Can a Commercial Diagnostic Ultrasound Device Accelerate Thrombolysis? An In Vitro Skull Model

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Background and Purpose—Recently, 3 clinical trials revealed encouraging results in recanalization and clinical outcome in acute stroke patients when 2-MHz transcranial Doppler monitoring was applied. This study investigated whether a 1.8-MHz commercial diagnostic ultrasound device has the potential to facilitate thrombolysis using an in vitro stroke model.

Methods—Duplex-Doppler, continuous wave-Doppler, and pulsed wave (PW)-Doppler were compared on their impact on recombinant tissue plasminogen activator (rtPA)–mediated thrombolysis. Blood clots were transtemporally sonicated in a human stroke model. Furthermore, ultrasound attenuation of 5 temporal bones of different thickness was determined.

Results—In comparison, only PW-Doppler accelerated rtPA–mediated thrombolysis significantly. Without temporal bone, PW-Doppler plus rtPA showed a significant enhancement in relative clot weight loss of 23.7% when compared with clots treated with rtPA only (33.9/5.5% versus 27.4/5.2%; \( P < 0.0005 \)). Ultrasound attenuation measurements revealed decreases of the output intensity of 86.8% (8.8 dB) up to 99.2% (21.2 dB), depending on temporal bone thickness (1.91 to 5.01 mm).

Conclusion—Without temporal bone, PW-Doppler significantly enhanced thrombolysis. However, because of a high attenuation of ultrasound by temporal bone, no thrombolytic effect was observed in our in vitro model, although Doppler imaging through the same temporal bone was still possible. (Stroke. 2005;36:124-128.)

Key Words: middle cerebral artery stroke thrombolysis ultrasonography, Doppler, tranascranial

Noninvasive high-frequency ultrasound (2 MHz) has been shown in several in vitro studies to accelerate thrombolysis enzymatically.\(^1\)\(^-\)\(^3\) However, these studies used transducers not certified for in vivo application. Limitations for in vivo usage of high-frequency ultrasound are potential tissue damage caused by heating,\(^4\)\(^-\)\(^5\) when applied at high ultrasound intensities, and further, a limited penetration of tissues, for example, the cranial bone.\(^6\)\(^-\)\(^7\) Advantages are a high-resolution for imaging and a possible combined diagnostic and therapeutic in vivo application using the same transducer,\(^8\) which could be implemented by the usage of commercially available ultrasound devices following US Food and Drug Administration output intensity limitations (maximum 720 mW/cm\(^2\)) to guarantee a safe application.

Currently, systemic thrombolytic therapy using recombinant tissue plasminogen activator (rtPA) has become a new option in treatment of patients experiencing acute ischemic stroke.\(^9\)\(^-\)\(^10\) Studies showed that similar to treatment strategies of myocardial infarction, long-term outcome could be improved in a preselected group of these patients.\(^11\) Noninvasive sonothrombolysis might be a promising approach for further advancement of recanalization rate and clinical outcome. First, Alexandrov published a study including 40 acute stroke patients with occlusions of the middle cerebral artery (MCA), internal carotid artery, or basilar artery, which revealed high rates of complete recanalization with dramatic clinical recovery when continuous transcranial Doppler (TCD) monitoring was used during tissue plasminogen activator (tPA) infusion.\(^12\) Another study by Cintas showed high rates of recanalization of the MCA during continuous TCD monitoring in 6 acute stroke patients, even without using tPA.\(^13\) Eggers reported a prospective randomized trial enrolling 25 acute stroke patients including a control group.\(^14\) Thereby, recanalization of the MCA and a positive tendency for all

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clinical outcome parameters after sonication during tPA infusion were observed. All these studies used commercial diagnostic Doppler ultrasound devices (2-MHz frequency). Thereby, sonication lasted between 30 and 60 minutes. But these studies are limited by comparatively small numbers of individuals and by the fact that 2 studies had no control group. However, very recently, data of the randomized phase II Combined Lysis of Thrombus in Brain Ischemia Using Transcranial Ultrasound and Systemic tPA (CLOTBUST) trial were published, providing clinical evidence for the existence of ultrasound-enhanced thrombolysis in humans that can amplify the existing therapy for ischemic stroke.15

So far, no in vitro study evaluated the thrombolytic effect of a commercially available 1.8-MHz high-frequency diagnostic ultrasound device (2-MHz range) in a transtemporal setting. Furthermore, no data are available comparing different Doppler qualities (Duplex- versus continuous wave [CW]- versus pulsed wave [PW]-Doppler) in the same context. Therefore, the goal of this study was to investigate the potential of such a diagnostic ultrasound device for therapeutic sonothrombolysis using an in vitro skull model and further to evaluate the influence of different temporal bone thickness on ultrasound attenuation.

