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# Ultrasound-Induced Blood Clot Dissolution without a Thrombolytic Drug Is More Effective with Lower Frequencies

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**Key Words**
Therapeutic ultrasound · Thrombolysis · Frequency dependence · Stroke

**Abstract**
***Background and Purpose:*** Therapeutic ultrasound as stand-alone therapy or in combination with rt-PA has proven to be an effective measure for recanalisation of acute vessel occlusion in different in vitro and in vivo studies. Uncertainty still exists concerning the optimal frequency and intensity with regard to the thrombolytic efficacy of ultrasound. The purpose of this study was a direct comparison of different ultrasound frequencies, when otherwise using identical measurement settings and parameters. ***Methods:*** Ultrasound-induced dissolution of fresh human blood clots was studied in a flow system using low-frequency continuous wave ultrasound of 20, 40 and 60 kHz. After calibration of each ultrasound probe, blood clots were exposed to local time average intensities of either 0.12 or 0.2 W/cm². Exposure time of the clots to ultrasound was 10 min, the number of treated clots in each experimental group was 12. ***Results:*** As tested with 0.2 W/cm², we found the most pronounced thrombolytic effect with the 20-kHz probe (weight loss of blood clots: 52.4%) and the 40-kHz probe (49.4%), as compared to the 60-kHz probe (21.4%) and the control group (18.5%). The difference between the 20- and 60-kHz probes was statistically significant ($p < 0.001$). The treatment effect was clearly intensity dependent with a less pronounced, but still significant treatment effect at 0.12 W/cm² (24.5% at 20 kHz; $p < 0.001$ compared to 0.2 W/cm²; $p = 0.045$ compared to controls). ***Conclusions:*** These data show that therapeutic efficacy of ultrasound, in absence of a thrombolytic drug, is frequency and intensity dependent with best results at low frequencies. With continuous wave transmission, the benefit may be limited to the very low frequency range. The results are a basis for further evaluation in animal models.



## Introduction

There is strong experimental evidence that application of therapeutic ultrasound improves time to and rate of recanalisation in acute vessel occlusion, as compared to a fibrinolytic therapy with rt-PA. Different in vitro and animal studies have shown a thrombolytic potential of ultrasound in combination therapy with rt-PA as well as in stand-alone therapy [1–4]. Thrombolytic efficacy is de-

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**Table 1.** Influence of different ultrasound frequencies on thrombus weight loss

| Ultrasound frequency | Intensity level | Weight loss[1], % | Wilcoxon p value vs. control | Initial thrombus weight[2], mg | Residual thrombus weight[2], mg |
|---|---|---|---|---|---|
| Control | – | 18.5 (16.1–25.1) | – | 488 ± 31 | 386 ± 34 |
| 20 kHz | 0.2 | 52.4 (40.2–59.5) | <0.001 | 447 ± 43 | 213 ± 72 |
| 40 kHz | 0.2 | 49.4 (32.1–55.4) | <0.001 | 494 ± 51 | 270 ± 81 |
| 60 kHz | 0.2 | 21.4 (16.4–33.3) | 0.378 | 463 ± 52 | 351 ± 75 |
| 20 kHz | 0.12 | 24.5 (20.6–39.7) | 0.045 | 476 ± 29 | 340 ± 55 |
| 40 kHz | 0.12 | 29.8 (24.4–42.5) | 0.006 | 537 ± 44 | 362 ± 48 |
| 60 kHz | 0.12 | 24.4 (20.3–21.6) | 0.078 | 462 ± 69 | 342 ± 35 |

Ultrasound exposure time in each group was 10 min. The number of blood clots at each experimental group was 12.

[1] Values are medians and quartiles.

[2] Values are means ± SD.

pendent on intensity and on duration of exposure [5]. Combination with an ultrasound contrast agent may further enhance the therapeutic effect [6–9]. There are reports on clinical studies that have shown an improvement of recanalisation rates and clinical outcome of stroke patients treated with rt-PA when diagnostic ultrasound was applied simultaneously [10–12]. The progress in basic research and in preliminary clinical applications of therapeutic ultrasound has recently been reviewed [13].

However, there is still a great amount of uncertainty towards the optimal ultrasound frequency. Lower frequencies have the advantage of reduced heating and of improved tissue and bone penetration, which may be of special importance in transcranial application.

