Brain Edema and Intracerebral Necrosis Caused by Transcranial Low-Frequency 20-kHz Ultrasound

A Safety Study in Rats

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Background and Purpose—Ultrasound-accelerated thrombolysis is a promising approach toward acute stroke treatment. In previous in vitro studies, we demonstrated enhanced thrombus destruction induced by 20-kHz ultrasound. However, little is known about biological interactions of low-frequency ultrasound with brain tissue. The aim of this in vivo MRI study was to assess safety aspects of transcranial low-frequency ultrasound in rats.

Methods—The cranium of 33 male Wistar rats was sonificated for 20 minutes (20-kHz continuous wave). Power output was varied between 0 and 2.6 W/cm^2. Tympanal and rectal temperature was monitored. Diffusion-weighted imaging and T2-weighted imaging was performed before and 4 hours, 24 hours, and 5 days after sonification. Apparent diffusion coefficients (ADCs) and T2 relaxation time (T2-RT) were measured in regions of interest in the cortex and the basal ganglia. The animals were euthanized for histological evaluation thereafter.

Results—Tympanal temperature increased significantly during insonation with 1.1 and 2.6 W/cm^2. ADCs decreased significantly at 0.5 and 1.1 W/cm^2, indicating cytotoxic edema. T2-RT increased significantly in the 0.5 and 1.1 W/cm^2 group, consistent with vasogenic edema. No changes were detectable in the low-power output group (0.2 W/cm^2). After sonification with 2.6 W/cm^2, a significant loss of neurons could be detected on histopathology. Furthermore, 3 animals developed circumscript cortical lesions that could be identified as parenchymal necrosis.

Conclusion—Low-frequency ultrasound caused vasogenic and cytotoxic brain edema and intracerebral necrosis in a dose-dependent fashion. This study indicates therapeutic low-frequency ultrasound as being potentially harmful and underlines the necessity of careful evaluation in further animal models. **(*Stroke*. 2006;37:000-000.)**

Key Words: ultrasonography ■ thrombolysis ■ stroke

Efficiency of intravenous thrombolysis for acute stroke treatment is limited because of a relatively poor recanalization rate.[1] Therapeutic application of ultrasound has been shown to be an effective method to improve recanalization rates in acute cerebral vessel occlusion. Two different randomized clinical trials of recombinant tissue plasminogen activator (rtPA) therapy of middle cerebral artery (MCA) occlusion have shown significantly increased recanalization rates and an improved clinical outcome when treatment was monitored continuously by means of diagnostic transcranial Doppler[2] and duplex ultrasound.[3] The progress in basic research and in preliminary clinical applications of therapeutic ultrasound has been reviewed recently.[4]

There is extensive experimental evidence that the benefit of therapeutic noninvasive ultrasound strongly depends on different technical parameters, such as intensity and duration of ultrasound exposure,[5–7] the mode of application (continuous wave [cw] versus pulsed wave[8]), and, most important, the chosen ultrasound frequency. Different in vitro studies suggest superior efficacy of the lower (kHz) frequency range.[8–10] An in vivo study in rats has shown effective treatment with transcranially applied 25-kHz ultrasound.[11] These results encourage further studies on therapeutic ultrasound in the low-frequency range.

However, in vivo application of transcranial low-frequency therapeutic ultrasound is problematic for various reasons. Because there are no established transcranial applications of low-frequency ultrasound in medicine, only little is known about specific interactions with the skull and brain tissue. Recent results of the TRUMBI trial emphasize the potential

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harmfulness of low-frequency ultrasound. Transcranial insonation at 300 kHz was associated with increased rates of cerebral hemorrhage.[12] Safety issues are to be carefully addressed before experiments can be carried into further experiments in various occlusion models.

Therefore, the aim of this study was to gather information on intensity-dependent brain damage or blood–brain barrier disruption of low-frequency (20 kHz) ultrasound in rats.

Materials and Methods

Animal Preparation and Ultrasound Application

All procedures are in accordance with the standards of the animal research community (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

Thirty-three male Wistar rats weighing 250 to 350 g (Charles River Laboratory; Sulzfeld, Germany) were anesthetized with 5% isoflurane delivered in air. Anesthesia was maintained with 1.5% to 2.5% isofluran during surgery, insonation, and MRI scanning.

Experimental Setup

The animals were fixed in a stereotactic headframe. Tympanal temperature, reflecting the brain temperature,[13] was monitored using a thin probe that was placed within the right auditory canal (Oximetrix 3; Abbott Laboratories). Rectal temperature was monitored continuously (Harvard MBS 3).

After longitudinal incision, the skin was mobilized from the skull; the skin flaps were lifted and attached to a ring (1 cm above the skull, diameter 4 cm). The resulting basin was then filled with water. This arrangement was chosen to ascertain full transmission of sound to the skull. As a cooling device, a flexible rubber bag, normally used for intravaginal ultrasound, was placed to cover the tip of the ultrasound probe and filled with 15 mL water.