Materials and Methods

Preparation of Human Whole Blood Clots

Human venous blood was obtained from a male donor, not taking blood-anticoagulating or antithrombotic medications. Blood was drawn into citrate Vacutainer tubes, and clot formation was achieved by recalcification always 1 hour before each experiment by adding 60 μL CaCl₂ (756 μmol/L) to 1.5 mL of anticoagulated blood in 2.0-mL Eppendorf microtubes.2,3 Thereafter, blood was gently agitated and incubated at 37°C for 60 minutes. Then clots were weighed on an analytic balance before and after experiments.

Experimental Set-Up

Blood clots were exposed to ultrasound in a blood vessel–mimicking tubing (silicone-copolymer; C-Flex; Cole-Parmer) with an inner diameter of 4.8 mm and 0.8 mm wall thickness. The acoustical properties of this material are similar to human blood vessels.16 The tubing was filled with saline, with or without rtPA (ACTILYSE; Boehringer Ingelheim Pharma) at a final concentration of 3000 IU/mL. At a distance of 50 mm from the transducer, the tubing was located in the sonication chamber. The sonication chamber was filled with water and connected to a water bath (37°C). For experimental groups treated with temporal bone, half of a macerated human skull without calvaria was completely immersed into the sonication chamber. The tubing containing the clot was placed in typical location of the MCA (distance to the transducer 50 mm). The temporal bone area of the skull was located exactly 5 mm beneath the transducer. The angle between the central axes of the transducer and the tubing was 35° (Figure 1). Saline and water used for experiments were degassed to avoid gas bubble–associated phenomena during sonication.

Ultrasound Device and Sonication

A diagnostic ultrasound device (Sonos 4500; Philips), which is routinely used for neurological examinations, was used for experiments. The clot was identified by B-mode imaging (probe S4–21330A) because a diffusion model was used and thus no Doppler signal could be detected. Therefore, the temporal bone was cut out and secured with a small hinge to ensure that the bone could be exactly relocated after tubing and probe were placed in correct position. Mean ultrasound frequency was 1.8 MHz. Duplex-, CW- or PW-Doppler was selected, and when Duplex-Doppler or PW-Doppler was applied, the sound beam was focused on the clot using the sample volume function. As measured with a Pt-100 thermometer during sonication, there was no significant temperature rise at the location of the thrombus. An acoustic absorber was located opposite to the transducer. Clots were sonicated for 60 minutes.

Calculation of Ultrasound Attenuation in a Human Skull

Loss of intensity was calculated along a virtual line from the transducer to the thrombus (50 mm) by assuming a parallel arrangement of 4 homogeneous layers: water as coupling medium between transducer and temporal bone (5 mm), temporal bone (1.91 mm), again water (42.29 mm), and the vessel-mimicking tubing (0.8 mm). When ultrasound waves impinge on the boundary between 2 different media, they are partly reflected and partly transmitted. Ratio of transmitted ultrasound energy (into medium 2) to the incident ultrasound energy (from medium 1), is given by $4 \cdot Z_1 \cdot Z_2 / (Z_1 + Z_2)^2$.2
Ultrasound Attenuation Measurements of Temporal Bones

Attenuation properties of 5 human temporal bones of different thickness (see Results) were evaluated by hydrophone (NPL Beam Calibrator; Gammax-RMI) measurements. Thereby, attenuation of each bone was measured at different locations. Results are shown as intensity levels by 10log(Iout/Iclot) in decibels, as well as percentage loss of the output intensity (see Figure 3).

Evaluation of the Acoustic Window by Diagnostic Duplex-Doppler Sonography

An acoustic window exists if flow signals of intracranial blood vessels can be detected by diagnostic Doppler sonography. To determine whether temporal bones used in this study had an acoustic window, a perfusion set-up with tubing was installed in the water bath (depth 50 mm). The circulation was filled up with saline plus an ultrasound contrast agent (Levovist; Schering). Signals with and without the interference of the 5 temporal bones were detected by PW-Doppler (Figure 3). Therefore, experiments using the stroke model were performed with PW-Doppler.

Calculated Ultrasound Attenuation in a Human Skull

The decrease of ultrasound intensity along the way to the MCA bridging temporal bone was calculated as described in Methods. Calculations for increasing temporal bone thickness (1.91 ± 0.29 mm; 2.69 ± 0.34 mm; 3.07 ± 0.43 mm; 4.44 ± 0.65 mm; and 5.01 ± 0.59 mm) resulted in an intensity loss of 9.08 dB (87.63% attenuation of the output intensity), 10.94 dB (91.94%), 11.84 dB (93.46%), 15.11 dB (96.92%), and 16.47 dB (97.74%), respectively, at the thrombus site.

