Original Article

An *in vitro* Model for Experimental Evaluation of Sonothrombolysis under Tissue-mimicking Material Conditions

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Abstract

Background: The mechanical properties of therapeutic ultrasound (US) have attracted scientific interest for thrombolysis enhancement in combination with thrombolytic agents and microbubbles (MBs). The aim of the study was to develop an *in vitro* model to observe how the effects of sonothrombolysis change in the case where a tissue-mimicking material (TMM) is placed in the path of the US beam before the clot. **Methods:** Fully retracted blood clots were prepared and pulse sonicated for 1 h under various conditions. The system was in a state of real circulating flow with a branch of an open bypass and an occluded tube containing a blood clot, thus mimicking the case of ischemic stroke. The effectiveness of thrombolysis was quantified in milligrams of clots removed. An agar-based TMM was developed around the occluded tube. **Results:** The clot breakdown in a TMM was found to be more pronounced than in water, presumably due to the retention of the acoustic field. A higher level of acoustic power was required to initiate clot lysis (>76 W acoustic power) using only focused US (FUS). The greatest thrombolysis enhancement was observed with the largest chosen pulse duration (PD) and the use of MBs (150 mg clot mass lysis). The synergistic effect of FUS in combination with MBs on the enzymatic fibrinolysis enhanced thrombolysis efficacy by 260% compared to thrombolysis induced using only FUS. A reduction in the degree of clot lysis was detected due to the attenuation factor of the intervening material (30 mg at 1 and 4 ms PD). **Conclusion:** *In vitro* thrombolytic models including a TMM can provide a more realistic evaluation of new thrombolytic protocols. However, higher acoustic power should be considered to compensate for the attenuation factor. The rate of clot lysis is slow and the clinical use of this method will be challenging.

Keywords: Focused ultrasound, microbubbles, stroke, thrombolytic agent, tissue-mimicking material

Introduction

In the past several decades, therapeutic ultrasound (US) has played an important role in the enhancement of thrombolytic treatment. The mechanical properties of focused US (FUS) have been exploited to fully decode the ability of US waves to disrupt clots and address stroke diseases. The appearance of a clot in the brain vessels is extremely difficult to deal with, and new therapeutic strategies are needed in such cases. Unfortunately, the main challenge in thrombolytic procedures is the time factor, and thus, new methods are required to act promptly and effectively. Thrombolysis is usually applied on an urgent basis, and to be effective, it should be started as soon as possible before permanent damage occurs, and the thrombus hardens to such an extent that it cannot be dissolved.

Stroke is the world's second leading cause of morbidity and mortality (excluding cancer) and a fundamental contributor to disability.[1] There are over 13.7 million new cases of stroke each year worldwide, with one in four people over the age of 25 years having a stroke in their lifetime.[2] Age, cancer, surgery, obesity, injuries, and medications are all risk factors for stroke, with people in hospitals and those who underwent surgery having an 8- and 22-fold higher risk of stroke, respectively.[3] The vast majority of stroke events can be classified as ischemic stroke, which is accompanied by a blockage of an artery in the brain, and hemorrhagic stroke,

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which is triggered by a collapse of a blood vessel.[4] Ischemic strokes are more frequent, accounting for 87% of all strokes in the United States.[4]

Until now, the gold standard treatment of stroke is the use of thrombolytic agents which are plasminogen activators, and many of them have been already approved by the United States Food and Drug Administration.[5] All these fibrinolytic drugs are associated with a substantial hemorrhage threat, which is approximately equal for all agents.[5] There are two ways to administer thrombolytics: through a peripheral intravenous line (systemic thrombolysis) or a catheter (thin tube), that is directed and placed inside the clot, which is considered a minimally invasive procedure. Therefore, thrombolytic drugs have to be administered to the patient within 4.5 h from the onset of stroke symptoms.[6]

The primary benefits of using FUS in combination with the existing thrombolytic agents are the higher possibility of blood clot dialysis in a much less treatment time, the minimization of side effects (especially vessel wall bleeding), and the full revascularization and recanalization, which lead to a normal healthy blood flow posttreatment. The use of FUS to produce cavitation (stable or inertial) for the clot lysis is known as "sonothrombolysis" and it is a promising technique that can act as a reinforcer to improve the enzymatic effect of thrombolytic agents.

