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Potentiating intra-arterial sonothrombolysis for acute ischemic stroke by the addition of the ultrasound contrast agents (Optison™ & SonoVue®)

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Abstract

Transcranial ultrasound in combination with intravenously administered ultrasound contrast agents (UCA) in the presence or absence of recombinant tissue plasminogen activator (rt-PA) has been widely evaluated as a new modality for treatment of ischemic stroke. Despite the successful demonstration of accelerated clot lysis there are inherent limitations associated with this modality such as inconsistency in temporal window thickness and/or potential serious cardiopulmonary reactions to intravenous administration of UCA that prevent broad application to ischemic stroke populations. As a complementary modality, we evaluated potential lysis enhancement by intraarterial ultrasound with concurrent intra-clot delivery of UCA and rt-PA. To this end, clots were formed with average pore diameter similar to clinically retracted clots by adjusting the thrombin concentration. Physical characteristic and retention of UCA after delivery through the catheter as a function of clinically relevant flow rates of 6, 12, 18 ml/h were determined using a microscopic method. The ability of the UCA employed in this study, Optison and SonoVue, to penetrate into the clot was verified using ultrasound B-mode imaging. Clot lysis as a function of rt-PA concentration, 0.009 through 0.5 mg/ml, in the presence and absence of UCA diluted to 1:10, 1:100, and 1:200 v/v at two Peak rarefaction acoustic pressures of 1.3 and 2.1 MPa were evaluated using a weighing method. The study results suggest the addition of only 0.02 ml of 1:100 diluted UCA to rt-PA of 0.009, 0.05, 0.3, and 0.5 mg/ml can enhance the lysis rate by 3.9, 2.6, 1.9 and 1.8 fold in the presence of peak rarefaction acoustic pressure of 1.3 MPa and by 5.1,

3.4, 2.6, 3.1 in the presence of peak rarefaction acoustic pressure of 2.1 MPa, respectively. In addition, Optison and SonoVue demonstrated comparable effectiveness in enhancing the clot lysis rate. Addition of UCA to intra-arterial sonothrombolysis could be considered as a viable treatment option for ischemic stroke patients.

Keywords

Sonothrombolysis; Optison™; SonoVue®; Intra-arterial treatment; Acute ischemic stroke; Microbubbles

Introduction

Early arterial recanalization is strongly associated with improved short- and long-term clinical outcome in ischemic stroke [1,2]. This correlation is even stronger for recanalization achieved within 6 h of symptoms onset while substantial penumbral tissues can be salvaged [2]. Clinical studies supported the 3 h time window for effective intravenous (IV) rt-PA therapy [3,4]. Recent published guidelines by American Heart and Stroke Association, additionally, recommended expanding of the time window for administration of IV rt-PA to 4.5 h and utilizing intraarterial (IA) thrombolysis for early management of ischemic stroke for the treatment of selected patients who have a major stroke of <6 h duration due to occlusions of the middle cerebral artery who are not otherwise candidates for IV rt-PA [5,6].

The effectiveness of IA thrombolysis has been demonstrated in the Phase II of Intra-arterial Pro-Urokinase for Acute Ischemic Stroke (PROACT-II) clinical trial [7]. Although the number of subjects (46) enrolled in PROACT II was too small to establish statistical significance, there appeared to be a 10–12% absolute increase in excellent neurological outcome in the IA group over placebo at 90 days even though the treatment window was extended 5 h beyond the IV rt-PA treatment modality [7]. The most important limitation of IA therapy has been the time it takes from the onset of symptom to the initiation of therapy (due to setup and preparation for angiographic catheter placement) and the time to recanalization once therapy has begun. For example, in the PROACT II study, the median time from onset of symptom to initiation of IA treatment was 5.3 h. In addition, the median time for recanalization is about 2 h in IA studies in which times are reported. Thus, using current IA strategies and systems, the time to recanalization is usually beyond 6 h, and often at 7 or 8 h from symptoms onset. New treatment modalities are needed to minimize the time to recanalization once IA therapy has begun.

Increased logistical problems due to inherent delays in initiation of IA therapy compared to IV therapy have lead clinicians to investigate combined IV/IA thrombolysis as a way to bridge the gap between early initiation of IV therapy and faster recanalization achieved by IA therapy. The IV/IA combination was evaluated in the Interventional Management of Stroke (IMS) I and II clinical studies. In IMS I a partial dose of rt-PA (0.6 mg/kg) was administered intravenously followed by an IA dose of ≤22 mg administered by a standard microcatheter. The clinical outcome in IMS I trended toward improved outcomes when compared to the IV rt-PA group in the NINDS trial [8]. However, only 50% of patients were totally cleared of clot at the targeted treatment zone after 2 h of IA infusion. Hence, in an attempt to reduce recanalization time, the IMS II study introduced the option of using the EKOS ultrasound infusion system (Bothell, WA, USA) in addition to IA therapy using standard microcatheter. It is well-known that enzyme mediated thrombolysis is enhanced in the presence of ultrasound [9]. The EKOS ultrasound infusion system allows for local delivery of thrombolytics in the presence of a high frequency, low intensity, pulsed ultrasound generated by the catheter mounted transducer. In IMS II, 41% of patients were

completely recanalized at 1 h of therapy and 68.9% at 2 h when treated with EKOS ultrasound catheters. In comparison, complete recanalization with a standard microcatheter was seen in only 30% of patients at 1 h and 53.3% at 2 h. IMS II subjects had significantly better outcomes at 3 months than NINDS placebo treated subjects for all end points and better outcomes than NINDS rt-PA treated subjects as measured by the Barthel Index and Global test statistic [10].