Ultrasound Attenuation Measurements of Five Temporal Bones by Hydrophone

Measurements resulted in an intensity loss of 8.79 ± 0.04 dB (86.79 ± 0.39% attenuation of the output intensity), 10.07 ± 0.04 dB (90.16 ± 0.36%), 14.29 ± 2.71 dB (96.28 ± 18.26%), 16.39 ± 3.87 dB (97.70 ± 23.07%), and 21.18 ± 3.87 dB (99.24 ± 18.13%), when compared with the output intensity at the transducer (Figure 3).

Results

Clot Weight Measurements Comparing Duplex-, CW-, and PW-Doppler

Weight reductions of clots treated with different Doppler modes in the presence of rtPA were compared with clots that were treated with rtPA alone. Relative weight reduction in rtPA treated clots was 27.4 ± 5.2%. Duplex-Doppler (30.9 ± 5.7%; \( P = 0.69 \)) and CW-Doppler (32.0 ± 3.1%; \( P = 0.12 \)) enhanced rtPA–mediated thrombolysis slightly but not significantly. PW-Doppler (33.9 ± 5.5%; \( P = 0.00015 \)) showed a significant additional effect of 23.7% on rtPA–mediated lysis rate (Figure 2). Therefore, experiments using the stroke model were performed with PW-Doppler.

Statistical Analysis

Clot weight losses are shown as means plus SEMs in Figure 2 (and as means plus SDs in Figure 4). Attenuation measurements are also shown as means plus SDs (Figure 3). Data were compared statistically by 1-factor ANOVA and Bonferroni’s post hoc test. Values of \( P < 0.05 \) were considered significant. Calculated and measured intensity losses were compared using Pearson correlation.

Figure 2. Comparison in clot weight reduction of rtPA alone with rtPA plus Duplex-, CW-, and PW-Doppler (n=10).

27.4 30.9 32.0 33.9
rtPA rtPA + Duplex rtPA + CW rtPA + PW

Figure 3. Correlation between temporal bone thickness and intensity loss. The intensity loss is given as a linear function in dB and as a polynomial function (fourth order) in percentage. Hydrophone measurements were obtained at 20°C using a frequency of 1.8 MHz, and the sonication angle was 35°.
Data from calculations correlated well with data obtained from hydrophone measurements, and differences can be assigned to stated limitations. Pearson correlations for calculated and measured intensity losses in dB were $r=0.961; P=0.0093$; and in percent $r=0.958; P=0.0104$. For a further approximation to an in vivo situation, attenuation of brain tissue (attenuation coefficient 0.067 cm$^{-1}$) also should be considered, which results in an additional attenuation of $\approx 2$ dB for a distance of 40 mm.

**Acoustic Window**

The threshold for obtaining flow signals (acoustic window) was at a temporal bone thickness of $\approx 4$ mm (Figure 3). Therefore, for weight loss experiments in the stroke model, the thinnest temporal bone (1.91 mm) resulting in the lowest attenuation with a pronounced acoustic window was used.

**Clot Weight Measurements After Application of PW-Doppler in a Stroke Model**

Clots treated with saline only resulted in a weight loss of 21.8±5.2%. When additionally treated with PW-Doppler (23.0±3.8%), no significant change in relative weight loss was found. Similarly, treatment with PW-Doppler, including the interference of temporal bone (21.0±4.5%), did not significantly change clot weight loss (Figure 4).

Clots treated with rtPA only showed a significant clot weight reduction of 27.4±5.2% when compared with clots treated with saline only ($P<0.05$). Clots treated with rtPA and PW-Doppler (33.9±5.5%; $P=0.00015$) revealed a significant enhancement of clot lysis when compared with clots treated with rtPA only. Combined treatment of rtPA and PW-Doppler, including the interference of temporal bone, resulted in a clot weight loss of 26.2±3.3%, which was not significantly different when compared with clots treated with rtPA only (Figure 4).

**Discussion**

In this experiment, an oblique angulation of a PW 1.8-MHz ultrasound beam at temporal bones $>1.9$ mm thickness resulted in no significant clot weight reduction than can be achieved with tPA treatment alone. However, comparing Duplex-, CW-, and PW-Doppler sonication modes, PW-Doppler revealed to exert the highest impact on rtPA-mediated thrombolysis.

In vitro studies with human skulls showed that even with low intensities of 0.5 W/cm$^2$, enough energy for enzymatic thrombus dissolution can be delivered transcranially to the thrombus. These studies used frequencies from 33.3 kHz up to 1 MHz. Thereby, thrombolysis was also accelerated when frequencies of 1 MHz were applied, but in comparison, low frequencies clearly revealed to be more efficient. Different from our study, Behrens et al used an in vitro perfusion model. Similar results were reported by Akiyama, who compared ultrasound frequencies of 212 kHz versus 1.03 MHz and found significantly higher ultrasound transmission rates in the lower frequency group. Using an in vivo setting, Ishibashi found significant higher recanalization rates in a rabbit femoral artery occlusion model when comparing 490 kHz ultrasound in combination with tPA versus tPA alone. However, so far, no study investigated the impact of high ultrasound frequencies, which are typically used for diagnostic ultrasound purposes. In addition, none of these studies implicated different temporal bone thickness or the existence of an acoustic window, which are important assessment parameters for the study outcome, as shown in the present article. However, this is a critical aspect because no therapeutic effects can be expected, unless at least diagnostic imaging is possible.