In combination with rt-PA, i.e., as an enhancement of pharmacological thrombolysis, in vitro studies suggest that lower-frequency (kHz) ultrasound applications may also have a superior effect as compared to higher (MHz) frequencies [14, 15].

Low-frequency ultrasound leads to significant clot dissolution even in the absence of a thrombolytic drug. This effect is used in catheter-based ultrasound thrombolysis [16]. Effective clot dissolution also occurs when ultrasound is applied over a distance, with a more pronounced effect in the presence of an echo contrast agent [4–6, 17]. This opens up the possibility of external, i.e., non-invasive application of therapeutic ultrasound without a thrombolytic drug in acute vessel occlusion. A preferable frequency range for this application has not yet been identified. However, identification of an optimal frequency will be critical in developing externally applied ultrasound for therapeutic purposes.

Therefore, the aim of this study was a direct comparison of the thrombolytic potential of different ultrasound frequencies, when otherwise using identical parameters.

## Materials and Methods

*Thrombus Formation*

Whole human blood was drawn into 1-ml syringes prepared with 10 IU of thrombin (Amersham Pharmacia, USA; 1 unit is approximately equivalent to 0.2 NIH units). The blood was allowed to clot for 3 h at room temperature at 15 rpm, resulting in stable red blood clots with a mean thrombus weight of 480 ± 53 mg.

Before experiments, each thrombus was washed three times in sodium phosphate buffer. Supernatant buffer was carefully removed. The clot weight was determined directly before and after each insonation experiment. The extent of thrombolysis is expressed as a percentage of decrease in thrombus weight. Additionally, mean values of the initial and the residual thrombus weight of the different experimental groups are stated in table 1. Statistical calculation is based on the median of the percent weight loss of the different experimental groups. The age of the blood clots at the beginning of the insonation experiments varied from 3 to 6 h. To achieve comparability, the blood clots of the different experimental groups were age matched.

*Experimental Set-Up and Calibration*

We used 20-, 40- and 60-kHz ultrasound generators (KLN Ultraschall GmbH, Heppenheim, Germany). The generators operate in a continuous wave mode at variable intensity outputs. For each experiment, the corresponding transducer was arranged in an experimental chamber as previously described [5]. In short, the blood clot was placed on a thin, acoustically transparent polyethylene membrane held by a glass cylinder (3.5 cm inner diameter), which then was placed in a larger water tank (20 × 24.5 × 16 cm). The distance from the transducer surface to the blood clot was 3 cm in all experiments.

Experiments were carried out in degassed 0.02 *M* sodium phosphate buffer at pH 7.4. The whole set-up was arranged as a flow system with a flow rate of the buffer of 15 ml/min. The temperature was adjusted to 37°C at the site of the clot. For each transducer, the ultrasound effect was studied at different local time average intensities (0.12 and 0.2 W/cm²) at an exposure time to ultrasound of 10 min (all transducers) and 20 min (20 and 60 kHz).

The number of tested thrombi in each experimental group and the control group was 12. As control, thrombi were placed in the flow system and exposed to the buffer without insonation. All experiments were performed separately with a single thrombus in the glass cylinder. After the experiment, the residual blood clot was carefully removed by cutting the polyethylene membrane, after gently lifting the experimental chamber out of the water tank. Without regarding ultrasound exposure times, blood clots in all experimental groups and in the control group were left in the flow system for 20 min.

In order to achieve comparable local time average intensities for each ultrasound generator, the acoustic fields of the different transducers were measured by the use of a calibrated needle-type hydrophone (hydrophone TC4013, Reson, Kiel, Germany). The transducer face was placed directly under the surface of a water tank and the hydrophone arranged at the experimental distance of 3 cm. From the resulting continuous sinus wave, time average intensity was calculated. The intensity output of the generators was adjusted so as to result in local time average intensities of 0.12 and 0.2 W/cm².

*Statistical Analysis*

Data description was based on means and standard deviations for normally distributed measurement series, but on medians and quartiles for measurement series with outliers (which were observed for weight reduction distributions in most constellations). Graphic representations were based on box-whisker plots, accordingly. Significance comparisons were performed by means of two-sample Wilcoxon tests, whose results were summarized as p values. $p < 0.05$ was regarded as an indicator of local statistical significance.