To further shorten time to lysis during IA treatment and increase the number of patients that could benefit from IV/IA combination therapy, we propose that UCA could be utilized during IA therapy with an endovascular ultrasound system. The impact of UCA in further accelerating sonothrombolysis in combination with a transcranial Doppler (TCD) monitoring system was demonstrated in subjects with middle cerebral artery occlusions. 73 subjects were allocated to receive rt-PA plus continuous 2 h TCD or rt-PA alone. 38 subjects were treated with rt-PA plus continuous 2 h TCD monitoring plus three doses of 2.5 g (400 mg/ml) of UCA given at 2, 20, 40 min after treatment initiation. The 2 h complete recanalization rate was significantly higher in the rt-PA + TCD + UCA group (54.5%) compared with rt-PA + TCD (40.8%) and rt-PA (23.9%) groups [11]. In a different study it was demonstrated that high recanalization rates, clinical course and long-term outcome in ischemic stroke are independent of the characteristics of the UCA [12]. However; this approach is associated with two major limitations: the high operator dependency of TCD and inability to transmit sufficient ultrasound energy across the cranium in 10–15% rate of patients who have an excessive bone thickness in the temporal area of the head; the so-called *temporal window* [13].

As previously demonstrated by our group, UCA can significantly enhance thrombolysis in a static clot model with a strong indication that stable cavitation is the dominant responsible mechanism [14]. Furthermore, our group demonstrated that the stability of rt-PA in the presence of activated UCA by ultrasound with peak rarefaction pressures up to 3.5 MPa is not compromised [15]. The purpose of the current study is to develop an IA treatment modality for faster dissolution of blood clots in neurovasculature by concurrent delivery of rt-PA and commercially available UCA to the interior of the clot in the presence of endovascular ultrasound in a dynamic thrombolysis model.

The administration of UCA along with a thrombolytic drug directly into the interior of the clot, places the UCA at the site where it can be most effective. It is likely, however, that the dense fibrin matrix of the clot presents a mechanical barrier to the UCA microbubbles, impeding their ability to migrate through the clot. The extent to which UCA moves through the clot depends on the physical properties of the clot structure (pore diameter and elasticity of fibrin matrix), the physical properties of the UCA microbubbles (bubble diameter, concentration, stiffness of bubble shell, compressibility of interior gas), and the injection parameters (flow rate, pressure). Hence, average clot pore diameter as a function of thrombin concentration, UCA physical properties at the time of entering the interior of the clot as a function of flow rate, and the extent of penetration of UCA into the clot were also investigated in this study. In addition, thrombolysis was evaluated as a function of rt-PA concentration ranging from 0.009 to 0.5 mg/ml and Optison and SonoVue concentrations ranging from 1:10 to 1:200 v/v, in the presence of two peak acoustic rarefaction pressures of 1.3 and 2.1 MPa.

Methods

UCA physical characteristics after delivery through a catheter

For diagnostic purposes, the UCA are characterized by their respective manufacturers for bolus IV administration. For our proposed technique of intra-clot delivery, UCA will be

infused continuously through the 0.45 mm diameter inner lumen of a 140 cm long catheter. To examine the physical endurance of Optison™ (Mallinckrodt Inc., St. Louis, MO) and SonoVue® (Bracco Diagnostics, Milano, Italy) following catheter delivery, their concentration and size distribution was determined microscopically as a function of clinically relevant flow rates.

Quantification method—A hemacytometer (Hausser Scientific, Horsham, PA) with a 125 mm² micro cover glass on top and 0.1 mm depth was used to sample consistent volumes of the UCA solutions. A microscope (BX60; Olympus) with 400× total magnification was used to view the UCA solution. Digital images (2048 × 1536) were acquired using a camera (Coolpix 885; Nikon) attached to one of the microscope eyepieces.

Due to buoyancy of UCA bubbles the focal plane visualizing the bubbles differed from the focal plane visualizing the Neubauer cell boundaries. Hence, two images at each focal plane of the same cell were obtained. Using Neubauer ruling of the counting chamber the pixel to micron scale was determined by dividing the specified length of one cell side (50 μm) by the number of pixels along the side. Size distribution for each UCA was determined by measuring the diameter (in pixels) of all bubbles within an image. UCA concentrations for the various dilutions were determined by counting the number of bubbles within a specified volume, and dividing by that volume. The adequacy of this method in determining UCA concentration was verified by measuring Optison concentrations for various dilutions ranging from 1:1 to 1:1000 and comparing the result to the average expected concentration calculated by using the middle of the range specified by the manufacturer (6.5×10^6 bubbles/ml). The Log-Log plot (not shown) of measured and calculated Optison concentration versus dilution fraction resulted in linear trendlines of $Y = 6.817 \times 10^8 X$ ($R^2 = 0.9976$) and $Y = 7 \times 10^8 X + 2 \times 10^{-8}$ ($R^2 = 1$), respectively. The finest resolution of this method in measurements of bubble diameter was 0.5 μm, which is comparable to the finest resolution reported for Coulter counters (Particle Technology Lab, Ltd., Downers Grove, IL; Beckman Coulter, Inc., Brea, CA), 0.4–1 μm. The advantage of this method is that the sample volume required for each quantification test is 0.01 ml, as compared to the 100–500 ml sample volume required for each quantification test by Coulter counters.

UCA delivery through the EKOS small vessel catheter—UCA solution was drawn from its original vial into a 10 ml syringe (BD, Franklin Lakes, NJ), which was then placed *horizontally* on a syringe pump. The microbubbles in UCA solutions were allowed to rise in the syringe for 10 min prior to initiating delivery through the catheter. The UCA solutions were delivered over 2 min at flow rates of 6, 12, and 18 ml/h. For the purposes of this experiment only, a 7 cm long polyimide tube was attached as an extension to the tip of the catheter to enable sample collection while the catheter transducer was submerged in a 37°C water bath (Fig. 1). The extended tip was placed through a Touhy- Borst adaptor mounted on the water tank wall to collect UCA sample solution. The experiment was performed in the presence and absence of ultrasound transmission.