Early diagnosis plays a vital role in the successful treatment of stroke. Diagnostic transcranial Doppler US was proven as a useful diagnostic real-time tool, as well as an integral part of a therapeutic system for ischemic stroke.[7] In the effort of expanding the applications of therapeutic US in the treatment of ischemic stroke, various US-enhanced thrombolytic systems have been developed and tested in clinical trials.[8-12] The proposed devices differ in their operating frequency and type of image guidance. For instance, in a multicenter Phase II trial called TRUMBI,[8] ischemic patients were treated with transcranial US -mediated thrombolysis. Low-frequency US was used for better penetration of ultrasonic waves through the skull, whereas the procedure was monitored with *magnetic resonance imaging*. Unfortunately, the procedure induced intracerebral hemorrhage, and its safety was called into question. It was later stated that hemorrhage was related to abnormal permeability of the human blood–brain barrier caused by the wide beam selection.[9]

Researchers have, thus, concentrated their studies on the thrombolytic effects of low-intensity, high-frequency (2 MHz) US.[10-12] One of the clinical trials using high frequency was the CLOTBUST randomized trial.[10] In this trial, it was shown that the use of recombinant tissue plasminogen activator (rt-PA) combined with transcranial Doppler US within 2 h from the onset of stroke recovers the blood flow at a significant level, with 49% of the patients presenting complete reperfusion. TUSCON[11] was another randomized clinical trial that combined systemic tissue plasminogen activator (t-PA) and US. In this clinical study, the continuous intravenous injection of a safe dose of microbubbles (MBs) induced higher recanalization of the intracranial occlusions, compared to the standard t-PA therapy. In addition, the EKOS Endowave[12] system is currently undergoing testing in clinical trials for catheter-directed sonothrombolysis for various types of clots, including pulmonary and deep vein thromboses.

Several *in vitro* models have been developed to test the efficacy of sonothrombolysis for human or animal blood clot lysis under various conditions and US protocols.[13] So far, experimental studies have suggested that US enhances the enzymatic fibrinolysis by boosting the catalyst transmission via a cavitation-related mechanism. As a result, thrombolytic drugs penetrate the clot more deeply, thus leading to a larger lysis zone.[14,15]

In the last decade, research studies have been concentrated on the development of substances that could improve sonothrombolysis.[16-24] The efficacy of sonothrombolysis has been investigated by varying the size and shell thickness of MBs.[16-18] In a study, human blood clots were exposed to pulse sonications with a 1 MHz single-element transducer, and the effect of MB size on the blood clot lysis was evaluated.[16] A catheter-based approach was followed for circulating the MBs through the blood clots, which is considered more invasive than the traditional intravenous therapies. The findings suggested that the use of larger MBs significantly improves sonothrombolysis rates when compared to cases where therapeutic doses of rt-PA alone or combined with small MBs were used. Borrelli *et al*.[17] reported that MBs of 3 μm at 1 MHz and 1 μm at 3 MHz were more effective for clot lysis. Thick-shelled MBs demonstrated no enhancement in clot lysis, while US combined with thin-shelled MBs was shown to accelerate the fibrinolytic activity of clot lysis.[18] Moreover, magnetic MBs and nanodroplets have been proposed to be used along with US for boosting *in vitro* lysis of blood clots.[19-24] Intravascular droplets demonstrated enhanced clot lysis even at reduced acoustic powers.[21]

