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In-vitro assessment of the thrombolytic efficacy of therapeutic ultrasound

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ABSTRACT

Background: Ultrasound is mainly used as a diagnostic tool. Several studies demonstrated that therapeutic ultrasound (TUS) can enhance thrombolysis, but the optimal mechanical parameters to achieve this biological effect are still unknown.

Methods: We assembled 46 blood clots in a closed *in-vitro* circulatory model. Clots were randomly divided into 7 groups, control group and six TUS groups of three frequencies (0.3, 0.5, 0.7 MHz) and six intensities (0.75, 1.5, 3, 237.7, 475, 950 W/cm^2). Treatment was composed of 12 repetitions, 5 min US application and 3 min pause, lasting 93 min in total. Clots' weight and flow rate were measured before and after the treatment.

Results: Mean initial clot weight (0.318 ± 0.129 g) and flow (0.53 ± 0.31 ml/min) were comparable among the experimental groups. We found a final clot weights reduction (0.15 ± 0.05, 0.16 ± 0.06, 0.09 ± 0.07, 0.21 ± 0.09, 0.17 ± 0.09, 0.17 ± 0.07 and 0.18 ± 0.02 g in groups 1 through 6, respectively) and a flow increase (30.61 ± 19.76, 52.1 ± 25.44, 28.78 ± 8.15, 43.93 ± 20.03, 40.86 ± 18.25 and 45.10 ± 22.20 ml/min in groups 1–6, respectively) in all TUS groups. Clot weight change (%) and flow increase reveals that the TUS profile f = 0.5 MHz I = 1.5 W/cm^2 was most efficacious. In the control group, clot weight change was +6.3% of baseline and flow increase of 4.4% of baseline, whereas −75.4% of baseline and 209.3% of baseline in the f = 0.5 MHz I = 1.5 W/cm^2 profile were noted, respectively.

Conclusions: Our study proved that TUS at low frequency (0.5 MHz) is most effective, whereas changing the intensity of TUS has only a minor effect on clot lysis magnitude.

1. Introduction

Thrombotic vascular occlusion, venous or arterial, is a common event [1]. Blood clots can form *de novo* in both vascular systems, or travel as emboli into those systems [2]. More than 25% of deaths worldwide are caused by the development of arterial blood clots, most of them in the coronary or cerebral systems [1]. Moreover, venous thromboembolism (VTE) is the third most common cardiovascular disease [3].

The current treatment consists of either thrombolytic therapy, percutaneous intervention or surgical procedures [4]. The percutaneous interventions include local injection of thrombolytic drugs or catheter thrombectomy, and sometimes mechanical fragmentation of the blood clot by balloon inflation. Surgical thrombectomy or bypass (venous or arterial) grafting, are usually the final alternative. The most feasible, non-invasive and rapid treatment seem to be thrombolytic treatment.

The most common fibrinolytic drugs used today are streptokinase, tissue plasminogen activator (tPA) and urokinase [5]. These drugs activate internal components of the clotting system, resulting in conversion of an inactive plasminogen to active plasminogen, which then breaks down fibrin and increases the solubility of the degradation products which are later removed by phagocytes [5]. Nevertheless, this treatment endures disadvantages and risks, including treatment resistance (in nearly 25% of patients), delayed vessel opening and bleeding complications resulting from the non-specific mechanism of the thrombolytic drugs [5]. Almost 40% of myocardial infarction patients treated with thrombolytic drugs fail to reperfuse [4,6–8]. On the other hand, using a higher dose of the thrombolitic agents increases the risk of bleeding. Most of the efforts to improve the efficacy and safety of thrombolytic therapy are currently focused on two areas: 1) Development of high-efficacy thrombolitic drugs, with a short half-life and as few side effects as possible; 2) Targeted, local drug injection, adjacent to the clot.

Therapuetic ultrasound (TUS) may accelerate thrombolysis and increase the efficacy of thrombolytic therapy [9–11]. Currently, ultrasound is primarily used as a diagnostic tool, but may also exert a

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therapeutic effect [12,13]. Since 1927 it has been found that changes in biological systems can be achieved by ultrasound at varying frequencies, intensities and differing time of exposure [13]. Since then, several TUS profiles have been found to be useful in areas such as physiotherapy, fusion of fractures, enhancement of local drug uptake, gene therapy, lithotripsy and more [13,14].

