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● *Original Contribution*

SONOTHROMBOLYSIS: THE CONTRIBUTION OF STABLE AND INERTIAL CAVITATION TO CLOT LYSIS

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Abstract—Microbubble-mediated sonothrombolysis (STL) is a remarkable approach to vascular occlusion therapy. However, STL remains a complex process with multiple interactions between clot, ultrasound (US), microbubbles (MB) and thrombolytic drug. The aim of this study was to evaluate the ability of combining US and MB to degrade fibrin and, more specifically, to assess the roles of both stable (SC) and inertial (IC) cavitation. Human blood clots containing radiolabeled fibrin were exposed to different combinations of recombinant tissue plasminogen activator (rtPA), US (1 MHz) and phospholipid MB. Three acoustic pressures were tested: 200, 350 and 1,300 kPa (peak-negative pressure). Clot lysis was assessed by diameter loss and release of radioactive fibrin degradation products. The combination rtPA + US + MB clearly revealed that IC (1,300 kPa) was able to enhance fibrin degradation significantly (66.3 ± 1.8%) compared with rtPA alone (51.7 ± 2.0%, $p < 0.001$). However, SC failed to enhance fibrin degradation at an acoustic pressure of 200 kPa. At 350 kPa, a synergistic effect between rtPA and US + MB was observed with an absolute increase of 6% compared to rtPA alone ($p < 0.001$). Conversely, without rtPA, the combination of US + MB was unable to degrade the fibrin network (0.3 ± 0.1%, $p > 0.05$ vs. control), but induced a distinct loss of red blood cells throughout the entire thickness of the clot, implying that MB were able to penetrate and cavitate inside the clot. (E-mail: Eric.allemann@unige.ch) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ischemic stroke, Human blood clot, Recombinant tissue plasminogen activator, Ultrasound, Microbubbles, Sonothrombolysis, Radiolabeled fibrinogen, Fibrinolysis, Stable cavitation, Inertial cavitation.**

INTRODUCTION

Microbubble-enhanced sonothrombolysis (STL) is a promising approach to the treatment of vessel occlusions. The use of microbubbles (MB) to accelerate STL was first described by Tachibana and Tachibana (1995) 20 years ago. Since then, many other studies have highlighted the strong potential of MB as STL enhancers, both *in vitro* (Cintas et al. 2004; Datta et al. 2008; Flores et al. 2011; Hitchcock et al. 2011; Petit et al. 2012; Prokop et al. 2007) and in animal studies (Brown et al. 2011; Culp et al. 2011; Fatar et al. 2008; Nedelmann et al. 2010; Shi et al. 2010; Xie et al. 2011). In addition, several clinical trials have already been conducted with promising results (Molina et al. 2006, 2009; Perren et al. 2008).

As revealed by the analysis of clots retrieved from ischemic stroke patients (Liebeskind et al. 2011; Marder et al. 2006), the main components of clots are red blood cells (RBC), fibrin and platelets. An important issue in the clot lysis process is the effect of STL on the fibrin network. Fibrin strands provide a 3-D scaffold for the intravascular clot and ensure its stability against mechanical stress and degradation (Mosesson 2005; Weisel 2007). Therefore, degradation of the fibrin network is a key factor in the success of STL therapy, and its monitoring is essential.

In a previous study (Petit et al. 2015), we used two different quantification techniques to assess fibrin degradation: measurement of radiolabeled-fibrin degradation products (FDP) and D-dimer assay. It was found that at high acoustic pressure, the combination of ultrasound (US) and MB was not able to degrade the fibrin network in the absence of a thrombolytic drug. On the contrary, a

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clear synergistic effect was observed on fibrin degradation when US, MB and recombinant tissue plasminogen activator (rtPA) were associated.