Similarly, Shekhar *et al*. used an *in vitro* system to demonstrate whether the combination of US with rt-PA-loaded echogenic liposomes (t-ELIP) that entrain octafluoropropane MBs (t-ELIP) can enhance the enzymatic lysis of human blood clots.[24] Recently, an *in vitro* clot model was developed using a lysis catheter embedded into human blood clots to investigate the dissolution of clots under various treatment conditions (US only, rt-PA only, US in synergy with rt-PA) and other experimental parameters such as the ultrasonic frequency and the distance from the probe to the clot.[25] The application of US combined with rt-PA for 1 h using an US planar high-frequency transducer of 10 MHz achieved a significantly higher lysis rate and clot volume reduction than any other condition. Yang and Zhou[26] estimated the extent of clot lysis induced by high-intensity focused US at varied pulse repetition frequencies (PRFs) in an *in vitro* model. The thrombolytic efficiency was significantly lower at 1 kHz, while it remained constant below 100 Hz. In addition, thrombi displacement was found to be more frequent at lower PRFs. Another *in vitro* model was developed using a plastic skull phantom, and dissolution of blood clot has been achieved using FUS in combination with rt-PA.[27]

The objective of the current study was to develop a realistic *in vitro* pulsatile circulating flow model to examine the efficacy of pulsed FUS in breaking artificial clots when combined with thrombolytic drugs or MBs. Blood clots were formed in a thin plastic transparent tube. Pulsed sonications were initially performed with the plastic tube being immersed in degassed water as other researchers have already done to investigate sonothrombolysis.[16,24-26,28] Sonications were also performed in a more realistic model, in which a tissue-like medium was developed around the plastic tube that hosted the clot. Specifically, the intervening medium was an agar-based tissue-mimicking material (TMM) with acoustic properties close to human tissue as reported in previous studies.[29,30] In contrast to other *in vitro* models, a bypass free-clot tube was included in this study, thus replicating the physiological situation during an ischemic stroke. Specifically, a combination of a circulating flow model and a tissue-like medium was used to evaluate the efficacy of sonothrombolysis under various treatment conditions compared to other studies that have not used this combination which fully mimics the real conditions during a treatment to occlude a thrombus. The effect of experimental ultrasonic parameters such as the acoustic power and pulse duration (PD) on thrombolysis efficacy was initially investigated by applying stand-alone US. In this case, the treatment time, PRF, dose of the thrombolytic drug, and continuous injection rate of MBs remained constant. Subsequently, after the production of a TMM around the plastic tube, the effectiveness of sonothrombolysis was assessed using FUS in synergy with a thrombolytic agent and MBs. A single-element spherically focused transducer was used and the mass clot removal for each experimental case was estimated.

Materials and Methods

The evaluation of sonothrombolysis using the proposed *in vitro* model was performed in porcine blood clots. A TMM was developed around the blood clots. No patient data were included in this study. Therefore, no informed consent from patients or approval from an ethics committee was required.

Experimental apparatus

All experiments were conducted in an acrylic tank (17 cm^3 × 14 cm^3 × 23 cm^3) that was filled with degassed/deionized water to avoid any interaction of the US waves with air bubbles. Pulse FUS waves were generated by a single-element spherically focused transducer operating at a central frequency of 1.05 MHz (50 mm diameter, 70 mm focal length, Medsonic Ltd., Limassol, Cyprus), which was powered by a radio frequency generator/amplifier (1000 W, JJ and A Instruments, Duvall, WA, USA). An US power meter (UPM-DT100N, Ohmic Instruments Co., Easton, MD, USA) was used to estimate the acoustic power of the transducer. Three-dimensional-printed acrylonitrile butadiene styrene structures were used for the complete stabilization of the transducer, plastic tube (with the thrombus), and agar-based TMM. The computer-aided design drawing of the experimental setup that was used to conduct the experiments with and without the TMM is shown in Figure 1a and b, respectively. It is noted that the water was adjusted at a reasonable level to minimize reflections from the plastic tube or phantom interface.

