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● *Original Contribution*

SHAKEN AND STIRRED: MECHANISMS OF ULTRASOUND-ENHANCED THROMBOLYSIS

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Abstract—The use of ultrasound and microbubbles as an effective adjuvant to thrombolytics has been reported *in vitro*, *ex vivo* and *in vivo*. However, the specific mechanisms underlying ultrasound-enhanced thrombolysis have yet to be elucidated. We present visual observations illustrating two mechanisms of ultrasound-enhanced thrombolysis: acoustic cavitation and radiation force. An *in vitro* flow model was developed to observe human whole blood clots exposed to human fresh-frozen plasma, recombinant tissue-type plasminogen activator (0, 0.32, 1.58 or 3.15 μg/mL) and the ultrasound contrast agent Definity (2 μL/mL). Intermittent, continuous-wave ultrasound (120 kHz, 0.44 MPa peak-to-peak pressure) was used to insonify the perfusate. Ultraharmonic emissions indicative of stable cavitation were monitored with a passive cavitation detector. The clot was observed with an inverted microscope, and images were recorded with a charge-coupled device camera. The images were post-processed to determine the time-dependent clot diameter and root-mean-square velocity of the clot position. Clot lysis occurred preferentially surrounding large, resonant-sized bubbles undergoing stable oscillations. Ultraharmonic emissions from stable cavitation were found to correlate with the lytic rate. Clots were observed to translate synchronously with the initiation and cessation of the ultrasound exposure. The root-mean-square velocity of the clot correlated with the lytic rate. These data provide visual documentation of stable cavitation activity and radiation force during sub-megahertz sonothrombolysis. The observations of this study suggest that the process of clot lysis is complex, and both stable cavitation and radiation force are mechanistically responsible for this beneficial bio-effect in this *in vitro* model. (E-mail: kenneth.bader@uc.edu)

Key Words: **Acute ischemic stroke, Ultrasound, Ultrasound contrast agents, Acoustic cavitation.**

INTRODUCTION

Stroke is currently the fourth leading cause of death in the United States (Go et al. 2013). At present, the only thrombolytic therapy that is approved by the U.S. Food and Drug Administration for the treatment of ischemic stroke is recombinant tissue-type plasminogen activator (rt-PA). However, this potent thrombolytic is administered in only 1.5% of cases (Go et al. 2013) because of potential bleeding complications and strict contraindication criteria (Turi et al. 1993). Adjuvant therapies that lower the dose of rt-PA or increase efficacy would represent an important breakthrough. Ultrasound-enhanced thrombolysis (UET) has exhibited the potential for both.

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The efficacy of UET has been demonstrated *in vitro* (Cheng et al. 2005; Datta et al. 2008; Prokop et al. 2007) and in clinical trials (Alexandrov et al. 2004; Molina et al. 2009), but the specific mechanisms underlying UET have yet to be elucidated. Enhancement of thrombolysis is thought to be primarily mechanical in nature (Blinc et al. 1993; Francis et al. 1992; Shaw et al. 2007). Acoustic streaming, generated by absorption of the ultrasonic energy (Nyborg 1953), increases penetration of rt-PA into the clot (Francis et al. 1995) or radiation force displaces the clot (Devcic-Kuhar et al. 2002). Acoustic cavitation, or the nucleation and generation of bubble activity by an acoustic source (Apfel 1981), has been found to enhance thrombolytic efficacy (Everbach and Francis 2000). Acoustic emissions from stable cavitation, characterized by gentle non-linear oscillations of the bubbles (Flynn 1964), have previously been correlated with the enhancement of rt-PA thrombolysis (Datta et al. 2008; Prokop et al. 2007). These stable

bubbles are known to induce small-scale streaming, termed *microstreaming* (Elder 1959), which is thought to facilitate increased penetration of rt-PA into the thrombus (Datta et al. 2008). Additionally, microstreaming is thought to remove fibrin degradation products, which increases the availability of plasminogen binding sites for rt-PA (Sutton et al. 2013).

Previous studies did not, however, provide real-time measurement of the lytic rate, which would enable identification of the mechanisms underlying thrombolytic enhancement. To overcome this difficulty, Cheng et al. (2005) developed an *in vitro* model to observe thrombolysis in real time. This model was extended by Gruber et al. (2014) to include fluid flow past the clot, a potentially important aspect of the lytic rate (Bajd and Serša 2012), to allow replenishment of cavitation nuclei (Hitchcock et al. 2011). The ability to detect bubble activity optically and track the movement of the clot has recently been integrated into this measurement system, to probe the relationship between lytic rate, cavitation and clot movement.

