MPa, 0.35 MPa, 0.48 MPa, 0.70 MPa and 0.79 MPa) using a center frequency of 120 kHz and a fixed duty cycle of 80% ($N=6$ each). The pressures were selected based on the stable and inertial cavitation thresholds measured for 120-kHz pulsed ultrasound with the same duty cycle [22]. In addition, the effect of pulsed 120-kHz ultrasound (0.35 MPa, 80% duty cycle) and rt-PA (0.107 mg/ml) exposure duration on clot mass loss was studied between 15 min and 2 h ($N=5$ each). Cerebral angiographic studies of stroke victims treated with rt-PA indicate that intravenous rt-PA partially reopens only 30 to 40% of occluded major intracranial trunk arteries within the first 1 to 2 h after initiation of treatment [32]. Thus the maximum treatment time was chosen to be 2 h. Clot mass loss data over time were fitted to a function of the form At^b , where t is time and A and b are constants, using Microsoft Excel.

Results

The degree of enhancement of rt-PA thrombolysis with 120-kHz ultrasound is shown in Fig. 4. With 120-kHz ultrasound exposure, clot mass loss increased significantly ($p<0.001$) over sham (rt-PA alone). However, ultrasound alone did not enhance thrombolysis in the absence of rt-PA. A lack of ultrasound-enhanced clot mass loss is evident at duty cycles less than 80% ($p>0.05$) for a peak-to-peak pressure amplitude of 0.35 MPa. Also, no ultrasound enhancement of rt-PA thrombolysis was observed at any duty cycle for a peak-to-peak pressure amplitude of 0.7 MPa ($p>0.05$). Note that an 80% duty cycle or CW 120-kHz ultrasound was required to enhance rt-PA-mediated thrombolysis in this model ($p<0.01$). The maximum mass loss was observed for an 80% duty cycle. However, 120-kHz ultrasound alone did not enhance mass loss significantly at 0.7 MPa ($p=0.42$) for any duty cycle. As shown in Fig. 5, 1.0-MHz ultrasound enhancement of rt-PA thrombolysis was observed at both peak-to-peak pressure amplitudes of 0.35 MPa and 1.0 MPa. At both acoustic pressure outputs significant enhancement of thrombolysis ($p<0.001$) was achieved over sham treatment (rt-PA alone).

Relative thrombolytic enhancement as a function of ultrasound duty cycle, defined by Eq. (1) using the data shown in Figs. 4 and 5, is shown in Fig. 6. The largest thrombolytic enhancement, 104% (which represents more than a doubling of the clot mass loss due to rt-PA alone), was noted for 1-MHz CW insonation at a 1.0 MPa peak-to-peak pressure amplitude. The largest thrombolytic enhancement for 120-kHz ultrasound, 56%, was found at 80% duty cycle and 0.35 MPa peak-to-peak pressure amplitude.

The data shown in Fig. 7 exhibit the effect of peak-to-peak pressure amplitude on 120-kHz ultrasound-enhanced rt-PA thrombolysis for a fixed duty cycle of 80%. The maximum mass loss was observed at a peak-to-peak pressure amplitude of 0.48 MPa. The enhancement in clot mass loss over sham was significant ($p<0.005$ Student's *t*-test) for all ultrasound pressure amplitudes except 0.30 MPa.

Shown in Fig. 8 is the effect of exposure duration of 120-kHz pulsed ultrasound and rt-PA on clot mass loss. Clot mass loss increased monotonically with treatment duration for both sham and combined rt-PA and ultrasound-treated clots. The mass loss was significantly higher ($p<0.05$) for ultrasound-exposed clots for each treatment duration compared to sham. In addition, the degree of enhancement increases with the square root of exposure time for longer treatment durations.