TUS exerts both thermal and mechanical effects on biological tissues. The thermal effect causes an increase in the treated tissue temperature, and results in beneficial physiological effects (*e.g.* increasing blood supply to the treated area). The mechanical effect is caused by the sound waves which pass through the tissue [13,14]. TUS increases clot dissolution [6,7,11,12,14–18], shortens latency to reprefusion, and allows the use of a lower drugs doses. The acceleration of clot dissolution is caused by increased enzymatic activity together with the vibrational effect which increases the surface area of the blood clot with which the drugs come in contact with [6,12,14,18]. Thus, TUS optimizes both the effect of the drugs and increases the depth of the drug's penetration into the clot [6,14]. An additional mechanism is known as the 'cavitation effect'. Specificaly, during the negative phase of the sound wave, microbubbles are created inside and around the clots, and during the positive phase of the sound wave the microbebbles are destructed, creating cavitations on the clots' surface [3].

Futhermore, high intensity ultrasound levels have been shown to increase clot lysis even independently of medications [3,6,7,12,14–17,19]. It was suggested that TUS lyses clots by increasing drug transport [3]. At low frequencies, its efficacy may even increase and collateral damage may be reduced due to higher tissue permeability and lower thermal effect [3]. Finally, it was suggested that TUS may also create structural changes of proteins [3]. All these effects were correlated with the intensity of the sound wave and the duration of exposure [16,17].

Nevertheless, up to now, TUS is not commonly used in the clinical arena. Its main disadvantage relies on the uncertainty regarding the optimal TUS profile. The TUS profile is a combination of several variables: the type of the ultrasound transducer, the intensity of the sound wave, US frequency, duty cycle, duration of treatment, *etc.* [12,16,17,20].

We hypothesized that different TUS profiles of intensity and frequency will have different efficacy on clot lysis. We therefore attempted to find an optimal combination of intensity and frequency that will increase the flow in a blocked tube by building an *in-vitro* system model of a clotted blood vessel.

2. Material and methods

Blood clots from rat blood were prepared within sections of artificial grafts used for human vascular surgery. Each graft was attached to a closed circular flow system, according to a known model (Fig. 1) in which saline (0.9% NaCl) was recirculated [20]. For each clot, we applied one of several ultrasonic treatment profiles that differ in intensity and frequency (Table 1). The weights of the clots, before and after the treatment, were measured, and from these measures the percentage of clot decomposition was calculated according to the following formula: $\frac{(initial\ weight - final\ weight)}{initial\ weight} \times 100$. The saline flow rate was recorded before and after treatment by Laser Doppler system (Advance laser flowmeter alf21d, Tokyo, Japan), and the difference between the final flow rate and the initial flow rate during the experiment was calculated.

2.1. Blood clots and graft sections preparation

Eight-mm diameter polytetrafluoroethylene (PTFE) grafts were cut into 5 cm sections. Each graft was sealed by two Luer locks and Cyanoacrylate glue. The graft was clumped in the center, and above the clump the clot was artificially created. Specifically, 0.7 ml of rat blood with 0.3 ml $CaCl_2$ solution at 2 M concentration was injected into the graft for activation of clotting reaction. The graft was placed vertically for 16 h at room temperature. During this time a clot that blocked the entire diameter of the tube was formed. The purpose of the graft blocking (the entire diameter) was to reduce the flow through the graft in the initial conditions and thus simulate a completely blocked blood vessel. Before connecting the graft to the system, the remaining empty space was filled with 1.5–2 ml of 0.9% saline to prevent air from entering the system.

2.2. The flow system

The flow system (Fig. 1) consisted of a peristaltic pump (MasterFlex L/S, Illinois, USA), which was connected to a pipe system (total volume of 40 ml). Luer valves were assembled in the flow system to enable three flow directions. By the split, we created a detour, which enabled the saline to flow in the system even when the graft was completely blocked, thus prevented the clot from breaking by the hydrostatic pressure which might have been created in the system.