The 20 kHz cw ultrasound probe (KLN Ultrasound) was then placed into the basin at a distance of 5 mm to the skull. The transducer had a plane circular surface with an area of 3.5 cm^2.

Magnetic Resonance Imaging

The animals were fixed in a body restrainer with tooth-bar and a cone-shaped head holder and were placed in an MRI spectrometer (Bruker PharmaScan 70/16; 7.0T; 16 cm). Respiratory rate was monitored with a pressure probe placed between the restrainer and the animal's thorax. Temperature was monitored using a rectal probe and maintained at 37°C by a thermostatically regulated water flow system during the entire imaging protocol. The restrainer was then placed into a custom-designed linear polarized volume resonator (diameter 60 mm) and tuned and matched manually.

Diffusion-Weighted Imaging

To map the apparent diffusion coefficient (ADC) of water, diffusion-weighted images were acquired with the use of a fat-suppressing echo planar imaging sequence. A volume shim with a volume selective double spin echo sequence (repetition time [TR]=1 s; echo time [TE]=30 ms; voxel size $10\times8\times15$ mm^3) was performed before the acquisition of the first diffusion series to optimize imaging quality. The achieved full width at half maximum of the water line was ≈25 to 35 Hz.

Six contiguous coronal slices with a thickness of 2 mm were collected with a field of view of 32×32 mm and a matrix size of 128×128 (TR=3003 ms; TE=38.6 ms; numbers of excitations [NEX]=4). A 4-fold segmentation was used to reduce image artifacts caused by local field inhomogeneity. Five sets of coronal images were recorded for quantitative determination of ADCs, with equidistant diffusion gradient values of 10, 40, 70, 100, and 130 mT/m and with a diffusion gradient duration (δ) of 9 ms and a gradient separation time (Δ) of 15 ms. This results in 5 b-values of 6.96, 111.3, 340.8, 695.6, and 1175.5 s/mm^2. The acquisition time for each echo planar imaging sequence was 4.5 minutes.

T2 Imaging

T2 relaxation time (T2-RT) was mapped using a CPMG (Carr Purcell Meiboom Gill) spin echo sequence. Six slices with a thickness of 2 mm were acquired with a field of view of 37×37 mm and a matrix size of 512×256 (TR=3833.5 ms; 90° excitation and 180° rephasing pulses; NEX=1). Twelve echoes were collected, starting with TE=18 ms (step size ΔTE=18 ms), resulting in a range from 18 to 216 ms. Acquisition time for each CPMG sequence was 16 minutes and 21 s.

The ADC and T2 maps were calculated by a least squares fit with use of the Image Processing Tool of the Paravision 2.1 software (Bruker).

T2-RT and ADC were calculated from regions of interest (ROIs) that were placed within the cortex and the basal ganglia on both hemispheres 4 and 8 mm posterior to the frontal pole. Mean T2-RT and ADC values were calculated per animal.

Histological Evaluation

One week after insonation, animals were submitted to perfusion fixation with 4% paraformaldehyde under deep anesthesia. The brain was removed carefully and embedded in paraffin to obtain coronal sections (3 μm). Sections were stained with hematoxylin/eosin. The histological evaluation was performed by a light microscopy equipped with a 20× lens (Zeiss) and a charge-coupled device camera (SCC-C370P; Sony) that was connected to a calibrated image analysis software (OPTIMAS 6.51; Bioscan). For quantitative assessment of brain damage, neurons were assessed in the cortex and the hippocampus on the ipsilateral and contralateral hemisphere. Cells were counted in 4 cortical and 4 subcortical xy calibrated frames (220×200 μm for the cortex and 220×100 μm for the hippocampus) in 2 different slices.

Three animals showed a localized damage of superficial brain tissue and were investigated with usual qualitative histopathological methods.

Study Design

Baseline MRI was performed in all animals before surgery. The rats were then randomly assigned to 5 experimental groups (n=6 each) with different intensity output levels (0, 0.2, 0.5, 1.1, and 2.6 W/cm^2). Insonation was performed for 20 minutes. Follow-up MRI was performed 4 hours, 24 hours, and 5 days after insonation. One week after insonation, the animals were euthanized for histopathology.

Statistical Analysis and Data Presentation

Before data analysis, all data were *z*-transformed with baseline values of each experimental group as reference. After checking assumptions of normality, a 3-way ANOVA for repeated measurements (ultrasound intensity, time, ROIs [global, cortical, and subcortical]) was performed using Statistica 6.0 software (STATSOFT). A P value <0.05 was considered to be statistically significant. Transformed ADC- and T2-weighted MRI data (Z scores, standard normal distribution) are graphically presented as mean±SEM. The gray zone indicates the normal ranges (95% CI) as derived from baseline values (mean±1.96×SD).

