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Sonoreperfusion Therapy Kinetics in Whole Blood using Ultrasound, Microbubbles and tPA

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Abstract

Coronary intervention for myocardial infarction often results in microvascular embolization of thrombus. Sonoreperfusion therapy (SRP) using ultrasound and microbubbles restored perfusion in our *in vitro* flow model of microvascular obstruction. In this study, we assessed SRP efficacy using whole blood as the perfusate with and without tissue plasminogen activator (tPA). In a phantom vessel bearing a 40- μ m pore mesh to simulate the microvasculature, microthrombi were injected to cause microvascular obstruction and were treated using SRP. Without tPA, the lytic rate increased from 2.6 ± 1.5 mmHg/min with 1000 cycles to 7.3 ± 3.2 mmHg/min with 5000 cycles ultrasound pulses ($p < 0.01$). The lytic index was similar between tPA-only [$(2.0 \pm 0.5) \times 10^{-3}$ mmHg⁻¹min⁻¹] and 5000 cycles without tPA [$(2.3 \pm 0.5) \times 10^{-3}$ mmHg⁻¹min⁻¹] ($p = 0.5$) but increased [$(3.6 \pm 0.8) \times 10^{-3}$ mmHg⁻¹min⁻¹] with tPA in conjunction with 5000 cycles ultrasound ($p < 0.01$). In conclusion, SRP restored microvascular perfusion in whole blood and SRP lytic rate in experiments without tPA increased with ultrasound pulse length and efficacy increased with the addition of tPA.

Keywords

Sonothrombolysis; Microcirculation; Cardiac diseases; Ultrasound contrast agents; Microbubbles; Thromboembolic events

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Introduction

ST elevation myocardial infarction (STEMI) is caused by the acute thrombotic occlusion of an epicardial coronary artery. Contemporary treatment for restoration of epicardial coronary artery patency is primary percutaneous coronary intervention (PCI). However, despite restoration of epicardial coronary artery patency with PCI, adequate microvascular perfusion is often not restored, a phenomenon known as microvascular obstruction (MVO) or no-reflow. This occurs in up to 55% of patients following PCI and portends poor clinical outcome (Kloner et al. 1974; van Kranenburg et al. 2014; Wu et al. 1998). MVO, largely caused by obstruction of the microcirculation with atherothrombotic debris, results in local inflammation, platelet aggregation, myocardial edema due to dysfunctional endothelium, and formation of *in situ* microvascular thrombi (Ito 2014; Robbers et al. 2013). While many preventative and curative strategies have been employed (Bernink et al. 2014; Fröhlich et al. 2013; Galasso et al. 2014), there has been no consistently efficacious therapeutic approach.

Tissue plasminogen activator (tPA) has long been used as part of the treatment regimen in ischemic stroke, ischemic coronary events and peripheral arterial occlusions (Papadopoulos et al. 1987) but while very potent, is prone to cause hemorrhage when administered systemically (Giugliano et al. 2001). Recently, it was found that half-dose tPA was not associated with increased hemorrhage in patients undergoing treatment for submassive pulmonary embolism (Sharifi et al. 2013). This supports the use of lower dose tPA for thrombolysis as a safer alternative. It has also been shown that the therapeutic efficacy of tPA can be enhanced by combining tPA with therapeutic ultrasound (US) and microbubbles (MB).

MB are micron sized (1–5 μm) gaseous spheres encapsulated in a stabilizing shell made of phospholipid, polymer or protein (Stride 2009). MB subjected to an ultrasound (US) pulse expand and compress and can lead to non-linear (stable cavitation) oscillations at moderate pressure levels (Frinking et al. 2000). Increasing the US pressure causes the microbubble to oscillate more violently, known as inertial cavitation. These processes involving stable and inertial cavitation causes microstreaming, fluid jets and a focal temperature increase resulting in bioeffects (Price et al. 1998). Strategies utilizing these potent sources of energy to effectively disrupt thrombi have been mainly focused on recanalizing large vessels in patients with large thrombi in ischemic stroke and STEMI patients (Roos et al. 2014) and is known as sonothrombolysis. Therapy utilizing this lytic effect to restore microvascular perfusion during MVO is called sonoreperfusion (SRP) therapy (Pacella et al. 2015).

We and others have recently shown that SRP using MB and long tone burst US could restore microvascular perfusion in a rodent model of MVO (Pacella et al. 2015; Porter et al. 2001; Porter et al. 2016), but the optimal US therapeutic conditions for microvascular SRP remain largely unknown. Our *in vitro* platform offers an opportunity to investigate the mechanisms leading to efficient sonothrombolysis and the kinetics of SRP therapy using US+MB and tPA in whole blood. Whole blood, in contrast to PBS, has higher viscosity and contains endogenous tPA and other blood components, such as RBCs, WBCs, and platelets, all of which could affect MB oscillations and hence SRP efficacy. Accordingly, we examined the hypothesis that US mediated MB cavitation, as quantified by stable and inertial cavitation

doses, could cause microvascular clot lysis in our *in vitro* MVO system in whole blood. We explored whether endogenous tPA in whole blood is sufficient to induce SRP with MB and US in our *in vitro* model. Finally, we compared the kinetics of MB and US SRP with tPA and a combination therapy of tPA, MB and US.

