

Therapeutic Application of 20-kHz Transcranial Ultrasound in an Embolic Middle Cerebral Artery Occlusion Model in Rats

Safety Concerns

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Background and Purpose—Therapeutic application of diagnostic ultrasound has been shown to improve recanalization rates in patients with acute cerebral vessel occlusion. There is experimental evidence that low-frequency ultrasound may be superior. This study was designed to evaluate the therapeutic efficacy and safety of low-frequency ultrasound in an embolic middle cerebral artery occlusion model in rats. A parameter setting was used that had not previously shown any side effects and interactions with healthy rat brain tissue.

Methods—Male Wistar rats were submitted to middle cerebral artery clot embolism and transcranial treatment with 20-kHz continuous-wave ultrasound (0.2 W/cm^2), either alone or in combination with recombinant tissue-type plasminogen activator. Control groups received no treatment or recombinant tissue-type plasminogen activator alone. Outcome assessment consisted of determination of infarct volume and neurological evaluation.

Results—Eleven animals treated with ultrasound died during the follow-up period of 7 days, compared with 2 animals in the control groups ($P=0.028$). In 3 animals, subarachnoid hemorrhage was detected (1 in the control group). The other animals that died displayed secondary worsening after an initial period of normal vigilance. Histological examination revealed massive edema formation. In surviving animals, no benefit of treatment could be demonstrated.

Conclusions—In this study, 20-kHz continuous-wave ultrasound caused death in a significant number of animals. Ultrasound at 20 kHz does not seem to be suitable for transcranial therapeutic cerebral application. The data underline the necessity to obtain further animal data to establish the safety limits of frequency and power output. (*Stroke*. 2007; 38:000-000.)

Key Words: efficacy ■ safety ■ stroke ■ thrombolysis ■ ultrasound

The efficacy of intravenous thrombolysis for acute stroke is limited owing to a relatively poor recanalization rate¹ and incomplete functional recovery in the majority of treated patients.² There is broad experimental evidence that noninvasively applied ultrasound may improve the effect of recombinant tissue-type plasminogen activator (rt-PA).³ Several clinical trials on thrombolytic treatment of intracranial vessel occlusion reported significantly increased recanalization rates when rt-PA treatment was monitored with diagnostic Doppler and duplex ultrasound.^{4–6}

The benefit of therapeutic ultrasound depends on ultrasound intensity and the duration of treatment,^{7–9} the mode of application (continuous wave [CW] or pulsed wave),¹⁰ and most important, ultrasound frequency. In vitro studies have shown a superiority of lower-frequency ultrasound (kHz) in comparison with higher frequencies (MHz), as are used in

diagnostic ultrasound.^{10–12} In a rat model, transcranial insonation at a frequency of 25 kHz has been an effective thrombolytic measure.¹³

However, application of low-frequency ultrasound in vivo remains problematic for various reasons. Not much is known about its interactions with brain tissue. Because of brain tissue's increased mechanical properties and a lower cavitation threshold, the potential for deleterious interactions may be increased compared with higher ultrasound frequencies. Accordingly, a recent clinical multicenter study on transcranial therapeutic application of 300-kHz ultrasound demonstrated an increased rate of cerebral hemorrhage.¹⁴

In a previous study, we defined the safety limits for transcranial application of 20-kHz CW ultrasound on healthy brain tissue in a rat model.¹⁵ A clearly dose-dependent damaging effect was found, beginning with an intensity of 0.5

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W/cm². Intensities below this threshold caused no pathological findings on magnetic resonance imaging scans and histology specimens. Therefore, the purpose of this study was to evaluate the safety and efficacy of transcranial 20-kHz CW ultrasound within these safety limits in an embolic middle cerebral artery (MCA) occlusion model in rats.

Materials and Methods

Animal Preparation

Sixty-two male Wistar rats (Charles River Laboratory, Sulzfeld, Germany) weighing 361 ± 54 g (mean ± SD) were used in the present study. All experiments were performed in accordance with German animal protection legislation and were approved by the regional ethics committee (Az 1.5 177-07/051-43).

After ether anesthesia, the rats were anesthetized further by intraperitoneal injection of chloral hydrate (initially, 36 mg/100 g body weight; maintenance, 36 mg/h), orally intubated, and mechanically ventilated throughout the entire operation procedure.

A thermostatically regulated heating pad was used to maintain rectal temperature at 37.0°C. The tail artery was cannulated with a PE-50 tube (0.58-mm inner diameter) for continuous monitoring of arterial blood pressure and blood sampling for blood gas analysis, determination of pH, hemoglobin, electrolytes, glucose, and lactate and for clot incubation. The submandibular gland vein was cannulated for rt-PA application. Animals were weighed at baseline and before perfusion-fixation.