A pressure microsensor was placed at the proximal end of the catheter lumen to measure the maximum pressure applied to UCA solution in above described delivery system. Using a Codman® ICP Express™ Monitoring system (J&J Medical Ltd., Berkshire, UK) pressure of 27.6 ± 2.7 , 42.9 ± 2.5 , and 57.3 ± 3.21 mmHg was measured for flow rates of 6, 12, and 18 ml/h, respectively.

Average clot pore diameter determination as a function of thrombin concentration

In intra-clot delivery, it is likely that clot pore diameter influences the extent of penetration of UCA into the clot and hence, impacts the extent of bioefficacy. For realistic bioefficacy

evaluation of this modality, it is essential that the in vitro clots are formed with clinically relevant pore diameter ranging from 0.1 to 1.0 μm .

Clot formation—A perfusion setup based on the system described by Carr and Hardin [16] was used to determine adequate thrombin concentration to form a whole blood clot with clinically relevant average pore diameter. Citrated human blood samples (Innovative Research, Southfield, MI) were drawn from 20 different individuals and pooled. The blood was refrigerated and used within 3 days from draw. During this time period the red blood cells (RBC) count was confirmed to remain within normal range of human blood, from 4.0×10^9 – 5.8×10^9 cells/ml, using the hemocytometric method. Clots were formed consistent with the method described in our previous publication [17].

To test the effect on clot pore diameter, the concentration of the added thrombin ranged from 0.0 to 1,000 IU/ml, where 0.0 IU/ml corresponded to pure 1% w/v NaCl.

Perfusion procedure—To measure the pore diameter of the fibrin network, erythrocytes trapped within fluid-filled spaces between the fibrin networks, were eliminated by perfusing the clot with distilled water until the dark hemolysed region had been eliminated from the clot [16]. For a water column of ~20 cm above the clot, the total water perfusion time ranged from 20 min to 3 h depending on the permeability of the clot, which depended on thrombin concentration. After hemolysis the clot was perfused with Phosphate Buffer Saline (PBS) to determine the rate of permeating flow. The average clot pore diameter, d (cm), was determined upon calculation of Darcy permeability, k (cm^2), of clot as described by Carr and Hardin [16].

Proper functioning of the perfusion apparatus was verified by comparing measured Darcy permeability and average pore diameter of a 1 μm glass fiber filter (data not shown) to respective values calculated from specifications provided by manufacturer (Pall Corporation, Port Washington, NY). The microscopic structure of this material consists of randomly arranged fibers, which is similar to the structure of a fibrin clot.

Statistical analysis was performed on pore diameter data using Student's *T*-test and One-way ANOVA (SPSS 12 for Windows; SPSS Inc., Chicago, IL) at a 0.05 level of significance.

Ultrasound imaging of UCA distribution in the clot

Using ultrasound imaging, 2-D cross-sectional B-mode images of UCA penetration into the clot were obtained. Due to the high echogenicity of RBC in in vitro clots [18] and an inability to distinguish them from UCA on B-mode images, pooled human plasma was used for this experiment.

Clot formation—Individual vials of frozen citrated human pooled platelet-poor plasma (Precision Biologic, Nova Scotia, Canada) were thawed for 4 min at 37°C before use and 1.0 ml of plasma was placed in the custom-made polyolefin test tube. Clotting was initiated by adding 0.1 ml of 0.25 M CaCl_2 (Sigma-Aldrich, St. Louis, MO) and 0.1 ml of 25 IU/ml bovine thrombin (VWR, West Chester, PA). The mean pore diameter was measured to be 0.87 μm . Immediately after thrombin addition, a 0.45 mm ID (same as catheter ID) polyimide delivery tube was inserted into the forming clot since platelet-poor plasma clots are difficult to penetrate without causing the clot structure to collapse. The tip of polyimide delivery tube was placed ~9.5 mm below the clot surface. The clot was allowed to form for 10 min at 37°C.

UCA preparation and delivery—Two types of UCA were tested: (1) Optison: albumin-shelled octafluoropropane-filled microbubbles; and (2) SonoVue: lipid-shelled

sulfurhexafluoride-filled microbubbles. The properties of these microbubbles are listed in Table 1. Both UCA were prepared according to their respective manufacturer-specified instructions. Prior to each experiment, each vial was vented to a reservoir of the appropriate gas (C3F8 for Optison, or SF6 for SonoVue) during UCA removal.

A volume of 0.12 ml of the diluted UCA solution (1:10 v/v with PBS) was delivered through polyimide delivery tube into the clot at a flow rate of 1.0 ml/h. Further dilution steps of UCA resulted in low contrast images and suboptimum visualization.

Ultrasound imaging of delivered microbubbles—A portable diagnostic ultrasound machine (Sonosite; Model 180) was used to obtain 2-D cross-sectional B-mode images of the clot. The clot was placed approximately 1 cm in front of a broadband linear array probe (Sonosite; Model L38) that was submerged in a room temperature (~22°C) water bath. An image depth of 2.2 cm was used for these tests. The probe was initially aligned ($z = 0$ mm) with the tip of the delivery tube. After UCA delivery, various crosssections of the clot (i.e. at $z = -1, 0, +1$ mm) were imaged. An external USB video capture device (Dazzle DVC 170) was used to capture digital images with 640×480 pixel resolution of the Sonosite video output. Pinnacle Studio 10 software was used to control image capture.

Thrombolysis procedure

An in vitro flow model similar to the one described by Harpaz et al. [19] was utilized to evaluate UCA impact on sonothrombolysis under dynamic flow conditions.

