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Combination Treatment with rt-PA is More Effective than rt-PA Alone in an *in Vitro* Human Clot Model

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Abstract

Incidence of intra-cranial hemorrhage linked to treatment of ischemic stroke with recombinant tissue plasminogen activator (rt-PA) has led to interest in adjuvant therapies such as ultrasound (US) or plasminogen, to enhance rt-PA efficacy and improve patient safety. High-frequency US (~MHz) such as 2-MHz transcranial Doppler (TCD) has demonstrated increased recanalization *in situ*. Low-frequency US (~kHz) enhanced thrombolysis (UET) has demonstrated higher lytic capabilities but has been associated with incidence of intracerebral hemorrhage in some clinical trials. *In vitro* studies using plasminogen have shown enhancement of lysis. This study compared rt-PA-induced lysis using adjuvant therapies, with 120-kHz or 2-MHz pulsed US, or plasminogen, in an *in vitro* human whole blood clot model. Blood was drawn from 30 subjects after local institutional approval. Clots were exposed to rt-PA at concentrations of 0 to 3.15 µg/ml. Clots were exposed to rt-PA alone (**rt-PA**) or in combination with plasminogen (**Plg**), 120-kHz US (**120-kHz**), or 2-MHz US (**2-MHz**). Thrombolytic efficacy was determined by assessing the percent fractional clot loss (FCL) at 30 minutes using microscopic imaging. There was no enhancement of lysis for combination therapy with [rt-PA]=0 µg/ml. Adding rt-PA increased lysis for all groups. As [rt-PA] increased, lysis tended to increase for **120-kHz** and **Plg** (FCL: from 50% to 70%, **120-kHz**; 65% to 83%, **Plg**) but not for **2-MHz** (58% to 52%). Lytic efficacy in combination therapy depends on rt-PA concentration and the adjuvant therapy type. For non-zero rt-PA concentrations, all combination therapies produced more lysis than rt-PA alone.

Keywords

Plasminogen; thrombolysis; tissue plasminogen activator; transcranial doppler; ultrasound

INTRODUCTION

Thrombolytic therapy is widely used for a variety of clinical cardiovascular disorders such as acute ischemic stroke [1], myocardial infarction [2], peripheral vascular disease, and deep

venous thrombosis [3]. Current therapy utilizes thrombolytic medications such as urokinase, streptokinase or recombinant tissue plasminogen activator (rt-PA). In general, these medications are plasminogen (Plg) activators that transform the zymogen plasminogen into plasmin. Plasmin actively cleaves the fibrin mesh of the culprit clot. The clinical utility of such medications is well-established. However there can be substantial side effects. For example, in the treatment of acute ischemic stroke, the use of rt-PA can cause intracerebral hemorrhage (ICH) in about 6% of patients [1]. The rate of ICH is about 1% for thrombolytic treatment of acute myocardial infarction [4]. The risk of hemorrhage is related to the dosage of plasminogen activators [4]. Thus, there is interest in combination therapies that will increase thrombolysis with decreased administered rt-PA dose.

The efficacy of plasminogen activators can be limited by a lack of substrate plasminogen [5-7]. This suggests the idea of using plasmin as a direct-acting lytic [5, 8-14]. However, plasmin has some disadvantages in that it is prone to autodegradation and is not stable at normal pH [8], making this lytic difficult to use in the clinical environment. In contrast, plasminogen is stable *in vivo*, does not require special handling, and would improve substrate availability as a supplement to rt-PA [15]. However, there is little data on the effect of combined rt-PA and plasminogen on thrombus size: to date no clinical trials have examined direct plasminogen supplementation for ischemic stroke.

Previous studies have shown that ultrasound (US) enhances thrombolysis when used with rt-PA [16-24]. The “Combined Lysis of Thrombus in Brain Ischemia Using Transcranial Ultrasound and Systemic t-PA” (CLOTBUST) trial demonstrated that transcranial doppler (TCD) ultrasound can successfully improve the rate of early recanalization when used in conjunction with rt-PA [21-22]. The trial, employing 2-MHz ultrasound exposure via TCD during rt-PA administration, showed a greater incidence of complete recanalization or dramatic recovery between treatment (with TCD) and placebo (no TCD) groups [21]. However, the difference of long-term neurologic outcomes at three months was not significant [21]. Also, studies have shown that MHz-frequency US suffers more attenuation through the skull than lower frequencies, likely limiting the efficacy of such treatment [25, 26]. The kHz range of frequencies has been investigated with the “Transcranial Low-Frequency Ultrasound-Mediated Thrombolysis in Brain Ischemia” (TRUMBI) trial [27]. Coupling rt-PA and 300 kHz ultrasound, the study was prematurely stopped due to safety concerns [27, 28]. It is possible that the high intensity used in TRUMBI could have yielded high pressure magnitudes, which may have increased incidences of negative bioeffects [19, 28]. Despite poor clinical trial results, significant *in vitro* studies have shown the efficacy of kHz-frequency US in enhancement of thrombolysis [16-19, 27]. Thus, the ideal frequency for *in situ* thrombolysis with ultrasound has yet to be determined.

The objective of this study was to compare the thrombolytic efficacy of representative alternative lytic therapies currently under investigation, in a well-defined *in vitro* human clot model. The primary hypothesis of this work is that either plasminogen or US significantly enhances rt-PA-induced thrombolysis, compared with rt-PA treatment alone.

MATERIALS AND METHODS

Preparation of rt-PA, Plasminogen, and Human Plasma

The rt-PA was obtained from the manufacturer (Activase®, Genentech, San Francisco, CA) as a lyophilized powder. Each vial was mixed with sterile water to a concentration of 1 mg/ml as per manufacturer’s instructions, aliquoted into 1.0 ml centrifuge tubes (Fisher Scientific), and stored at –80°C. The enzymatic activity of rt-PA is stable for years when stored in this fashion [29, 30]. The plasminogen was obtained from the manufacturer (Molecular Innovations, Novi, MI) in 1 mg aliquots of 0.423 ml solution, and was also

stored at -80°C . Human fresh-frozen plasma (hFFP) was procured from a blood bank in 250-300 ml units. Each unit was briefly thawed, aliquoted into 50 ml polypropylene centrifuge tubes (Model 05-538-68, Fisher Scientific Research, Pittsburgh, PA), and stored at -80°C . Aliquots of rt-PA, plasminogen, and hFFP were allowed to thaw for experiments, and the remaining amounts discarded following completion of each experiment.