The blood clots were exposed to a unidirectional circulating pulsatile flow at a flow rate of 50 mL/min using a peristaltic pump (7518-40, Cole-Parmer, Vernon Hills, IL, USA), and a bypass tube was also added to reproduce the real physiological situation of stroke. Specifically, the plastic tube with the clot was carefully filled with degassed water without displacing the clot. The circulating flow system including the bypass tube was connected to a reservoir which was also filled with degassed water. The thrombolytic drug and MBs (SonoVue, Bracco, Milan, Italy) were added at a later stage in the reservoir to examine their thrombolytic effects when combined with the pulsed US. The transducer was mounted on the holder facing upward at a distance from the clot equal to the focal length of the transducer. For precise focal spot positioning, a plastic X-shaped structure was used to perfectly align the clot with the center of the transducer's element. After the precise positioning of the focal spot on the targeted clot, the plastic structure was removed.

Preparation of *in vitro* blood clot

Fresh blood was collected from healthy slaughter pigs and artificial clots were formed by natural coagulation of blood. Empty plastic transparent tubes (inner diameter: 3.6 mm, thickness: 0.7 mm) were preweighted and then the blood clots were carefully transferred into the tubes. Each plastic tube was then incubated in a 37°C water bath for 3 h before refrigeration at 5°C for 72 h to achieve maximum clot retraction.[31] Following the creation of a clot, serum was cautiously aspirated from the tubes, and the occluded tubes were reweighted to quantify each clot's mass. Upon each experiment execution, the plastic tube was left to dry for 30 min before being weighed again to determine the clot mass loss due to each thrombolytic treatment type. Finally, the efficacy of thrombolytic treatment was estimated in milligrams of clots removed using a digital balance precision scale (JSR100, J-Scale, Phoenix, Arizona, United States).

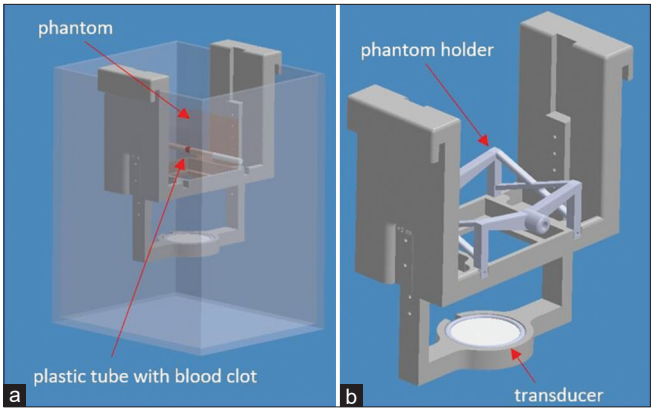

Figure 1: CAD drawing of the experimental apparatus that was used for sonothrombolysis evaluation (a and b) without the TMM. CAD: Computer-aided design, TMM: Tissue-mimicking material

Development of tissue-like medium around the plastic tube

An agar-based TMM (6 cm^3 × 6 cm^3 × 4 cm^3) was prepared in a mold. It consisted of 6% weight per volume of agar (Merck KGaA, EMD Millipore Corporation, Darmstadt, Germany) in degassed/deionized water. This phantom has been proven suitable to mimic the ultrasonic parameters of soft tissue.[29,30] The preparation procedure has been previously described in other studies.[32-35] The mold was designed to allow the insertion of the plastic tube with the blood clot at the center of the phantom. In addition, it was made out of composite pieces to allow for easy removal of the phantom. Figure 2 shows the mold with the occluded plastic tube before the TMM development. The final phantom that replicates a clogged artery in tissue is shown in Figure 3. The distance from the focused transducer to the bottom interface of the developed phantom was set at 5 cm, thus adjusting the focal depth to 2 cm where the blood clot was located. Figure 4 shows the experimental apparatus that was used to evaluate the efficiency of thrombolysis for different experimental protocols.