Methods

In vitro system of SRP

We used our previously described *in vitro* model of MVO (Leeman et al. 2012), with a minor modification regarding flow speed (1.5 ml/min previously) to allow for whole blood perfusion. Briefly, the model comprised a phantom vessel containing an intraluminal mesh with 40 μm pores to simulate a cross section of the microcirculation (Figure 1). The system, maintained at 37°C, was perfused with whole bovine blood at a lower constant flow rate of 0.75 ml/min which is an approximation of the physiological flow in small arterioles (Bedgood and Metha 2012; Marieb 2015). Bovine microthrombi were added to increase upstream pressure to 30 mmHg (range 25–35 mmHg) to mimic myocardial MVO. Upstream pressure was monitored as a surrogate marker of thrombus burden. Passive cavitation detection (PCD) was used to quantify MB activity. Depending on the experimental group, MB (2×10^6 MB/ml) were added to the blood perfusate. US (1 MHz) was delivered with a variable pulse length (1000, 3000 or 5000 cycles) and a peak negative pressure of 1.5 MPa. The US pulses were applied for 20 minutes at a repetition rate of 0.33 Hz to allow MB replenishment to the treatment area between pulses. Experiments without MB or US were performed as control conditions. A new mesh was mounted and exposed to microthrombi as described above for each experiment.

Perfusate

Heparin (1 IU/ml) and acetylsalicylic acid (0.06 mg/ml) were added to fresh citrated bovine blood (LAMPIRE Biological Laboratories, Pipersville, PA, USA) (< 96 h of venipuncture), to simulate the clinical presentation of acute coronary syndrome, during which patients are given heparin (5000 IE) and ASA (300 mg). tPA at 2.5 $\mu\text{g/ml}$, consistent with the steady state plasma concentration in humans during the first 30 min of tPA infusion phase (Tanswell et al. 1992), was added based on the experimental grouping.

Microbubbles

MB were fabricated by sonicating a mixture of 1,2-distearoyl-sn-glycero-3-phosphocholine (Avanti polar lipids, Alabaster, AL), polyoxyethylene (40) stearate (Sigma-Aldrich, St Louis, MO) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (Avanti polar lipids, Alabaster, AL) in a 2:1:1 weight ratio in the presence of perfluorobutane gas (FluoroMed, Round Rock, TX). After sonication using a 20 kHz probe (Heat Systems Ultrasonics, Newtown, CT), the MB were washed and resuspended in saline saturated with perfluorobutane and stored at 4°C until use. This procedure produced MB with a mean diameter of 3 ± 1 μm and a concentration of $1\text{--}2 \times 10^9$ MB/ml, as measured by Multisizer-3 Coulter counter (Beckman Coulter, Brea, CA) (Leeman et al. 2012).

Microthrombi

Thrombi were created by adding CaCl_2 (25 mM) to citrated bovine whole blood and incubating at room temperature for 3 h in type 1 borosilicate glass vials. The vial was then shaken for 20 s in a vial mixer (Vialmix, Bristol-Myers Squibb Medical Imaging, New York, NY). The thrombi were filtered through 200 μm mesh pores to produce $\text{MT} < 200 \mu\text{m}$ (Leeman et al. 2012). The bovine blood was chosen for this study as it has been determined previously that bovine clots treated with plasmin most closely resemble the lysis observed with human clots (Landskroner et al. 2005).

Pressure monitoring

A fluid filled pressure transducer (BD DTX plus, Becton Dickinson Co., Franklin Lakes, NJ) was positioned to monitor pressure upstream of the mesh. Baseline upstream pressure during constant flow (0.75 ml/min) and without clot, was calibrated to 0 mmHg.

Ultrasound

US was delivered from a 1 MHz focused single element transducer (A302S-SU-F1.63-PTF, 1 inch/1.67 inch focus, Olympus, Waltham, MA) driven by a pulse generator (33250A, Agilent technologies, Santa Clara CA) and a power amplifier (100A250A, Amplifier Research, Souderton, PA). The US field was calibrated with a 200- μm capsule hydrophone (HGL-0200, Onda Corp, Sunnyvale, CA). The -6 dB beam width was 3.5 mm and therefore covered $>90\%$ of the area of the mesh.

Passive cavitation detection

A focused single element broadband transducer with a center frequency of 3.5 MHz (V383-SU-F1.00IN-PTF, 0.375 inch/1 inch focus, Olympus, Waltham, MA) was confocally aligned with the treatment transducer on the mesh for PCD. The detected radio frequency signal was amplified (5073PR, Olympus, Waltham, MA), band-pass filtered (2–20 MHz cutoffs) and digitized on a digital oscilloscope (WaveRunner 6051A, Lecroy, Chestnut Ridge, NY) at 50 MHz sampling rate for off-line processing. Data corresponding to up to 5000 cycles of treatment were analyzed using joint time frequency analysis, with a window size of 250 μs and a time step of 100 μs (60% overlapping) in MATLAB (The MathWorks Inc., Natick, MA) software. The acoustic energy between 3.2–3.8 MHz, but excluding the band between 3.4–3.6 MHz, integrated over the whole tone-burst, was defined as inertial cavitation dose (ICD). The energy in the peak at the ultraharmonic band (3.48–3.52 MHz) above the broadband signal, integrated over the whole tone-burst, was defined as the stable cavitation dose (SCD) (Chen et al. 2003). The bandwidth chosen for SCD corresponded to the -6 dB bandwidth in the fundamental peak (Black et al. 2016; Datta et al. 2008).

