

Effect of modulated ultrasound parameters on ultrasound-induced thrombolysis

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Abstract

The potential of ultrasound to enhance enzyme-mediated thrombolysis by application of constant operating parameters (COP) has been widely demonstrated. In this study, the effect of ultrasound with modulated operating parameters (MOP) on enzyme-mediated thrombolysis was investigated. The MOP protocol was applied to an *in vitro* model of thrombolysis. The results were compared to a COP with the equivalent soft tissue thermal index (TIS) over the duration of ultrasound exposure of 30 min ($p < 0.14$). To explore potential differences in the mechanism responsible for ultrasound-induced thrombolysis, a perfusion model was used to measure changes in average fibrin pore size of clot before, after and during exposure to MOP and COP protocols and cavitation activity was monitored in real time for both protocols using a passive cavitation detection system. The relative lysis enhancement by each COP and MOP protocol compared to alteplase alone yielded values of $33.69 \pm 12.09\%$ and $63.89 \pm 15.02\%$ in a thrombolysis model, respectively ($p < 0.007$). Both COP and MOP protocols caused an equivalent significant increase in average clot pore size of $2.09 \times 10^{-2} \pm 0.01 \mu\text{m}$ and $1.99 \times 10^{-2} \pm 0.004 \mu\text{m}$, respectively ($p < 0.74$). No signatures of inertial or stable cavitation were observed for either acoustic protocol. In conclusion, due to mechanisms other than cavitation, application of ultrasound with modulated operating parameters has the potential to significantly enhance the relative lysis enhancement compared to application of ultrasound with constant operating parameters.

Introduction

It has been demonstrated that ultrasound application enhances enzyme-mediated thrombolysis *in vitro* (Blinic *et al* 1993, Francis *et al* 1992, Harpaz *et al* 1993, Lauer *et al* 1992), *in vivo*

(Kashyap *et al* 1994, Kornowski *et al* 1994, Riggs *et al* 1997) and in clinical studies (Alexandrov *et al* 2004, The IMS II Trial Investigators 2007, Mahon *et al* 2003, Motarjeme 2007, Wissgott *et al* 2007).

To enhance thrombolysis, ultrasound has to affect one or a combination of components involved in the thrombolytic process namely, clot structure, fluid dynamics within the clot structure and exogenous thrombolytic agents. Previously, we demonstrated that low energy ultrasound does not directly increase the potency of thrombolytic agents (Soltani and Soliday 2007). Temperature rise due to absorption of ultrasound can enhance the biochemical reaction of these enzymes and accelerate the lysis, however ultrasound induced lysis cannot be completely attributed to the temperature rise (Sakharov *et al* 2000). Hence, ultrasound must be interacting with the clot by means of modifying structure and/or fluid flow to enhance thrombolysis.

In two independent studies, Siddiqi *et al* (1995, 1998) demonstrated clot structure modification by ultrasound. The authors demonstrated that fluid permeability induced by hydrostatic pressure through the clot was accelerated by an ultrasound field (1 MHz at 2 W cm^{-2} , duty cycle of 50%) perpendicular to the fluid direction. The authors also showed ultrasound of the same parameters changed fibrin structure reversibly to increase binding sites of fibrin to the molecules of a thrombolytic agent, tissue plasminogen activator (tPA), which in turn accelerated thrombolysis.

Other studies measured fluid velocity in blood clots due to ultrasound absorption, which is known as acoustic streaming (Starritt *et al* 2000). A self-measurement of acoustic streaming by pulsed Doppler ultrasound at diagnostic power levels (acoustic power of 3.5 mW and I_{SPPA} of 38 W cm^{-2} at 20 MHz or acoustic power of 4.1 mW and I_{SPPA} of 99 W cm^{-2} at 10 MHz) resulted in a velocity of nearly zero (Hartley 1997). Streaming velocity generated by high intensity focused ultrasound (HIFU) at 5 MHz with focal intensity of 31 W cm^{-2} and duty cycle of 1% in blood clot was 1.6 cm s^{-1} with zero velocity for reverse flow (Shi *et al* 1999).

