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Stability of alteplase in presence of cavitation

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Abstract

Several experimental studies have demonstrated that ultrasound (US) can accelerate enzymatic fibrinolysis and this effect is further enhanced in the presence of ultrasound contrast agents (UCA). Although UCA have been shown to be safe when administered to ischemic stroke patients, safety information of these agents in the thrombolysis setting is limited. Therefore, in this study we investigated potential adverse effects of acoustic cavitation generated by UCA on alteplase (t-PA), the drug used for treatment of ischemic stroke patients. A volume of 0.9 mL of alteplase was dispensed into a custom-made polyester sample tube. For treatments in the presence or absence of cavitation either 0.1 mL Optison or phosphate buffer saline was combined with alteplase. Three independent samples of each treatment group were exposed to ultrasound of 2 MHz frequency at three different peak negative acoustic pressures of 0.5, 1.7, and 3.5 MPa for a duration of 60 min. All treatments were carried out in a cavitation detection system which was used to insonify the samples and record acoustic emissions generated within the sample. After ultrasound exposure, the treated samples and three untreated drug samples were tested for their enzymatic activity using a chromogenic substrate. The insonified samples containing Optison demonstrated cavitational activity proportional to acoustic pressure. No significant cavitation activity was observed in the absence of Optison. Enzymatic activity of alteplase in both insonified groups was comparable to that in the control group. These tests demonstrated that exposure of alteplase to 60 min of 2 MHz ultrasound at acoustic pressures ranging from 0.5 MPa to 3.5 MPa, in the presence or absence of Optison had no adverse effects on the stability of this therapeutic compound.

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Keywords: Acoustic cavitation; Sonothrombolysis; Enzymatic stability; Sonochemistry; Alteplase

1. Introduction

It is well known that ultrasound (US) can accelerate clot dissolution by plasminogen activators (PA) [1–3]. Although the mechanisms are not fully understood, it is speculated that ultrasound accelerates enzyme mediated thrombolysis primarily through mechanical effects, by increasing transport of drug molecules into the clot [4–6]. In particular, cavitation has been identified as a mechanism that can significantly enhance this process [7]. The addition of ultrasound contrast agents (UCA), which act as cavitation nuclei, has been shown to increase the effectiveness of ultrasound-accelerated thrombolysis [8–11]. The feasibility of this approach has been demonstrated in the treatment of ischemic stroke by Molina et al. [12] and Viguier et al. [13]. Molina et al. reported that administration of UCA induces further acceleration of US-enhanced thrombolysis in acute ischemic stroke leading to a more complete recanalization. Although UCA have been shown to be safe when administered to acute ischemic stroke patients [14,15], there is limited information available as to the impact of UCA on alteplase stability in the presence of ultrasound at the acoustic pressure levels relevant to ischemic stroke treatment. Smikahl et al. [16] demonstrated that alteplase

Abbreviations: US, Ultrasound; ANOVA, ANalysis Of VAriance; OP, Optison; UCA, Ultrasound contrast agent; PA, Plasminogen activator; TCD, Transcranial Doppler.

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exposed to a 20 kHz vibrating wire for 6 min remained fully active and stable. Although the presence of cavitation was not directly verified during the tests reported by Smikahl et al., it was proposed as the principle mechanism for this device [17].

In a previous study we demonstrated that ultrasound (1 MHz, 2.5–3.1 W/cm^2) did not affect the biological activity of four plasminogen activators, including alteplase [18]. Meanwhile, we learned that the acoustic output used in the previous study was not high enough to generate cavitation in a clean, particulate-free, solution. Considering that cavitation is known to be mechanism responsible for causing biochemical reactions in sonochemistry [19] by causing bond dissociations in molecules and produce free radicals that can react with biomolecular materials [20–22], we designed the current study to investigate the impact of acoustic cavitation arising from UCAs on the stability of alteplase. Any adverse effect caused by ultrasound to alteplase that would lead to inactivation, denaturation, or fragmentation due to high temperature, microjets, free radical generation, acoustic streaming and increased shear stress, will lead to a decrease in enzymatic activity of this enzyme which could be detected using a well established chromogenic assay [1,23–28]. Enzymatic activity was evaluated after 60 min of exposure to 2 MHz ultrasound at various acoustic pressure amplitudes in the presence and absence of a UCA called Optison while the type (stable, inertial), relative quantity and duration of cavitation was acoustically monitored in real-time.