METHODS

Preparation of human fresh-frozen plasma and recombinant tissue-type plasminogen activator

Human fresh-frozen plasma (hFFP) was procured from a blood bank (Hoxworth Blood Center, Cincinnati, OH, USA). Thirty-milliliter aliquots of the hFFP were thawed for each experiment and allowed to reach atmospheric gas equilibrium at 37°C in an open container for 2 h. Recombinant tissue-type plasminogen activator (rt-PA) was obtained from the manufacturer (Activase, Genentech, San Francisco, CA, USA) as lyophilized power. Each vial was mixed with sterile water to a concentration of 1 mg/mL as per manufacturer instructions, aliquoted into 1.0-mL centrifuge tubes and stored at −80°C. The enzymatic activity of rt-PA is stable over a period of 7 y using this protocol (Shaw et al. 2009b).

Preparation of blood clots

Human whole blood clots were manufactured around silk sutures according to a protocol developed by Shaw et al. (2008). After local institutional review board approval and written informed consent, venous human whole blood was drawn from a pool of five healthy volunteers. Aliquots of 500 μL were transferred to sterile glass tubes containing borosilicate glass micropipets (1.12-mm inner diameter, World Precision Instruments, Sarasota, FL, USA), pre-threaded with 7-O silk sutures (Ethicon Industries, Cornelia, GA, USA). The blood was allowed to clot around the silk suture at 37°C for 3 h. After clot formation, the tubes were stored at 5°C for a minimum of 3 d to allow for maximal clot retraction (Shaw et al. 2009a), lytic resistance and stability (Shaw et al. 2006). Before each measurement, the micropipet was removed to produce a cylindrical clot adherent to the suture. The initial clot (550 ± 43 μm) was smaller than the middle cerebral artery (2.4–4.6 mm) (Ng et al. 2007; Saqqur et al. 2007), the site of occlusion for the majority of ischemic strokes (Gibo et al. 1981). However, the clot is comparable in size to intracerebral perforating branches of the middle cerebral artery (80–840 μm) (Marinkovic et al. 1985), which are highly vulnerable to occlusion.

Preparations of ultrasound contrast agents

Vials of Definity (perflutren lipid microspheres; Lantheus Medical Imaging, North Billerica, MA, USA), microbubbles consisting of octofluoropropane encapsulated by a lipid shell monolayer, were activated according to the manufacturer's instructions. Vials were stored at 5°C until needed. The vials were allowed to warm to room temperature (20°C–24°C) for 1 h before activation by shaking for 45 s using a Vial-Mix (Lantheus Medical Imaging). The agent was diluted to a final concentration of 2 μL/mL (1×10^4 particles/mL). This number density is consistent with the manufacturer's recommended dose (Lantheus Medical Imaging) for left ventricular opacification.

In vitro *flow phantom*

The *in vitro* flow model based on Cheng et al. (2005) and Gruber et al. (2014) used to quantify thrombolytic efficacy and bubble activity is depicted in Figure 1. An acrylic tank (16 × 33 × 9 cm) was filled with approximately 3 L of degassed (20 ± 5% dissolved oxygen), reverse-osmosis water heated to 37.3 ± 0.3°C. The water was filtered (0.2 μm), and the gas content and temperature were maintained throughout the experiment with a custom-built recirculation system. The walls of the tank were lined with a 1-cm-thick acoustic absorber (Aptflex F48, Precision Acoustics, Dorchester, Dorset, UK).

The flow channel consisted of low-density polyethylene tubing (inner diameter 1.6 mm, outer diameter 3.2 mm; Part 1 J-109-10, Freelin Wade, McMinnville, OR, USA) to direct the perfusate from a reservoir to a glass micropipet (2.15-mm inner diameter, 0.3-mm wall thickness; Part 5-000-2200, Drummond Scientific, Broomall, PA, USA). A clot was mounted along the central axis of the micropipet by snuggly fitting the suture at the ends of the micropipet with latex tubing. The micropipet was positioned over a microscope slide (Fisherbrand 12-550 C, Fisher Scientific, Pittsburg, PA, USA) in the bottom of the tank to allow imaging of the clot with an inverted microscope (IX71, Olympus, Center Valley, PA, USA). The focal area of the objective (UPlanFLN 10 × , 10-mm working distance, Olympus)