In the flow-line to which the graft was connected, two tubes were attached with Luer adapters that enabled the graft to be connected to the system. After attaching the graft, we placed it on the bottom of a water bath directly above the ultrasonic transducer for TUS (see below). In the continuation of this branch a flow sensor was placed (Fig. 1).

2.3. The treatment system

2.3.1. Preparation

We performed the ultrasonic treatment in the water medium. The water medium contained degassed water, which transfer the ultrasonic energy more efficiently due to the absence of bubbles in the water. For this purpose, a bath made of polycarbonate, which is transparent to the ultrasonic energy, was filled with 2 l of double distilled water (DDW). To remove the gas bubbles from the water, the water was pumped through two 10 μm diameter filters (CellTrics® 10 μm, Sysmex Partec GmbH, Goerlitz, Germany) for about 12 h at a rate of 200 rpm.

2.3.2. TUS Assembly

The ultrasound system was composed of a signal generator (50MS/s Single-Channel Arbitrary Waveform/Function Generator, Lahat Technologies, Kfar-Saba, Israel) that generates electrical signals according to the treatment profiles (Table 1). The electrical signal was simultaneously transmitted to a scope (Picoscope 5243A 100 MHz, Pico Technology, Cambridgeshire, United Kingdom) and to the ultrasonic transducer that turns the electrical signal to the ultrasonic signal. We transferred the signal from the generator, as required, through an amplifier (RF power amplifier, Electronics & Innovation, Rochester, NY, USA) to increase the signal intensity (Table 1). Finally, the ultrasonic transducer was attached to the bottom of the water bath after being smeared with ultrasonic gel (Konix Ultrasound gel, Turkiaz, Istanbul, Turkey).

2.3.3. The treatment profiles

During the treatment, the saline was rotated in the closed circuit by the peristaltic pump at constant, low velocity of 10 rpm (the low velocity was aimed to prevent it from decomposing the clot by itself). The transducer (V318-SU, Olympus, Walthman, MA, USA) was operated in one of the 6 profiles that are listed in Table 1. Each treatment lasted 93 min in total, composed of 12 repetitions, divided into 5 min of activity and 3 min of intermissions. During the 5 min of activity the transducer worked continuously (continuous wave, duty cycle −100%). For control purposes, clots were assembled to the system in the same manner, but without ultrasound treatment (profile 0).

In order to evaluate the efficacy of the TUS, the weight of each clot was measured before and after the treatment. Subsequently, the percentage of clot lysis was calculated. The flow at the beginning of the experiment was measured (with the assembly of the clot into the

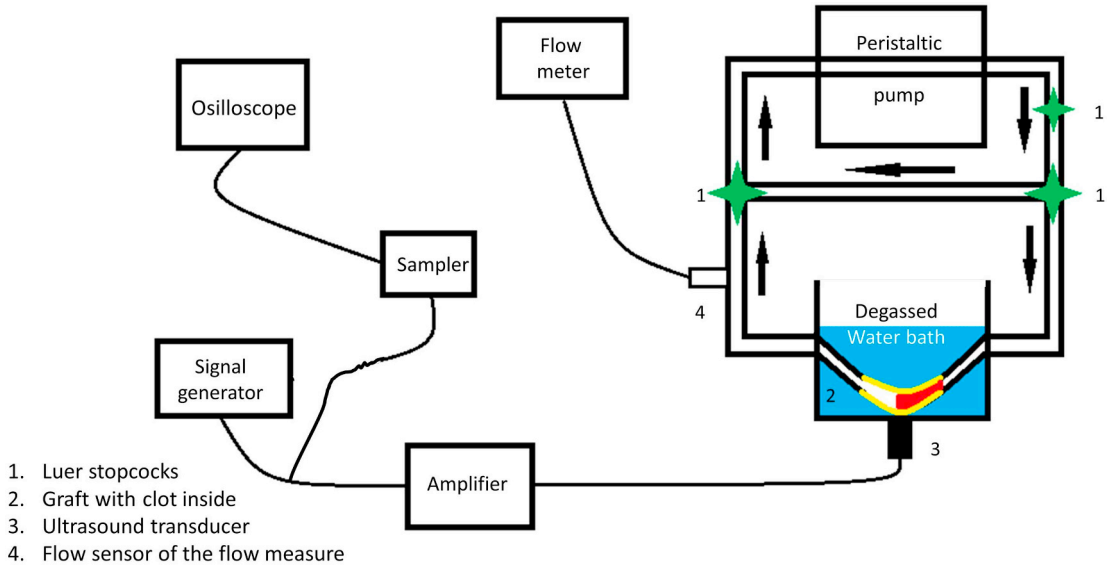

Fig. 1. The experiment system.