Induction of Brain Ischemia and Treatment

Thromboembolic cerebral ischemia was produced as previously described.¹⁶ In short, for the preparation of the blood clot, 200 μL of blood was mixed with 4 NIH units of thrombin (human α-thrombin, Enzyme Research Laboratories, South Bend, Ind). The blood was delivered into a 20-cm-long piece of a PE-50 catheter (0.86 mm) and left at room temperature for 2 hours. The resulting blood clot was rinsed from the catheter and gently washed in isotonic saline. The required length of a 40-mm blood clot was cut from the string and drawn into the injection catheter.

The right and left common carotid arteries (CCAs), the right external carotid artery (ECA), and the right internal carotid artery (ICA) were exposed. ECA branches were cauterized, the distal ECA was ligated, and the stump was mobilized. Both CCAs and the ICA were temporarily clamped, and a modified PE-50 catheter (0.28- to 0.30-mm outer tip diameter) was inserted into the ECA and gently advanced ≈ 15 mm into the ICA, thereby approaching the MCA origin by ≈ 2 mm. The previously prepared blood clot was injected into the catheter and placed in the distal ICA. The catheter was withdrawn and the puncture site cauterized. The vascular clip on the left CCA was removed 5 minutes and the vascular clips on the right ICA and CCA were removed 10 minutes after clot application. The animals were fixed in a stereotactic headframe. Tympanic temperature as a correlate of brain temperature¹⁷ was monitored in the right auditory canal. The scalp was mobilized from the skull by longitudinal incision. The skin flaps were attached to a ring (diameter of 40 mm, 10 mm above the skull) to form a basin that was then filled with water. The ultrasound probe (Bandelin Electronic, Berlin, Germany) was placed 5 mm above the skull and into the basin to ascertain full transmission of sound to the skull.

In the ultrasound treatment group, 20-kHz CW ultrasound was applied at 0.2 W/cm² for 20 minutes. This intensity was chosen because it was the lowest intensity tested that had previously not produced brain damage in the healthy rat brain.¹⁵ The transducer had a plane circular surface with an area of 3.5 cm², which is similar to the one used in our previous study.¹⁵ Intensity was spatial average, time average, and was regulated by a power analyzer connected to the ultrasound probe. The control group underwent the same ultrasound setup; however, the ultrasound probe was not switched on. Both groups consisted of 20 animals that survived the follow-up period and were divided into 2 subgroups of 10 animals each. The death rate of animals that died during the observation period was

determined separately. One subgroup received rt-PA treatment next to the site of ultrasound application, and the other subgroup received a placebo injection. The application of rt-PA or isotonic saline as a placebo was done in a blinded manner. rt-PA (Actilyse; Boehringer Ingelheim, Ingelheim, Germany) was given at a dose of 5 mg/kg body weight, with 10% as a bolus and the remainder as a 1-hour perfusion. This dose was chosen because it induces incomplete recanalization in the embolic MCA occlusion model, which is a situation that mimics thrombolytic therapy in human stroke and is a prerequisite to study recanalization therapies as add-ons to rt-PA.¹⁸ Treatment (ultrasound and rt-PA) started 40 minutes after vessel occlusion. The group of sham-operated animals consisted of 6 animals.

Seven days after surgery, the animals were deeply anesthetized with ether and chloral hydrate and submitted to transcardial perfusion-fixation with 4% paraformaldehyde. The brain was removed carefully, embedded in paraffin, and cut into 3-μm-thick coronal sections at 400-μm intervals. Sections were stained with hematoxylin and eosin. Histological evaluation was performed with a light microscope equipped with a 20× lens (Zeiss, Wetzlar, Germany) and a charge-coupled device camera (SCC-C370P, Sony) that was connected to calibrated image analysis software (OPTIMAS 6.51; Bioscan, Atmonds, Wash). The brain slices were blinded for histological examination. Only areas of pannecrosis consisting of the loss of affinity for hematoxylin, which affects all cell types (neuroglial, glial, and vascular), were measured. Infarct volume was calculated by multiplying the infarct area of each slice by the distance (400 μm) between successive slices and adding the determined volume per slice.

Functional Evaluation

All behavioral tests were performed between 1 PM and 4 PM. A 5-minute rest period was given between each test. The test battery included a Neuro score and a parallel-bar crossing test. Functional evaluation was performed on the preoperative day (baseline) and on a daily basis from postoperative day 1 to 6. Training of the animals on the parallel bars was performed on 2 consecutive days before baseline.