2. Materials and methods

2.1. Sample preparation

Alteplase (Activase®; Genentech Inc., South San Francisco, CA) was reconstituted to a concentration of 580,000 IU/mL. 0.9 mL of the alteplase was dispensed into a custom-made, 9.4 mm ID, thin-walled (0.0020″ wall), polyester test tube chosen for its acoustic transparency. For treatments in which cavitation was desired, 0.1 mL Optison (Amersham Health Inc., Princeton, NJ, USA) was added to the alteplase to give an Optison volume concentration of 10% v/v, which is approximately 50 times greater than the specified maximum total dose for intravenous application of Optison, which is 0.2% v/v or 8.7 mL in ~4.7 L blood. For treatments without contrast agent, 0.1 mL phosphate buffered saline was combined with the alteplase. In all cases, the final alteplase concentration was 522,000 IU/mL. The reason for choosing higher concentrations of alteplase and Optison than used in a clinical setting was to increase the measurement sensitivity. A high Optison concentration will enhance the cavitational activity [29] and a high concentration of alteplase molecules will increase the molecular interaction [30].

2.2. Treatment protocols

Three main treatment protocols were tested: (1) A—alteplase-only control; (2) A + US—alteplase exposed to ultrasound in the absence of Optison; and (3) A + US + OP—alteplase exposed to ultrasound in the presence of Optison. For both protocols with ultrasound, three different acoustic pressure levels were investigated (resulting in a total of seven treatment protocols). Three independent samples were tested for each of the treatment protocols [25,26].

2.3. Experimental setup

All treatments were carried out in a cavitation detection system, which was used to induce cavitation, and record scattered acoustic emissions generated within the sample. Fig. 1 shows a diagram of the experimental setup. The water bath was heated to 37 °C, filtered to 0.2 μm, and degassed to less than 36% of saturation. The test tube containing alteplase sample was lowered into the water bath where it remained for the duration of the 60 min treatment. A magnetic, stir bar (5 mm length) at the bottom of the sample tube, controlled by a magnetic stir plate positioned 4 cm beneath the sample tube base, rotated at 400 rpm to

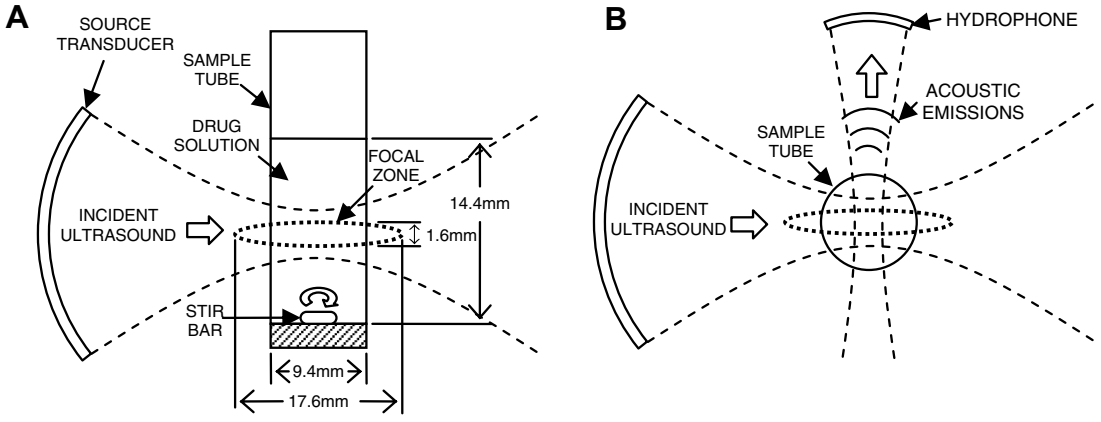

Fig. 1. Diagram of experimental setup: (A) side view; (B) top view.

maintain constant mixing of the alteplase solution. Mixing was particularly important for the treatment with Optison to ensure that the microbubbles remained suspended throughout and continuously replenished the transducer focal zone.