Ultrasound protocols

Each sonication set included a pulse repetition period (PRP) of 10 ms, a PD of 1 and 4 ms (duty factor of 10% and 20%), and an operating frequency of 1.05 MHz. The total treatment time for all cases was 1 h. The effect of acoustic power on the grade of thrombolysis was investigated with and without a TMM around the plastic tube. The low duty factor selected in the experiments led to low energy deposition rates in water and TMM and hence minimal thermal rise at beam focus (≤1°C), making sure that no bioeffects were generated from thermal mechanisms.

Thrombolytic agent and microbubbles

FUS pulsed waves were used in combination with a thrombolytic agent (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) to investigate the enhancement of thrombolysis with US. As stated by the manufacturer, the agent contains the active ingredient alteplase. The agent is supplied as a powder along with sterilized water which serves as the solvent for injection purposes. Before use, the water is added to the powder to form a solution ready for administration. In this study, the concentration of the thrombolytic agent in the flow system was 1 mg/mL.

Furthermore, the effect of FUS combined with MBs (SonoVue, Bracco) was examined. The specific microbubbles, coated with a very thin lipid monolayer membrane shell encapsulating the gas SF_6, have a mean radius of around 1.5 μm and a concentration of (2–5) × 10^8 bubbles ⁄ ml. The same type of MBs was previously tested and the outcomes demonstrated that MBs improve thrombolysis efficiency when thrombolytic enzymes are used.[28] In the current study, the effect of MBs on thrombolysis efficiency was compared for the cases where a medium was present or not between the transducer and the blood clot. During treatment, MBs were continuously injected into the circulating flow system at a constant rate of 0.5 mL/5 min to replace the destroyed ones, thus keeping the cavitation mechanisms active.

Results

In all experiments, the values of thrombolysis are presented as mean ± standard deviation and each experiment was conducted on 10 blood clots (n = 10). The clot mass loss was estimated for several treatment protocols. First, the blood clot was sonicated without any medium around the plastic tube and the degree of clot lysis was determined using stand-alone FUS at acoustical power of 38, 76, 114, and 152 W. Figure 5 shows the clot mass loss in milligrams versus acoustic power when no medium was included around the plastic tube for a PD of 1 ms. The treatment with FUS alone required >76 W acoustic power to initiate clot lysis while using FUS combined with a thrombolytic agent initiated clot lysis at 38 W.

The enhancing effect of FUS on the drug-induced thrombolysis was assessed for the same values of acoustic power of 38, 76, 114, and 152 W. The procedure was repeated with an acoustic power of 152 W but a higher PD (4 ms) using FUS alone, as well as in synergy with the thrombolytic drug. Furthermore, the

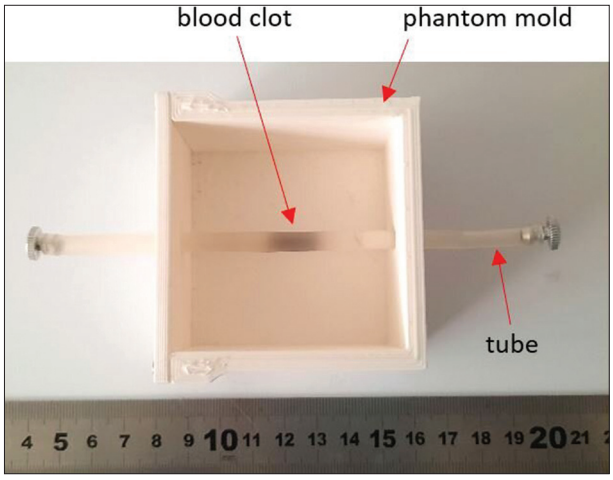

Figure 2: Placement of the tube with an induced blood clot in the mold for phantom development