## Results

The use of continuous-wave ultrasound at 20 kHz resulted in a significant thrombolytic effect at the intensity level of 0.2 W/cm² and a 10-min exposure to ultrasound. The median weight loss of the blood clots was 52.4% (interquartile range 40.2–59.5%), as compared to a control group with blood clots that showed a spontaneous median weight loss of 18.5% (interquartile range 16.1–25.1%; $p < 0.001$). This effect was clearly intensity dependent with a less pronounced, but still significant treatment effect at the reduced intensity level of 0.12 W/cm² (median reduction 24.5%, $p = 0.045$ vs. controls). The difference between the two intensity levels was statistically significant ($p < 0.001$).

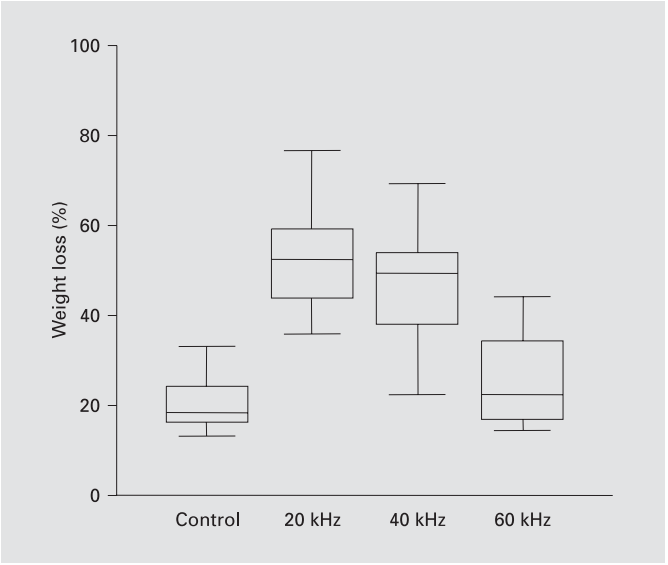


**Fig. 1.** Box-whisker plots for the distribution of weight reduction to illustrate the effect of different ultrasound frequencies on clot dissolution (0.2 W/cm², 10 min exposure time). Compared with the control group, the treatment effect was statistically significant at 20 and 40 kHz ($p < 0.001$). The observed frequency-dependent decrease in efficacy was highly significant ($p < 0.001$ from 20 to 60 kHz).

Thrombolytic efficacy decreased with increasing ultrasound frequencies. At the intensity level of 0.2 W/cm², 10 min exposure to ultrasound at 40 kHz still resulted in a statistically significant weight reduction of the blood clots (49.4%; $p < 0.001$ compared to control). However, there was no statistically significant treatment effect observed at 60 kHz (21.4%, $p = 0.378$ vs. controls; fig. 1). This decrease in efficacy with increasing frequencies showed a non-significant trend from 20 to 40 kHz, but was highly significant between 40 and 60 kHz ($p < 0.001$) and between 20 and 60 kHz ($p < 0.001$; table 1).

Similar to the results with the 20-kHz ultrasound probe, the treatment effect at 40 kHz was dependent on the chosen intensity level. The difference between the observed thrombus weight reductions at 0.12 W/cm² (29.8%, 24.4–42.5%) and at 0.2 W/cm² (49.4%, 32.1–55.4%) was statistically significant ($p = 0.016$). At 60 kHz and 10 min exposure to ultrasound, no significant treatment effects were found at any of the two tested intensity levels.

The use of ultrasound at the lower intensity of 0.12 W/cm² still showed a significant weight reduction at 20 and at 40 kHz as compared to control, but in general revealed far less pronounced treatment effects (table 1).

At a prolonged 20-min exposure time to ultrasound, a comparable frequency dependency was observed. At 0.2 W/cm$^2$, there was a significant treatment effect for both, 20 kHz (66.2%, 48.2–76.5%) and for 60 kHz (31.2%, 24.8–44.2%; $p < 0.001$ and $p = 0.006$ vs. controls, respectively). The difference in efficacy of clot dissolution between 20 and 60 kHz was statistically significant ($p < 0.001$).

## Discussion

This study demonstrates that ultrasonic clot dissolution is frequency dependent with best results at very low frequencies. In our experimental setting, ultrasound transducers of three different frequencies were compared towards their therapeutic efficacy. A set-up was chosen with constant operating parameters, including identical local time average intensities for each ultrasound transducer. Thus, the influence of ultrasound frequency on clot dissolution was directly studied. The direct comparison revealed superior efficacy of 20 kHz ultrasound, as compared to 40 and 60 kHz. This effect was consistently observed at different ultrasound exposure times (10 and 20 min).