Discussion

Some of the parameters required for ultrasonic enhancement of thrombolysis were explored in this study to determine an optimal strategy for therapeutic benefit. These experimental studies elucidated the effects of acoustic parameters, such as center frequency, duty cycle and peak-to-peak pressure amplitude, and treatment duration on rt-PA-mediated clot mass loss. To facilitate the comparison of treatment protocols, a standard clot model was developed by analyzing the effect of clot age and pig-to-pig variability on rt-PA-induced clot mass loss. Freshly formed clots exhibit greater mass loss compared to fully retracted blood clots as shown in Fig. 2. Sabovic et al. [33] also found that serum-poor blood clots were more resistant to rt-PA thrombolysis and that plasminogen recruited from surrounding plasma contributes significantly to clot lysis. The extent of thrombolysis is dependent on rt-PA concentration and increases with increasing thrombolytic concentration until saturation above 0.05 mg/ml as shown in Fig. 3. This trend probably shows the limit of concentration-dependent diffusion and penetration of rt-PA across the clot surface. A higher concentration of rt-PA beyond 0.05 mg/ml does not promote further degradation of the fibrin mesh and concomitant clot mass loss. Similar saturation trends were noted by Onundarson et al. [34] at 0.005 mg/ml and by Trusen

et al. [35] at 0.003 mg/ml in human whole blood clots. The difference in rt-PA saturation concentration could be attributed to a significantly lower concentration of plasminogen in porcine blood than human blood [36].

Ultrasound without the presence of rt-PA did not enhance thrombolysis compared to control treatment (see Figs. 4 and 5). In addition, treatment with rt-PA alone produced more thrombolysis than ultrasound alone, both at 50% duty cycle and CW, even at the highest peak-to-peak pressure amplitude (1.0 MPa). Schäfer et al. [37] noted significant enhancement of thrombolysis (without the presence of a thrombolytic drug) in fresh human whole blood clots exposed to 2 to 4.5 MHz pulsed and continuous wave ultrasound. However, we did not observe such enhancement with ultrasound alone at 120 kHz or 1.0 MHz for similar treatment durations. It should be noted that the pressures reported by Schäfer et al. were an order of magnitude larger than the peak-to-peak pressures explored in our study.

Although the largest clot mass loss, 25.0%, was measured using 1.0-MHz CW ultrasound with a peak-to-peak pressure amplitude of 1.0 MPa, 22% clot mass loss was noted using 1.0-MHz pulsed ultrasound with a 10% duty cycle and a 0.35 MPa peak-to-peak pressure amplitude. The potential for harmful bioeffects in the brain due to ultrasound exposure is reduced for pulsed ultrasound at lower peak pressure amplitudes. The largest clot mass loss noted using 120 kHz ultrasound, 19.1%, was observed using 80% duty cycle at a peak-to-peak amplitude of 0.35 MPa. The difference between the highest mean clot mass loss at 1.0 MHz and the highest mean clot mass loss at 120 kHz, however, is not statistically significant ($p > 0.05$). Note also that the attenuation of ultrasound through human temporal bone is much higher at 1.0 MHz than at 120 kHz, 1.5 vs. 0.12 Np/cm, respectively [38]. A larger amount of attenuation and heating at the surface of the cranial bone would be expected at higher frequencies.

Clots exposed to rt-PA and ultrasound for 30 min exhibited enhanced thrombolysis over clots exposed to rt-PA alone, as much as 56% for 120-kHz ultrasound and 104% for 1-MHz ultrasound, as shown in Fig. 6. Curiously, no definitive trends in thrombolytic enhancement as a function of duty cycle were observed. In a recent study, Meunier et al. observed a linear increase in initial lytic rate as a function of duty cycle in human clots exposed to 120 kHz pulsed ultrasound [39]. However, after 30 min the thrombolytic enhancement measured by these investigators as a decrease in clot width did not increase linearly with duty cycle. The clot mass loss data plotted in Fig. 8 as a function of time demonstrate a square root of time dependence. A similar time dependence has been predicted by a theoretical model of rt-PA thrombolysis developed by Shaw et al. [40]. The phenomenon of *in vitro* rt-PA induced clot lysis is predominantly controlled by plasminogen-and fibrin-specific chemical reactions and, importantly, the diffusion of exogenous rt-PA and plasminogen into the clot sample. Thus it is reasonable that the enhancement of clot mass loss with time would follow a square root of time dependence.

The effect of pressure amplitude on 120-kHz ultrasound-enhanced thrombolysis is plotted in Fig. 7. A statistically significant increase ($p < 0.05$) in mass loss over sham (rt-PA alone) was noted for clots exposed to 0.35 MPa and 0.48 MPa peak-to-peak pressure amplitude only. This result is in agreement with a previous study that demonstrated a correlation of enhanced thrombolysis with stable cavitation [22]. Pressure amplitudes greater than 0.60 MPa were shown to promote inertial cavitation, which “destabilized” the stable cavitation and reduced the degree of thrombolytic enhancement.