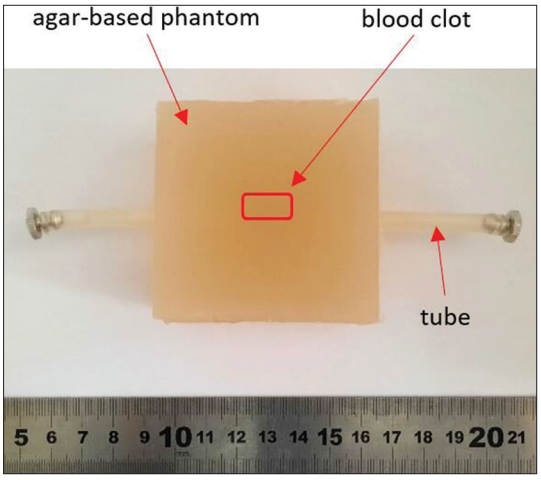

Figure 3: Agar-based phantom as developed around the plastic tube with the thrombus

role of cavitation nuclei was investigated by injecting MBs into the circulating flow system at acoustic power of 152 W for a PD of 1 and 4 ms. Figure 6 shows the clot mass loss in milligrams versus PD when no medium was included around the plastic tube for acoustic power of 152 W. The synergistic effect of FUS in combination with MBs on the enzymatic fibrinolysis for a PD of 1 ms enhanced thrombolysis efficacy by 260% compared to thrombolysis induced using only FUS. By the presence of the TMM, the clot lysis was reduced by 30% compared to thrombolysis induced using FUS in combination with MBs.

A medium (agar-based TMM) was developed around the clogged plastic tube to imitate the normal tissue anatomy. Finally, FUS in combination with MBs was applied at acoustic power of 152 and 228 W for 1 and 4 ms PD. Treatment was performed without the addition of a thrombolytic drug. The clot mass loss in milligrams versus acoustic power and PD, when a TMM was added around the plastic tube, is shown in Figure 7. A 20% rise of the clot lysis was detected by increasing the PD from 1 to 4 ms at 152 W acoustic power, whereas at 228 W acoustic power, the rise of the clot lysis was only 7%.

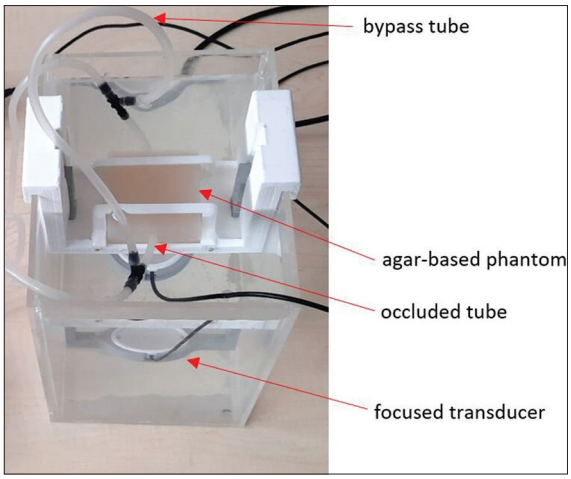

Figure 4: Experimental apparatus with the TMM placed above a single-element spherically focused 1.05 MHz transducer. TMM: Tissue-mimicking material

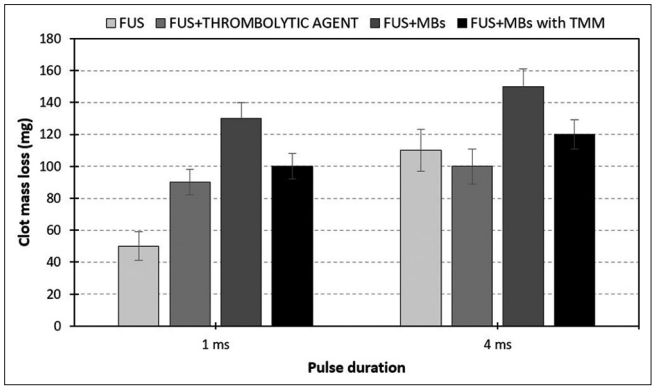

Figure 6: Effect of PD on clot mass loss for various treatments using FUS in combination with a thrombolytic agent and MBs without a TMM placed around the plastic tube and treatment with FUS + MBs with the presence of a TMM. Parameters: acoustic power = 152 W, PRP = 10 ms, *n* = 10. PD: Pulse duration, FUS: Focused ultrasound, MBs: Microbubbles, TMM: Tissue-mimicking material, PRP: Pulse repetition period