2.4. Acoustic source

A spherically-focused (3.5 cm diameter; 5.5 cm radius of curvature) ultrasound transducer (Sonic Concepts, WA, USA) was used to generate an acoustic wave of precise pressure amplitude in the sample tube. The transducer was positioned so that the focal zone was centered in the tube, both laterally and vertically. The −6 dB focal width and depth were 1.6 mm and 17.6 mm, respectively. Assuming a cylindrical focal zone that intersected the sample tube walls, the volume of alteplase solution enclosed by the focal zone was ~20 μl. Thus, at any given time during the treatment, approximately 2% of the total fluid volume was exposed to ultrasound.

The transducer was driven at 2 MHz in pulsed mode using 10,000-cycle (5 ms) bursts. The amplitude of the input drive voltage was adjusted to produce one of three acoustic peak-negative pressures at the focal zone: 0.5 MPa, 1.7 MPa, and 3.5 MPa. The peak negative acoustic pressure at the focus of the 2 MHz transducer was measured using a bilaminar PVDF membrane hydrophone (Model 804, Sonora Medical Systems) which had a frequency response that was smooth and flat up to 20 MHz. The waveforms corresponding to the three pressure amplitudes that were tested are shown in Fig. 2. Amplitude asymmetry due to nonlinear distortion was observed at the highest pressure (3.5 MPa).

2.5. Cavitation detection system and signal analysis

Bubbles oscillating in a sound field emit acoustic waves. Depending on the type of cavitation, the frequency spectrum of the acoustic emissions may contain components that are specific to the bubble activity (i.e. are not present in the spectrum of the driving sound field). Bubbles undergoing a nonlinear stable pulsation can generate subharmonics at half the insonation frequency. Inertial cavitation produces broadband noise, a result of the rapidly changing bubble radius

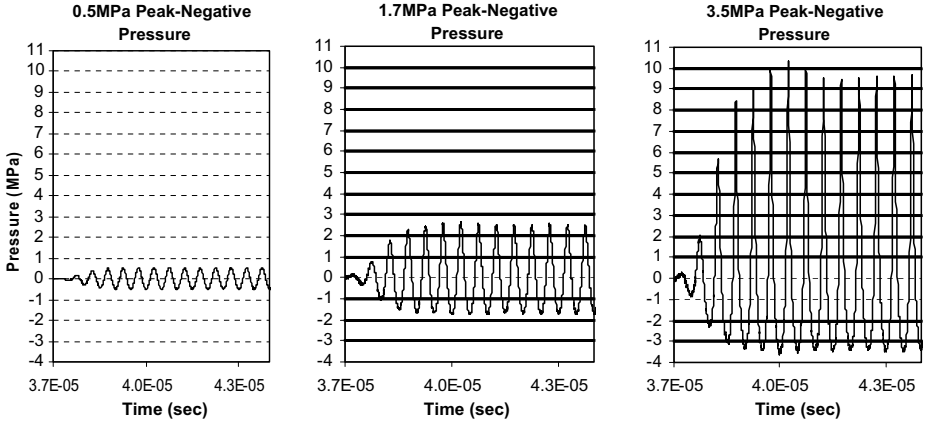

Fig. 2. Acoustic pressure waveforms measured at focus of 2 MHz transducer in degassed water.