In the kilohertz frequency range several nonthermal mechanisms related to the transport of fibrinolytic enzymes into the clot have been proposed [12,41,42]. Nahrinyak et al. [38] have shown that for 120-kHz and 1-MHz exposed clots at a peak-to-peak pressure amplitude of 0.25 MPa, the temperature rise in the center of the clot was less than 1 °C, suggesting that a thermal

mechanism does not contribute significantly to the thrombolytic enhancement. Some studies have shown that ultrasound accelerates enzymatic fibrinolysis by increasing the transport of reactants through cavitation-related mechanisms [16-18]. Soltani and Soliday [43] elucidated the lack of a direct effect of ultrasound on the enzymatic activity of rt-PA and other thrombolytics. Rather, ultrasound appears to facilitate the penetration of rt-PA into the fibrin structure of the clot [41,44]. In addition, Datta et al. [22] demonstrated the correlation of enhanced thrombolysis with the presence of stable cavitation without inertial cavitation.

Curiously, no clear trend in clot mass loss as a function of duty cycle is evident in the data either at 120 kHz or at 1 MHz. If stable cavitation were present throughout the entire pulse duration, then one would expect an increase in thrombolysis for increased duty cycle. However, without continuous nucleation, stable cavitation may not occur throughout the entire pulse duration. The peak-to-peak pressure amplitude threshold of stable cavitation in this *in vitro* system has been shown to be 0.4 MPa [22]. Also, the threshold of inertial cavitation was determined to be 0.6 MPa [22]. The presence of inertial cavitation prevents the sustainment of stable cavitation by definition. Therefore, the lack of duty cycle dependence on thrombolytic enhancement for ultrasound exposures below the stable cavitation threshold (0.35 MPa) or above the inertial cavitation threshold (0.70 MPa) suggests a specific dependence on sustained stable cavitation. Further thrombolytic enhancement might be possible with controlled nucleation of stable cavitation and careful prevention of inertial cavitation throughout the entire pulse duration.

Härdig et al. [45] measured a 31.2% enhancement of streptokinase thrombolysis in human whole blood clots exposed to 1.0-MHz pulsed ultrasound with a duty cycle of 10% and a spatial average, temporal average intensity of 0.5 W/cm². These investigators also noted a decrease in ultrasound enhancement of streptokinase thrombolysis at higher exposure level (above 1 W/cm²). A similar trend was noted in our clots exposed to 1.0-MHz pulsed ultrasound with a 10% duty cycle. The enhancement in mass loss is particularly high for duty cycles greater than 50%. It is hypothesized that the lower amplitude ultrasound exposure promoted stable cavitation and the higher amplitude exposure level promoted both stable and inertial cavitation [22]. The amount and duration of stable cavitation appears to correlate with rt-PA thrombolytic enhancement and the presence of inertial cavitation in this case serves to decrease the overall stable cavitation activity [22,44].

As a clot is not an actively incorporating thrombus, the use of an ex vivo porcine clot model for these studies represents a limitation of the study. However it embodies a necessary first step, particularly for porcine *in vivo* studies of ultrasound-enhanced rt-PA thrombolysis. The thrombolytic drug used in this study, rt-PA, may very likely have a different effect on human thrombi *in vivo* than the efficacy demonstrated in this *in vitro* porcine clot study [46-48]. In addition, the amount of endogenous plasminogen in humans and pigs differs by as much as an order of magnitude. The normal human range of plasminogen concentration in blood is 0.80–1.20 U/ml and the range of plasminogen concentration in pigs is 0.08 to 0.5 depending on the species [36]. Because rt-PA works by converting plasminogen to plasmin, the overall availability of plasminogen is crucial to its effectiveness. Thus the clot mass loss observed in this porcine *in vitro* study may be lower than that expected in human *in vivo*. Note also the concentration of rt-PA in this *in vitro* study is greater than that used in humans.