Statistical and computational analysis

Data were plotted as upstream pressure (normalized to pressure at $t=0$) as a function of time. The lytic efficacy was quantified by the final pressure drop, the initial lytic rate (rate of pressure drop in the first 4 minutes) and the lytic index (inverse of the area under the pressure-time curve). The lytic rate indicates the initial rate of sonoreperfusion, while the lytic index indicates the overall integrated decrease of clot burden over time. All parameters

were tested for significance using analysis of variance (ANOVA) with Bonferroni *post hoc* correction or student's t-test when applicable. Statistical analysis was performed using SPSS 22 (IBM, USA) and statistical significance was defined as $p < 0.05$. Results are expressed as mean $\pm$ standard deviation.

Results

SRP in whole blood without additional tPA

Upstream pressure decreased during SRP therapy, indicating decreased thrombus burden, and SRP efficacy varied by the specific US regime with and without MB (Figure 2). With US alone (1.5 MPa, 5000 cycles, no MB) pressure did not decrease after 20 min of treatment ($n=3$, $P=3.9 \pm 16.9\%$, $p=0.576$). However, sonoreperfusion was achieved when MB were added. Using 1000 cycles US at 1.5 MPa for 20 minutes with MB, there was a significant reduction in upstream pressure ($n=7$, $P=33.7 \pm 21.7\%$, $p=0.005$). Increasing the pulse length to 3000 cycles yielded a further increase in SRP efficacy ($n=5$, $P=57.7 \pm 13.6\%$, $p < 0.001$). A 5000 cycle pulse at 1.5 MPa also produced a significant reduction in pressure ($n=5$,

$P=59.2 \pm 13.8\%$, $p=0.013$), but this was not significantly greater than with 3000 cycles ($p=0.906$). In the presence of MB, the lytic rate increased with cycle length: the lytic rate increased from 2.6 ± 1.5 mmHg/min at 1000 cycles to 7.3 ± 3.2 mmHg/min at 5000 cycles ($p < 0.01$). Removing MB from the treatment protocol resulted in a very low lytic rate (0.5 ± 0.1 mmHg/min), which was significantly lower than that for 5000 cycle US+MB therapy ($p < 0.01$).

SRP in whole blood supplemented with tPA

Compared to previous experiments using PBS perfusate (Leeman et al, 2012), upstream pressure did not fully return to baseline during SRP therapy in whole blood perfusate without tPA. However, a further pressure reduction was achieved with the addition of tPA. When tPA was administered during 1.5 MPa and 5000 cycles, a marked reduction in upstream pressure was observed ($n=5$, $P=87.6 \pm 8.2\%$, $p < 0.001$). Using tPA alone (no MB, no US), upstream pressure decrease compared to when tPA and US+MB therapy were applied together was similar ($p=0.655$). The pressure versus time curve for tPA alone was notable for the absence of an initial rapid descent (decreased lytic rate) seen in the US+MB curves (Figure 3). With tPA alone, the lytic rate (1.2 ± 1.3 mmHg/min) was lower than 5000 cycles US+MB ($p < 0.01$). With 5000 cycles US+MB therapy, the lytic index was significantly higher with tPA [$(3.5 \pm 0.8) \times 10^{-3}$ mmHg $^{-1}$.min $^{-1}$] than without tPA [$(2.3 \pm 0.6) \times 10^{-3}$ mmHg $^{-1}$.min $^{-1}$] ($p < 0.01$) (Table 1). The lytic rate for US+MB 5000 cycles and US+MB+tPA 5000 cycles was not statistically different ($p=NS$). In the presence of tPA, the lytic index was significantly higher with 5000 cycles MB+US [$(3.5 \pm 0.8) \times 10^{-3}$ mmHg $^{-1}$.min $^{-1}$] compared to tPA only [$(2.0 \pm 0.5) \times 10^{-3}$ mmHg $^{-1}$.min $^{-1}$] ($p < 0.05$). Overall, the most effective SRP regime (greatest lytic index and lytic rate) consisted of a combination of tPA and US+MB.