Production of Blood Clots

Human whole blood was obtained from healthy volunteers by sterile venipuncture following local Institutional Review Board approval and written informed consent. In order to approximate the acute care environment in which most of these therapies are initiated, the only exclusion criterion was the use of aspirin within 72 hours of the blood draw. Blood from a total of 30 subjects was used in these studies. Samples of 1-2 ml were placed in sterile glass tubes (VWR, Batavia, IL; 3 ml) and allowed to form clots in and around a small diameter ($\sim 600\text{ }\mu\text{m}$) micropipette (Becton, Dickinson and Company, Franklin Lakes, NJ; 20 λ) through which a segment of 7-0 silk suture (Ethicon Industries, Cornelia, GA) had been threaded. This is similar to clot production methods used in imaging studies by Winter [31] and Yu [32]. The clots were incubated for three hours at 37°C , and refrigerated at 5°C for 3 days ensuring maximal clot retraction, lytic resistance and stability [33-35]. Before each experiment, the micropipette was removed to produce a cylindrical clot adherent to the suture. The clot was typically 5-8 μl in volume and approximately 200 - 300 μm in width. At this size the clots were similar in diameter to the intracerebral segments of the middle cerebral arteries (80 to 840 μm in diameter) or other cerebral vessels such as the recurrent artery of Heubner and its perforators ($643 \pm 237\text{ }\mu\text{m}$ in diameter) [36, 37]. Additional details and justification of the clot sample preparation are discussed elsewhere [16, 17, 38].

Ultrasound Parameters

In select treatment regimes, clots were exposed to pulsed US via one of two transducers. The first transducer was supplied as part of the E-Z Dop® pulsed-wave diagnostic focused US system (Compumedics DWL, Singen, Germany), which is one of the 2-MHz TCD systems used during the CLOTBUST trial [21]. The second transducer was a custommade unfocused 120-kHz transducer (Sonic Concepts Inc, Woodburn, WA) [16].

For each trial involving US, a transducer was mounted at one end of the tank at a 30° angle to the tank bottom thus allowing US exposure of the sample clot. Sound absorbing material (rho-c rubber, Precision Acoustics, Dorchester, UK) was placed at the end of the tank opposite the transducer to inhibit acoustic reflections. (Fig. 1) The transducers were calibrated with a 0.2-mm needle hydrophone (Reson, Goleta, CA; TC4038). The geometric focus of the 2-MHz transducer is 41 mm from the transducer face. The 2-MHz ultrasound parameters used were a peak free-field rarefactional acoustic pressure amplitude (P_R) of 0.47 MPa, a pulse repetition frequency (PRF) of 10.5 kHz and a duty cycle of 13%. Others have measured an increase in thrombolysis with increasing duty cycle, so the apparatus settings that produce the largest duty cycle of 13% were employed to maximize thrombolytic effects [16, 39-41]. Pressure attenuation of 2-MHz pulsed US through the micropipette wall is $41 \pm 5\%$. For the 120-kHz transducer, parameters of a 50% duty cycle, a PRF of 1.667 kHz, and a free-field P_R of 0.18 MPa were used. The pressure attenuation of 120-kHz pulsed US through the micropipette wall was found to be less than 1% [42]. These parameters have been shown to produce substantial clot lysis at 120 kHz [16-17, 19].

Visualization of the Clot

For each experiment, the clot attached to the suture was placed in a clean micropipette (Drummond Scientific Company, Broomall, PA), and inserted into a U-shaped sample holder composed of hollow luer lock connectors and silicone tubing (Cole Parmer, Vernon

Hills, IL; outer diameter 0.125"). The sample holder was placed in a custom-made acrylic water tank with a microscope slide at the bottom.

Water in the tank was maintained at a temperature of $37 \pm 1^\circ\text{C}$ during all experiments using two heating elements (Hagen A721, Mansfield, MA; 25 W). The tank was placed over the objective of an inverting microscope (Olympus, Melville, NY) to visualize the clot. The field of view in the image was $340 \mu\text{m} \times 260 \mu\text{m}$ (640 pixels $\times$ 480 pixels). In all trials the full width of the clot was within the microscope field of view. The entire apparatus was placed on top of a vibration isolation table (Newport, Irvine, CA; XL-G) for mechanical isolation. Fig. (1) shows a schematic of the experimental apparatus. Images were recorded at 6 frames/min using a CCD camera (Hitachi, Woodbury, NY; KP-M1A), and data was stored for later analysis on a desktop computer (Dell, Round Rock, TX; Intel Pentium). A complete description of the experimental system can be found elsewhere [16, 17].

Experimental Protocol

Initially clots were exposed to one of four treatment regimens: (1) hFFP and rt-PA (**rt-PA**); (2) hFFP, 120-kHz ultrasound, and rt-PA (**120-kHz**); (3) hFFP, plasminogen ([plasminogen]=167 $\mu\text{g/ml}$), and rt-PA (**Pig**); or (4) hFFP, 2-MHz TCD ultrasound, and rt-PA (**2-MHz**). The rt-PA concentrations for all treatments were 0.00, 0.50, 1.00, or 3.15 $\mu\text{g/ml}$. This range was chosen to span the therapeutic concentration of rt-PA in humans [43, 44]. The plasminogen concentration of 167 $\mu\text{g/ml}$ was chosen to be similar to that occurring naturally in human plasma [6]. The addition of the exogenous plasminogen would be expected to approximately double the plasminogen contained within the hFFP.