Conclusions

We have demonstrated that 120-kHz or 1.0-MHz ultrasound used as an adjuvant to rt-PA can increase clot dissolution in an *in vitro* model. Clot mass loss due to rt-PA alone is dependent on rt-PA concentration and increases with increasing thrombolytic concentration until saturation above 0.05 mg/ml. Exposure of the clots to ultrasound in the presence of rt-PA

resulted in enhancement of thrombolysis greater than 50% over treatment with rt-PA alone at both frequencies. The largest thrombolytic enhancement, 104%, was observed for 1-MHz CW ultrasound exposure at a 1.0 MPa peak-to-peak pressure amplitude. For 120-kHz exposures, the largest thrombolytic enhancement, 56%, was measured using 80% duty cycle and 0.35 MPa peak-to-peak pressure amplitude. This enhancement increased for longer treatment durations at 120 kHz. Higher pressure amplitudes at 120 kHz beyond 0.48 MPa did not necessarily lead to increased clot mass loss. Further improvement of thrombolytic enhancement may be possible if stable cavitation is both nucleated and sustained and inertial cavitation is avoided throughout the entire pulse duration. These *in vitro* data may help guide the development of an optimal strategy for an ultrasound-assisted thrombolysis system that promotes rapid restoration of blood flow in the treatment of myocardial infarction or ischemic stroke.

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References

1. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, et al. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics-2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007;115:e69–171. [PubMed: 17194875]
2. Broderick J, Brott T, Kothari R, Miller R, Khoury J, Pancioli A, et al. The Greater Cincinnati/Northern Kentucky Stroke Study: preliminary first-ever and total incidence rates of stroke among blacks. *Stroke* 1998;29:415–21. [PubMed: 9472883]
3. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med* 1995;333:1581–8. [PubMed: 7477192]
4. Wolpert SM, Bruckmann H, Greenlee R, Wechsler L, Pessin MS, del Zoppo GJ. Neuroradiologic evaluation of patients with acute stroke treated with recombinant tissue plasminogen activator. The rt-PA Acute Stroke Study Group. *AJNR Am J Neuroradiol* 1993;14:3–13. [PubMed: 8427107]
5. Mueller HS, Dyer A, Greenberg MA. The thrombolysis in myocardial infarction (TIMI) trial. Phase I findings. *N Engl J Med* 1985;312:932–6. [PubMed: 4038784]
6. Marder VJ, Sherry S. Thrombolytic therapy: current status (1). *N Engl J Med* 1988;318:1512–20. [PubMed: 3285216]
7. Marder VJ, Sherry S. Thrombolytic therapy: current status (2). *N Engl J Med* 1988;318:1585–95. [PubMed: 3287158]
8. Gore JM, Sloan M, Price TR, Randall AM, Bovill E, Collen D, et al. Intracerebral hemorrhage, cerebral infarction, and subdural hematoma after acute myocardial infarction and thrombolytic therapy in the Thrombolysis in Myocardial Infarction Study. *Thrombolysis in Myocardial Infarction, Phase II, pilot and clinical trial. Circulation* 1991;83:448–59. [PubMed: 1899364]
9. Braunwald E. Effects of tissue plasminogen activator and a comparison of early invasive and conservative strategies in unstable angina and non-Q-wave myocardial infarction: results of the TIMI IIIB trial. *Circulation* 1994;89:1545–56. [PubMed: 8149520]
10. Cannon CP. Exploring the issues of appropriate dosing in the treatment of acute myocardial infarction: potential benefits of bolus fibrinolytic agents. *Am Heart J* 2000;140:S154–60. [PubMed: 11100010]
11. Sobbe A, Stumpff U, Trubestein G, Figge H, Kozuschek W. Thrombolysis by ultrasound (author's transl). *Klin Wochenschr* 1974;52:1117–21. [PubMed: 4444229]
12. Siegel RJ, Ariani M, Forester JS, Hashemi Z, De Castro E, Don Michael TA, et al. Cardiovascular applications of therapeutic ultrasound. *J Invasive Cardiol* 1989;4:219–29.
13. Rosenschein U, Bernstein JJ, DiSegni E, Kaplinsky E, Bernheim J, Rozenzajn LA. Experimental ultrasonic angioplasty: disruption of atherosclerotic plaques and thrombi in vitro and arterial recanalization in vivo. *J Am Coll Cardiol* 1990;15:711–7. [PubMed: 2137480]
14. Ariani M, Fishbein MC, Chae JS, Sadeghi H, Michael AD, Dubin SB, et al. Dissolution of peripheral arterial thrombi by ultrasound. *Circulation* 1991;84:1680–8. [PubMed: 1914107]