Passive cavitation detection

Typical time-frequency analysis and corresponding ICD and SCD calculations are reported in Figure 4. For 1000 and 3000 cycles (Fig. 4a and 4b), cavitation activity was detected

throughout the duration of the pulse and cumulative ICD and SCD power plateaued at around 1 ms and 2.5 ms. For the 5000 cycles pulse, cavitation activity persisted up to 5000 cycles as observed on the spectrogram (Fig. 4c). The corresponding cumulative ICD plateaued at 3.5 ms but the cumulative SCD continued to increase beyond 3 ms. The corresponding ultraharmonic peaks after 4 ms are clearly visible on the spectrogram. There was no detectable cavitation activity nor ICD or SCD without MB (Fig. 4d). The aggregate results over repeated experiments are summarized in Figure 5. In the presence of MB, inertial cavitation dose increased with US pulse length up to 3000 cycles (Figure 5a). ICD was significantly higher for 3000 and 5000 cycle (respectively $14.5 \pm 2.0 \text{ mV}^2.\text{ms}$ and $16.8 \pm 2.6 \text{ mV}^2.\text{ms}$) compared with the 1000 cycle experiments ($3.5 \pm 0.7 \text{ mV}^2.\text{ms}$, $p < 0.001$). ICD did not differ significantly between 3000 and 5000 acoustic cycles regimen. In addition, upstream pressure drop, lytic rate and lytic indices were positively correlated with ICD ($r^2 > 0.92$, $p < 0.05$). The stable cavitation dose also increased with pulse length and reached its highest value with 5000 cycles ($0.5 \pm 0.2 \text{ mV}^2.\text{ms}$), which was significantly higher than for that for 3000 cycles ($0.3 \pm 0.1 \text{ mV}^2.\text{ms}$, $p < 0.05$) (Figure 5b). SCD also vanished in the absence of MB. Lytic rate, but not lytic index or pressure drop, correlated with SCD ($r^2 = 0.99$, $p < 0.05$).

Discussion

There have been numerous *in vitro* studies using petri dishes, beakers, open or closed loop systems filled with PBS or plasma addressing sonothrombolysis of large clots, generally showing a synergistic effect of US+MB with tPA on clot dissolution (Bader et al. 2015; Datta et al. 2008; Hitchcock et al. 2011; Holscher et al. 2009; Mizushige et al. 1999; Nishioka et al. 1997; Petit et al. 2012; Petit et al. 2015; Porter et al. 2001; Prokop et al. 2007; Sharifi et al. 2013; Sutton et al. 2013). Our results indicate that SRP of MVO could be achieved *in vitro* using whole bovine blood perfusate. In this study, SRP efficacy increased with US pulse length up to 3000 cycles in the presence of MB (Figure 2), similarly to our previous findings using the non-cellular perfusate PBS (Leeman et al. 2012). In addition, SRP efficacy correlated with ICD, which also increased with pulse length up to 3000 cycles and then reached a plateau (Figure 5a). Interestingly, stable cavitation dose continued to significantly increase beyond 3000 cycles (Figure 5b), but this did not translate into an increase in SRP efficacy. It is not clear what caused SCD to persist while ICD decreased beyond 3000 cycles. The acoustic activity of daughter bubbles and clusters could be in play (Chen et al. 2003; Chen et al. 2016; Tu et al. 2006). It is unlikely that misalignment between the transmitting and receiving transducers could explain these results as it would affect both ICD and SCD similarly. It is important to point out that the signal level of the SCD was much weaker than that of the ICD, suggesting that the inertial cavitation activity was dominant under high pressure insonation, as would be expected. Our findings therefore suggest that MB inertial cavitation was directly related to the disruption of the microthrombi in our microvascular model with whole blood and without the addition of tPA.

It has been demonstrated previously that without tPA, the macroscopically observed clot size reduction was the result of RBC hemolysis, as the fibrin content of their clots only decreased in the presence of tPA (Petit et al. 2012). From this perspective, our results suggest that US +MB disruption of RBC in microthrombi could be sufficient to restore microvascular

perfusion, potentially by reducing the size of the microthrombi to less than 40 μm , thus allowing their dislodgement and passage through the mesh. We have shown in a separate study that SRP using long tone burst ultrasound could restore perfusion in a hindlimb model of MVO (Pacella et al. 2015), supporting SRP *in vivo* efficacy, but the mechanisms of action remain to be elucidated, as other factors such as vasodilation following SRP could also be playing a role.

In humans, the capillary bed has a flow speed of 0.3 mm/s and mean capillary pressure ranging from 19 to 30 mm Hg (Marieb 2015; Starling 1896; Williams et al. 1988). We created a unique *in vitro* microvascular model that operates with similar parameters, mimicking a situation with clinical MVO. In a previous study, microthrombi were seeded onto a 40- μm pore mesh in this constant flow system, resulting in increased upstream pressure (Leeman et al. 2012). In that study the kinetics of pressure drop was measured during US+MB therapy, as a surrogate marker of clot burden reduction during therapy and demonstrated the efficacy of US+MB in achieving SRP within a PBS perfusate. As earlier studies have already shown that endogenous tPA is present in bovine blood (Karges et al. 1994), we were interested in determining whether US+MB could achieve more efficacious SRP in our whole blood system compared to PBS. Our current data indicates that SRP efficacy was reduced in whole blood, as evidenced by an incomplete upstream pressure drop at 20 minutes and lower lytic index and lytic rate, compared with previous experiments conducted in PBS (Leeman et al. 2012). Although we cannot directly compare the two experiments (see limitations below), we surmise that this apparent reduced SRP efficacy is due to the presence of RBCs in the perfusate, which are the major contributors to blood viscosity (Baskurt and Meiselman 2003). This increased viscosity results in damped MB oscillations compared to PBS, as confirmed in a recent study using high speed imaging (Helfield et al. 2016). Reduced SRP efficacy was also found when plasma viscosity was adjusted to mean blood viscosity of 4 cP for venous and arterial type microthrombi in the same model of MVO (Black et al. 2016). Our results also support that the addition of tPA was necessary to achieve SRP efficacy similar to that obtained in PBS perfusate as our control experiments without US, MB and without the addition of tPA suggested that endogenous tPA was insufficient to cause effective SRP. This is consistent with results found by Sutton et al, who demonstrated in an *ex-vivo* artery setup that endogenous endothelial tPA was insufficient to improve sonothrombolysis in the presence of MB and US (Sutton et al. 2013). Overall, the mitigating effect of blood should be taken into consideration when extrapolating *in vitro* data using non-blood perfusates to predict *in vivo* efficacy of a given sonothrombolytic regimen. Additional studies using blood perfusion in this *in vitro* MVO model while manipulating acoustic and microbubble parameters should help define optimal conditions for maximizing microvascular sonothrombolysis.