Therefore, ultrasound attenuation of 5 human temporal bones of different levels of thickness was evaluated. Experiments with diagnostic PW-Doppler showed that temporal bone thickness exceeding 4 mm becomes critical for gaining diagnostic signals. Interestingly, temporal bone thickness increases with age, and therefore, the number of patients having no acoustic window is higher in elderly patients. For clot weight loss experiments, the thinnest temporal bone with a pronounced acoustic window was chosen to study if, in PW-Doppler mode, sufficient ultrasound energy could be transmitted through the skull to accelerate thrombolysis at the position of the MCA. The experiment revealed no benefit of ultrasound, which can be explained by ultrasound attenuation properties of temporal bones as measured. Thereby, an intensity loss of 86.8% (8.8 dB) even of the thinnest bone was recorded, which was confirmed by calculations. This indicates that cranial bone thickness, as typical for human skulls, always exerts a major influence on ultrasound attenuation; only very thin bones ($<1$ mm) would lead to an acceptable attenuation, which is not a realistic scenario in human skulls. Furthermore, it must be recognized that after temporal bone passage, ultrasound is further attenuated by brain tissue, so that only $\approx 10\%$ of the output intensity is able to affect the thrombus even when a thin temporal bone is bridged. To deliver energies effective for sonothrombolysis through the temporal bone, much higher output intensities would be required. This would be technically possible even in commercial diagnostic ultrasound devices, but safety concerns prevent such manipulations.

Our data are contradictory to the findings of clinical studies published recently. Of course, our data are limited by the fact of an in vitro setting. No muscle layer between transducer and temporal bone was used, which leads to an additional loss of ultrasound energy of 4% by reflection. Furthermore, possible positive in vivo influences of ultrasound, such as direct thrombolytic effects on the endothelium, local renewing of plasminogen and tPA, or evacuation of dissolved thrombotic material by collaterals, could not be investigated. Hereby, it has to be stated that patients with no acoustic windows (ie, no detectable signals at all) were excluded from cited clinical studies, and therefore, the effect observed at bedside was likely
limited to patients with either thinner temporal bones or bones with variable thickness but areas containing good insonation windows. Alexandrov et al reported a trial studying 40 acute stroke patients. Early dramatic recovery during tPA infusion was seen in 20%, and at 24 hours, 40% of the patients showed a clear clinical improvement. In another study, Cintas found high rates of early recanalization applying transcranial ultrasound (2 MHz; 415 mW/cm²; application time between 30 and 45 minutes; PW mode) in 6 acute stroke patients without the use of thrombolytics. However, the authors used no control group in this study. In contrast, Culp applied an in vivo swine model with an occlusion model of the ascending pharyngeal artery and the rete mirabilis. They found a significant effect of ultrasound in combination with an ultrasound contrast agent. The first study, including a control group, was published by Eggers. Thereby, 11 patients received ultrasound treatment plus rtPA versus 14 patients receiving only rtPA. The crucial parameter of recanalization was determined by the TIBI (Thrombolysis in Brain Ischemia) score and showed no significant difference. In fact, all clinical outcome parameters showed a positive trend toward ultrasound application, but these findings are limited by a small sample size and a higher number of intraparenchymal bleedings. CLOTBUST is the first randomized clinical trial with larger patient numbers investigating the effect of TCD monitoring on rtPA-induced thrombolysis in stroke patients. The trial showed a significantly higher rate of complete recanalization or dramatic clinical recovery from ischemic stroke in patients whose tPA infusion was continuously monitored with TCD compared with patients who received tPA without ultrasound monitoring. Furthermore, the trial revealed a trend toward sustaining complete recovery after ischemic stroke after 3 months in the former group of patients. However, high-frequency diagnostic ultrasound has the potential to accelerate thrombolysis significantly when used without bridging bone tissue. Our data suggest that target vessels up to a depth of 50 mm could be visually displayed, and a thrombus in this area might be detected and successfully sonicated, leading to a shorter recanalization time when treated together with rtPA. This might be the situation in peripheral arterial thrombosis or in shunt thrombosis.

In conclusion, diagnostic ultrasound has the potential to enhance enzymatic thrombolysis. However, because of an at least 86.8% reduction of ultrasound energy at temporal bone passage, as shown in our in vitro model, this method seems not to be feasible for transcranial thrombolysis.

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