15. Lauer CG, Burge R, Tang DB, Bass BG, Gomez ER, Alving BM. Effect of ultrasound on tissue-type plasminogen activator-induced thrombolysis. *Circulation* 1992;86:1257–64. [PubMed: 1394932]
16. Francis CW, Onundarson PT, Carstensen EL, Blinc A, Meltzer RS, Schwarz K, et al. Enhancement of fibrinolysis in vitro by ultrasound. *J Clin Invest* 1992;90:2063–8. [PubMed: 1430229]
17. Blinc A, Francis CW, Trudnowski JL, Carstensen EL. Characterization of ultrasound-potentiated fibrinolysis in vitro. *Blood* 1993;81:2636–43. [PubMed: 8490172]
18. Francis CW, Blinc A, Lee S, Cox C. Ultrasound accelerates transport of recombinant tissue plasminogen activator into clots. *Ultrasound Med Biol* 1995;21:419–24. [PubMed: 7645133]
19. Everbach EC, Francis CW. Cavitation mechanisms in ultrasound-accelerated thrombolysis at 1 MHz. *Ultrasound Med Biol* 2000;26:1153–60. [PubMed: 11053750]
20. Akiyama M, Ishibashi T, Yamada T, Furuhashi H. Low-frequency ultrasound penetrates the cranium and enhances thrombolysis in vitro. *Neurosurgery* 1998;43:828–32. [PubMed: 9766310]discussion 832-3
21. Behrens S, Daffertshofer M, Spiegel D, Hennerici M. Low-frequency, low-intensity ultrasound accelerates thrombolysis through the skull. *Ultrasound Med Biol* 1999;25:269–73. [PubMed: 10320316]
22. Datta S, Coussios C, McAdory LE, Tan J, Porter T, De Courten-Myers G, et al. Correlation of cavitation with ultrasound enhancement of thrombolysis. *Ultrasound Med Biol* 2006;32:1257–67. [PubMed: 16875959]
23. Siegel RJ, Atar S, Fishbein MC, Brasch AV, Peterson TM, Nagai T, et al. Noninvasive, transthoracic, low-frequency ultrasound augments thrombolysis in a canine model of acute myocardial infarction. *Circulation* 2000;101:2026–9. [PubMed: 10790341]
24. Alexandrov AV, Molina CA, Grotta JC, Garami Z, Ford SR, Alvarez-Sabin J, et al. Ultrasound-enhanced systemic thrombolysis for acute ischemic stroke. *N Engl J Med* 2004;351:2170–8. [PubMed: 15548777]
25. Molina CA, Ribo M, Rubiera M, Montaner J, Santamarina E, Delgado-Mederos R, et al. Microbubble administration accelerates clot lysis during continuous 2-MHz ultrasound monitoring in stroke patients treated with intravenous tissue plasminogen activator. *Stroke* 2006;37:425–9. [PubMed: 16373632]
26. Daffertshofer M, Gass A, Ringleb P, Sitzler M, Sliwka U, Els T, et al. Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: Increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator—results of a phase II clinical trial. *Stroke* 2005;36:1441–6. [PubMed: 15947262]
27. Shaw GJ, Hahn NL, Wagner KR, Kanter DS, Holland CK. Ultrasound assisted clot lysis for stroke therapy. *J Acoust Soc Am* 2001;109:2456.
28. Holland CK, Vaidya SS, Coussios CC, Shaw GJ. Thrombolytic effects of 120-kHz and 1-MHz ultrasound and tissue plasminogen activator on porcine whole blood clots. *J Acoust Soc Am* 2002;112:2370.
29. Shaw GJ, Vaidya SS, Wagner KR, Holland CK. 1-MHz ultrasound assists clot lysis. *J Ultrasound Med* 2002;21:S10.
30. Coussios CC, Holland CK, Shaw GJ. Transmission of a large unfocused 120-kHz and 1-MHz ultrasound beam through the human skull. *J Acoust Soc Am* 2002;112:2433.
31. Barford, NC. Experimental measurements: precision, error and truth. John Wiley & Sons; 1985.
32. Broderick JP, Hacke W. Treatment of acute ischemic stroke: Part I: recanalization strategies. *Circulation* 2002;106:1563–9. [PubMed: 12234965]
33. Sabovic M, Lijnen HR, Keber D, Collen D. Effect of retraction on the lysis of human clots with fibrin specific and non-fibrin specific plasminogen activators. *Thromb Haemost* 1989;62:1083–7. [PubMed: 2515603]
34. Onundarson PT, Francis CW, Marder VJ. Depletion of plasminogen in vitro or during thrombolytic therapy limits fibrinolytic potential. *J Lab Clin Med* 1992;120:120–8. [PubMed: 1613318]
35. Trusen B, Ries M, Zenker M, Rauh M, Beinder E, Kuiper H, et al. Whole blood clot lysis in newborns and adults after adding different concentrations of recombinant tissue plasminogen activator (Rt-PA). *Semin Thromb Hemost* 1998;24:599–604. [PubMed: 10066156]
36. Lewis, JH. Comparative hemostasis in vertebrates. New York-London: Plenum Press; 1996.