Neurological Score

Neurological deficits at baseline and after induction of ischemia were studied by use of a Neuro score with 10 different motor, coordinative, and sensory items. Each item scored 0 (no impairment) or 10 (impairment), except for spontaneous walking (0=normal gait, 5=drifting/circling, 10=unable to walk on ground) and parallel-bar traversing (to test for motor functions under more difficult circumstances; 0=no impairment, 5=impairment [eg, slowing or foot faults], 10=unable to cross). Thus, overall functional impairment was scored from 0 (no impairment) to 100 (no reaction to any stimulus).

Single items included the inability to fully extend the left forelimb, instability to a lateral push from the right, and a tail suspension test. In this test, animals were gently lifted by the tail, and impairment was assumed when the animals flexed their body to the left and remained in that position for 3 consecutive attempts; also included were walking on the ground (see previous section), semiquantitative assessment of walking on parallel bars (see previous section), whisker movements on the left side (present or absent), consciousness (normal or no reactions to stimuli), hearing (normal or no reaction to acoustic stimuli), sensory (normal or no reaction to a left-sided touch or prick), and left-sided hemianopia (normal or repeatedly absent reaction to visual stimuli approaching from the left).

Parallel-Bar Crossing Test

Two parallel wooden bars (diameter, 1.8 cm; length, 1 m; distance between bars, 2.5 cm) were positioned horizontally, 50 cm above a soft pad. The room was quiet and darkened. After placing the rat on 1 side of the bars, a light and noise source behind the rat was switched on, serving as an adverse stimulus. By crossing the bars, the rat reached a dark chamber and the adverse stimulus was switched

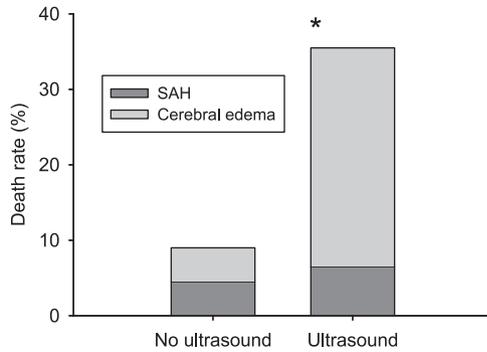


Figure 1. Death rate of animals in experimental groups treated with ultrasound compared with that in animals that were not exposed to ultrasound treatment. Most animals showed massive signs of cerebral edema within ischemic tissue. A minority had subarachnoid hemorrhage (SAH) as the suspected cause of death. * $P=0.028$ compared with animals without ultrasound treatment.

off. The time required to cross the bar and the number of foot faults were counted, with a maximum of 60 seconds and 15 foot faults. Inability to cross the bars and falling were counted as 60 seconds and 15 foot faults. With each rat, 3 trials were performed in sequence and the scores of each trial were averaged.

Statistical Analysis

Statistical analysis was performed with SPSS 12.0 for Windows. The χ^2 test was used for evaluation of death rates. Comparisons of infarct volume between the different experimental groups and the sham group and differences in functional outcome between the experimental and control groups were assessed by ANOVA, followed by a post hoc (least significant difference) test. Values are expressed as mean \pm SE, except where otherwise stated. $P < 0.05$ was considered statistically significant.

Results

Physiological Variables and Mortality

A total of 61 rats was included in this study, resulting in 10 surviving animals per experimental group and 6 animals in the sham group that completed the observation period of 7 days. The physiological parameters of these animals remained within normal limits during the whole experimental procedure. Tympanic temperature remained unchanged throughout the surgical procedure and the insonation period.

Two animals that died of a cause clearly attributable to the operational procedure (rupture of the extracranial ICA during placement of the catheter, massive intraperitoneal bleeding

before rt-PA administration) were excluded from evaluation. Of the remaining animals, 13 did not survive the observation period (Figure 1 and the Table). Eleven of these animals had been treated with ultrasound (4 animals in combination with rt-PA, 7 with ultrasound only), resulting in a death rate of 35.5%. Two animals in the control group died, which corresponds to a death rate of 9.1%. One of these animals had been treated with rt-PA, but the other animal had not been treated at all. The difference in death rate between animals treated with ultrasound compared with no ultrasound treatment was statistically significant ($P=0.028$). There were no deaths in the group of sham-operated animals.