Flow system—The flow system was an open circuit consisting of 6 mm (internal diameter) flexible Tygon tubing. One end was connected to a reservoir filled with PBS. The tube system has a bifurcation to two parallel arms (Fig. 2). One of the arms was submerged in a 37°C water bath (not shown in schematic) and occluded with a human blood clot. A programmable hot plate (Thomas Scientific, Swedesboro, NJ) maintained the PBS temperature at 37°C upon reaching the bifurcation site as measured by an inline thermistor. A peristaltic pump (Cole-Parmer, Vernon Hills, IL) provided constant pulsating flow at 150 ml/h to the system. A pressure transducer connected upstream of the bifurcation site allowed constant monitoring of the perfusion pressure in the flow loop. A metal pinch-tight tube clamp placed distal to the bifurcation site was used to define the flow resistance and maintain pressure between 20 and 25 mmHg in the loop. During the lysis procedure a flow through heater (Cole-Parmer, Vernon Hills, IL) maintained the water bath temperature at 37°C.

rt-PA preparation—rt-PA (Activase®: Genentech, South San Francisco, CA), was reconstituted and frozen after splitting in 0.35 ml aliquots. The rt-PA was thawed prior to each experiment and diluted with PBS to obtain the ultimate concentrations of 0.009, 0.05, 0.3, 0.5 mg/ml. The diluted rt-PA solution was delivered at a flow rate of 12 ml/h over 10 min. The total rt-PA dose administered was 0.017, 0.1, 0.6, and 1 mg.

UCA preparation for thrombolysis—Both Optison and SonoVue were prepared as described in “ultrasound imaging of UCA in the clot” section above. Upon removal of 0.1 ml volume of each vial, the solution was diluted to 1:10, 1:100, and 1:200 v/v in two steps. In the first step each type of UCA was diluted with PBS and in the second step they were diluted with rt-PA solution.

Clot preparation—Citrated pooled human blood was used to form clots in custom tubes made by cutting a 10 ml serological pipette with 8 mm inner diameter to 6.5 cm flat-ended segments. Parafilm was used to form the base of the tube at one end of the segment. Clotting

was initiated by adding 0.2 ml of 0.25 M calcium chloride and 0.2 ml of 12.5 U/ml bovine thrombin to 2.0 ml of blood. Upon 10 min incubation at 37°C, the clot was removed from the tube, washed in saline and cut to 1 cm segments. Each clot was dried on three layers of filter paper for 20 s, rolling it over every 5 s and weighed on a precision balance prior to treatment.

Clot placement—After recording the clot weight prior to treatment, the clot was submerged in saline and then drawn into a piece of polyimide tube with 6 mm inner diameter using a syringe. Polyimide was chosen due to low acoustic absorbance of this material. The polyimide tube with blood clot was integrated in the main arm of the loop (the arm submerged in 37°C water bath) using two polypropylene barbed female luer-locks with two different strainers attached to the end of the barbs (Fig. 3). The two strainers serve to hold the catheter in the middle of the clot, to hold the clot in place physically and by restricting the flow through the clot served to mimic a vascular stenosis condition. Strainer 1 restricts the flow by approximately 54% of initial flow and is placed inline with the flow. Strainer 2 restricts the flow by approximately 85.6% of the initial flow. The 30% flow restriction difference serves to generate a clinically relevant pressure gradient across the clot.

Clot lysis procedure—The ultrasound tip catheter primed with infusion solution was placed into the main arm holding the clot, by introducing it through a hemostasis valve and guiding it through the central hole of strainer 1. The catheter was advanced until the ultrasound element at the tip was positioned inside the clot. Then, the catheter was secured in place by tightening the hemostasis valve around the catheter shaft. Subsequently, ultrasound was turned on for 10 min, during which time the catheter was advanced inside the clot in 2 mm increments every 2.5 min. Concurrent to ultrasound exposure, the clot was infused with 2 ml solution containing rt-PA, UCA or rt-PA plus UCA delivered via the catheter drug lumen at 12 ml/h. A miniature T-type thermocouple placed at the proximal end of transducer was used to monitor catheter surface temperature during ultrasound treatment.

Measurement method—At the end of the 10 min treatment period, the clot was removed from the tubing, washed in saline, dried and weighed. The clot weights recorded before and after treatment were used to calculate the percentage of weight loss which was used as measure of lysis.

The percentage of clot weight loss for each treatment group is defined as:

$$\frac{W_b - W_a}{W_b} \times 100 \quad (1)$$

where W_b and W_a are the average clot weight (mg) of any clot sample group before and after treatment. A minimum of five data points were generated for each treatment group.

There were a total of seven treatment groups in this study:

1. Clots treated with rt-PA: T
2. Clots treated with rt-PA plus ultrasound with peak rarefaction pressure of 1.3 MPa: T + US₁
3. Clots treated with rt-PA plus ultrasound with peak rarefaction pressure of 2.1 MPa: T + US₂

4. Clots treated with rt-PA plus ultrasound with peak rarefaction pressure of 1.3 MPa and Optison: $T + US_1 + OP$
5. Clots treated with rt-PA plus ultrasound with peak rarefaction pressure of 2.1 MPa and Optison: $T + US_2 + OP$
6. Clots treated with rt-PA plus ultrasound with peak rarefaction pressure of 1.3 MPa and SonoVue: $T + US_1 + SO$
7. Clots treated with rt-PA plus ultrasound with peak rarefaction pressure of 2.1 MPa and SonoVue: $T + US_2 + SO$

Statistical analysis was performed using Student's *T*-test and One-Way ANOVA at a 0.05 level of significance.

Acoustic source and parameters—A cylindrical piezoceramic transducer mounted along the distal tip and coaxial with the central lumen of the catheter (EKOS Corporation, Bothell, WA) was used for this study. The nominal dimensions of the radiating cylindrical surface were 0.81 mm outer diameter, 0.45 mm inner diameter, and 2.1 mm length. Due to the absence of an ultrasound transmission barrier built along the inner diameter of this cylindrical transducer, some inward ultrasound transmission is to be expected. The characteristic of this inner acoustic field can not be directly measured; however, as compared to 81% efficiency of outward radial transmission measured by a radiation force balance, it could be considered negligently small.