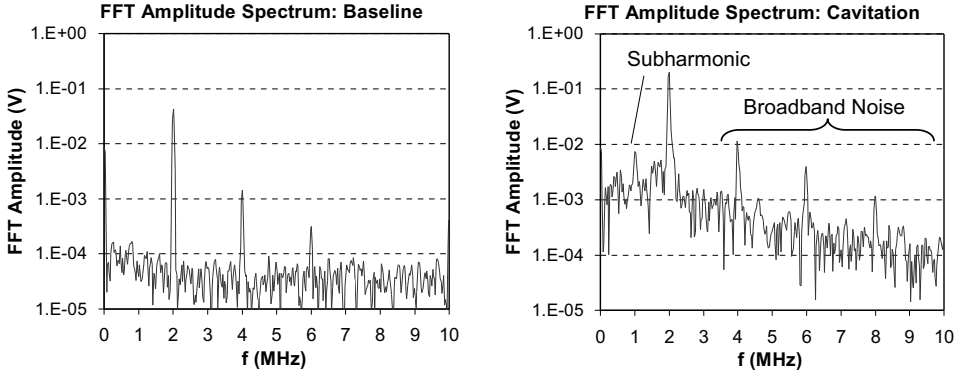

Fig. 3. FFT amplitude spectra of detected acoustic signal in the absence of cavitation (left), and in the presence of both stable and inertial cavitation (right), as indicated by a subharmonic peak located at 1 MHz, and broadband noise elevation. The measurements were taken from the same sample before and after addition of Optison. Note that peaks at the fundamental frequency (2 MHz) and its harmonics (4 MHz, 6 MHz, etc.) are observed in both the baseline and cavitation signals.

[31]. Passive cavitation detection involves "listening" for these acoustic emissions (Fig. 3).

A passive cavitation detection scheme [32] was used to monitor cavitation generated within each sample during treatment. A broadband (flat frequency response up to 10 MHz), spherically-focused (1.3 cm diameter, 5.5 cm radius of curvature), polyvinylidene fluoride (PVDF) hydrophone (Sonic Concepts, WA, USA) was used to monitor acoustic emissions from the sample. The hydrophone voltage signal passed through an anti-aliasing 10 MHz lowpass filter, was digitized at 50 MHz sampling rate using an 8 bit analog-to-digital converter board, and stored on a computer for offline analysis. Software programs written in LabVIEW v.7.1 were used to control signal acquisition and to perform signal analysis. During data collection, recording of the hydrophone signal was synchronized to the source transducer driving pulse. To limit the collected data to manageable file sizes, signals were recorded for 1 out of every 30 bursts, corresponding to an acquisition rate of 1 signal per second. Hydrophone data was collected over 5-min intervals (resulting in 300 captured bursts per interval) at five different times during the treatment: at 0 min (start of exposure), 5 min, 15 min, 30 min, and 55 min of elapsed treatment time.

Data analysis consisted of tracking and quantifying certain frequency components detected in the scattered acoustic emissions that are characteristic of cavitation activity. Subharmonic emission at one-half the driving frequency, a characteristic of stable nonlinear bubble oscillation [33,34], was used as a general indicator for stable cavitation activity. Broadband noise was used as an indicator for inertial cavitation activity [35]. Analysis was performed on a small segment of the recorded burst, 41 μs in duration, beginning 19 μs after the start of the burst. The time-domain hydrophone signal corresponding to each burst was converted to the frequency domain using a 2048-point Fast Fourier Transform (FFT). A Blackman–Harris window was used to reduce spectral leakage in the frequency domain, providing increased signal-to-noise ratio.

2.6. Broadband noise quantification

Broadband cavitation noise corresponded to an elevation in signal amplitude between the harmonic peaks in the FFT magnitude spectrum. The broadband noise amplitude, NA, in a particular signal was quantified by integrating the "inter-peak" noise between 4 and 10 MHz. To reference the noise amplitude to a non-cavitating "baseline" signal (i.e. the background noise level), a relative noise enhancement, RNE, was calculated as the increase in noise amplitude relative to the average baseline noise amplitude, $\langle \mathrm{BNA} \rangle$ (Eq. (1)).

$$\mathrm{RNE} = \frac{\mathrm{NA} - \langle \mathrm{BNA} \rangle}{\langle \mathrm{BNA} \rangle} \tag{1}$$

The baseline signal was collected from the sample tube filled with 0.2 μm filtered, degassed water, and exposed to ultrasound. Separate baselines were obtained for each pressure level. To quantify the average noise level over a 5-min data collection interval, the noise quantities per burst were averaged over the 300 recorded bursts to yield the average relative noise enhancement, $\langle \mathrm{RNE} \rangle$. Subsequently, these values were averaged for each treatment group. The groups were subject to statistical analysis using One-Way ANOVA and a post-hoc student's *T*-test with Tukey's correction.