Three of these animals died shortly after the operational and treatment procedures, 2 of which had been treated with ultrasound alone, but 1 animal had not been treated at all. These animals showed signs of massive subarachnoid hemorrhage on inspection of postmortem brains. The majority of deceased animals shared a common pattern. After treatment, they initially recovered to normal vigilance. However, compared with animals that survived the observation period, they showed stronger impairment on the Neuro score (65.0 ± 3.2 vs 39.0 ± 4.3) and were unable to successfully cross the parallel bars. After this period, there was a clinical deterioration between 24 and 48 hours after treatment, resulting in death. Nine of these animals had been treated with ultrasound (4 in combination with rt-PA), and 1 animal had been treated with rt-PA alone. Postmortem histological examination revealed massive infarction and edema of the right hemisphere (Figure 2). Only for these animals with infarction and edema formation as the cause of death, there was a statistically significant difference between animals treated and animals not treated with ultrasound ($P=0.022$).

Infarct Volume and Functional Testing

Ten surviving animals per experimental group were included in the histological and outcome assessment (for details, see the Table). There was no statistically significant difference in infarct volume between the experimental groups and the control groups. With regard to the different functional tests performed during the follow-up period of 6 days, there were no statistically significant differences between the experimental and control groups on each of the observation days. The Table shows the results of functional testing on day 6 of follow-up.

Outcome Characteristics of the Different Experimental Groups

| | No Ultrasound | | Ultrasound | | Sham |
|---------------------------------|----------------|----------------|----------------|-----------------|---------------|
| | No rt-PA | With rt-PA | No rt-PA | With rt-PA | |
| Death rate, % | 9.1 | 9.1 | 41.2* | 28.6* | 0 |
| Infarct volume, mm ³ | 35.6 \pm 9.3 | 35.8 \pm 8.3 | 39.3 \pm 9.4 | 52.0 \pm 11.4 | 1.5 \pm 0.5 |
| Neuro score | 24.0 \pm 4.8 | 21.5 \pm 5.5 | 25.5 \pm 5.8 | 19.5 \pm 5.2 | 0 |
| Parallel bar foot faults, No. | 1.8 \pm 0.8 | 1.8 \pm 1.5 | 2.8 \pm 1.4 | 5.0 \pm 2.2 | 0.5 \pm 0.2 |
| Parallel bar time, s | 9.0 \pm 1.9 | 10.8 \pm 5.5 | 11.8 \pm 5.5 | 20.8 \pm 8.0 | 3.1 \pm 0.6 |

Values are mean \pm SE. Results of functional testing are from day 6 of the follow-up period. No significant differences were found between the treatment groups with regard to lesion volume and the functional test results.

*The difference in death rate between the animals treated with ultrasound compared with the animals without ultrasound treatment was statistically significant ($P=0.028$).

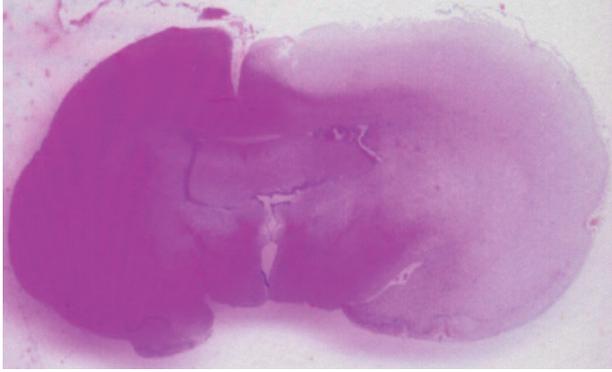


Figure 2. Postmortem histology section from an animal treated with 20-kHz CW ultrasound (no rt-PA was given). Hematoxylin/eosin staining reveals massive infarction and edema of the right hemisphere.

Discussion

This study was designed to evaluate the safety and efficacy of low-frequency, 20-kHz CW ultrasound in an embolic occlusion model in rats. Our principal finding is a significantly increased mortality rate in animals treated with transcranial ultrasound. Animals that survived the observation period did not show a benefit of treatment with regard to lesion volume and functional outcome.

Our results have to be viewed in the context of previously published data on the safety of 20-kHz ultrasound.¹⁵ Exposure of healthy brain tissue leads to clearly dose-dependent side effects, including induction of cytotoxic and vasogenic edema. Higher ultrasound intensities may even cause hemorrhage and necrosis. In this study, ultrasound was applied at 0.2 W/cm², an intensity that previously had not shown any side effects on healthy brain tissue. This suggests that there are deleterious interactions specifically between ultrasound and ischemic brain tissue that are not found after such exposure in the healthy rat brain. Accordingly, our main observation was excessive ultrasound-induced swelling and infarction of the animals' brain hemispheres that had been subjected to ischemia. The exact cause of this deleterious effect cannot be derived from our data. Ischemic tissue may be more vulnerable to the mechanical properties of low-frequency ultrasound than is healthy tissue, resulting in disruption of the blood-brain barrier and edema formation, as was seen in our animals. In a recent research report, disruption of the blood-brain barrier has been suggested as a potential mechanism of low-frequency ultrasound-related side effects.¹⁹ A temperature effect related to ultrasound is unlikely, as registration of tympanic temperature did not detect any temperature elevation. Also, previous work on temperature side effects of 20-kHz¹⁵ and 340-kHz²⁰ ultrasound did not detect relevant temperature increases in this low-intensity ultrasound range. However, because tympanic temperature reflects a more global increase in brain temperature¹⁷ and the cited previous studies determined temperature effects in nonischemic brain tissue, a circumscribed temperature elevation within the ischemic lesion cannot be excluded with certainty.