A function generator (Agilent, Palo Alto, CA,) connected to a RF power amplifier (ENI Technology Inc., Rochester, NY) was used to drive the transducers at 1.7 MHz in pulsed mode. The catheter transducer was operated using two acoustic protocols. The first protocol is the operating parameters used in the IMS II clinical trials with a calculated peak rarefaction acoustic pressure of 1.3 MPa (US_1). The peak rarefaction acoustic pressure of the second protocol was modestly increased to 2.1 MPa (US_2). To equalize the thermal impact of these two acoustic protocols on thrombolysis, the temporal average acoustic power of US_2 was reduced by decreasing the duty cycle from 8.5 to 4%. Soft tissue thermal index, a relative indicator of temperature increase in soft tissue by ultrasound, is correlated with temporal average acoustic power [20]. The details of both acoustic protocols are described in Table 2.

Results

UCA physical characteristics after catheter delivery

The percent retentions of Optison microbubbles delivered at 6, 12, and 18 ml/h flow rates in absence of ultrasound transmission were 5.9, 5.4, and 8.9%, and in the presence of ultrasound transmission, US_1 , were 1.5, 0.8, and 0.12%, respectively.

Similar reduction in percent retention was observed for SonoVue microbubbles. The percent retention of SonoVue microbubbles delivered at 6, 12, and 18 ml/h flow rates in absence of ultrasound transmission were 3.5, 6.4, and 14%, and in the presence of ultrasound transmission, US_1 , were 0.7, 1, and 0.5%, respectively. Due to the low retention rates, the original concentration of UCA for this experiment was not diluted to achieve the lower concentrations used in thrombolysis experiments.

The microbubble size distributions for both Optison and SonoVue as a function of flow rates are demonstrated in Fig. 4a–c and d–f, respectively. As shown in these frequency histograms, upon delivery through the catheter there is a significant shift towards smaller diameter microbubbles for both Optison and SonoVue. This is most likely the result of the

elevated static pressure in the delivery system forcing gas into solution [21, 22]. When UCA solutions pass through the catheter lumen while exposed to the inward ultrasound field of the cylindrical transducer, the local acoustic stress can also disrupt the encapsulating shells of bubbles, further resulting in the reductions in the bubble populations post sonication reported above.

Average clot pore diameter determination as a function of thrombin concentration

Mean pore diameter decreased with increasing thrombin concentration for both whole blood and platelet poor plasma clots as demonstrated in Fig. 5. One-Way ANOVA applied to each group showed that a significant difference existed among the different thrombin concentrations for each clot type. The resulting F value and its corresponding significance level for blood and plasma clots were 104.3 ($P < 0.001$) and 86.5 ($P < 0.001$), respectively. Mean pore diameter, d (μm), decreased as an inverse power of thrombin concentration, C (IU/ml):

$$\text{Blood clot: } d = 1.3099 \times C^{-0.3014} \quad (R^2 = 0.9980) \quad (2)$$

$$\text{Plasma clot: } d = 1.8068 \times C^{-0.2251} \quad (R^2 = 0.9999) \quad (3)$$

The mean pore diameter of plasma clot was greater than whole blood clot as shown in detail in Table 3. Student's T -test showed that a statistically significant difference existed at each thrombin concentration ($P < 0.0001$).

The majority of blood clots formed with the highest thrombin concentration (1,000 IU/ml) tended to break during the flow measurement. Plasma clots demonstrated similar tendency at the lowest (0.0 IU/ml) as well as the highest (1,000 IU/ml) thrombin concentrations. Therefore, no reliable data were obtained for blood clots at 1,000 IU/ml and plasma clots at 0.0 and 1,000 IU/ml of thrombin.

Consistent with the purpose of each experiment, different clot types, blood or plasma, had to be used in the current study. The required thrombin concentration to maintain the average clot pore diameter in the range of 0.1–1.0 μm for each clot type was determined by utilizing Eqs. 2 and 3.

Carr and Hardin [16] measured the mean pore diameter of human plasma and blood clot (formed with 1 IU/ml thrombin concentration) to be 3.3 and 5.1 μm . The pore diameter measured in the current study for clots made with comparable thrombin concentrations (0.2–2 IU/ml final thrombin concentration) are a factor of ~2 to 4 times lower for plasma and ~5 to 10 times lower for blood compared to the values reported by Carr and Hardin. This discrepancy may be due to the fact that Carr and Hardin formed clots by mixing plasma or whole blood in a 1:1 dilution with the thrombin solution which caused reduction of the fibrinogen concentration and increased the mean pore diameter of the clot. In addition, Carr and Hardin found that clots had larger pores when formed in the presence of erythrocytes: 3.3 μm for plasma clots and 5.1 μm for clots formed with 20% erythrocytes. In the current study the opposite trend was observed: clots formed from whole blood had smaller pores than clots formed from plasma. The reason for this discrepancy is unclear.

Ultrasound imaging of the UCA spatial distribution within the clot

Figure 6 shows ultrasound 2-D cross-sectional B-mode images of either Optison ($\text{OP}_{0,1,2,3}$) or SonoVue ($\text{SO}_{0,1,2,3}$) injected into plasma clot. The accumulated UCA inside the delivery

tube aids in locating the delivery point in the center of the clot prior to UCA delivery. The delivered UCA appear as bright region around the point of delivery ($z = -1, 0, +1$ mm). Due to the heterogeneity nature of clots, UCA distributions were not always uniform which made the quantification of penetration depth difficult. However, as shown in Fig. 6 both Optison and SonoVue have the physical ability to penetrate and distribute into the clot. Figure 6 displays the first frame of each imaging sequence, for after 5 s exposure to ultrasound imaging field the contrast enhancement induced by the UCA would disappear, presumably due to bubble destruction.