2.7. Subharmonic emission quantification

Subharmonic emission corresponded to an elevation in the signal amplitude at half the fundamental frequency in the FFT magnitude spectrum. To quantify the subharmonic content in a recorded burst, the subharmonic amplitude, SA, was taken to be the FFT magnitude at 1 MHz. To reference the subharmonic amplitude to the baseline signal, the relative subharmonic enhancement, RSE, was calculated as the increase in subharmonic amplitude relative to the average baseline subharmonic amplitude, $\langle \mathrm{BSA} \rangle$ (Eq. (2)).

$$\mathrm{RSE} = \frac{\mathrm{SA} - \langle \mathrm{BSA} \rangle}{\langle \mathrm{BSA} \rangle} \tag{2}$$

To quantify the average subharmonic level over a 5 min data collection interval, the subharmonic quantities per burst were averaged over the 300 recorded bursts to yield the average relative subharmonic enhancement, $\langle \mathrm{RSE} \rangle$. Subsequently, these values were averaged for each treatment group. The groups were subject to statistical analysis using One-Way ANOVA and a post-hoc student's *T*-test with Tukey's correction.

2.8. Enzymatic activity analysis

After treatment with ultrasound, all alteplase samples were placed in the refrigerator and allowed to stabilize to the same temperature, ~4 °C. Each alteplase sample was diluted using three different volumes of Tris buffer (pH = 8.4), so that absorbance of each diluted sample would fall within the dynamic range of this measurement technique that can detect any difference in the enzymatic activity. The dilutions for these tests were 1:190, 1:380, and 1:500.

Each treatment protocol was performed on three independent alteplase samples. For repeatability and accuracy, each of the dilutions was tested in triplicate, which resulted in a final number of 27 data points per treatment protocol.

Diluted samples were centrifuged at 3000 rpm for 2 min to insure proper mixing of sample with Tris buffer and removal of Optison residuals. After preparation of all dilutions, 190 μl of each sample was dispensed into a 96 well plate. Samples were incubated in a microplate reader for 3 min and 30 s at 37 °C. After the incubation period, 20 μl of 10 mM chromogenic substrate H-D-Isoleucyl-L-prolyl-L-arginine-*p*-nitroaniline dihydrochloride (S-2288; DiaPharma Group Inc., West Chester, OH) was added into

each of the sample wells on the plate. The reading of the plate was started immediately following the addition of the chromogenic substrate. The plates were read on a microplate reader (Model: ELx-808-I, Bio-Tek Instruments, Winooski, VT). The microplate reader was set for a kinetic reading at 405 nm wavelength at 37 °C. The light absorbance through the each sample was read at 30 s intervals for 5 min. To insure uniform color distribution in each well, the samples were shaken by the plate reader, at an intensity setting of 2 for 10 s prior to each reading.

At the conclusion of the sample readings the change in absorbance per minute (ΔA/min) for each sample was plotted using Microsoft Excel®. The best-fit line was then determined by linear regression for each sample and the slope of this line per volume (in microliters) of each sample was calculated. The slope of each ΔA/min graph was divided by the volume of undiluted alteplase sample that was used in each of the three dilutions to account for diluted alteplase concentration. The change in absorbance per minute per microliter (ΔA/min/μl) was calculated as a specific measure of enzymatic activity.

Subsequently, these values were averaged for each treatment group. The groups were then subject to statistical analysis by One-Way ANOVA and the student's T-test with Tukey's correction using SPSS statistical software version 12.0.

3. Results

3.1. Cavitation activity

For treatments with Optison, the alteplase solution was optically opaque (cloudy) prior to ultrasound exposure, a result of the high concentration of suspended Optison microbubbles. Upon commencing ultrasound exposure, the solution changed from being cloudy to optically clear after a time that was inversely proportional to the acoustic peak pressure. The solution cleared after ~50 s, ~10 s, and ~5 s for acoustic peak pressures of 0.5 MPa, 1.7 MPa, and 3.5 MPa, respectively. The observed optical clearing of the solution was qualitative confirmation of the destruction of initial Optison microbubbles by the ultrasound.