Thrombolysis kinetics

One major advantage of our *in vitro* model is the possibility to quantify reperfusion kinetics, which allows us to compute parameters such as the lytic rate and the lytic index during treatment. Our results clearly indicate that tPA and US+MB thrombolysis individually operate with different kinetic responses. As seen in Figure 3, US+MB mediated reperfusion is a faster process albeit incomplete in terms of terminal pressure drop, compared to the tPA

treatment. Conversely, tPA alone induced a more complete reperfusion at 20 minutes of treatment but had a slower therapeutic onset. This is reflected quantitatively in the lytic rates and indices reported in Table 1. The combination of tPA and US+MB achieved both a fast onset reperfusion and a complete terminal pressure drop. This observation holds a promising potential for *in vivo* translation of the approach by combining the apparent synergistic tPA and locally targeted MB activity. This synergistic effect may be caused by an enhanced penetration of tPA into the clot, by local MB oscillations creating tunnels in the microthrombi. This allows for a tPA to have an effect locally, circumventing the need for a systemic lytic state with a regular dose of tPA.

In areas where PCI is the gold standard in STEMI treatment, concomitant pharmacotherapy includes anticoagulant/antiplatelet agents such as bivalirudin and glycoprotein IIB/IIIA receptor antagonists, respectively, in place of tPA (Mehran et al. 2009). While the results in this study do not reflect a situation in which these drugs are used, combined dual antiplatelet therapy and bivalirudin might prove to be beneficial when combined with sonoreperfusion in the treatment of MVO after STEMI.

Safety is also an important consideration. In STEMI, every second of delayed reperfusion causes more tissue damage in the ischemic region of the myocardium. Pre-treatment using therapeutic ultrasound on top of regular treatment might enhance reperfusion prior to coronary intervention, but could also be cause for side-effects. It has already been shown that an increase in pulse duration might be responsible for coronary vasoconstriction in the clinical application of SRP therapy in human STEMI patients (Roos et al. 2016). While our experiments showed that 5000 cycles was optimal *in vitro*, the effects of ultrasound on living tissue, such as possible hemolysis and hemorrhaging, should not be discarded and future studies will have to consider safety as an important trial outcome.

Limitations

The biggest drawback of our model, like any model for STEMI therapy, is not being able to fully replicate the true process of atherosclerosis, plaque rupture and atherosclerotic thrombus formation. There is a limited number of patho-physiological processes that we can mimic and take into account. Blood was anticoagulated and therefore not all factors of the intrinsic and extrinsic coagulation cascade are present in this *in vitro* model. Also, our *in vitro* vascular model does not account for biological effects of SRP on the vascular tone as endothelial and smooth muscle cells are not present. In order to accommodate experiments in whole blood, we had to modify our previously used protocol (Leeman et al. 2012) to correct for viscosity differences between whole blood and PBS. These changes included a reduction in flow rate from 1.5 ml/min to 0.75 ml/min and a decrease in initial upstream pressure from 40 to 30 mmHg, which make direct comparisons between SRP studies in PBS and whole blood imperfect.

Whole bovine blood instead of human blood was used in our experiments as it does not aggregate (Baumler et al. 2001). This reduced the complexity of our setup, but might also create a bias in results as the formation of RBC aggregates in human blood might further decrease the efficacy of SRP therapy

Conclusion

Sonoreperfusion therapy was achieved in our *in vitro* model of MVO in the presence of whole blood. SRP efficacy without exogenous tPA increased with US pulse length but plateaued at 3000 cycles, consistent with the inertial cavitation dose measurements. tPA in combination with US+MB showed potential for synergistic therapeutic effects, as US+MB favored a rapid therapeutic onset while the addition of tPA was necessary to achieve optimal therapeutic efficacy. Future preclinical studies are needed to validate and build upon these results.