37. Schäfer S, Kliner S, Klinghammer L, Kaarmann H, Lucic I, Nixdorff U, et al. Influence of ultrasound operating parameters on ultrasound-induced thrombolysis in vitro. *Ultrasound Med Biol* 2005;31:841–7. [PubMed: 15936499]
38. Nahirnyak VM, Mast TD, Holland CK. Ultrasound-induced hyperthermia in clotted blood and cranial temporal bone. *Ultrasound in Med Biol* 2007;33:1285–95. [PubMed: 17490808]
39. Meunier JM, Holland CK, Lindsell CJ, Shaw GJ. Duty cycle dependence of ultrasound enhanced thrombolysis in a human clot model. *Ultrasound Med Biol* 2007;33(4):576–83. [PubMed: 17337113]
40. Shaw GJ, Dhamija A, Bavani N, Wagner KR, Holland CK. Arrhenius Temperature Dependence of In-vitro Tissue Plfaminogen Activator Thrombolysis. *Phys Med Biol* 2007;52:2953–67. [PubMed: 17505082]
41. Siddiqi F, Odrliin TM, Fay PJ, Cox C, Francis CW. Binding of tissue-plasminogen activator to fibrin: effect of ultrasound. *Blood* 1998;91:2019–25. [PubMed: 9490686]
42. Suchkova V, Siddiqi FN, Carstensen EL, Dalecki D, Child S, Francis CW. Enhancement of fibrinolysis with 40-kHz ultrasound. *Circulation* 1998;98:1030–5. [PubMed: 9737524]
43. Soltani A, Soliday C. Effect of ultrasound on enzymatic activity of selected plasminogen activators. *Thromb Res* 2007;119:223–8. [PubMed: 16545433]
44. Datta S, Vaidya SS, Beiler S, de Courten-Meyer G, Holland CK. Ultrasound-assisted penetration of rt-PA into clots to enhance thrombolysis. *J Acoust Soc Am* 2006;120:3003.
45. Härdig BM, Persson HW, Olsson SB. Low-energy ultrasound exposure of the streptokinase molecule may enhance but also attenuate its fibrinolytic properties. *Thromb Res* 2006;117:713–20. [PubMed: 15993473]
46. Niessen F, Hilger T, Hoehn M, Hossmann KA. Differences in clot preparation determine outcome of recombinant tissue plasminogen activator treatment in experimental thromboembolic stroke. *Stroke* 2003;34:2019–24. [PubMed: 12843350]
47. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* 2005;115:3355–62. [PubMed: 16322780]
48. Marder VJ, Chute DJ, Starkman S, Abolian AM, Kidwell C, Liebeskind D, et al. Analysis of thrombi retrieved from cerebral arteries of patients with acute ischemic stroke. *Stroke* 2006;37:2086–93. [PubMed: 16794209]

Abbreviations

A-PTT	activated partial thromboplastin time
CW	continuous wave
PFFP	porcine fresh frozen plasma
PRF	pulse repetition frequency
rt-PA	recombinant tissue plasminogen activator
TE	thrombolytic enhancement
US	ultrasound

Figure 1. Schematic of ultrasound exposure apparatus. A porcine clot is placed in an acoustically transparent latex sample holder located at the experimentally determined Rayleigh distance of the source transducer.