The **rt-PA**, **120-kHz**, and **2-MHz** trials began by introducing 1 ml hFFP containing the appropriate concentration of rt-PA. The **Pig** trials began by introducing 1 ml of hFFP containing 167 $\mu\text{g/ml}$ of plasminogen immediately followed by adding the amount of rt-PA needed to achieve the desired rt-PA concentration. At time equal to 0, the hFFP solution was in contact with the clot in all treatment groups. The ends of the sample holder were exposed to atmospheric pressure, and the clot surface to a static fluid column. Clots were exposed to a specific treatment regimen for 30 minutes. Previous studies have shown that the majority of thrombolysis occurs within this time period [16-20]. Each regimen used an average of 32 clots (range 8-158) from 6 subjects (range 3-24).

Determination of Lytic Efficacy

Light intensity transmitted through the sample clot is reduced with increased clot thickness or clot density. The CCD camera records image light intensity $I(x,z)$ at each pixel (x,z) where z is along the axis of the clot and x is along the clot diameter. By analyzing the light intensity in each pixel, the clot edges can be identified, thus enabling measurement of clot width. In brief, an edge detection routine written in Matlab 6.5 R13 (Mathworks, Inc., Natwick, MA) is used to determine the positions of the two clot-plasma interfaces. The average clot width for each image was calculated by averaging the width over all z values. The clot width data were subsequently corrected for the average suture width used in these experiments ($95 \pm 15 \mu\text{m}$, based on analysis of 252 samples of the 7-0 suture), and normalized to the initial value of the clot width during the first minute (the average over the first six frames). Note that the mean of this corrected normalized clot width (CW_{NORM}) is equal to 1 during the first minute, and becomes equal to zero if the clot is totally lysed, thus leaving only the suture visible. The parameter CW_{NORM} is then averaged for all clots in a given treatment group. The fractional clot loss (FCL, expressed as %) is defined as

$$FCL \equiv (1 - CW_{NORM}(30\text{min})) * 100\% \quad (1)$$

where CW_{NORM} (30 min) is the average normalized clot width over the final minute of treatment. Note that as a given sample clot is imaged 6 times per min, there are 6 measurements of CW_{NORM} (30 min) for each clot trial. A complete description of these image analysis methods can be found in previous works [16-19, 38].

Statistical Analysis

Data are presented as mean values with standard deviations. Mean FCL values, standard error of the means (SEM) of FCL values, and 95% confidence intervals (CIs) are used to report the effects of rt-PA and treatment combinations.

RESULTS

Fig. (2) shows the average CW_{NORM} versus time for several treatment groups including two representative **rt-PA** treatment groups ([rt-PA]= 0.00 and 3.15 $\mu\text{g/ml}$), the **Plg** treatment groups for the same concentrations ([rt-PA]=0.00, 3.15 $\mu\text{g/ml}$), and the **120-kHz** and **2-MHz** groups with [rt-PA]=3.15 $\mu\text{g/ml}$. The initial diameter for clots in all sixteen groups was 230 μm (95% CI: 227-233 μm , N=510 clots). The time course of the clot width for **Plg**([rt-PA] = 0 $\mu\text{g/ml}$, or **Plg** (0 $\mu\text{g/ml}$)) trials is similar to that of **rt-PA** (0 $\mu\text{g/ml}$). The clot width decreases to a greater extent for the **rt-PA** (3.15 $\mu\text{g/ml}$) trials, and even further in the **120-kHz** (3.15 $\mu\text{g/ml}$) and **2-MHz** (3.15 $\mu\text{g/ml}$) groups. The ultrasound groups also have a faster initial decrease in clot width, compared to the **rt-PA** (3.15 $\mu\text{g/ml}$) group. The final curve, **Plg** (3.15 $\mu\text{g/ml}$), although initially slower than the **120-kHz** and **2-MHz** groups at the same rt-PA concentration, shows the largest overall decrease in clot width.

Substantial lysis was observed in many individual trials, but particularly in the **Plg** (3.15 $\mu\text{g/ml}$) group. In three of the eighteen trials, the clot was completely lysed within the thirty minute exposure period by the combination of rt-PA and plasminogen. The mean dissolution time for these three trials is 24 ± 9 minutes. Also, in four cases of the fifteen **120-kHz** (3.15 $\mu\text{g/ml}$) trials, the clot was completely lysed within the thirty minute exposure period, with a mean dissolution time of 16 ± 4 minutes. There were no instances of complete clot dissolution within the **120-kHz** or **Plg** groups at other rt-PA concentrations, nor within any of the **rt-PA** alone or **2-MHz** groups.

Fig. (3) exhibits the average FCL (Equation 1) values for all sixteen treatment groups. There was no significant difference in FCL for clots not exposed to rt-PA ($p > 0.5$). FCLs are noticeably greater as the rt-PA concentration increases to 0.5 $\mu\text{g/ml}$, typically at least double the FCL at 0 $\mu\text{g/ml}$. For the **rt-PA** (0.50 $\mu\text{g/ml}$) group, FCL rises to 44% (37-52%), and then remains slightly below this level at 1 and 3.15 $\mu\text{g/ml}$. FCLs in the **2-MHz** groups also reach a peak at 0.5 $\mu\text{g/ml}$, at 58% (50-65%), and then remain relatively constant up to 3.15 $\mu\text{g/ml}$. In contrast, FCLs for trials with rt-PA and 120-kHz US (**120-kHz**) continue to rise with increasing rt-PA concentration. Among the four treatment regimes, the **Plg** combination typically shows the highest FCL of any regime at a specific concentration of rt-PA. The **Plg** (3.15 $\mu\text{g/ml}$) group achieves the maximum FCL among all sixteen groups, at 83% (80-86%). Overall, lysis increases dramatically in each combination group as rt-PA is added.

Table 1 lists the average FCL values along with the standard deviation, 95% CI, number of donors, and number of clots for each group. Using a linear mixed model to account for multiple clots from the same subjects, the statistical tests compare the three combination treatments to the **rt-PA** treatment within each concentration. Significant differences between FCLs of a combination treatment and the **rt-PA** group at the same concentration ($p < 0.05$, 0.01, and 0.001) are noted.