Even though the respective roles of microstreaming and microjets in MB-enhanced STL remain unclear, it is commonly recognized that cavitation-based phenomena play an important role in the clot dissolution process, as reported by some authors (Datta et al. 2008; Hitchcock et al. 2011; Prokop et al. 2007; Shi et al. 2010; Xie et al. 2011). Indeed the argument for using MB to enhance US action is that MB dramatically reduce the acoustic cavitation threshold by providing cavitation nuclei in the medium. Cavitation could induce direct mechanical damage to the clot as well as positively affect the action of rtPA by improving access to fibrin strands and drug transport. For example, Prokop et al. (2007) reported that in the presence of a thrombolytic drug, the enhancement of clot lysis is related to the cavitation activity caused by the combination of US and MB. Historically, cavitation activity has been classified as either stable or inertial (Neppiras 1980). Stable cavitation (SC) designates the stable oscillation of MB over time in response to applied acoustic waves (Miller et al. 1996) and induces microstreaming (Collis et al. 2010; Leighton 1994; Wu and Nyborg 2008). At higher acoustic pressures, inertial cavitation (IC) occurs, and MB undergo a rapid increase in size followed by violent bubble collapse (Miller et al. 1996). Inertial cavitation produces a more violent mechanical action, such as microjets, but within a much shorter period than SC action (Holland and Apfel 1990; Miller et al. 1996).

Whether SC or IC would be more appropriate for STL is still debated. For instance, Datta et al. (2008) observed a significant correlation between clot lysis and SC activity with 120-kHz US and MB. Hitchcock et al. (2011) later reported significant enhancement of rtPA-induced lysis by US and MB, with US parameters selected to have maximal SC exposure. Thus, these studies emphasize the contribution of SC in MB-enhanced STL. Although the experiments were designed to maximize SC phenomena, IC was also present in these studies, which underlines the complexity of discriminating the effect of each type of cavitation.

Because no cavitation measurements were performed in our previous study, in the work described here, we attempted to evaluate the respective contributions of SC and IC to the fibrin degradation process during MB-enhanced STL treatment. For that purpose, cavitation thresholds were determined first; then blood clots containing radiolabeled fibrin were exposed to acoustic conditions with either SC, IC or a combination of both cavitation types. Lysis was assessed by clot diameter loss and release of radioactive FDP.

METHODS

Materials

Trizma hydrochloride buffer solution, sodium chloride, sodium citrate tribasic dihydrate, bovine serum albumin, calcium chloride dihydrate and 6-aminocaproic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Plasminogen-depleted human fibrinogen was purchased from Calbiochem (Merck, Darmstadt, Germany). Iodine-125 (^{125}I) radionuclide was purchased from Perkin Elmer (Waltham, MA, USA). Pierce iodination tubes were from Thermo Scientific (Rockford, IL, USA). Frozen citrated human plasma was purchased from Milan Analytica (Magden, Switzerland), defrosted at 37°C, filtered through 0.22-μm Steripak GP20 filters (Millipore, Billerica, MA, USA), aliquoted and stored at –20°C. Plasma was defrosted at 37°C before use in STL experiments. The rtPA used was the commercial Actilyse (Boehringer Ingelheim, Basel, Switzerland). It was reconstituted according to the supplier's indications at 1 mg/mL and stored at –80°C (Petit et al. 2012; Shaw et al. 2009). BR38 phospholipid microbubbles were from Bracco Suisse (Geneva, Switzerland) (Schneider et al. 2011).

Preparation of radioactive human blood clots

The preparation of radioactive human blood clots was described in our previous study (Petit et al. 2015). The study protocol was approved by the institutional ethics committee. Briefly, 9 mL of fresh venous human blood, drawn from healthy volunteers who signed an informed consent, were mixed with 200 μL of ^{125}I-fibrinogen solution (0.3 mg/mL in 25 mM Tris–HCl, 0.4 M NaCl, 20 mM citrate, pH 7.5) and 20 μL of 0.5 M $CaCl_2$. Calcium was added to annihilate the effect of citrate present in the fibrinogen buffer. Then, 4 mL of radioactive blood were transferred into a glass test tube (Pyrex, 16 × 100 mm) containing five 3.4-mm-i.d. glass tubes through which a silk suture thread had previously been stretched. Radioactive clots were obtained after a 6-h incubation at 37°C. They were cylindrical in shape with an initial mean diameter and length of 2.15 ± 0.08 mm and 25 ± 2 mm, respectively. To avoid inter-donor variability, we chose to use clots from the blood of a single donor for all experiments; however, STL experiments were repeated with another donor, and all results followed the same trend (data not shown).

In vitro sonothrombolysis

The experimental setup used for STL experiments was described previously (Petit et al. 2012, 2015). Briefly, clots were positioned in a transparent 3.2-mm-i.d. polyvinyl chloride (PVC) tube in a 37°C thermostated