Results

Three animals died during induction of anesthesia and thus were excluded from analysis. All 30 animals that were included in the analysis survived the observation period.

Temperature

The changes of rectal and tympanal temperature are shown in the Table. Rectal temperature remained within the physiological range in all but the high-intensity group (2.6 W/cm^2). Tympanal temperature increased mildly in animals subjected to higher power output, with significant changes at 1.1 (+0.6°C; $P=0.003$) and 2.6 W/cm^2 (+1.1°C; $P=0.013$).

Intensity-Dependent Increase of Body Temperature (rectal) and Brain Temperature (tympanal) in °C (values are mean±SD)

	0 W/cm^2	0.2 W/cm^2	0.5 W/cm^2	1.1 W/cm^2	2.6 W/cm^2
Body temperature	0.0±0.18	−0.30±0.38	0.0±0.18	0.28±0.30	0.47±0.42*
Brain temperature	0.05±0.19	−0.10±0.55	0.2±0.19	0.60±0.30*	1.10±0.85*

*Significant at $P<0.05$.

Magnetic Resonance Imaging

In 3 of 6 animals subjected to ultrasound at 2.6 W/cm^2, cortical lesions could be detected that appeared hyperintense on T2-weighted imaging (T2WI) and hypointense on diffusion-weighted imaging (DWI). The lesions were not visible on baseline images but could be seen on all follow-up scans (most clearly after 24 hours). The lesion size was calculated as 0.7, 0.7, and 19.7% of the hemispheric volume, respectively (Figure 1). Because of the obvious structural damage caused by 2.6 W/cm^2, this experimental group was excluded from evaluation of predefined ROIs. In 3 of 6 animals exposed to 1.1 W/cm^2, small blurred hyperintense lesions could be detected on T2WI after 4 and 24 hours but not at baseline or after 5 days. DWI appeared normal in these animals. T2-RT values in ROIs within these lesions were excluded from further evaluation. In the remaining animals, no overt pathological findings could be detected by gross inspection of the DWI and T2 parameter maps.

The time course of the changes of T2-RT and of ADC values is presented in Figure 2. ADC and T2-RT did not change in the low-intensity group (0.2 W/cm^2) and in controls. Four and 24 hours after sonification, T2-RT was significantly increased in rats subjected to 1.1 W/cm^2, consistent with vasogenic edema. After 5 days, prolongation of T2-RT normalized partially.

A significant ADC decline could be observed in animals subjected to sonification with 0.5 and 1.1 W/cm^2, indicating cytotoxic edema. ADC values returned to normal after 5 days in the 0.5 W/cm^2 group but remained decreased in rats treated with 1.1 W/cm^2.

Figure 3 shows the subgroup analysis of the cortical and subcortical ROIs at different ultrasound intensities and time points after insonation. In direct comparison of cortical and subcortical ROIs, no significant differences could be detected (ADC $P=0.694$; T2-RT $P=0.667$). Furthermore, we did not observe any interhemispheric differences.

Histopathology

Results of the histological evaluation are shown in Figure 4. Up to 1.1 W/cm^2, we found no significant changes in the number of intact cortical and subcortical neurons 7 days after ultrasound exposure. At 2.6 W/cm^2, there was a significant decrease of surviving cortical cells in the predefined cortical regions ($P=0.003$). The loss of subcortical cells at this high-power output was not significant ($P=0.196$).

The cortical lesions at 2.6 W/cm^2, as detected by MRI, were further analyzed in histology. In 1 animal, no structural