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References

- Bader KB, Gruber MJ, Holland CK. Shaken and stirred: mechanisms of ultrasound-enhanced thrombolysis. *Ultrasound Med Biol*. 2015; 41:187–96. [PubMed: 25438846]
- Baskurt OK, Meiselman HJ. Blood rheology and hemodynamics. *SeminThrombHemost*. 2003; 29:435–450.
- Baumler H, Neu B, Mitlohner R, Georgieva R, Meiselman HJ, Kiesewetter H. Electrophoretic and aggregation behavior of bovine, horse and human red blood cells in plasma and in polymer solutions. *Biorheology*. 2001; 38:39–51. [PubMed: 11381164]
- Bedgood P, Metha A. Direct visualization and characterization of erythrocyte flow in human retinal capillaries. *Biomed Opt Express*. 2012; 3:3264–77. [PubMed: 23243576]
- Bernink FJP, Timmers L, Beek aM, Diamant M, Roos ST, Van Rossum aC, Appelman Y. Progression in attenuating myocardial reperfusion injury: An overview. *Int J Cardiol*. 2014; 170:261–269. [PubMed: 24289874]
- Black JJ, Yu FTH, Schnatz RG, Chen X, Villanueva FS, Pacella JJ. Effect of Thrombus Composition and Viscosity on Sonoreperfusion Efficacy in a Model of Micro-Vascular Obstruction. *Ultrasound Med Biol*. 2016 accepted.
- Chen W-S, Brayman AA, Matula TJ, Crum LA. Inertial cavitation dose and hemolysis produced in vitro with or without Optison. *Ultrasound Med Biol*. 2003; 29:725–37. [PubMed: 12754072]
- Chen X, Wang J, Pacella JJ, Villanueva FS. Dynamic Behavior of Microbubbles during Long Ultrasound Tone-Burst Excitation: Mechanistic Insights into Ultrasound-Microbubble Mediated Therapeutics Using High-Speed Imaging and Cavitation Detection. *Ultrasound Med Biol*. 2016; 42:528–538. [PubMed: 26603628]
- Datta S, Coussios C-C, Ammi AY, Mast TD, de Courten-Myers GM, Holland CK. Ultrasound-enhanced thrombolysis using Definity as a cavitation nucleation agent. *Ultrasound Med Biol*. 2008; 34:1421–33. [PubMed: 18378380]
- Frinking PJ, Bouakaz A, Kirkhorn J, Ten Cate FJ, de Jong N. Ultrasound contrast imaging: current and new potential methods. *Ultrasound Med Biol*. 2000; 26:965–75. [PubMed: 10996696]
- Fröhlich GM, Meier P, White SK, Yellon DM, Hausenloy DJ. Myocardial reperfusion injury: Looking beyond primary PCI. *Eur Heart J*. 2013; 34:1714–1724. [PubMed: 23536610]
- Galasso G, Schiekofer S, D'Anna C, Gioia GD, Piccolo R, Niglio T, Rosa RD, Strisciuglio T, Cirillo P, Piscione F, Trimarco B. No-reflow phenomenon: pathophysiology, diagnosis, prevention, and treatment. A review of the current literature and future perspectives. *Angiology*. 2014; 65:180–189. [PubMed: 23362304]