It seems unlikely that rt-PA contributed to the induction of side effects. Brain swelling and death randomly occurred in

animals treated with ultrasound exclusively and in animals treated in combination with rt-PA. Subarachnoid hemorrhage was found in 3 animals, 2 of which were treated with ultrasound and none with rt-PA. It is unclear whether there is a connection between the finding of subarachnoid hemorrhage and ultrasound treatment. Subarachnoid hemorrhage is a typical side effect of the procedure of experimental MCA occlusion in rats. It has been specifically described as a frequent side effect of the intraluminal thread model²¹ and may as well be related to the catheter technique that was used in our model. Further research is required to investigate these specific interactions of ultrasound with ischemic brain tissue.

Our study did not look for a direct effect of ultrasound on recanalization. Postmortem vascular evaluation would not have been helpful, because the embolic model has a high rate of spontaneous recanalization after a follow-up period of 7 days.²² Laser Doppler evaluation of ischemia and of recanalization was not included in the study, as this would have required thinning of the skull, which would have been incompatible with the transcranial therapeutic approach. A potential positive impact of low-frequency ultrasound on clot lysis may thus have remained undetected, as it may have been counterbalanced by the deleterious effects on ischemic brain tissue that we observed.

The main side effects observed in our study are clearly different from those seen in the TRUMBI trial.¹⁴ The TRUMBI trial had to be terminated because of an increased rate of intracranial hemorrhage. Hemorrhage occurred in atypical locations and was clearly attributable to treatment with 300-kHz ultrasound. These differences in pathology may be explained by the much higher total power output that was used in the TRUMBI trial (time-averaged intensity, 0.7 W/cm², resulting in much higher peak intensities, with respect to the duty cycle of 5%, and a larger power output, with respect to the large surface of the probe). Also, the different ultrasound frequencies (300 vs 20 kHz) may have contributed to the differences in pathology.

Research on specific ultrasound side effects will have to be a main issue in the development of therapeutic ultrasound. Because such effects are strongly dependent on ultrasound frequency, intensity, and the modality of application,^{15,20} these specific factors will have to be taken into account. Evaluation of different ultrasound frequencies (from kHz to MHz) will be of prominent interest. There are different theoretical advantages and disadvantages to low-frequency applications. Low-frequency ultrasound irradiated from a common-size probe as in this experiment can propagate only spherically. As a consequence, there is no need for exact localization of the occlusion (which may facilitate sonification of branches of the MCA). On the other hand, many "healthy" structures lie within the sound field and are exposed to potential side effects. Also, tissue attenuation decreases and bone penetration increases with lower frequencies. This may allow the use of reduced intensities. On the other hand, owing to the spherical nature of the low-frequency sound field, structures close to the surface may receive stronger irradiation. Because these factors are contrary, theoretical deduction of an optimal frequency is difficult. Further experimental studies will therefore be necessary

to more clearly identify the exact mechanisms and parameters that lead to improved ultrasound efficiency and help minimize deleterious effects on healthy and ischemic brain tissue.

In our study, evaluation of animals that survived the observation period did not show any beneficial treatment effects. This is in contrast to other reports that have shown therapeutic efficacy of low-frequency ultrasound in *in vitro* and *in vivo* experimental settings.^{10–13} It might be discussed that the 5-kHz difference from the 25-kHz probe used by Daffertshofer et al¹³ may be responsible for the difference in results by significantly changing the therapeutic characteristics and biologic side effects of ultrasound. We believe that this small frequency difference does not play a significant role in the context of very-low-frequency ultrasound interactions with brain tissue. However, this discrepancy between our data and those of Daffertshofer et al merits further comparative studies. The lack of benefit possibly reflects the very low intensities that were used in our study. However, because higher intensities had shown side effects, even in healthy tissue,¹⁵ side effects seem to outweigh potential treatment benefits at this very-low-ultrasound frequency range. In conclusion, our data emphasize that 20-kHz CW ultrasound does not seem suitable for transcranial therapeutic purposes.

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Disclosures

None.

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