In all aforementioned studies, the measured outcome was the result of ultrasound with constant operating parameters (COP) applied to a thrombolytic process. To the authors' knowledge, the effect of modulating ultrasound operating parameters on thrombolysis has not been explored before. Therefore, in this study we investigated the influence of ultrasound with modulated operating parameters (MOP) on the thrombolysis process by measuring the changes in the rate of clot lysis, clot porosity and acoustic signatures indicating cavitation activity.

The MOP protocol was developed by varying the spatial-peak pulse-average intensity (I_{SPPA}) from 28 to 138 W cm^{-2} during application of 1.7 MHz ultrasound. The pulse width and pulse repetition frequency of MOP protocol were also varied from 1.16 to 8.06 ms and from 10 to 39 Hz, respectively, to maintain time average acoustic power of 0.34 W over each 2.5 min of ultrasound exposure similar to time average acoustic power generated by the COP protocol. The same time average acoustic power results in equal soft tissue thermal index for both protocols and maintains equivalent average temperature rise in clots over the course of treatment.

We applied the COP and MOP protocols to an *in vitro* thrombolysis model and compared the enhancement in lysis by measuring clot weight reduction. In an attempt to elucidate the mechanism, the changes in average clot pore size were measured during exposure to COP and MOP protocols. The difference between changes in average clot pore size can indicate whether the clot structure is affected differently while exposed to these protocols. In addition, the real-time acoustic emissions from clots exposed to COP and MOP protocols were monitored and the signals were evaluated for cavitation signatures such as broadband noise and subharmonics frequency content.

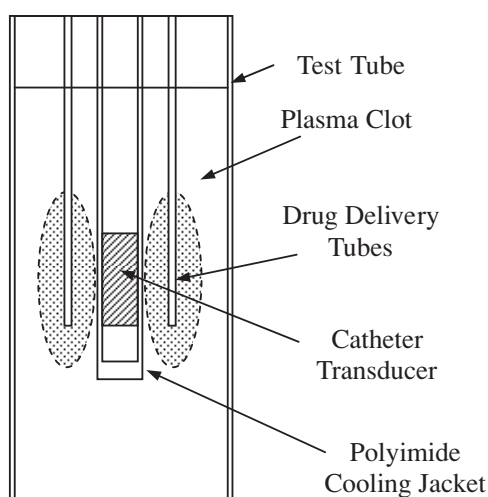


Figure 1. Schematic of *in vitro* thrombolysis model.

Materials and methods

Thrombolysis model

Clot model

1 mL of citrated human pooled plasma (Precision BioLogic, Dartmouth, Nova Scotia, Canada) was added to a polystyrene culture tube. Clotting was initiated by the addition of 100 μL each of 0.25 M calcium chloride and 100 μL of 12.5 U mL^{-1} bovine thrombin. Immediately after the addition of thrombin, a fixture containing two drug delivery lumens and the distal portion of an EKOS NeuroWave research unit (EKOS Corp., Bothell, WA) enclosed in a cooling jacket was placed into the clot, thereby allowing the clot to form around the fixture (figure 1) for 10 min while incubated in a water bath at 37 °C. The cooling jacket enclosure served to prevent clot heating due to imperfect electrical-to-ultrasound energy conversion. The drug lumens and cooling jacket were made of ultrasound transparent polyimide tubing. The internal diameter of the cooling jacket was 0.042 inch with a 0.001 inch wall. One end of the cooling jacket tube was sealed closed using UV glue to prevent direct contact of the coolant, degassed water, with the clot. After 10 min of incubation, 0.04 mL of alteplase (Genentech, Vacaville, CA) of 5000 U mL^{-1} concentration was delivered through each drug delivery lumen at a flow rate of 0.48 mL h^{-1} . The clot was exposed to ultrasound for 30 min after the drug was delivered. This duration was sufficient to result in substantial clot lysis from alteplase alone and in combination with ultrasound (Francis *et al* 1992). This process was conducted semi-automatically by a robotic arm to reduce data variability due to operator skill level. The transducer surface temperature was monitored and the flow rate of coolant was adjusted to maintain the temperature at 43 ± 2 °C. The temperature of 43 °C is the maximum allowable temperature on the patient contact surface with ultrasonic transducers (IEC 60601-2-37:2001).