system) and at the end of treatment, from these results, the change in flow through the experiment was calculated as the difference between the final flow and the initial one.

2.4. Statistical analysis

All data presented as mean ± SD. To compare the treatment groups, an analysis of variance (ANOVA) in which the profile treatment was the independent variable and the measures (the percentage of change in the clot weight and the change in flow) were the dependent variables. The initial and final clot weights and the initial and final flow velocities were examined by ANOVA with repeat measurements and two independent variables (measurement time and ultrasound treatment profile). The probability of type 1 error which was < 5% ($P < 0.05$), was considered as statistically significant.

3. Results

Forty-six clots were assembled in the system (Fig. 1) and treated with TUS. The clots were randomly assigned to one of 7 groups (Table 1) according to the treatment protocol (n = 7 in groups 0–3 and n = 6 in groups 4–6).

The mean initial clot weight was 0.318 ± 0.129 g, with no significant difference between the groups ($P > 0.05$) (Fig. 2A). The mean weight of clots at the end of TUS was 0.15 ± 0.05 g, 0.16 ± 0.06 g, 0.09 ± 0.07 g, 0.21 ± 0.09 g, 0.17 ± 0.09 g, 0.17 ± 0.07 g and 0.18 ± 0.02 g in groups 0 through 6, respectively. Only in groups 1, 2, 3 and 5, there was a significant ($P < 0.05$) decrease in the weight of the clot following TUS (Fig. 2A and B).

In the calculation of the % of clot lysis, an insignificant, but worth mentioning, increase in clot weight was demonstrated in the control group (group 0) (Fig. 2C), probably due to absorption of water into the clot in the experimental setup (see Material and methods section). Specifically, in this group, clot weight increased by 9.46 ± 70.63%. In contrast, in the TUS groups (1–6) a decrease in clot weights were noted. Explicitly, −33.80 ± 41.21%, −75.37 ± 19.99%, −41.90 ± 12.63%, −45.45 ± 12.59%, −42.20 ± 20.00% and −26.75 ± 14.56% in groups 1–6, respectively. Statistically, the change in the weight of the clot was highest in group 2, which was also significantly different from the control group ($P < 0.001$, Fig. 2C).

An initial flow of 0.53 ± 0.31 ml/min was recorded at baseline at all groups ($P > 0.05$). At the end of the treatment, an average flow of 4.2 ± 3.01 ml/min was observed in the control group, flow of 30.61 ± 19.76 ml/min in group 1, 52.1 ± 25.44 ml/min in group 2, 28.78 ± 8.15 ml/min in group 3, 43.93 ± 20.03 ml/min in group 4, 40.86 ± 18.25 ml/min in group 5 and 45.10 ± 22.20 ml/min in group 6. All the groups undergoing ultrasonic therapy (1–6) had a significantly increased flow ($P < 0.05$, within group 3, $P < 0.01$ within group 1 and $P < 0.001$ within groups 2, 4, 5 and 6, relative to the initial flow of the same group). Furthermore, in groups 2, 4, 5 and 6, a greater flow was observed relative to the control group (0, $P < 0.05$ *vs.* groups 4, 5 and 6 and $P < 0.001$ *vs.* group 2) (Fig. 3C). The flow in group 2 was also increased relative to groups 1 and 3 ($P < 0.05$) (Fig. 3A and B).