Thrombolysis in a dynamic model

rt-PA mediated thrombolysis—By increasing the rt-PA concentration from 0.009 to 0.05 mg/ml the increased lysis rate of the clot was three-fold. The percent clot lysis was increased from 3.1 ± 2.6 to $9.7 \pm 2.14\%$ ($P < 0.017$). Further increasing the rt-PA concentration to 0.3 and 0.5 mg/ml demonstrated a positive trend toward increased lysis rate; however, this trend was not statistically significant as shown in Fig. 7. The percent clot lysis achieved with 0.3 and 0.5 mg/ml were 16.4 ± 2.9 and $17.7 \pm 3.4\%$, respectively.

Ultrasound-enhanced rt-PA mediated thrombolysis—For all four rt-PA drug concentrations, both US₁ (IMS II clinical setting) and US₂ protocols (Table 2) significantly enhanced clot lysis compared to rt-PA group as shown in Fig. 7. The US₁ protocol augmented rt-PA mediated thrombolysis by 3.9, 2.6, 1.9 and 1.8 fold at rt-PA concentrations of 0.009, 0.05, 0.3, and 0.5 mg/ml, respectively. With respect to the same rt-PA concentrations US₂ protocol enhanced thrombolysis by 5.1, 3.4, 2.6, and 3.1 fold. The clot lysis enhancement of US₂ over US₁ protocol was statistically significant for all rt-PA concentrations with exception of 0.009 mg/ml ($P < 0.123$). The detailed values are summarized in Table 4.

UCA impact on thrombolysis in presence and absence of rt-PA—In the absence of rt-PA, Optison diluted 1:10, 1:100, and 1:200 was administered to the clot at 12 ml/h while ultrasound was on. Optison alone did not induce any detectable clot weight reduction when exposed to the US₁ protocol. This outcome was in contradiction with a previous report [23]; hence we decided to investigate the impact of a protocol generating higher acoustic pressure (US₂) as well. For Optison diluted to 1:10, 1:100, and 1:200 exposed to US₂ protocol for 10 min a percent clot lysis of $12.6 \pm 2.4\%$ ($P < 0.002$), $13.9 \pm 1.6\%$ ($P < 0.0001$), and $7.2 \pm 2.5\%$ ($P < 0.030$) was achieved, respectively (Fig. 8a).

In the presence of rt-PA at a 0.05 mg/ml concentration, Optison was administered to the clot. Clots exposed to the US₁ protocol demonstrated clot lysis values of 22.5 ± 2.4 , 43.2 ± 2.4 , and $27.2 \pm 2.1\%$ for administered Optison diluted to 1:10, 1:100, and 1:200, respectively. With respect to the same Optison dilution, the clots exposed to US₂ protocol shown slightly higher clot lysis values $27.6 \pm 2.6\%$ ($P < 0.003$), $45.7 \pm 3.0\%$ ($P < 0.056$) and $30.7 \pm 1.3\%$ ($P < 0.001$). For both ultrasound protocols, the clot lysis values showed similar trends with respect to Optison concentration and reached a peak at 1:100 dilution as shown in Fig. 8b. However, taking the dilution one step further resulted in a decreased clot lysis. The higher concentration of Optison bubbles surrounding the catheter resulted in an acoustic impedance mismatch that limited the transducers ability to transmit sound into the clot volume, in essence shielding the clot from the ultrasound [24]. It appears that selecting the correct UCA concentration is an optimization problem, and the lower lysis rate for an Optison dilution factor of 1:10 could be explained by this shielding effect.

Conversely, the concentration of Optison bubbles at 1:200 dilution factor is shown to be lower than the required concentration to reach maximum bioeffect. The trend shown by our

data suggests that at 1:10 dilution, Optison microbubbles may cause a shielding effect preventing ultrasound to reach effectively beyond the bubble cloud and at 1:200 dilution, the concentration of Optison microbubbles are lower than optimum.

In the presence of rt-PA concentrations of 0.05 and 0.5 mg/ml SonoVue diluted to 1:100 was also administered to the clot. Clots exposed to US₁ and US₂ protocol demonstrated a clot lysis of 36.1 ± 2.6 and $53.1 \pm 2.6\%$ at rt-PA concentration of 0.05 mg/ml and 37.5 ± 2.6 and $62.6 \pm 5.7\%$ at rt-PA concentration of 0.5 mg/ml, in a respective manner.

As demonstrated in Fig. 9a and b clot lysis achieved by Optison and SonoVue of similar concentrations did not differ statistically ($P > 0.05$).

Discussion

Ultrasound, applied transcranially or intra-arterially, is known to accelerate clot lysis in treatment of ischemic stroke [10,25-27]. In combination with IV rt-PA, intravenous infusion of bolus UCA in the presence of transcranial ultrasound demonstrated significant increase in complete recanalization without an increased risk of symptomatic Intracerebral hemorrhage when compared to rt-PA alone or combined rt-PA with transcranial ultrasound [28]. However, issues which limit broader use of this modality include: extreme operator dependency of transcranial ultrasound, inconsistency in temporal window of patients, treatment window limited to 3 h after symptom onset and occurrence of serious cardiopulmonary reaction of some patients to intravenous administration of UCA. Alternatively, the combination modality of rt-PA and UCA can be applied to intra-arterial treatment by delivering rt-PA, UCA and ultrasound concurrently to the interior of the clot. The EKOS 3 Fr ultrasound catheter is comparable to standard microcatheters used by interventional neuroradiologists for IA access, as IA treatment it can be applied up to 5 h post symptom onset, and the required UCA for 2 h treatment will be approximately <1% of the UCA used in clinical studies with transcranial ultrasound [12]. The extremely low concentration of Optison or SonoVue required by intraclot delivery will significantly reduce cardiopulmonary reaction of patients to these agents and will dramatically reduce the cost of administered agents.