Clot lysis

At the end of ultrasound exposure, the clot was removed from the test tube and placed between two blocks covered with filter papers and pressed with 10 lb (4.5 kg) weight for 5 min to expel the serum. The residual fibrin film was removed and weighed on a precision balance. This experiment consisted of four independent sample groups: untreated clots, clots treated with alteplase alone, clots treated with alteplase and COP protocol and clots treated with alteplase and MOP protocol. For each sample group an average clot weight, W (mg), was compiled from a minimum of 20 independent samples. The average clot weight for the sample groups treated with alteplase alone, W_D (mg), or in combination with COP or MOP protocols, W_i (mg), and average clot weight of untreated clots, W_C (mg), was used to calculate the percent clot lysis, L_i (%), as shown in equation (1) (Pfaffenberger *et al* 2005):

$$L_i(\%) = \left[\frac{W_C - W_i}{W_C} \right] \times 100. \quad (1)$$

For clots treated with alteplase combined with COP and MOP protocols, the lysis enhancement relative to alteplase alone, LE_i (%), was calculated as the percent increase in clot lysis relative to the clot lysis by alteplase alone (L_A) as shown in equation (2) (Prokop *et al* 2007):

$$LE_i(\%) = \left[\frac{L_i - L_A}{L_A} \right] \times 100 = \left[\frac{W_D - W_i}{W_C - W_D} \right] \times 100. \quad (2)$$

Statistical analysis was performed on the raw clot weight data using Microsoft Excel, two-sided Student's *t*-test.

Acoustic source

Catheter-mounted ultrasound transducers (EKOS Corporation, Bothell, WA) were used in this experiment. The device consisted of a single piezoelectric ceramic cylinder (L : 0.21 cm, OD: 0.08 cm) mounted coaxially along the distal tip of the catheter. A function generator (33 120 A; Agilent, Palo Alto, CA) connected to a RF power amplifier (2100 L; ENO Technology Inc., Rochester, NY) was used to drive the transducers at 1.7 MHz in pulsed mode. Due to geometric spreading of the field, acoustic pressure was greatest at the surface of the transducer and decreased with radial distance away from the transducer. A thermocouple attached to the external surface of each transducer was used to monitor the temperature continuously.

Perfusion model

The perfusion setup (figure 2) is based on the system described by Carr and Hardin (1987) with the difference that in this study we integrated the perfusion setup in an acoustic test cell (Sonic Concepts, Bothell, WA) to provide a consistent ultrasound exposure for facilitating direct comparison of bioeffects. The test cell consists of a LexanTM water tank ($15.5 \times 11 \times 11$ cm³) with an acoustic transducer holder on one wall and an acoustic absorber ($2.5 \times 11 \times 11$ cm³) on the opposite wall.

Briefly, a plasma clot was formed in a polyolefin tube and perfused under constant hydrostatic pressure (1.7 kPa) while the flow rate was monitored. The inner wall of the polyolefin tube was sanded using 600-grit sandpaper to increase clot adhesion. A piece of parafilm was pulled across one end of the polyolefin tube to serve as a removable base for this tube using an o-ring as a seal. The clot was prepared by adding 1 mL of citrated human pooled plasma (Precision BioLogic, Dartmouth, Nova Scotia, Canada) to the polyolefin tube. Clotting was initiated by the addition of 100 μ L each of 0.25 M calcium chloride and 100 μ L