- Giugliano RP, McCabe CH, Antman EM, Cannon CP, Van de Werf F, Wilcox RG, Braunwald E. Lower-dose heparin with fibrinolysis is associated with lower rates of intracranial hemorrhage. *AmHeart J.* 2001; 141:742–750.
- Helfield B, Black JJ, Qin B, Pacella J, Chen X, Villanueva FS. Fluid Viscosity Affects the Fragmentation and Inertial Cavitation Threshold of Lipid-Encapsulated Microbubbles. *Ultrasound Med Biol.* 2016; 42:782–794. [PubMed: 26674676]
- Hitchcock KE, Ivancevich NM, Haworth KJ, Caudell Stamper DN, Vela DC, Sutton JT, Pyne-Geithman GJ, Holland CK. Ultrasound-enhanced rt-PA thrombolysis in an ex vivo porcine carotid artery model. *Ultrasound Med Biol.* 2011; 37:1240–51. [PubMed: 21723448]
- Holscher T, Raman R, Ernstrom K, Parrish J, Le DT, Lyden PD, Mattrey RF, Ernström K, Parrish J, Le DT, Lyden PD, Mattrey RF. In vitro sonothrombolysis with duplex ultrasound: first results using a simplified model. *CerebrovascDis.* 2009; 28:365–70.
- Ito H. Etiology and clinical implications of microvascular dysfunction in patients with acute myocardial infarction. *IntHeart J.* 2014; 55:185–189.
- Karges HE, Funk KA, Ronneberger H. Activity of coagulation and fibrinolysis parameters in animals. *Arzneimittelforschung.* 1994; 44:793–7. [PubMed: 8053983]
- Kloner RA, Ganote CE, Jennings RB. The “no reflow” phenomenon after temporary coronary occlusion in the dog. *J Clin Invest.* 1974; 54:1496–1508. [PubMed: 4140198]
- Landskroner K, Olson N, Jesmok G. Cross-Species Pharmacologic Evaluation of Plasmin as a Direct-Acting Thrombolytic Agent: Ex Vivo Evaluation for Large Animal Model Development. *J Vasc Interv Radiol.* 2005; 16:369–377. [PubMed: 15758133]
- Leeman JE, Kim JS, Yu FTH, Chen X, Kim K, Wang J, Chen X, Villanueva FS, Pacella JJ. Effect of Acoustic Conditions on Microbubble-Mediated Microvascular Sonothrombolysis. *Ultrasound Med Biol.* 2012; 38:1589–1598. [PubMed: 22766112]
- Marieb, EN. *Essentials of Human Anatomy & Physiology.* 11. San Francisco: Pearson Education, Inc; 2015.
- Mehran R, Lansky AJ, Witenbichler B, Guagliumi G, Peruga JZ, Brodie BR, Dudek D, Kornowski R, Hartmann F, Gersh BJ, Pocock SJ, Wong SC, Nikolsky E, Gambone L, Vandertie L, Parise H, Dangas GD, Stone GW. Bivalirudin in patients undergoing primary angioplasty for acute myocardial infarction (HORIZONS-AMI): 1-year results of a randomised controlled trial. *Lancet.* 2009; 374:1149–1159. [PubMed: 19717185]
- Mizushige K, Kondo I, Ohmori K, Hirao K, Matsuo H. Enhancement of ultrasound-accelerated thrombolysis by echo contrast agents: dependence on microbubble structure. *Ultrasound Med Biol.* 1999; 25:1431–7. [PubMed: 10626631]
- Nishioka T, Luo H, Fishbein MC, Cercek B, Forrester JS, Kim CJ, Berglund H, Siegel RJ. Dissolution of thrombotic arterial occlusion by high intensity, low frequency ultrasound and dodecafluoropentane emulsion: an in vitro and in vivo study. *JAmCollCardiol.* 1997; 30:561–568.
- Pacella JJ, Brands J, Schnatz FG, Black JJ, Chen X, Villanueva FS. Treatment of microvascular micro-embolization using microbubbles and long-tone-burst ultrasound: an in vivo study. *Ultrasound Med Biol.* 2015; 41:456–64. [PubMed: 25542487]
- Papadopoulos SM, Chandler WF, Salamat MS, Topol EJ, Sackellares JC. Recombinant human tissue-type plasminogen activator therapy in acute thromboembolic stroke. *JNeurosurg.* 1987; 67:394–398. [PubMed: 3112328]
- Petit B, Bohren Y, Gaud E, Bussat P, Arditi M, Yan F, Tranquart F, Allémann E. Sonothrombolysis: The Contribution of Stable and Inertial Cavitation to Clot Lysis. *Ultrasound Med Biol.* 2015; 41:1402–1410. [PubMed: 25601463]
- Petit B, Gaud E, Colevret D, Arditi M, Yan F, Tranquart F, Allemann E. In vitro sonothrombolysis of human blood clots with BR38 microbubbles. *Ultrasound MedBiol.* 2012; 38:1222–1233.
- Porter TR, Kricsfeld D, Lof J, Everbach EC, Xie F. Effectiveness of transcranial and transthoracic ultrasound and microbubbles in dissolving intravascular thrombi. *J Ultrasound Med.* 2001; 20:1313–1325. [PubMed: 11762543]
- Porter TR, Radio S, Lof J, Everbach C, Powers JE, Vignon F, Shi WT, Xie F. Diagnostic Ultrasound High Mechanical Index Impulses Restore Microvascular Flow in Peripheral Arterial Thromboembolism. *Ultrasound Med Biol.* 2016; 0:461–466.