In this study, the maximum clot lysis rate using rt-PA was achieved by using a concentration of 0.5 mg/ml. However, the increased lysis rate from increasing the rt-PA concentration from 0.3 to 0.5 mg/ml was minor and statistically not significant (Table 4). Application of 1.7 MHz ultrasound with a calculated acoustic peak rarefaction pressures of 1.3 MPa (US₁) and 2.1 MPa (US₂) increased the rt-PA induced lysis rate at 0.5 mg/ml by 1.8 and 3.1 fold, respectively.

There is a general consensus that high-frequency (>1 MHz), low-pressure ultrasound (≤ 5.2 MPa) enhances thrombolysis via direct absorption of ultrasound energy by clot components and does not involve thermal or cavitation effects [14,17,29-33]. Acoustic cavitation is defined as the generation and volumetric oscillation of small gas bubbles in an acoustic field [24]. In the absence of cavitation, it is the absorption of ultrasound energy by blood clot components that has the greatest potential for inducing bioeffects. In fact, the noncavitation ultrasound phenomena reported to enhance rt-PA thrombolysis: (1) increase porosity of the fibrin matrix scaffolding the clot; and (2) enhanced fluid streaming within the clot; both are coupled to ultrasound absorption [17,32-35]. The highest peak rarefaction acoustic pressure used in this study was 2.1 MPa (US₂) which is less than reported 5.2 MPa cavitation threshold pressure in blood clots reported by Deng et al. [29]. Hence, in this study we could safely assume that absorption rather than cavitation is responsible for increased rt-PA lysis by ultrasound in the absence of UCA.

In absence of cavitation, thermal effects could not explain further augmented rt-PA lysis achieved by US₂ as compared to US₁ protocol either, since similar temporal average acoustic power of 0.3 W was used for both ultrasound protocols (Table 2). The lysis rate advantage of US₂ protocol could be explained by utilizing higher acoustic pressure.

Due to geometrical spreading, the acoustic pressure generated by an acoustic transducer drops with increasing distance from the transducer surface [36]. Studies at EKOS, consistent with other studies, have shown that US-enhanced thrombolysis is dependent on acoustic pressure generated by transducer [37,38]. Therefore, ultrasound efficiency in enhancing enzymatic thrombolysis will decrease with distance from the transducer. Thus, there will be a limited volume of the clot treated with “effective ultrasound pressures” for thrombolysis. The higher the acoustic pressure on the acoustic source surface, the larger the clot volume that can be treated with “effective ultrasound pressures”.

The addition of UCA stabilized bubbles to an acoustic field eliminates the need to employ the high rarefaction pressure amplitudes needed to “nucleate” them heterogeneously; by “seeding” the clot with bubbles we enable cavitation effects at lower acoustic pressures [30]. It is useful to consider the nature of the cavitation activity generated in such a system and its impact on clot lysis. When exposed to a sound field, a bubble can undergo forced, repetitive linear or nonlinear volume pulsations about an equilibrium radius, a phenomenon known loosely as stable cavitation. At sufficiently large pressure amplitudes, the same bubble can experience unstable growth followed by a rapid, energetic collapse, a process termed inertial cavitation [24]. It is less likely that stable cavitation alone can cause significant mechanical disruption of the clot. The violent collapse of an inertial bubble, however, releases a significant amount of energy in the form of an acoustic shock wave and can induce supplemental mechanical stresses from high velocity shearing flows; both physical effects could potentiate clot fragmentation [39]. These two classes of cavitation phenomena could be used to explain the differences in clot lysis by US₁ and US₂ protocols in combination with Optison of various concentrations and in absence of rt-PA. The lack of detectable clot weight reduction using the US₁ protocol suggests that stable cavitation may be the dominant cavitation mechanism at peak rarefaction acoustic pressure of 1.3 MPa, whereas at the 2.1 MPa inertial cavitation sets in and enhanced lysis ensues.

The possible correlation between stable cavitation and the rt-PA increased lysis by US₁ in the presence of Optison was demonstrated in an in vitro static model by our group previously [14]. The statistically significant clot weight reduction with US₂ suggests that the peak rarefaction pressure threshold for inertial cavitation of Optison bubbles is probably higher than 1.3 MPa and ≤ 2.1 MPa. The direct correlation of inertial cavitation dose with acoustic pressure has been demonstrated by Lai et al. [40].

Bubbles undergoing stable cavitation in a viscous medium experience boundary stresses that result in the generation of a steady streaming flow in the surrounding fluid, a phenomenon known as cavitation microstreaming [41]. These oscillating bubbles act as small microscopic pumps that promote local mass transfer and thus facilitate deeper penetration of rt-PA into the clot matrix. This would explain the further increased clot lysis rate from 1.8 to 2.6 fold by US₁ and from 3.1 to 3.7 by US₂ protocol in presence of rt-PA of 0.5 mg/ml once 1:100 diluted Optison was added.

The presence of a large concentration of UCA microbubbles in the immediate volume around the catheter-mounted ultrasound transducer introduces an acoustic impedance mismatch that changes the load characteristics of the transducer. This could result in electrical impedance shift, decreased efficiency and increased temperature of transducer. However; low power impedance measurements of the catheter-mounted transducers in a

plasma clot seeded with Optison (diluted 1:100 v/v) was similar to plain plasma clot (data not shown). Considering that Optison diluted 1:100 v/v represents the initial concentration prior to the catheter delivery and upon delivery through the catheter into the clot this concentration is reduced to 1% of initial concentration in presence of ultrasound transmission, it is safe to assume that the final Optison concentration in the clot does not have an unloading effect on transducer during sonothrombolysis in vitro or in clinical setting. The fact that continuous monitored surface temperature of the transducers for the UCA treatment groups showed no temperature increase as compared to other groups on account of reduced transducer efficiency due to the unloading, further verifies the absence of an unloading effect of low UCA concentration on catheter mounted transducers.