Discussion

The purpose of this study was the development of an *in vitro* model for a simplified and effective evaluation of sonothrombolysis in two different situations. The first situation was the most common *in vitro* model that has been previously used to conduct experiments.[16,24,26-28,36,37] The model involves sonicating a tube with an embedded artificial blood clot in degassed water, which is weakly attenuating. The tube was immersed in degassed water, without any interfering tissue-like medium along the US beam. Advantageously, a more realistic model in which a TMM was developed around the vessel mimicking the tube containing the clot was also developed. In a study by Wright *et al*.,[38] an agar-based TMM was developed to solely investigate the clot displacement by the acoustic radiation force mechanism. Porcine blood clots were exposed to a unidirectional flow rate, with the inclusion of a bypass tube to imitate real conditions of a stroke. As a

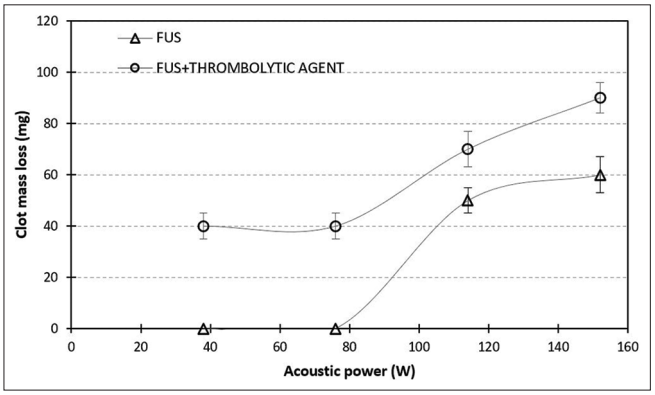

Figure 5: Effect of acoustic power on clot mass loss when stand-alone FUS and FUS + thrombolytic drug was used. Parameters: duty factor = 10%, PD = 1 ms, PRP = 10 ms, *n* = 10. FUS: Focused ultrasound, PD: Pulse duration, PRP: Pulse repetition period

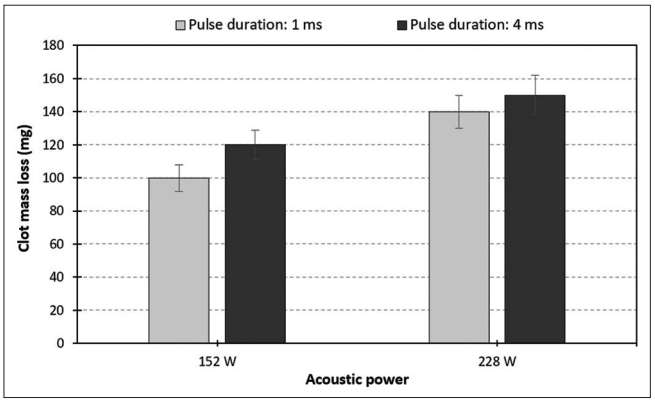

Figure 7: Effect of acoustic power and PD on clot mass loss after treatment using FUS in combination with MBs while a TMM was developed around the plastic tube. The pulse repetition period was 10 ms and n = 10. PD: Pulse duration, FUS: Focused ultrasound, MBs: Microbubbles, TMM: Tissue-mimicking material

result, the authors were able to compare the outcomes in the case where FUS directly interacted with the clot, with those in the case where an attenuated medium was included in the path of the beam. The clot breakdown in a brain TMM was found to be more pronounced than in water, presumably due to the retention of the acoustic field.[39]

Clinical studies have demonstrated that the selection of an operating frequency in the order of kHz does not sufficiently enhance thrombolysis and induces vessel wall hemorrhage.[8] In other clinical trials,[10-12] a higher frequency of 2 MHz was used. However, it is almost impossible for high-frequency US waves to penetrate the skull, especially when using a single element transducer. Therefore, in the current study, a frequency of 1.05 MHz was chosen to conduct the experimental trial. In addition, the size of the clots was around 10 mm and a wide beam transducer was required. Thereby, high-frequency transducers that sharply focus the ultrasonic energy were not suitable. A limitation of the lower frequency transducer is that it generates lower intensities, and for that reason, higher acoustic power was used. However, the localized temperature increase at beam focus was kept below 1°C to ensure safe treatment.