In an attempt to find a correlation between clot weight change and flow rate, we presented them on the same Cartesian coordinates (Fig. 4). Specifically, percent change in clot weight was set on the abscissa while the percent flow change was set on the ordinate. Finally, Fig. 4 depicts the correlation between the level of clot lysis and the change in flow rate. A statistically significant correlation of $R^2 = 0.76$ ($P < 0.05$) was found between the two parameters. The relation between clot lysis and flow increase can be formulated by the following

Table 1
Ultrasound treatment profiles.

Treatment #	Frequency [MHz]	Intensity [W/cm^2]	Intensity after amplification [W/cm^2]	Remarks
0	0	0	0	Control
1	0.3	0.75	0.75	Without amplifier
2	0.5	1.5	1.5	Without amplifier
3	0.7	3	3	Without amplifier
4	0.3	0.75	237.5	With amplifier 50 dB
5	0.5	1.5	475	With amplifier 50 dB
6	0.7	3	950	With amplifier 50 dB

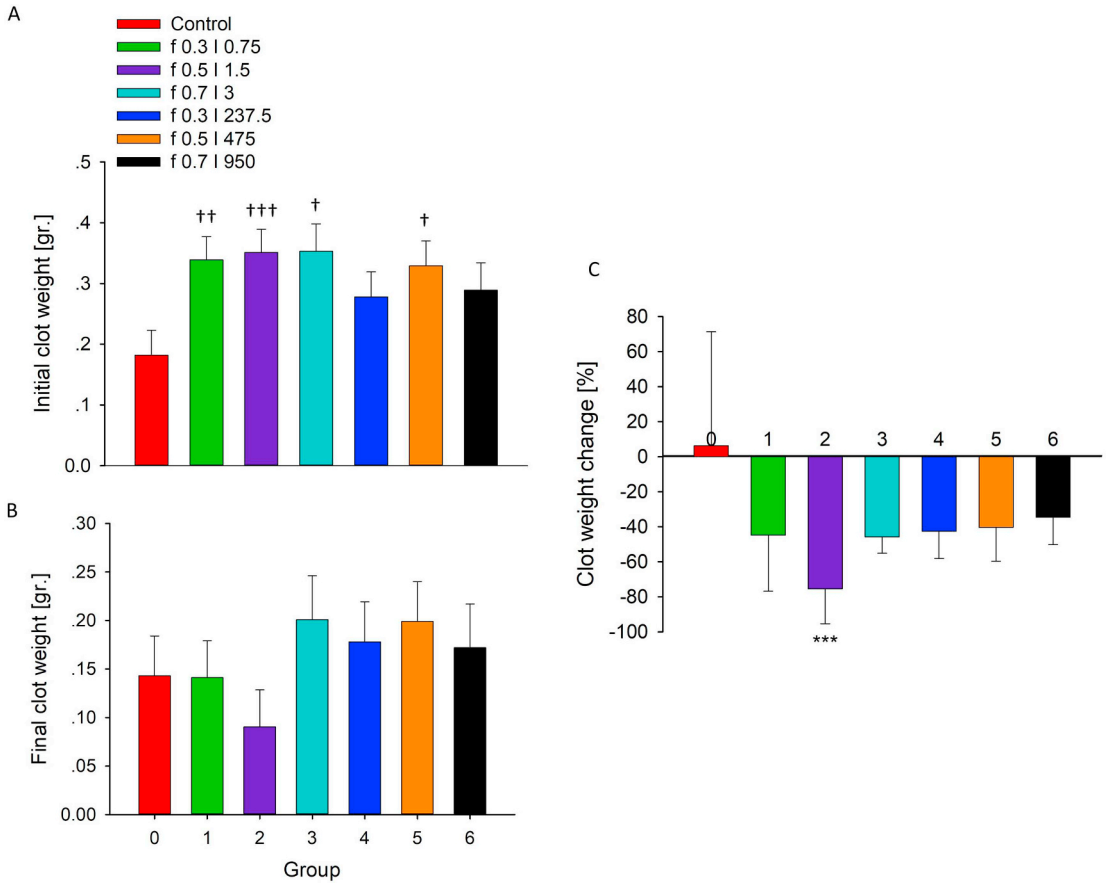

Fig. 2. The weight of blood clots. A) The weight of blood clots in the various groups before treatment, B) after treatment C) percentage of change in clot weight. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ *vs.* after treatment (A *vs.* B, in the same treatment group). ***$P < 0.001$ compared with the control (0) group).

equation (Fig. 4):

Flow change (in%) = 2.34 × weight change (in%) − 13.65.