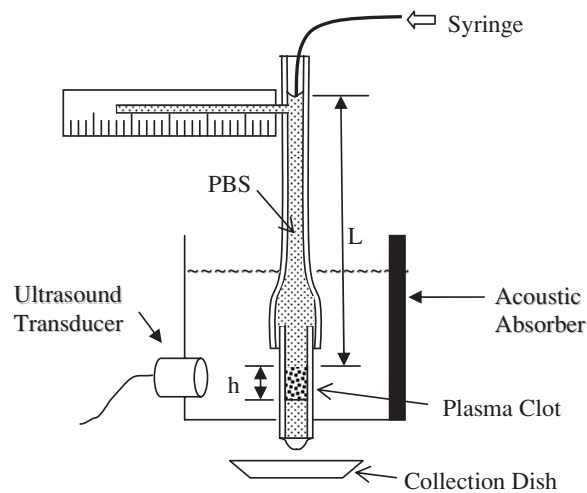


Figure 2. Diagram of perfusion model.

of 12.5 U mL^{-1} bovine thrombin. The clot was then incubated in a water bath at 37°C for 1 h prior to integration in the perfusion setup.

Average clot pore size evaluation

To observe the course of flow through plasma clot and ensure that the fluid is permeating the clot and does not pass through gaps between the clot and the tube, dye was added to permeating fluid, phosphate buffered saline (PBS), prior to and after flow rate measurement. The average clot pore size was determined upon calculation of Darcy permeability of clot as described by Carr and Hardin (1987).

Prior to ultrasound exposure, stability of each clot was ensured by taking sets of three measurements at 10 min intervals. During ultrasound exposure a total of 12 measurements were taken. The average pore size of clots during ultrasound OFF and ON time, P_{OFF} and P_{ON} (μm), was compiled from 10 independent samples.

Statistical analysis was performed using paired *t*-test as described by Devore and Peck (1993).

Acoustic source

An unfocused external piezoceramic transducer (Sonic Concepts, Bothell, WA) with an active diameter of 20 mm was used for this experiment. This transducer was operated at 2.1 MHz frequency and was positioned 3 cm distant from the middle of the clot. The acoustic field at the working distance in the present setup was determined based on measurements of the transverse beam profile at a distance of 3.0 cm from the transducer surface. This measurement was used to adjust the electrical operating parameters to achieve a comparable peak negative pressure used for clot treatment with catheter-mounted transducer.

Cavitation detection system

The cavitation detection apparatus and data collection and evaluation are described previously by our group (Prokop *et al* 2007) with the exception of using an external acoustic source instead of a catheter-mounted ultrasound transducer. Briefly, a passive cavitation detection scheme (Holland and Apfel 1990, Datta *et al* 2006) was used to monitor and analyze scattered acoustic signals from the clot exposed to two different acoustic protocols for the presence of inertial (Chen *et al* 2003) and stable (Eller and Flynn 1968, Lauterborn 1976) cavitation signatures. A total number of five clots were exposed to each treatment protocol. A sample size of five for both clot and non-cavitating control was exposed to each treatment protocol. Previous cavitation detection testing has shown this sample size to be sufficient for determining a statistically significant difference between the acoustic signals of plasma clot and the control.

Cavitation data collection and analysis

Data collection consists of monitoring and recording the acoustic signal emitted from the plasma clot during ultrasound exposure. Acquisition of the hydrophone signal is performed every 5 s. During each acquisition, a single ultrasound burst is recorded at a sampling rate of 50 MHz.

Data analysis consists of quantifying certain frequency components in the recorded signal that are characteristic of cavitation activity. The time-domain waveform is converted to the frequency domain using a 2048-point fast Fourier transform (FFT). The FFT amplitude at the subharmonic frequency (1.0 MHz) is used to indicate stable cavitation, and the broadband noise integrated from 4 to 10 MHz is used to indicate inertial cavitation.

A straightforward method for determining presence or absence of cavitation is to statistically compare the quantified cavitation values obtained for the test sample to the values obtained for the non-cavitating control. Cavitation will be deemed present in the test sample if the average quantified stable and inertial cavitation values are statistically greater (i.e. $p < 0.05$ using two-tailed Student's *T*-test) than the non-cavitating control values.