Safe and effective application of therapeutic ultrasound requires optimised technical and procedural parameters. More important and interesting aspects include the question about an optimum ultrasound frequency, since different ultrasound frequencies have some very different characteristics as to their interactions with tissue. Tissue and bone penetration is much better with low frequencies, allowing higher ultrasound energy levels at the site of the vessel occlusion [14, 18]. This may be of special importance in non-invasive transcranial application of therapeutic ultrasound, which requires transmission of ultrasound with sufficient energy through bone and brain tissue. This transcranial approach, which may be understood as a potential alternative or extension of established treatment strategies in acute cerebral vessel occlusion, has become an interesting field of research. Using low-frequency (33 kHz) ultrasound, Behrens et al. [19] have shown a significant transcranial augmentation of rt-PA mediated fibrinolysis in an in vitro model. These results were confirmed in a transcranial rat model of embolic ischemic stroke (25.6 kHz) [20] and, using mid-kHz ultrasound, in a rabbit femoral artery model with transosseous application of 490 kHz ultrasound [21].

Besides possible advantages of lower frequencies as to their tissue penetration characteristics, only very little is known about differences in treatment effect. In combination with rt-PA, i.e., as an enhancement of pharmacological thrombolysis, an in vitro study suggested that lower-frequency ultrasound (kHz) may be superior to higher (MHz) frequencies [15]. However, this study did not check for identical intensity levels at the site of the clot. Since reported intensity levels were higher at the lower-frequency probe, this may have led to a relative overestimation of the effect at 185 kHz as compared to 1 MHz.

The potential use of non-invasive therapeutic ultrasound as 'stand-alone' therapy, i.e., without concomitant use of rt-PA, may well be limited to the lower-frequency ranges. In a previously published study, using 20 kHz continuous-wave ultrasound, we found that a significant treatment effect with statistically significant weight reduction of treated blood clots could be achieved with low ultrasound intensities of about 0.15 W/cm$^2$, even when applied over a distance of several centimetres. The treatment effect showed an intensity dependency. A complete dissolution of blood clots was found at 1.2 W/cm$^2$ [5]. The results of our study demonstrate that application of therapeutic ultrasound may be most efficacious in the very low frequency range. We found a significant increase in efficacy from 60 to 20 kHz. Moreover, the intensities required for treatment effects were relatively low. In a safety study in rats, ultrasound exposure at this intensity level resulted in no relevant increase in intracranial temperature [22] and no evidence of cerebral damage in histology and on MRI (20 min transcranial insonation with continuous-wave ultrasound at 20 kHz) [23]. We therefore conclude that the use of very low frequencies may be preferential in testing therapeutic ultrasound as 'stand-alone' therapy in animal models.

Our study did not test for specific treatment effects of higher (mid-kHz and MHz) frequencies. Comparison of low- and mid-kHz with MHz ultrasound will be an important question for future (animal) studies, as clinical data with diagnostic MHz Doppler and duplex probes are promising [10–12]. Unwanted side effects of ultrasound therapy are not only dependent on intensity, but may also strongly depend on the chosen frequency. Cavitation phenomena, for example, are more likely to occur at lower frequencies. Furthermore, side effects may arise from the formation of standing waves after reflexion of sonic energy once the ultrasound passed the brain and before it exits the skull. Due to specific tissue penetration characteristics, the formation of standing waves may be a distinct problem of lower-frequency applications.

Therefore, future studies comparing the different frequency ranges should be designed with regard to specific

frequency-dependent intensity thresholds for the occurrence of side effects. A model mimicking biological conditions would require the use of an undamaged skull. In a first step, these questions can be addressed most appropriately in experimental animal models [20]. In a second step, these data must be transferred to research on human tissue.

Our study was designed to investigate treatment effects of continuous-wave ultrasound. Specific differences in treatment efficacy due to the application of ultrasound in a pulsed wave mode will have to be taken into consideration in future studies.

A key question will be whether ultrasound, in a magnitude which is sufficient for a satisfactory treatment effect, is safe as to the occurrence of unwanted side effects. Animal studies are a prerequisite before extending treatment studies into a clinical setting.

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