The thrombolytic effects of a stand-alone FUS were firstly investigated for varying acoustical power without the addition of a TMM. FUS alone was not capable to induce clot lysis for acoustical power lower than 76 W. However, a higher level of acoustic power was required to initiate clot mass lysis. A significant increase in clot mass loss of up to 60 mg was measured at acoustical powers in the range between 76 and 152 W. It has been shown from other studies that stand-alone FUS thrombolysis at 0.5–1 MHz operating frequency is feasible and able to dissolve blood clots and restore normal flow at high enough intensities.[40-42] In that case, the acoustic radiation force induced clot displacement leading to tube occlusion. A linear dependency of the peak axial clot displacement to the applied acoustic power was reported by Wright *et al*. for acoustical powers up to 60 W, using an approximately similar frequency transducer (1.51 MHz).[38]

Recent research studies have reported the ability of FUS to induce clot lysis without the need for a thrombolytic agent in both *in vitro* and *in vivo* animal trials.[26,43-45] Since for clinical applications, high intensities should be avoided to ensure the safety of the treatment, the use of a low dose of the thrombolytic drug along with lower intensity is ideal. By retaining the dose of thrombolytic agent (alteplase) constant, it was demonstrated that with an acoustic power lower than 76 W, a 40 mg clot lysis is achieved. These findings are supported by other studies reporting that the combination of FUS with a drug is more effective than a thrombolytic agent dose or FUS alone.[46,47] Previous studies, in which a 1 MHz focused transducer was also used, confirmed the increase in thrombolysis efficiency with the increase of acoustic power (or intensity) when using US in combination with a thrombolytic agent at a lower range of acoustic intensity (<1447 W/cm^2).[38,48,49]

In an additional investigation, the quadruple increase of PD (1–4 ms) resulted in a higher thrombus dissolution in all cases (FUS only, FUS + thrombolytic drug, FUS + MBs). When clots were treated with FUS and MBs, a higher thrombolysis enhancement was observed at the largest chosen PD. The efficacy of MBs in combination with FUS for successful blood clot occlusion has been studied,[16,36] with the outcomes suggesting that the addition of thrombolytic agents is not particularly necessary.

The difference in clot lysis efficacy of FUS combined with MBs when a TMM was present as a medium along the focal beam was then evaluated. A reduction in the degree of clot lysis was detected due to the attenuation factor of the material. By comparing the results with those in the case where no material was included along the focal beam, it is concluded that there was no significant reduction and the enzymatic lysis was adequate to remove most of the initially induced thrombus. In addition, the placement of a TMM did not change the effect of acoustic power and PD on the efficiency of clot mass removal. In the case of an acute ischemic stroke, the presence of the skull is a challenge due to the high attenuation and distortion of the US focal waves. Even though the suggested *in vitro* model does not provide the condition where the skull barrier exists, the partial removal of a piece of the skull approaches this model that resembles brain tissue with a branch of a normally open and clogged artery.

Conclusion

An apparent limitation of sonothrombolysis remains the required long treatment procedure. A 1-h sonothrombolysis treatment was able to dissolve only about 100 mg of blood clot and the method was confirmed to be slow. One concern about the findings of thrombolysis using stand-alone FUS was that a small percentage of thrombus removal was achieved. This leads to the conclusion that pulsed therapeutic US should be used even with a low dose of thrombolytic agents or continuous MBs administration.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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