- Price RJ, Skyba DM, Kaul S, Skalak TC. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation*. 1998; 98:1264–1267. [PubMed: 9751673]
- Prokop AF, Soltani A, Roy RA. Cavitational mechanisms in ultrasound-accelerated fibrinolysis. *Ultrasound Med Biol*. 2007; 33:924–33. [PubMed: 17434661]
- Robbers LFHJ, Eerenberg ES, Teunissen PF, Jansen MF, Hollander MR, Horrevoets AJG, Knaapen P, Nijveldt R, Heymans MW, Levi MM, Van Rossum AC, Niessen HWM, Marcu CB, Beek AM, Van Royen N. Magnetic resonance imaging-defined areas of microvascular obstruction after acute myocardial infarction represent microvascular destruction and haemorrhage. *Eur Heart J*. 2013; 34:2346–2353. [PubMed: 23594591]
- Roos ST, Juffermans LJM, Slikkerveer J, Unger EC, Porter TR, Kamp O. Sonothrombolysis in acute stroke and myocardial infarction: A systematic review. *IJC Hear Vessel*. 2014; 4:1–6.
- Roos ST, Juffermans LJM, van Royen N, van Rossum AC, Xie F, Appelman Y, Porter TR, Kamp O. Unexpected High Incidence of Coronary Vasoconstriction in the Reduction of Microvascular Injury Using Sonolysis (ROMIUS) Trial. *Ultrasound Med Biol*. 2016; 42:1919–28. [PubMed: 27160847]
- Sharifi M, Bay C, Skrocki L, Rahimi F, Mehdipour M. Moderate pulmonary embolism treated with thrombolysis (from the “MOPETT” Trial). *AmJCardiol*. 2013; 111:273–277.
- Starling EH. On the Absorption of Fluids from the Connective Tissue Spaces. *J Physiol*. 1896; 19:312–26.
- Stride E. Physical principles of microbubbles for ultrasound imaging and therapy. *Cerebrovasc Dis*. 2009; 27:1–13.
- Sutton JT, Ivancevich NM, Perrin SR, Vela DC, Holland CK, Perrin SR Jr, Vela DC, Holland CK. Clot retraction affects the extent of ultrasound-enhanced thrombolysis in an ex vivo porcine thrombosis model. *Ultrasound MedBiol*. 2013; 39:813–24.
- Tanswell P, Tebbe U, Neuhaus K-L, Gläsle-Schwarz L, Wojcik J, Seifried E. Pharmacokinetics and fibrin specificity of alteplase during accelerated infusions in acute myocardial infarction. *J Am Coll Cardiol*. 1992; 19:1071–1075. [PubMed: 1372625]
- Tu J, Matula TJ, Brayman AA, Crum LA. Inertial cavitation dose produced in ex vivo rabbit ear arteries with optison® by 1-mhz pulsed ultrasound. *Ultrasound Med Biol*. 2006; 32:281–288. [PubMed: 16464673]
- van Kranenburg M, Magro M, Thiele H, de Waha S, Eitel I, Cochet A, Cottin Y, Atar D, Buser P, Wu E, Lee D, Bodi V, Klug G, Metzler B, Delewi R, Bernhardt P, Rottbauer W, Boersma E, Zijlstra F, van Geuns R-J. Prognostic value of microvascular obstruction and infarct size, as measured by CMR in STEMI patients. *JACC Cardiovasc Imaging*. 2014; 7:930–9. [PubMed: 25212799]
- Williams SA, Wasserman S, Rawlinson DW, Kitney RI, Smaje LH, Tooke JE. Dynamic measurement of human capillary blood pressure. *Clin Sci*. 1988; 74:507–12. [PubMed: 3370917]
- Wu KC, Zerhouni Ea, Judd RM, Lugo-Olivieri CH, Barouch La, Schulman SP, Blumenthal RS, Lima Ja. Prognostic significance of microvascular obstruction by magnetic resonance imaging in patients with acute myocardial infarction. *Circulation*. 1998; 97:765–772. [PubMed: 9498540]

Figure 1.

Experimental setup. Whole blood mixed with MB was infused at a constant rate through the flow channel. Microthrombi were trapped onto the mesh with 40 µm pores, causing upstream pressure to rise. A 1 MHz treatment transducer aiming at the mesh was used to deliver the therapeutic ultrasound. Cavitation activity was detected by a 3.5 MHz transducer and digitized. Upstream pressure was monitored as a surrogate for thrombus burden.

Figure 2.

Upstream pressure with pulse length of 1000 ($n=7$), 3000 ($n=5$), 5000 ($n=5$) cycles US with MB, and 5000 cycles without MB ($n=3$) during SRP therapy in whole blood. US treatment started at $t=0$. All experiments were performed without added tPA.

Figure 3.

Upstream pressure during SRP in whole blood for tPA only ($n=3$), 5000 cycles US+MB with tPA ($n=5$), 5000 cycles US+MB ($n=5$) and 5000 cycles US No MB ($n=3$).

Figure 4.

Time frequency analysis, ICD and SCD for 1 MHz, 1.5 MPa, and (a) 1000 cycle, (b) 3000 cycles, (c) 5000 cycles pulse during SRP and (d) for a 5000 cycles pulse but without MB.

Figure 5.

(a) Inertial cavitation dose (ICD) at 1.5 MPa as a function of pulse length. ICD increased with pulse duration in the presence of MB and plateaued at 3000 cycles. (b) Stable cavitation dose (SCD) at 1.5 MPa as a function of pulse length. SCD increased with pulse length in the presence of MB and was significantly higher at 5000 cycles compared to 3000 cycles ($n=5$ per experimental condition, $*p<0.05$).

Table 1

Sonoreperfusion efficacy

	Terminal pressure drop (%)	Lytic rate (mmHg/min)	Lytic Index ($\times 10^{-3}$ mmHg $^{-1}$ min $^{-1}$)
US+MB 1000 cycles	33.7 $\pm$ 21.7 ⁺	2.6 $\pm$ 1.5	1.9 $\pm$ 0.5
US+MB 3000 cycles	57.7 $\pm$ 13.6 ⁺	4.6 $\pm$ 2.5	2.1 $\pm$ 1.1
US+MB 5000 cycles	59.2 $\pm$ 13.8 ⁺	7.3 $\pm$ 3.2	2.3 $\pm$ 0.6
US only 5000 cycles	3.9 $\pm$ 16.9	0.5 $\pm$ 0.1	1.8 $\pm$ 0.1
tPA only	84.0 $\pm$ 14.1 ⁺	1.2 $\pm$ 1.3	2.0 $\pm$ 0.5
US+MB+tPA 5000 cycles	87.6 $\pm$ 8.2 ⁺	4.3 $\pm$ 0.8	3.5 $\pm$ 0.8

US=Ultrasound, MB=Microbubbles, tPA= tissue plasminogen activator,

⁺ $p<0.05$ vs baseline;

* $p<0.05$