Cavitation noise was observed in the collected signals as increases in both broadband noise and subharmonic enhancement. Fig. 4 shows an example of noise-versus-time signal (for broadband noise) recorded at various times throughout the 60 min exposure. The temporal characteristics of the cavitation signals were similar for the various treatment protocols, and could be divided into two regimes: an initial period of high-amplitude, continuous noise (region #1 in Fig. 4) that lasted approximately 2 min, followed by a period of low-amplitude, intermittent noise (region #2 in Fig. 4) that persisted for up to 30 min.

In all treatment groups, the cavitation signals had returned to baseline before 30 min.

Statistical comparison of the treatment groups was performed on the quantified cavitation signals, ⟨RNE⟩ and ⟨RSE⟩, collected at the beginning (0–5 min) of the exposure, for which differences in cavitation activity were most pronounced (Fig. 5). The numerical values of the average quantified noise signals obtained for the different groups are listed in Table 1. One-Way ANOVA comparison of A + US + OP, A + US, and baseline was performed for each pressure level. For ultrasound alone, neither ⟨RNE⟩ nor ⟨RSE⟩ showed a statistically significant difference from baseline at any pressure, despite an apparent increase in both at the highest pressure, 3.5 MPa. When Optison was present, ⟨RNE⟩ was significantly greater than baseline at 1.7 MPa ($p < 0.001$) and 3.5 MPa ($p < 0.003$), and ⟨RSE⟩ was significantly greater than baseline at all pressures ($p < 0.003$ for 0.5 MPa, $p < 0.001$ for 1.7 MPa, and $p < 0.024$ for 3.5 MPa). Additionally, when the cavitation signal for A + US + OP was greater than baseline, it was also significantly greater than A + US (⟨RNE⟩: $p < 0.001$ for 1.7 MPa, $p < 0.007$ for 3.5 MPa; ⟨RSE⟩: $p < 0.002$ for 0.5 MPa, $p < 0.001$ for 1.7 MPa), except for ⟨RSE⟩ at 3.5 MPa ($p < 0.130$), which was a result of the increased noise level for A + US.

One-Way ANOVA showed that a statistically significant difference existed among the Optison groups at different

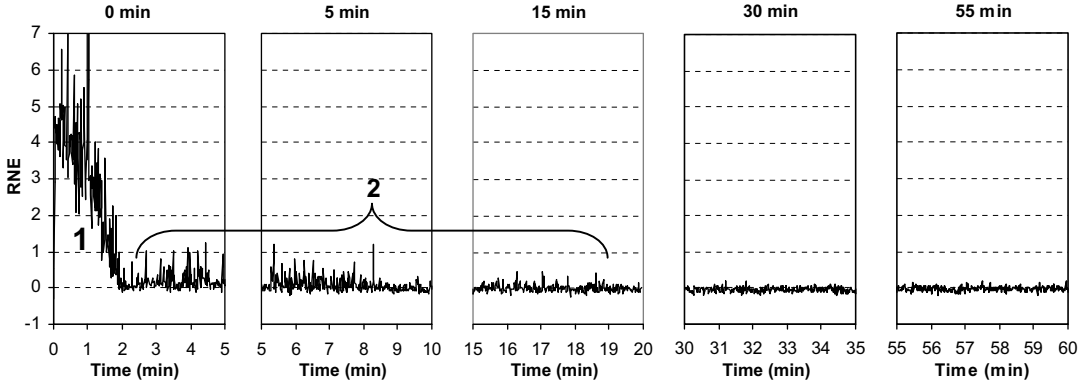

Fig. 4. Relative noise enhancement versus time during the 60 min ultrasound exposure, for Optison sample exposed to 3.5 MPa. (1) Region of high-amplitude, continuous noise. (2) Region of low-amplitude, intermittent noise.