DISCUSSION

It was shown that combination therapy with rt-PA and plasminogen, 120-kHz pulsed ultrasound, or 2-MHz pulsed ultrasound has greater lytic efficacy than rt-PA treatment alone ([rt-PA] = 0 – 3.15 µg/ml) in this *in vitro* human clot model. While a particular therapy without rt-PA causes no increased lysis, any single therapy combined with rt-PA at concentrations of 0.5 µg/ml or greater dramatically increases clot lysis, substantially reducing the clot diameter over that of rt-PA alone. Clot size is the parameter of greatest interest to a clinician treating a thrombotic disorder.

One observation of the study is that FCL for the **rt-PA** group does not increase as rt-PA concentration increases. As shown in Fig. (3), lysis using rt-PA alone reaches a maximum at 0.50 µg/ml, and then decreases at 1 µg/ml, with a slight increase following at 3.15 µg/ml. This may be due to the presence of multiple clot fragments at higher concentrations that “soak up” available rt-PA and plasminogen in the plasma; also, creation of plasmin by plasminogen and rt-PA more rapidly due to the higher concentration of rt-PA may be deactivated more rapidly by alpha-2 antiplasmin [38, 45]. However, these mechanisms are speculative.

There are further differences in FCL based on both the concentration of rt-PA and the type of combination therapy employed. For example, FCLs for the **Plg** group generally increase as rt-PA concentration increases. However, FCL for the **2-MHz** group remains relatively constant at 0.5 µg/ml and higher. This is probably due to differing mechanisms of thrombolytic enhancement. The **Plg** group increases lysis by increasing the supply of substrate available for rt-PA to activate into plasmin. With “plasminogen steal” no longer a limiting factor, the FCL tends to increase as the amount of rt-PA increases. In the US treatment groups, the lytic enhancement mechanism is likely stable cavitation [46], or the activation of microbubbles in the plasma by the ultrasound field.

Using an *in vitro* human whole blood clot model, Datta *et al.* demonstrated that 120-kHz ultrasound will not improve rt-PA-induced clot mass loss significantly if the magnitude of the applied ultrasound is below the threshold for initiating stable cavitation [46]. The peak negative pressure threshold for stable cavitation at 120 kHz in a medium of clot, plasma, and rt-PA is approximately 0.20 MPa [46, 47]. Other *in vitro* studies demonstrated significant enhancement of rt-PA-driven thrombolysis with 120-kHz ultrasound at a P_R of 0.18 MPa, close to the reported threshold [16-19]. At a frequency of 2 MHz, the calculated value for this stable cavitation threshold increases to approximately 0.50 MPa, which is slightly greater than the 0.47 MPa used in this study [46, 47].

The question now becomes, of the different therapies investigated in this study, which one is the most ideal for thrombolysis? Each treatment group has its advantages. Thrombolytic therapy using rt-PA by itself is the easiest to implement clinically as it is the current standard of care and has the fewest components to manage. The addition of ultrasound is attractive due to the increased thrombolysis and, with TCD units, the added benefit of monitoring intracranial blood flow. In CLOTBUST, there were more instances of early recanalization in treatment patients (those receiving TCD in addition to rt-PA) compared to non-treatment patients, demonstrated via TIBI flow. Several studies have demonstrated benefits to short-term patient recovery due to rapid recanalization following stroke [21, 48-53]. Similarly, therapy groups using rt-PA coupled with 120-kHz pulsed ultrasound had large FCLs, tending to increase as the concentration of rt-PA increased. Although not definitive, it should be noted that instances of complete clot dissolution were only observed using ultrasound at the lower frequency, though that phenomenon has yet to be demonstrated *in vivo* at low US frequencies.

The use of plasminogen in addition to rt-PA may be a useful therapy for a number of reasons. First, the addition of rt-PA or other plasminogen activators can deplete the supply of plasminogen both within the culprit clot and the surrounding plasma [5, 54]. Adding plasminogen thus increases the substrate supply, and the total amount of plasmin available to lyse the clot. Second, clots are more resistant to lysis with clot age likely due to clot retraction and a reduction in plasminogen contained within the clot [33]. In the current study, the combination of rt-PA and plasminogen tended to have the highest FCL at any rt-PA concentration, which is probably due in some part to the additional available substrate.

The ideal goal for the use of combination thrombolytic therapy with rt-PA is to maintain lytic efficacy while reducing the rt-PA dose. This would reduce the risk of hemorrhagic side effects as these are dose-related in plasminogen activators [4]. In this study, FCLs were very similar for the **120-kHz, Plg**, and **2-MHz** groups at 0.50 and 1.00 $\mu\text{g/ml}$. Each of the **120-kHz, Plg**, and **2-MHz** groups have roughly equivalent FCLs at 1.00 $\mu\text{g/ml}$, which is closest to the plasma concentration expected in acute ischemic stroke treatment. The use of combination therapy would thus allow rt-PA concentration to be reduced with no loss of thrombolytic efficacy, with choice of specific combination therapy determined by other factors *in situ*. Further, as FCLs increase in groups using combination therapy, the rate of lysis increases as well. This is demonstrated in the average CW_{NORM} versus time data shown in Fig. (2): groups using US or Plg achieve greater lysis in shorter periods of time. As mentioned previously, other studies have noted a more rapid recanalization rate *in vivo* (implying a faster rate of clot lysis) has implications on better short- and long-term patient outcomes; for example, Alexandrov et al. noted a trend toward reduced incidence of death following treatment with rt-PA and TCD vs. rt-PA alone, though the difference did not achieve statistical significance [21, 48-53]. Even in cases where the clot was not completely lysed, the use of combination therapy at lower rt-PA concentrations achieves greater lysis more quickly compared to use of rt-PA alone, and could therefore improve patient outcome, potentially minimizing incidence of hemorrhage or death and the severity of post-stroke disability.