The safety and efficacy of US₁ protocol in enhancing rt-PA mediated clot lysis in an IA approach was demonstrated in IMS II clinical trial [10]. In addition, using UCA in the neurovasculature of stroke patients to accelerate thrombolysis using TCD ultrasound was not associated with any increased risk as compared to standard treatments [2,11,12,26-28]. Hence, we propose an IA therapy consisting of intra-clot delivery of ultrasound (US₁), rt-PA, and UCA (diluted 1:100) can be safely applied and evaluated in ischemic stroke clinical trials.

The US₂ protocol in our in vitro dynamic setting demonstrated better potency in increasing the rt-PA mediated clot lysis rate in presence and absence of UCA. However, in vivo safety studies would be required to evaluate potential biological adverse effects of this protocol especially in combination with UCA due to its possible association with inertial cavitation.

Conclusion

The results of this study suggest that IA application of rt-PA and low dose of UCA in presence of ultrasound can be considered as a viable treatment for ischemic stroke patients. However; the extent of short and long term clinical benefits of this modality should be evaluated in clinical trials. Consistent with Rubiera et al. [12] the difference in physical characteristics of Optison and SonoVue had no impact on their respective clot lysis rates.

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Fig. 1. Schematic of experimental setup used to determine UCA retention after delivery through the catheter in presence and absence of ultrasound emission

Fig. 2.
Schematic of flow system

Fig. 3.
Female luer locks with strainers to simulate in vivo stenosis

Fig. 4.
a–f Size distribution for Optison and SonoVue. Each *histogram* depicts the size distribution (1) measured immediately out of the vial following manufacturer's specified procedure and after delivery through the catheter in the (2) presence and (3) absence of transmission of US₁ ultrasound protocol for flow rates of 6, 12, and 18 ml/h

Fig. 5. Clot pore diameter as a function of thrombin concentrations: Blood versus plasma (platelet poor)

Fig. 6.

Ultrasound B-mode images of Optison and SonoVue penetration into plasma clot. The periphery of the test tube (*black arrows*) and location of delivery tip ($z = 0$, *white arrows*) for both Optison and SonoVue prior to delivery into the clot are shown in OP_0 and SO_0 images, respectively. $OP_{1,2,3}$ and $SO_{1,2,3}$ images are taken after delivery of Optison and SonoVue, respectively, at cross sections $z = 0, +1, -1$ mm

Fig. 7. Percent clot lysis after 10 min treatment of blood clots (D: 6 mm $\times$ L: 10 mm) with rt-PA (T), rt-PA plus peak rarefaction acoustic pressure of 1.3 MPa (T + US₁), and rt-PA plus peak rarefaction acoustic pressure of 2.1 MPa (T + US₂)

Fig. 8.
a, b Percent weight reduction of clots treated with Optison and US₁, and Optison and US₂ ultrasound protocols **a** in absence of rt-PA and **b** in presence of rt-PA (0.05 mg/ml)

Fig. 9.
a, b Weight reduction comparison of clots treated with SonoVue and Optison at rt-PA concentrations of **a** 0.5 mg/ml and **b** 0.05 mg/ml after exposure to US₁ and US₂ ultrasound protocols

Table 1

Properties of Optison and SonoVue

MB	Shell	Gas	Mean concentration (bubbles/ml)	MB dia. size (μm)
Optison™	Albumin	Octafluoropropane	$5-8 \times 10^8$ (range) ^a	3.75 (mean) ^a
SonoVue®	Lipid	Sulfurhexafluoride	$2-5 \times 10^8$ (range) ^b	2.5 (mean) ^b

^aMallinckradt Inc, St. Louis, MO^bBracco Diagnostics, Inc., Princeton, NJ

Table 2

US₁ and US₂ acoustic protocols in details

Acoustic protocol	Duty cycle (%)	Pulse repetition frequency (Hz)	Temporal average acoustic power (W)	I _{SPPA} (W/cm ²)	Peak rarefaction pressure (MPa)
US ₁	8.5	30	0.3	52.9	1.3
US ₂	4	40	0.3	144.8	2.1

Table 3

Darcy permeability, k (AVG $\pm$ SD), and mean pore diameter, d (AVG $\pm$ SD), measured for blood and plasma clots

Thrombin conc. (IU/ml)	Blood clot		Plasma clot	
	k ($\text{cm}^2 \times 10^{10}$)	d (μm)	k ($\text{cm}^2 \times 10^{10}$)	d (μm)
0	12.62 ± 2.17	0.993 ± 0.087	–	–
2.5	12.34 ± 1.57	0.983 ± 0.064	27.80 ± 4.83	1.473 ± 0.129
25	3.42 ± 1.34	0.511 ± 0.094	9.95 ± 3.59	0.871 ± 0.157
100	1.33 ± 0.31	0.321 ± 0.041	5.28 ± 0.73	0.643 ± 0.045

Table 4Percent clot lysis (AVG $\pm$ SD) for each treatment group

rt-PA conc. (mg/ml)	T (%)	T + US ₁ (%)	T + US ₂ (%)	T + US ₁ + OP (%)	T + US ₂ + OP (%)
0.009	3.1 $\pm$ 2.6	12.1 $\pm$ 2.0	16.0 $\pm$ 2.6	23.9 $\pm$ 2.2	27.7 $\pm$ 1.4
0.05	9.7 $\pm$ 2.1	24.8 $\pm$ 3.2	33.1 $\pm$ 3.0	40.5 $\pm$ 4.9	48.3 $\pm$ 6.3
0.3	16.4 $\pm$ 2.9	30.4 $\pm$ 2.4	41.8 $\pm$ 2.9	44.9 $\pm$ 2.6	60.6 $\pm$ 2.1
0.5	17.7 $\pm$ 3.4	31.5 $\pm$ 2.5	54.0 $\pm$ 4.2	46.7 $\pm$ 2.9	64.7 $\pm$ 3.9