4. Discussion

We studied a number of TUS profiles, differing by their frequency and intensity, for their efficacy on clots lysis. For this purpose we built an *in-vitro* system that simulates a blood clot inside an artificial blood vessel in a closed flow system. We found that TUS, in the ranges examined, without the administration of any fibrinolytic drug, effectively lysed clots as compared to the control group (group 0, no treatment), while all the groups treated with TUS showed a significant increase ($P < 0.05$) in flow rate at the blocked branch of the *in-vitro* system. Further, among the TUS profiles, some had higher efficacy than others. In addition, all the groups that underwent TUS exposure showed a decrease in clot weights compared with the control group, in which the clot weight even increased.

Examining the difference between the various protocols, we have noted that a frequency of 0.5 MHz and intensity of 1.5 W/cm^2 (Group 2) resulted in the most significant improvement in flow rate compared to the control group ($P < 0.01$), and also showed the most significant decrease in weight clot *versus* the baseline weight ($P < 0.01$). Moreover, this specific profile had higher efficacy than the 0.3 MHz 0.75 W/cm^2 and the 0.75 MHz 3 W/cm^2 profiles. Finally, we found a positive correlation between the level of clot lysis and the increase in the flow rate it induces in the system (Fig. 4). We assume that this protocol (0.5 MHz, 1.5 W/cm^2) may also be the most likely to lyse clots most effectively in *in vivo* systems as well.

Many hypotheses were raised regarding the TUS mechanism of action on blood clots dissolution. As mentioned above, ultrasound has both thermal and mechanical effects. These effects depend on both the sound wave frequency and intensity. It is commonly assumed that the thermal effect of TUS increases enzyme activity in tissues, may alter the structure of enzymes, transforms them from their inactive form to an active one, promotes protein production and even affects the cell membrane [14,15]. Fischell et al. also demonstrated a vasodilatatory effect in the ultrasound treated tissue arterioles [21]. This may be an advantage when treating atherosclerotic patients who suffer from endothelial dysfunction that significantly reduces the flow rate in a diseased artery [14,15]. Certainly, all these effects are missing in our artificial system.

The mechanical effect is reflected in the acoustic pressure generated by the TUS. It is described using the following formula: P^2/f = constant, where f is the frequency, and P is the rarefactional pressure. According to this formula – the lower the frequency, the tissue stress will increase [22].

In studies currently being conducted, mainly for the treatment of stroke, researchers are using imaging ranges of TUS (*i.e.* 2 MHz). It was conjectured that there is an advantage of using the therapeutic range of < 1 MHz. Studies have shown that low-frequency TUS (under 1 MHz) has a better permeability to tissue [23,24], allowing higher levels of energy to reach the target [17]. Moreover, when using the range of kHz values there is less thermal effect [14].

In contrast to these advantages, as the TUS frequency decreases, the acoustic pressure increases [22], and may cause more cavitation and locally damage the clot. For example, in the TRUMBI clinical trial [25], which examined the efficacy of TUS in conjunction with tPA for treatment of stroke, a frequency of only 300 kHz was used. This study was discontinued prior to termination due to an increase in cerebral