Despite their advantages, each combination therapy has its drawbacks. Though the CLOTBUST trial showed safety and possibly improved efficacy of 2-MHz TCD ultrasound and rt-PA [21, 22], the FCLs for the **2-MHz** group lagged behind the other groups at most rt-PA concentrations tested here, particularly at higher concentrations ($[\text{rt-PA}] > 0.5 \mu\text{g/ml}$). In this case, total clot lysis may be limited by the lack of thrombolytic substrate such as plasminogen. In the case in which a finite amount of plasminogen is available, ultrasound could enhance the thrombolytic rate (Fig. 2), perhaps by enhancing diffusion of rt-PA or plasmin into the clot. But total lysis would not be significantly different due to the limit of the available thrombolytic substrate *in situ* [19, 55]. The lytic efficacy of 120-kHz ultrasound may have surpassed that of the 2-MHz ultrasound in trials due not to an ability to overcome substrate limitations but by some other factor. Possibly there was greater stable cavitation occurring at 120 kHz than at 2 MHz in this *in vitro* setting; studies measuring stable cavitation quantitatively are needed to confirm this hypothesis.

Combination therapy with US may be limited by anatomy as well. The human skull attenuates ultrasound as it passes through the temporal bone [25, 56]. The attenuation is dependent upon the ultrasound frequency. At 2 MHz, the pressure at the origin of the middle cerebral artery (MCA) can be reduced by 70-95% [56]. This reduction in pressure can have an unintended benefit. Harmful bioeffects can increase with increasing ultrasound pressure amplitude. Thus, a reduction in pressure within the skull may reduce incidence of ICH in patients receiving combination ultrasound therapy [57]. However, because the amount of attenuation varies with temporal bone thickness, the actual P_R *in situ* during clinical practice can not be easily determined [56]. A recent simulation study by Baron *et al.* calculated that,

given an approximate P_R of 0.385 MPa from a TCD device as used in CLOTBUST, the peak negative pressure inside the skull cavity would be at most 0.07 MPa [28]. This is below the threshold for opening the blood-brain barrier, but also below the threshold for stable cavitation, which may limit enhancement of thrombolysis [46, 57].

Despite the greater *in vitro* lytic efficacy of the **Plg** group, combination therapy with plasminogen could be problematic. The amount of plasminogen required to raise the normal human body's mean concentration of plasminogen is substantial. Also, there is rapid deactivation of plasmin due to scavenging by alpha-2 antiplasmin, occurring almost immediately following its creation from rt-PA and plasminogen [8, 10]. Plasmin is autolytic, and is not stable at normal pH [8]. In addition, whole blood can inactivate plasmin doses up to 12 mg/kg [58]. These limitations would likely require local administration of rt-PA and plasminogen to the clot, as the resulting plasmin would be quickly inhibited in the bloodstream [10-12].

A number of limitations concerning combination thrombolytic therapy for ischemic stroke are unable to be addressed in this study. First, this work is performed *in vitro*, and does not replicate the complex *in vivo* or clinical scenario of lytic therapy. In addition, the clots were manufactured from the blood of healthy donors, thus reducing the applicability of these results to individuals on anticoagulants or other medications, or those suffering from significant medical problems. The small size of the clots is a limitation in trial groups in which thrombolysis is extremely effective. For example, in the **Plg** (3.15 $\mu\text{g/ml}$) and **120-kHz** (3.15 $\mu\text{g/ml}$) groups, seven trials completely removed all clot from the suture. The amount of lysis that could have been accomplished in larger clots, where more clot material is available for lysis, is underestimated. However, the small size of the clots are necessary to detect minute changes in the clot diameter following delivery of lytics [19]. Utilizing clots of 200-300 μm diameter allows resolution of these small values of lysis.

The parameters for duty cycle and pulse repetition frequency in the 2-MHz TCD apparatus used in this study are set by the device manufacturers for optimal intracranial blood flow detection, and are not optimized for thrombolysis [21, 22, 27, 39, 59-61]. However, at the settings which produced the maximum output of 0.47 MPa peak rarefactional pressure, 2-MHz ultrasound enhanced thrombolysis when used with rt-PA. Thus, optimization of TCD parameters could further enhance thrombolysis at 2 MHz with rt-PA. Despite these limitations, this study shows the effects of combination rt-PA therapy on the physical size of human clots.

CONCLUSION

In conclusion, this study demonstrates that combination treatment with rt-PA is more effective than rt-PA alone in human whole blood clots. Although the clots respond to rt-PA alone at several concentrations, greater thrombolysis is achieved when rt-PA and plasminogen or ultrasound are used concurrently. The effect is dependent both on the type of combination therapy and the concentration of rt-PA. At clinically relevant dosages of rt-PA, the combination therapies tested here are almost equal in efficacy. Furthermore, the use of combination therapy would allow for a reduction in rt-PA concentration while maintaining, and in some cases improving, the efficacy of the treatment. Ultimately, this could reduce the risk for ICHs associated with rt-PA activity. As such, combination therapy using rt-PA shows potential to improve the clinical treatment of ischemic stroke, myocardial infarction, peripheral arterial disease, deep vein thrombus, and other thrombotic disorders.

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ABBREVIATIONS

CCD	Charge-coupled device
CW_{NORM}	Corrected normalized clot width
FCL	Fractional clot loss
hFFP	Human fresh-frozen plasma
ICH	Intracerebral hemorrhage
Plg	Plasminogen
rt-PA	Recombinant tissue plasminogen activator
TCD	Transcranial Doppler
US	Ultrasound
UET	Ultrasound enhanced thrombolysis