Figure 2. Effect of clot age on clot mass loss for sham treatment (rt-PA concentration is 0.05 mg/ml). Error bars represent the standard deviation ($N=5$ each).

Figure 3. Percent clot mass loss as a function of rt-PA concentration. Error bars represent the standard deviation ($N=5$ each).

Figure 4.

Effect of 120-kHz pulsed and CW ultrasound on clot mass loss. The concentration of rt-PA was 0.107 mg/ml. Control exposures (PFFP alone) are shown in the white bar with hatching ($N=34$). Sham exposures (rt-PA alone) are shown in the solid white bar ($N=35$). Ultrasound exposures with rt-PA at a peak-to-peak pressure amplitude of 0.35 MPa are shown in solid light gray bars ($N=10$). Ultrasound exposures with rt-PA at a peak-to-peak pressure amplitude of 0.70 MPa are shown in solid dark gray bars ($N=5$). Ultrasound exposures alone (no rt-PA) are shown in hatched light, and dark gray bars for 0.35 MPa ($N=10$) and 0.7 MPa ($N=5$) peak-to-peak pressure amplitudes, respectively. Error bars represent the standard deviation.

Figure 5.

Effect of 1-MHz pulsed and CW ultrasound on clot mass loss. The concentration of rt-PA was 0.107 mg/ml. Control exposures (PFFP alone) are shown in the white bar with hatching ($N=34$). Sham exposures (rt-PA alone) are shown in the solid white bar ($N=35$). Ultrasound exposures with rt-PA at a peak-to-peak pressure amplitude of 0.35 MPa are shown in solid light gray bars ($N=5$). Ultrasound exposures with rt-PA at a peak-to-peak pressure amplitude of 1.0 MPa are shown in solid dark gray bars ($N=5$). Ultrasound exposures alone (no rt-PA) at a peak-to-peak pressure amplitude of 1.0 MPa are shown in hatched dark gray bars ($N=5$). Error bars represent the standard deviation.

Figure 6. Plot of the thrombolytic enhancement as a function of ultrasound duty cycle, defined in (Eq. (1)) using data shown in Figs. 4 and 5. Error bars represent standard error of mean for the combined measurements.

Figure 7. Effect of 120-kHz pulsed ultrasound (80% duty cycle) on thrombolysis as a function of peak-to-peak pressure amplitude ($N=6$ each). Sham data represents treatment of the clots with rt-PA in plasma alone ($N=35$). The concentration of rt-PA used was 0.107 mg/ml. Error bars represent the standard deviation.

Figure 8.

Effect of 120-kHz pulsed ultrasound and rt-PA exposure duration on clot mass loss. The concentration of rt-PA was 0.107 mg/ml. Control exposures (handling only) are plotted with open circles ($N=5$ each). Sham exposures (rt-PA alone) are plotted with open diamonds ($N=5$ each). Pulsed ultrasound exposures at a peak-to-peak pressure amplitude of 0.35 MPa and 80% duty cycle with rt-PA are shown in open triangles ($N=5$ each). Error bars represent the standard deviation. The time dependence of the clot mass loss with rt-PA and ultrasound treatment follows an approximate square root of time trend.

Table 1

Control (no ultrasound, no rt-PA) and sham (rt-PA alone) mass loss data from 5 different pigs ($N=5$ each) for a fixed exposure time of 30 min with an rt-PA concentration of 0.107 mg/ml

Pig number	Clot mass loss (%)	
	Control	Sham
1	3.66 (± 2.43)	9.44 (± 4.40)
2	3.50 (± 2.11)	13.52 (± 1.95)
3	2.97 (± 1.82)	14.60 (± 3.03)
4	2.49 (± 1.73)	12.39 (± 1.33)
5	2.97 (± 1.82)	14.60 (± 3.03)

Data represent the mean and standard deviation of the clot mass loss measurements.