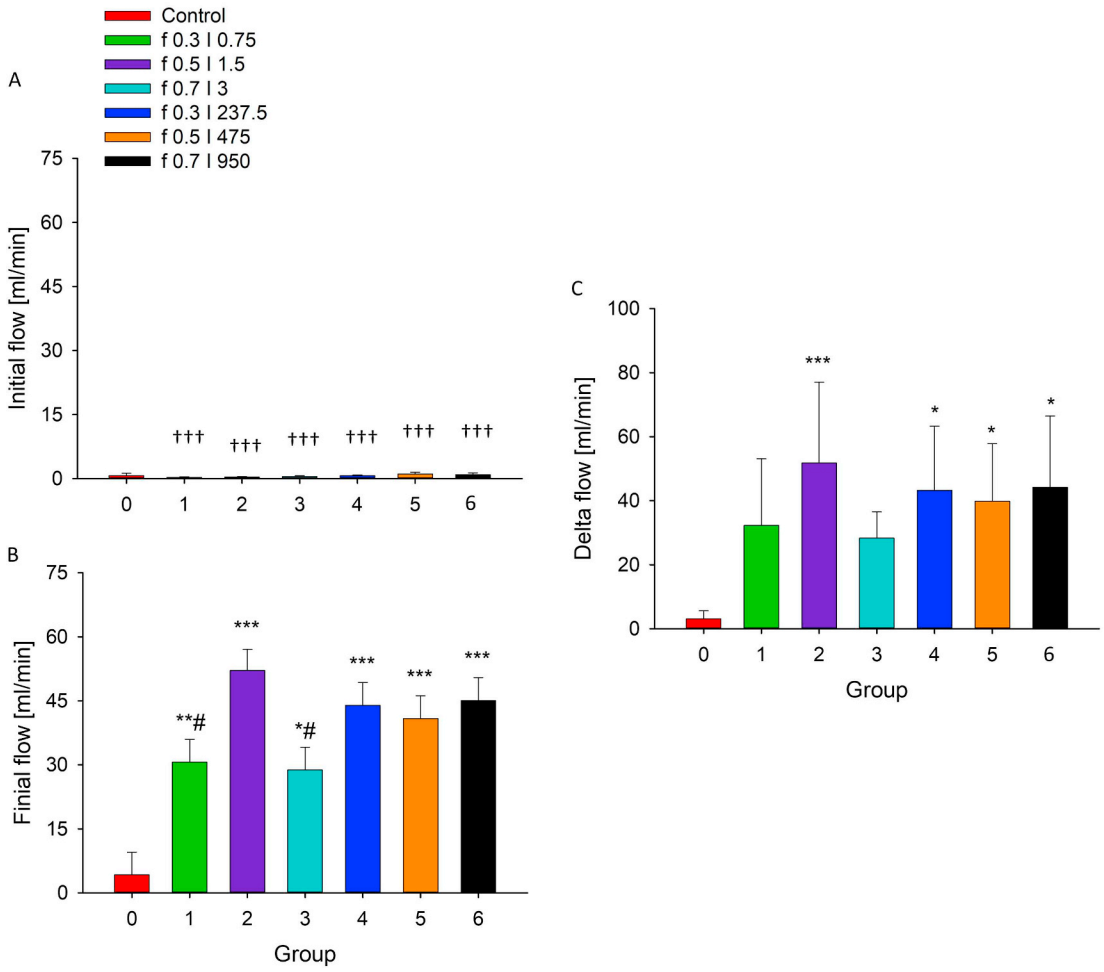

Fig. 3. Saline flow in the system. A. Primary flow in the system in the various groups, before treatment, B. After treatment, C. The flow change. $^{\dagger\dagger\dagger}P < 0.001$ *vs.* post-treatment (A *vs.* B, in the same treatment group). $^{*}P < 0.05$ *vs.* control group, $^{**}P < 0.01$ *vs.* control group, $^{***}P < 0.001$ *vs.* control group. $^{\#}P < 0.05$ compared with treatment no. 2.

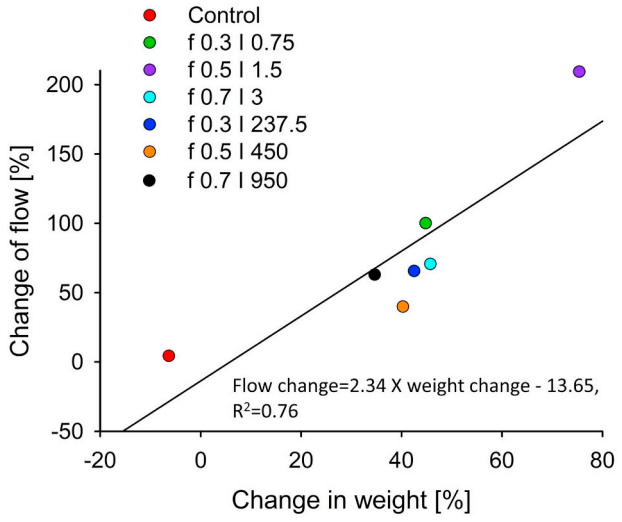

Fig. 4. Change of flow is presented as a function of % change in clot weight. Regression is significant $P < 0.05$.

hemorrhage [23,24,25].