REFERENCES

- [1]. Adams HP Jr, Brodt TG, Furlan AJ, et al. Guidelines for Thrombolytic Therapy for Acute Stroke: a Supplement to the Guidelines for the Management of Patients with Acute Ischemic Stroke. A statement for healthcare professionals from a Special Writing Group of the Stroke Council, American Heart Association. *Stroke*. 1996; 27:1711–8. [PubMed: 8784157]
- [2]. Dunn CJ, Goa KL. Tenecteplase: a review of its pharmacology and therapeutic efficacy in patients with acute myocardial infarction. *Am J Cardiovasc Drugs*. 2001; 1:51–66. [PubMed: 14728052]
- [3]. Alesh I, Kayali F, Stein PD. Catheter-directed thrombolysis (intrathrombus injection) in treatment of deep venous thrombosis: A systematic review. *Catheter Cardiovasc Interv*. 2007; 70:143–8. [PubMed: 17377992]
- [4]. Patel S, Mody A. Cerebral hemorrhagic complications of thrombolytic therapy. *Prog Cardiovasc Dis*. 1999; 42:217–33. [PubMed: 10598922]
- [5]. Onundarsen PT, Francis CW, Marder VJ. Depletion of plasminogen in vitro or during thrombolytic therapy limits fibrinolytic potential. *J Lab Clin Med*. 1992; 120:120–8. [PubMed: 1613318]
- [6]. Robbie LA, Bennett B, Croll AM, Brown PA, Booth NA. Proteins of the fibrinolytic system in human thrombi. *Thromb Haemost*. 1996; 75:127–33. [PubMed: 8713791]
- [7]. Potter Van Loon BJ, Rijken DC, Brommer EJP, Van Der Maas APC. The amount of plasminogen, Tissue-Type plasminogen activator and plasminogen activator inhibitor type 1 in human thrombi and the relation to Ex-Vivo lysis. *Thromb Haemost*. 1992; 67:101–5.
- [8]. Novokhatny VV, Jesmok GJ, Landskroner KA, Marder VJ, Zimmerman TP. Locally delivered plasmin: why should it be superior to plasminogen activators for direct thrombolysis? *Trends Pharmacol Sci*. 2004; 25:72–5. [PubMed: 15102492]
- [9]. Novokhatny V, Taylor K, Zimmerman TP. Thrombolytic potency of acid-stabilized plasmin: superiority over tissue-type plasminogen activator in an in vitro model of catheter-assisted thrombolysis. *Thromb Haemost*. 2003; 1:1034–41.

- [10]. Comerota AJ. Development of catheter-directed intrathrombus thrombolysis with plasmin for the treatment of acute lower extremity arterial occlusion. *Thromb Res.* 2008; 122:S20–S6. [PubMed: 18701142]
- [11]. Shlansky-Goldberg RD, Matsumoto AH, Baumbach GA, et al. A first-in-human phase I trial of locally delivered human plasmin for hemodialysis graft occlusion. *Thromb Haemost.* 2008; 6:944–50.
- [12]. Shlansky-Goldberg R. Phase 1 study of human plasma-derived plasmin (TAL-05-00018) in hemodialysis graft occlusion. *Thromb Res.* 2008; 122
- [13]. Stewart D, Kong M, Novokhatny V, Jesmok G, Marder VJ. Distinct dose-dependent effects of plasmin and tPA on coagulation and hemorrhage. *Blood.* 2003; 101:3002–7. [PubMed: 12446443]
- [14]. Marder VJ, Landskroner K, Novokhatny V, et al. Plasmin induces local thrombolysis without causing hemorrhage: a comparison with tissue plasminogen activator in the rabbit. *Thromb Haemost.* 2001; 86:739–45. [PubMed: 11583303]
- [15]. Bookstein JJ. Plasminogen-enriched pulse-spray thrombolysis with tPA: Further developments. *J Vasc Interv Radiol.* 2000; 11:1353–62. [PubMed: 11099249]
- [16]. Meunier JM, Holland CK, Lindsell CJ, Shaw GJ. Duty Cycle Dependence of Ultrasound Enhanced Thrombolysis in a Human Clot Model. *Ultrasound Med Biol.* 2007; 33:576–83. [PubMed: 17337113]
- [17]. Shaw GJ, Meunier JM, Huang SL, Lindsell CJ, McPherson DD, Holland CK. Ultrasound-enhanced thrombolysis with tPA-loaded echogenic liposomes. *Thromb Res.* 2009; 124:306–10. [PubMed: 19217651]
- [18]. Meunier JM, Holland CK, Pancioli AM, Lindsell CJ, Shaw GJ. Low Frequency Ultrasound Enhances Combined rt-PA and Eptifibatide Thrombolysis in Human Clots. *Thromb Res.* 2009; 123:528–36. [PubMed: 18619651]
- [19]. Shaw GJ, Meunier JM, Lindsell CJ, Holland CK. Tissue plasminogen activator concentration dependence of 120 kHz ultrasound-enhanced thrombolysis. *Ultrasound Med Biol.* 2008; 34:1783–92. [PubMed: 18468773]
- [20]. Shaw GJ, Porter TM, Meunier JM, Woo D, Holland CK. Low-intensity 120-kHz pulsed ultrasound “busts more clot” than 2 MHz transcranial Doppler in the presence of rt-PA. *Stroke.* 2007; 38:509.
- [21]. Alexandrov AV, Molina CA, Grotta JC, et al. Ultrasound-enhanced systemic thrombolysis for acute ischemic stroke. *N Engl J Med.* 2004; 351:2170–8. [PubMed: 15548777]
- [22]. Alexandrov AV, Wojner AW, Grotta JC. CLOTBUST: Design of a Randomized Trial of Ultrasound-Enhanced Thrombolysis for Acute Ischemic Stroke. *J Neuroimaging.* 2004; 14:108–12. [PubMed: 15095554]
- [23]. Dhond MR, Nguyen TT, Dolan C, Pulido G, Bommer WJ. Ultrasound-enhanced thrombolysis at 20 kHz with air-filled and perfluorocarbon-filled contrast bispheres. *J Am Soc Echocardiogr.* 2000; 13:1025–9. [PubMed: 11093105]
- [24]. Cintas P, Nguyen F, Boneu B, Larrue V. Enhancement of enzymatic fibrinolysis with 2-MHz ultrasound and microbubbles. *Thromb Haemost.* 2004; 2:1163–6.
- [25]. Coussios CC, Holland CK, Shaw GJ. Transmission of a large unfocused 120 kHz and 1 MHz ultrasound beam through the human skull. *J Acoust Soc Am.* 2002; 112:2433.
- [26]. Akiyama M, Ishibashi T, Yamada T, Furuhashi H. Low-frequency ultrasound penetrates the cranium and enhances thrombolysis in vitro. *Neurosurgery.* 1998; 43:828–32. [PubMed: 9766310]
- [27]. Daffertshofer M, Gass A, Ringleb P, et al. Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: Increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator - Results of a phase II clinical trial. *Stroke.* 2005; 36:1441–6. [PubMed: 15947262]
- [28]. Baron C, Aubry JF, Tanter M, Meairs S, Fink M. Simulation of Intracranial Acoustic Fields in Clinical Trials of Sonothrombolysis. *Ultrasound Med Biol.* 2009; 35:1148–58. [PubMed: 19394756]