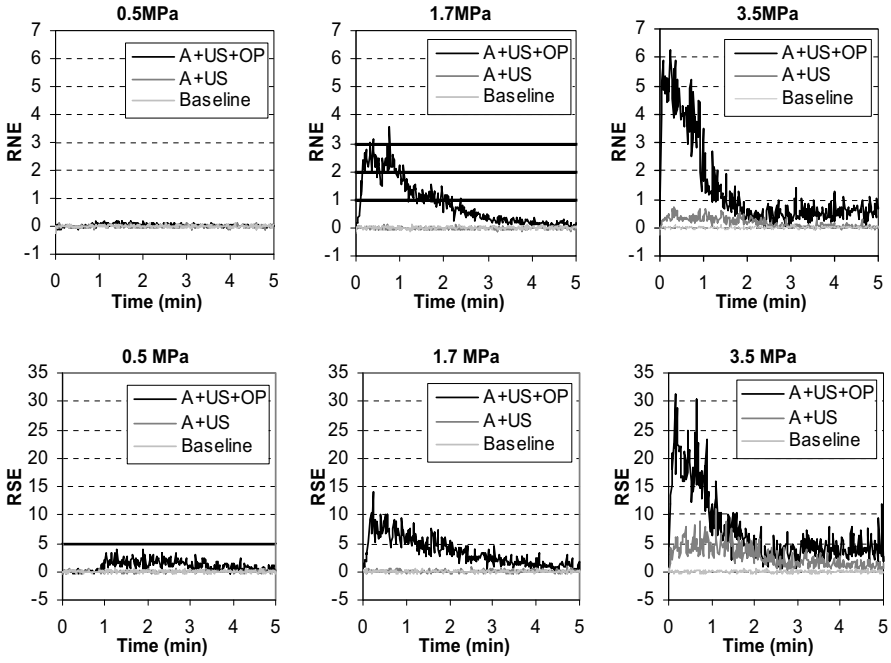

Fig. 5. Relative noise enhancement (top) and relative subharmonic enhancement (bottom) versus time at the beginning (0–5 min) of the 60 min ultrasound exposure. Each trace is an average over $N = 3$ independent snapshots.

Table 1
Noise enhancement and subharmonic enhancement (AVG ± standard deviation) at beginning (0–5 min) of ultrasound exposure for the various treatment groups

	⟨RNE⟩			⟨RSE⟩		
Peak pressure (MPa)	A + US + OP	A + US	Baseline	A + US + OP	A + US	Baseline
0.5	0.03 ± 0.04	−0.01 ± 0.04	0.00 ± 0.02	0.99 ± 0.35	0.02 ± 0.06	0.00 ± 0.05
1.7	0.85 ± 0.06	−0.02 ± 0.02	0.00 ± 0.02	3.46 ± 0.47	0.06 ± 0.06	0.00 ± 0.03
3.5	1.35 ± 0.49	0.18 ± 0.16	0.00 ± 0.02	7.28 ± 3.93	2.71 ± 1.45	0.00 ± 0.04

Table 2
Average values for specific enzymatic activity (ΔA/min/μl) for each treatment group

	A	A + US			A + US + OP		
	Baseline	0.5 MPa	1.7 MPa	3.5 MPa	0.5 MPa	1.7 MPa	3.5 MPa
Avg ± Std [ΔA/min/μl]	6.4 ± 0.5e−2	6.3 ± 1.2e−2	6.4 ± 0.4e−2	7.1 ± 0.4e−2	6.4 ± 1.2e−2	7.0 ± 0.7e−2	6.6 ± 1.0e−2

pressures for both ⟨RNE⟩ ($p < 0.004$) and ⟨RSE⟩ ($p < 0.041$). The quantified cavitation signals in Optison groups were increased with increasing peak pressure.

3.2. Enzymatic activity

The detailed numeric values for the specific enzymatic activity averages for each treatment protocol are listed in the Table 2.

One-Way ANOVA comparison of A + US + OP, A + US, and baseline was performed for each pressure level. The resulting significance levels were $p < 0.966$, $p < 0.364$, and $p < 0.508$ for pressure levels of 0.5, 1.7, and 3.5 MPa, respectively. This suggests that treatment of alteplase with US or US + OP did not affect the enzymatic activity of alteplase. In addition, One-Way ANOVA suggests no difference in enzymatic activity of alteplase treated at three different pressure levels of ultrasound in presence ($p < 0.988$) or absence ($p < 0.745$) of Optison. Additionally, Tukey's test showed no significant difference between individual groups.