Degassed, 0.2 μm filtered water served as non-cavitating control in this experiment since the cavitation pressure threshold of this medium is typically about 3.0 MPa at low megahertz frequencies (Atchley *et al* 1988).

Acoustic source

A 2 MHz, spherically focused, single element ultrasound transducer (Sonic Concepts, Bothell, WA) was used for this experiment. The element had a 3.5 cm diameter and 5.5 cm radius of curvature. 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. 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 peak negative pressure at the focus was scaled to match the acoustic output of catheter-mounted transducer.

Acoustic protocols

The COP protocol consisted of constant values of peak power (5.3 W), pulse width (2.8 ms) and pulse repetition interval (33.3 ms) resulting in a peak rarefactional pressure of 1.6 MPa during

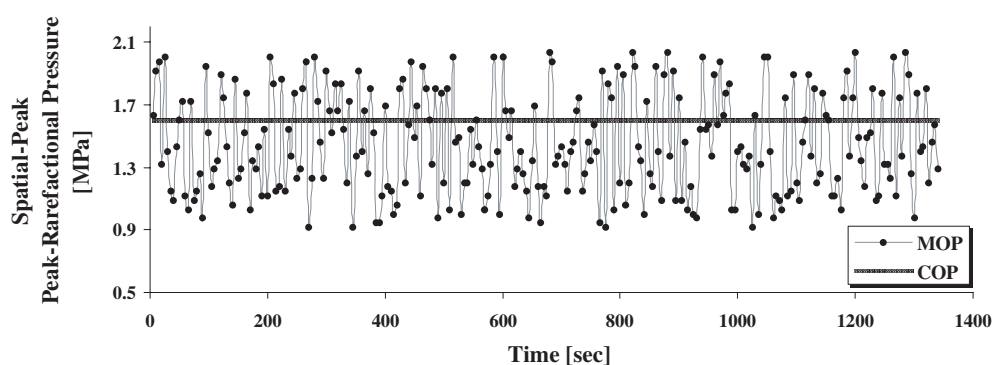


Figure 3. Spatial-peak peak-rarefactional pressure of MOP protocol is compared to COP over 30 min treatment time.

Table 1. The relevant acoustic parameters for mechanical effects, I_{SPPA} and MI, and for thermal effects, I_{SPTA} and TIS, for COP and MOP protocols are shown.

Acoustic parameters	I_{SPPA} ($W\ cm^{-2}$)	I_{SPTA} ($W\ cm^{-2}$)	MI	TIS
COP	85.7	7.28	1.26	2.64
MOP (min to max)	28.0 to 138	0.61 to 34.2	0.7 to 1.56	0.21 to 12.03
MOP (Avg \pm SD)	73.5 ± 32.1	8.1 ± 6.3	1.1 ± 0.25	2.84 ± 2.22

the 30 min of ultrasound exposure time. In the absence of knowledge regarding the relevant combination of acoustic parameters, effective dwell time and frequency of modulation, a sequence of non-repetitious pulse profiles was deemed to be more appropriate for the purposes of this feasibility study. The MOP protocol consisted of 268 combinations of three variables. The variation ranges for peak power, pulse width and pulse repetition interval were 1.6–7.9 W, 1.2–8.1 ms and 25.6–100 ms, respectively. Each combination of three variables created a pulse profile with a dwell time of 6.7 s. The resulting spatial-peak peak-rarefactional pressure ranged from 0.92 to 2.03 MPa. The sequence of pulse profiles in the MOP protocol was determined arbitrarily (figure 3) to achieve time average acoustic power of 0.34 W over each 2.5 min exposure time. This is equivalent to the time average acoustic power in COP and leads to equal soft tissue thermal index for both protocols and maintains the same average temperature rise in clots during the treatment time. The relevant acoustic parameters for mechanical effects, spatial-peak pulse-average intensity (I_{SPPA}) and mechanical index (MI), and thermal effects, spatial-peak time-average intensity (I_{SPTA}) and soft tissue thermal index (TIS), for both COP and MOP protocols are summarized in table 1.