- [29]. Jaffe GJ, Green GD, Abrams GW. Stability of recombinant tissue plasminogen activator. *Am J Ophthalmol.* 1989; 108:90–1. [PubMed: 2502019]
- [30]. Shaw GJ, Sperling M, Meunier JM. Long-term stability of recombinant tissue plasminogen activator at –80 C. *BMC Res Notes.* 2009; 2:117. [PubMed: 19566947]
- [31]. Winter PM, Shukla HP, Caruthers SD, et al. Molecular imaging of human thrombus with computed tomography. *Acad Radiol.* 2005; 12(Suppl 1):S9–13. [PubMed: 16106538]
- [32]. Yu X, Song SK, Chen J, et al. High-resolution MRI characterization of human thrombus using a novel fibrin-targeted paramagnetic nanoparticle contrast agent. *Magn Reson Med.* 2000; 44:867–72. [PubMed: 11108623]
- [33]. Francis CW, Totterman S. Magnetic resonance imaging of deep vein thrombi correlates with response to thrombolytic therapy. *Thromb Haemost.* 1995; 73:386–91. [PubMed: 7667820]
- [34]. Loren M, Garcia Frade LJ, Torrado MC, Navarro JL. Thrombus age and tissue plasminogen activator mediated thrombolysis in rats. *Thromb Res.* 1989; 56:67–75. [PubMed: 2512687]
- [35]. Shaw GJ, Bavani N, Dhamija A, Lindsell CJ. Effect of mild hypothermia on the thrombolytic efficacy of 120 kHz ultrasound enhanced thrombolysis in an *in vitro* human clot model. *Thromb Res.* 2006; 117:603–8. [PubMed: 15951005]
- [36]. Marinkovic SV, Miliavljjevic MM, Kovacevic MS, Stevia ZD. Perforating branches of the middle cerebral artery: Microanatomy and clinical significance of their intracerebral segments. *Stroke.* 1958; 15:1022–9.
- [37]. Tao X, Yu XJ, Bhattarai B, et al. Microsurgical anatomy of the anterior communicating artery complex in adult Chinese heads. *Surg Neurol.* 2006; 65:151–61.
- [38]. Shaw GJ, Meunier JM, Lindsell CJ, Pancioli AM, Holland CK. Making the right choice: Optimizing rt-PA and eptifibatide lysis, an *in vitro* study. *Thromb Res.* 2010; 126
- [39]. Suchkova V, Carstensen EL, Francis CW. Ultrasound enhancement of fibrinolysis at frequencies of 27 to 100 kHz. *Ultrasound Med Biol.* 2002; 28:377–82. [PubMed: 11978418]
- [40]. Rosenschein U, Furman V, Kerner E, Fabian I, Bernheim J, Eshel Y. Ultrasound imaging-guided noninvasive ultrasound thrombolysis: preclinical results. *Circulation.* 2000; 102:238–45. [PubMed: 10889137]
- [41]. Schäfer S, Kliner S, Klinghammer L, et al. Influence of ultrasound operating parameters on ultrasound-induced thrombolysis *in vitro*. *Ultrasound Med Biol.* 2005;31. [PubMed: 15653228]
- [42]. Cheng JY, Shaw GJ, Holland CK. *In vitro* microscopic imaging of enhanced thrombolysis with 120-kHz ultrasound in a human clot model. *Acoust Res Lett Online.* 2005; 6:25–9. [PubMed: 18080003]
- [43]. Tanswell P, Seifried E, Stang E, Krause J. Pharmacokinetics and hepatic catabolism of tissue-type plasminogen activator. *Arzneimittelforschung.* 1991; 41:1310–9. [PubMed: 1815534]
- [44]. Seifried E, Tanswell P, Ellbruck D, Haerer W, Schmidt A. Pharmacokinetics and haemostatic status during consecutive infusions of recombinant tissue-type plasminogen activator in patients with acute myocardial infarction. *Thromb Haemost.* 1989; 61:497–501. [PubMed: 2508258]
- [45]. Collet JP, Montalescot G, Lesty C, Weisel JW. A structural and dynamic investigation of the facilitating effect of glycoprotein IIb/IIIa inhibitors in dissolving platelet-rich clots. *Circ Res.* 2002; 90:428–34. [PubMed: 11884372]
- [46]. Datta S, Coussios CC, McAdory LE, et al. Correlation of cavitation with ultrasound enhancement of thrombolysis. *Ultrasound Med Biol.* 2006; 32:1257–67. [PubMed: 16875959]
- [47]. Datta, S. PhD dissertation. The University of Cincinnati; Cincinnati (OH): 2007. The role of cavitation in enhancement of rt-PA thrombolysis.
- [48]. Eggers J, Koch B, Meyer K, König I, Seidel G. Effect of ultrasound on thrombolysis of middle cerebral artery occlusion. *Ann Neurol.* 2003; 53:797–800. [PubMed: 12783427]
- [49]. Alexandrov AV, Burgin WS, Demchuk AM, El-Mitwalli A, Grotta JC. Speed of intracranial clot lysis with intravenous tissue plasminogen activator therapy: sonographic classification and short-term improvement. *Circulation.* 2001; 103:2897–902. [PubMed: 11413077]
- [50]. Molina CA, Alexandrov AV, Demchuk AM, et al. Improving the predictive accuracy of recanalization on stroke outcome in patients treated with tissue plasminogen activator. *Stroke.* 2004; 35:151–6. [PubMed: 14671245]