In our experiment, the TUS protocol that showed superiority over the other protocols was the 500 kHz (0.5 MHz), which is consistent with other experiments that showed efficacy on clot lysis at frequencies of 490 kHz and 490.6 kHz, although at lower intensities (0.2 W/cm^2) [17,22]. Furthermore, in our study we showed that intensity (I) has only a minute effect on clot lysis efficacy as shown by comparing the results between group 2 (1.5 W/cm^2) and group 5 (475 W/cm^2) (~300-fold intensity increase). In the use of high levels of TUS, the acoustic effect, *i.e.* the clot shaking, leads to significant dissolution into smaller fractions, which may lead to a secondary dissolution of blood clots downstream, leading to possibly additional downstream obstructions. Therefore, it is reasonable to prefer working at lower intensity levels [6,22].

Nevertheless, further research is needed to investigate the efficacy of the TUS treatment at the current frequency (500 kHz) with additional intensities and other factors affecting the TUS profile (duty cycle, pulse repetition frequency, number of treatment sessions, *etc.*). The 500 kHz frequency is in a range that may be free of the risks associated with the use of high frequency values, but higher than the range of values for secondary signs of damage (for example, brain hemorrhage in the TRUMBI study mentioned above).

5. Study limitations

This is an *in-vitro* experiment; accordingly, it models the clinical scenario in an artificial system. The relation between our system and the *in-vivo* environment is yet to be determined. This study dealt with one particular aspect of TUS, its ability to dissolve a blood clot within an artificial vascular graft. Our model has several technical limitations:

A. the clot and the TUS transducer were both static, one in front of the other, for over 90 min; B. such proximity can be found only with superficial clots (with *e.g.* clotted hemodialysis shunts); C. The efficacy of this treatment should be examined in shorter time intervals, so that it can be adapted to *in-vivo* experiments. We did not test other TUS parameters, such as different duty cycles and treatment durations, but these are planned for the near future. Specifically, we have studied thrombi formed nearly 16 h prior to treatment. Therefore, our results may not be applicable to thrombi of longer duration; further effective 'time post thrombus formation' was not addressed yet.

6. Clinical implications

The current study, though aimed at assessing the *in vitro* efficacy of TUS on clot lysis, has also clinical significance. As elaborated in the 'Material and methods' section, the clots were prepared within a polytetrafluoroethylene (PTFE) grafts which are frequently used for several indications. Among those, probably the most relevant, may be an A-V fistula (AVF) used in pre-dialytic patients. Stolic, in 2003, summarized the most important chronic complications of AVFs for hemodialysis [26]. According to his manuscript, thrombosis accounts for 17–25% of AVFs complications only second to stenosis (14–42%). Additionally, many of the AVFs include PTFE graft as well.

Another relevant medical problem that may be addressed TUS is superficial vein thrombosis (SVT). SVT may be hazardous, as it might develop into pulmonary embolism (PE) or deep vein thrombosis (DVT) [27]. SVT's prevalence is estimated to be twice the prevalence of PE and DVT combined. Furthermore, it is estimated that 6%–36% of patients with SVT may develop DVT as well [27].

Finally, both AVFs and SVTs are, by definition, superficial, thus very close to the skin. To our opinion, these are potential targets for the developed method.

7. Conclusions

In this study we showed that TUS, without the use of a fibrinolytic drug, is an effective method for clot lysis. Low-frequency TUS is more effective than high-frequency, and that using a frequency of 500 kHz has the highest efficacy. In addition, no additional benefit can be attributed to the TUS intensity, as even ~300-fold intensity increase, did not improve clot lysis.

Additional research is needed to test the efficacy of this frequency in combination with fibrinolytic drugs to enable lowering doses of the drug, thus raising its safety profile, and examining whether there is synergy between fibrinolytic therapy and TUS.

Statement of conflict of interest

The authors have no conflict of interest.

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