Results

Thrombolysis model

The resulting clot weights for each treatment protocol and the statistical analysis are listed in table 2. The relative clot lysis with alteplase alone was $5.72 \pm 0.53\%$ ($p < 0.0001$). The relative clot lysis was further increased to $7.64 \pm 0.50\%$ ($p < 0.0015$) and $9.37 \pm 0.56\%$ ($p < 0.0001$) after exposing the clot to COP and MOP protocols, respectively. The relative lysis enhancement of each COP and MOP acoustic protocol compared to alteplase alone yielded values of $33.69 \pm 12.09\%$ and $63.89 \pm 15.02\%$, respectively.

Table 2. Clot weight before and after treatment, percent lysis and percent lysis enhancement (all Avg \pm SD) obtained for each treatment protocol and *p*-values from statistical comparisons between groups are shown.

Treatment protocol	Average clot weight (mg)	Clot lysis (%)	Lysis enhancement (%)	Statistical comparison vs			
				Control	Alteplase	Alteplase + COP	Alteplase + MOP
Control	9.27 \pm 0.15	N/A	N/A				
Alteplase	8.74 \pm 0.20	5.72 \pm 0.53	N/A	0.000	1.000		
Alteplase + COP	8.56 \pm 0.16	7.64 \pm 0.50	33.69 \pm 12.09	0.000	0.0015	1.000	
Alteplase + MOP	8.40 \pm 0.19	9.37 \pm 0.56	63.89 \pm 15.02	0.000	0.000	0.007	1.000

Table 3. The average pore size of the clots while exposed to COP and MOP during ultrasound OFF and ON time, their difference and the statistical significance are shown.

Treatment protocol	Average clot pore size (μm)		Difference in average clot pore size (μm) US (ON – OFF)	Statistical comparison <i>p</i> -value
	US OFF	US ON		
COP	7.96 $\times 10^{-1} \pm 0.12$	8.16 $\times 10^{-1} \pm 0.12$	2.09 $\times 10^{-2} \pm 0.01$	0.000
MOP	7.48 $\times 10^{-1} \pm 0.07$	7.68 $\times 10^{-1} \pm 0.07$	1.99 $\times 10^{-2} \pm 0.004$	0.000

Table 4. Quantified broadband noise (BBN) values for plasma clot and control and the statistical significance are shown.

Protocol	No. of samples	$\langle \text{BBN} \rangle \times 10^{-5}$ (V MHz)		$\max\{\text{BBN}\} \times 10^{-5}$ (V MHz)		<i>p</i> -value (control versus clot)	
		Control	Plasma clot	Control	Plasma clot	$\langle \text{BBN} \rangle$	$\max\{\text{BBN}\}$
COP	5	7.04 ± 0.14	7.01 ± 0.06	8.53 ± 0.59	8.27 ± 0.12	0.6667	0.3776
MOP	5	7.01 ± 0.06	6.97 ± 0.04	8.20 ± 0.20	8.23 ± 0.15	0.2992	0.8014

Perfusion model

The resulting average pore size of the clots while exposed to COP and MOP during ultrasound OFF and ON time, their difference and the statistical significance is listed in table 3. The COP and MOP protocols increased the average clot pore size by 2.63% ($p < 0.000$) and 2.66% ($p < 0.000$), respectively. However, there was no statistically significant difference between the increase in clot pore size by either protocol.

Cavitation detection

Inertial cavitation

The average and maximum broadband noise for plasma clot and control and the *p*-values from the Student's *t*-test comparisons between plasma clot and control are listed in table 4. All statistical comparisons showed no significant difference from the control.

Table 5. Quantified subharmonic (SUB) values for plasma clot and control and the statistical significance are shown.