- [51]. Christou I, Alexandrov AV, Burgin WS, et al. Timing of recanalization after tissue plasminogen activator therapy determined by transcranial Doppler correlates with clinical recovery from ischemic stroke. *Stroke*. 2000; 31:1812–6. [PubMed: 10926939]
- [52]. Alexandrov AV, Grotta JC. Arterial reocclusion in stroke patients treated with intravenous tissue plasminogen activator. *Neurology*. 2002; 59:862–7. [PubMed: 12297567]
- [53]. Labiche LA, Al-Senani F, Wojner AW, Grotta JC, Malkoff M, Alexandrov AV. Is the Benefit of Early Recanalization Sustained at 3 Months? A Prospective Cohort Study. *Stroke*. 2003; 34:695–8. [PubMed: 12624293]
- [54]. Torr SR, Nachowiak DA, Fujii S, Sobel BE. ‘Plasminogen steal’ and clot lysis. *J Am Coll Cardiol*. 1992; 19:1085–90. [PubMed: 1532403]
- [55]. Shaw GJ, Meunier JM, Holland CK. Exogenous plasminogen increases tPA lytic efficacy in *in vitro* human clot. *Acad Emerg Med*. 2009; 16:S120.
- [56]. Ammi AY, Mast TD, Huang I-H, et al. Characterization of the ultrasound propagation through ex-vivo human temporal bone. *Ultrasound Med Biol*. 2008; 34:1578–89. [PubMed: 18456391]
- [57]. Hynynen K, McDannold N, Sheikov NA, Jolesz FA, Vykhodtseva N. Local and reversible blood-brain barrier disruption by noninvasive focused ultrasound at frequencies suitable for trans-skull sonications. *Neuroimaging*. 2005; 24:12–20.
- [58]. Banbula A, Zimmerman TP, Novokhatny VV. Blood inhibitory capacity toward exogenous plasmin. *Blood Coagul Fibrinolysis*. 2007; 18:241–6. [PubMed: 17413760]
- [59]. Frenkel V, Oberoi J, Stone MJ, et al. Pulsed high-intensity focused ultrasound enhances thrombolysis in an in vitro model. *Radiology*. 2006; 239:86–93. [PubMed: 16493016]
- [60]. Shaw GJ, Vaidya SS, Wagner KR, Holland CK. 1 MHz ultrasound assists clot lysis. *J Acoust Soc Am*. 2002; 21:S10.
- [61]. Schneider F, Gerriets T, Walberer M, et al. Brain edema and intracerebral necrosis caused by transcranial low-frequency 20-kHz ultrasound: A safety study in rats. *Stroke*. 2006; 37:1301–6. [PubMed: 16556877]

Fig. (1). Schematic diagram of experimental apparatus

The human whole blood clot is placed in U-shaped hollow tubing, which is placed within an acrylic tank filled with water maintained at 37°C. The tank is placed over an inverted microscopic objective, allowing visualization of the full width of the sample clot. For trials involving ultrasound, a transducer is placed at one end of the tank, and sound absorbing material at the opposite end.

Fig. (2). Clot width as a function of time for clots exposed to rt-PA, plasminogen, and US
 Normalized average clot width is shown during the 30 min treatment period for **rt-PA** ([rt-PA] = 0.00 µg/ml $\blacktriangle$, and 3.15 µg/ml $\circ$), and **Plg** ([rt-PA] (µg/ml) = 0.00, $\ast$; 3.15, ∇) trials. Also shown are normalized average clot width for **120-kHz** ($\square$) and **2-MHz** ($\triangle$) groups ([rt-PA] = 3.15 µg/ml). Vertical bars show representative standard deviations for the data. The clot widths for the **120-kHz**, **Plg** (3.15 µg/ml), and **2-MHz** groups decrease to a greater degree than those of the **rt-PA** or **Plg** (0 µg/ml) groups.

Fig. (3). The fractional clot loss (FCL) for each treatment group

The decrease in the normalized average clot width following 30 minutes of treatment, calculated using Equation (1), is given as means, in units of % of normalized clot width. Vertical bars show standard error of the sample means. Lysis increases dramatically in each combination group as rt-PA is added.

Table 1

Results for each Trial Group. Mean FCL (%) with standard deviation, 95% Confidence Intervals, Number of donors (NDonors), and Number of clots (NClots) for the combination rt-PA clot lysis measurements. Using a linear mixed model to account for the fact that multiple clots came from the same subjects, the statistical markers compare FCLs of the three combination treatments (120-kHz, Plg, 2-MHz) to the control treatment (rt-PA) within each level of rt-PA

Group	rt-PA Concentration (µg/ml)			
	0.00	0.50	1.00	3.15
rt-PA	(FCL) 14% (11) 95% CI [13-15%] (N _{Donors} /N _{Clots}) 21 / 158	44% (36) [37-52%] 6 / 14	30% (23) [27-33%] 8 / 42	38% (28) [36-40%] 24 / 136
120-kHz	16% (15) [13-19%] 4 / 16	50% (30) [42-59%] 3 / 8	58% (30) [51-65%] 3 / 11	70% (29) [§] [64-76%] 5 / 15
Plg	17% (11) [15-20%] 3 / 16	65% (27) [*] [59-71%] 3 / 13	58% (28) [§] [53-64%] 3 / 16	83% (16) [§] [80-86%] 4 / 18
2-MHz	12% (12) [8-15%] 3 / 8	58% (36) [50-65%] 4 / 14	53% (26) [†] [47-60%] 3 / 12	52% (26) [*] [47-58%] 5 / 13

^{*} $p < 0.05$ vs. rt-PA at the same concentration

[†] $p < 0.01$ vs. rt-PA at the same concentration

[§] $p < 0.001$ vs. rt-PA at the same concentration