4. Discussion and conclusion

Experimental and clinical studies have consistently demonstrated the capability of US to potentiate enzymatic thrombolysis [4,36–38]. In addition, recent clinical studies demonstrated that administration of UCA accelerates the

sonothrombolysis effect [12,13,39] possibly due to lowering the cavitation threshold. Culp et al. in a Phase I/II human feasibility study used MRX-815 with ultrasound both with and without alteplase for lysis of thrombosed dialysis grafts. They reported an improved luminal filling in both alteplase and non alteplase groups [39]. Molina et al. demonstrated that administration of UCA for sonothrombolysis in acute ischemic stroke, led to a more complete degree of arterial recanalization and a trend toward better short- and long-term outcomes. The authors used 2 MHz pulsed-wave transcranial Doppler (TCD) as the ultrasound source [12]. Apart from, Viguier et al.[13], who reported a high asymptomatic hemorrhagic transformation in their pilot study of treating 8 patients with TCD and UCA, the other aforementioned clinical studies suggested that this treatment modality is a safe means to enhancing the sonothrombolytic effect.

Although acoustic cavitation generated by UCA has the potential to cause bond dissociations in molecules and produce free radicals that can react with biomolecular materials [20–22], the investigation of the acoustic cavitation effect on PA used in sonothrombolysis has been confined to the study of Smikahl et al. [16]. These authors demonstrated that alteplase exposed to a 20 kHz vibrating wire for up to 6 min remained fully active and stable. Although the presence of cavitation was not directly verified during their study, it was proposed as the principle mechanism for thrombolysis in this device [17].

In the current study, we have investigated the potential adverse effect of UCA cavitational activity on alteplase, the drug used in ischemic stroke treatment. By monitoring the real-time cavitational signatures we were able to identify the duration and type of cavitation – inertial or stable – during the 60 min US exposure. We chose a frequency of 2 MHz and three acoustic peak negative pressures (0.3, 1.7, 3.5 MPa) that are relevant to the acoustic parameters used by ultrasonic devices in sonothrombolytic treatment of ischemic stroke namely TCD and EKOS Microlysus® Infusion system (EKOS Corp., Bothell, WA). The test condition described in this study presents a worst case scenario: (1) Pure alteplase solution was chosen, to avoid the limited half life seen in the presence of plasma proteins and fibrin. In addition, the solution presents a lower cavitation threshold than blood or blood clots [40]. (2) An Optison volume concentration of 10%v/v that is approximately 50 times greater than the specified maximum total dose for intravenous application of Optison, 0.2%v/v was used. This would increase the quantity of cavitation in the alteplase solution [29]. (3) The alteplase was not diluted, thus providing a maximum number of drug molecules surrounding the Optison microbubbles. Considering the extremely short half life of radicals [41], this fact should increase the chance of molecular interactions [30]. (4) An US exposure of 60 min was chosen in order to monitor the potential secondary activation of microbubbles. Although the initial Optison bubbles are mostly destroyed after 2 min of US exposure, the microbubble gas content and remaining shell could still act as cavitation nuclei in the alteplase solution [42]. (5) A 15% duty cycle was used, since the quantity of cavitation and severity of related bioeffects increases with burst duration [43].

In conclusion, US of 2 MHz and acoustic peak negative pressure up to 3.5 MPa did not generate statistically significant cavitation in alteplase test solution. However, addition of Optison lowered the cavitation threshold as expected. The presence of stable cavitation was verified for all acoustic pressures (0.5, 1.7, 3.5 MPa) and inertial cavitation was observed at an acoustic peak negative pressure of 1.7 MPa, and was quantitatively increased at 3.5 MPa. The temporal characteristics of the cavitation signals for both cavitation types were similar for the various treatment protocols, and can be divided into two regimes: an initial period of high-amplitude, continuous noise that lasted approximately 2 min, followed by a period of low-amplitude, intermittent noise that persisted for up to 30 min. However, the presence or absence of cavitation did not impact the stability of alteplase.

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