Protocol	No. of samples	$(\text{SUB}) \times 10^{-6} \text{ (V)}$		$\max\{\text{SUB}\} \times 10^{-6} \text{ (V)}$		$p\text{-value}$ (control vs clot)	
		Control	Plasma clot	Control	Plasma clot	$\langle \text{SUB} \rangle$	$\max\{\text{SUB}\}$
COP	5	6.24 ± 0.77	5.64 ± 0.56	35.5 ± 4.7	32.7 ± 2.1	0.1996	0.2757
MOP	5	6.45 ± 0.51	6.04 ± 0.53	33.0 ± 2.3	32.0 ± 3.5	0.2490	0.6173

Stable cavitation

The average and maximum subharmonic amplitude for plasma clot and control and the p -values from the Student's t -test comparisons between plasma clot and control are listed in table 5. All statistical comparisons showed no significant difference from the control.

Discussion

It has been demonstrated that high-frequency ultrasound (> 1 MHz), due to mechanisms other than thermal and cavitation, has the potential to increase enzymatic dissolution of clots (Prokop *et al* 2007, Everbach and Francis 2000). The presence of cavitation has been shown to further increase the thrombolysis rate; however, it was necessary to apply either high intensity or exogenous cavitation nuclei (microbubbles) to achieve cavitation. The percent clot lysis was shown to increase by 1.1% per 1 °C up to temperatures of 50 °C as reported by Francis *et al* (1992). The authors demonstrated that approximately 8% of total clot lysis was related to thermal increase due to ultrasound absorption.

The non-cavitation and non-thermal enhancement of sonothrombolysis could be due to an acoustic effect on fluid dynamics within the clot and/or on clot structure, since ultrasound does not seem to directly affect enzymatic activity of thrombolytic agents by modifying the structure of this protein (Soltani and Soliday 2007).

Thrombolysis is a complex process involving pressure gradient induced microfluid permeation in a nonuniform porous medium, clot structure, which is itself under dynamic modification during thrombolysis. Therefore, in this study the influence of ultrasound with modulated operating parameters on the thrombolysis process was investigated as compared to previously applied constant operating parameters.

Application of MOP and COP protocols both resulted in significant lysis enhancement compared to thrombolytic agent alone. However, the effectiveness of MOP protocol in thrombolysis (LE) was significantly higher as compared to COP ($p < 0.007$). To elucidate the mechanism responsible for this advantage, the effect of these protocols on average clot pore size was measured. Both protocols resulted in an equal significant increase in average clot pore size by approximately 0.02 μm . This suggests that the beneficial effect of MOP in clot lysis as compared to COP may not be due to ultrasound interaction with clot structure. However, in this study the size distribution of clot pores was not investigated.

The absence of cavitation activity of COP protocol when applied during thrombolysis has been demonstrated previously by our group (Prokop *et al* 2007). However, since the maximum acoustic pressures applied in MOP were higher than the pressures applied in COP, the scattered acoustic signal of samples was monitored in real time and evaluated for signatures of cavitation activities. The signals indicated the absence of stable and inertial cavitation for either protocol. This suggests that microstreaming associated with stable cavitation (Miller 1988) and microjets associated with inertial cavitation (Crum 1988) cannot be considered

as potential mechanisms for enhanced clot lysis by COP or explain the advantage of MOP over COP in thrombolysis. The thermal effect does not seem to be one of the responsible mechanisms either, since the heat generated by the transducer element for both protocols was maintained at 43 ± 2 °C by adjusting the coolant flow rate, the time average acoustic power for both acoustic protocols was 0.34 W for the period of each 2.5 min ultrasound exposure time, and average spatial-peak time-average intensity and soft tissue thermal index over 30 min ultrasound exposure time for both COP and MOP protocols were not significantly different (table 1). Whether fluid dynamics within clot structure is more efficiently affected by MOP and/or MOP creates a more effective distribution of increased clot pores as compared to COP, remains to be explored in future studies.

Conclusion

The influence of a modulated operating parameter on enzyme-mediated thrombolysis was determined and the responsible mechanism was explored. The MOP protocol demonstrated significantly higher effectiveness in accelerating thrombolysis as compared to COP. The experiment results suggested that the responsible mechanism is non-cavitation and non-thermal.

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