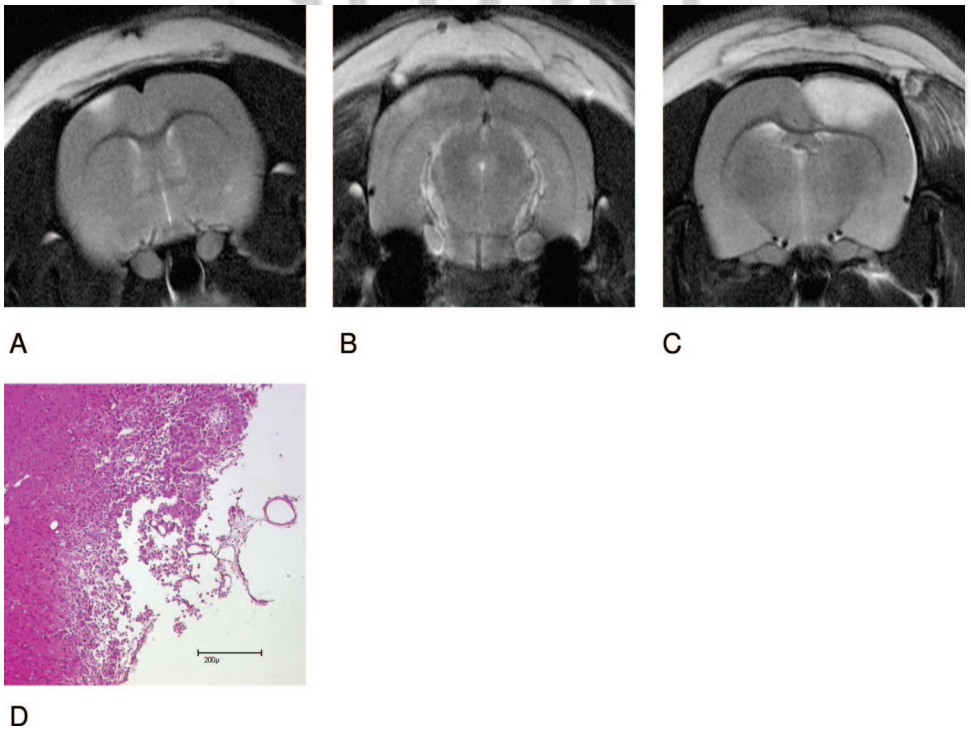

Figure 1. Structural lesions in 3 different animals (T2WI) 24 hours after insonation with 2.6 W/cm^2 (A through C). D shows histology corresponding to necrosis found in animal C (see text for further details).

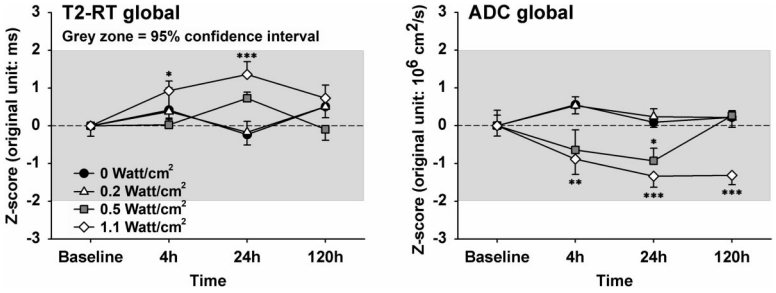

Figure 2. Time course of T2-RT and ADC after sonification with different ultrasound intensities. T2-RT and ADC values from predefined cortical and subcortical ROIs from both hemispheres were added, reflecting global deviations from baseline values. The gray zone marks the 95% CI (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

changes were found in histology (corresponding MRI is shown in Figure 1A). Another animal revealed a small cortical necrosis with signs of slight surrounding edema. Macrophages containing hemosiderin were found on the edge of the necrosis (corresponding MRI is shown in Figure 1B). Histology of the third animal showed an extended cortical necrosis with plenty of macrophages and some capillary proliferation as a sign of organization. There was only a slight edema surrounding the necrosis, reaching into the subcortical white matter. There were remnants of circumscribed hemorrhage in the subarachnoid space (Figure 1C; histology, Figure 1D).

Discussion

This is the first in vivo report demonstrating that low-frequency ultrasound represents a potential hazard to healthy brain tissue. After transcranial application of 20 kHz cw ultrasound, we found a damaging effect that was clearly dose dependent.

Using a low intensity of 0.2 W/cm^2, we did not observe any pathological changes on MRI and histology. Sonification with an intensity of $\geq$0.5 W/cm^2 resulted in magnetic resonance tomographic signal changes consistent with cytotoxic and vasogenic edema. Subgroup analysis revealed that these signal changes were found in cortical and subcortical regions. Circumscript structural damage, consistent with necrosis, and loss of neurons were detected at a high-power output (2.6 W/cm^2). The histological pattern of these animals treated with 2.6 W/cm^2 is neither typical for pure ischemic damage nor for coagulating necrosis, as seen in thermal damage. There were no signs of intensive hemorrhage (although some accompanying signs of hemorrhage were found), ruptured blood vessels, or thrombotic lesions. Some details of the histological alterations hint to a pathogenic pathway, quite different from a natural event, with similarities to a contusional brain lesion. Possibly the damaging event is a phenomenon of direct resonance of biological structures to the kinetic energy of ultrasound waves.

The observed bioeffect was nonthermal. For determination of cerebral temperature, we used tympanic temperature as a surrogate parameter. In a previous study, we showed that it reliably reflects basal brain temperature.[13] The determined ultrasound-induced temperature increment was moderate and within the range that will not lead to substantial thermal damage.[14] A statistically significant and possibly mildly clinically relevant temperature elevation of 1.1°C was only found in the highest-power output group. The ADC changes seen in the other experimental groups were global, with effects in all defined ROIs. If these changes were attributable to a temperature effect, this would have been reflected by a change in tympanic temperature. However, a focal increase in temperature may not necessarily be reflected by tympanic measurements. Therefore, circumscript adverse temperature effects are not excluded by our study design. However, as

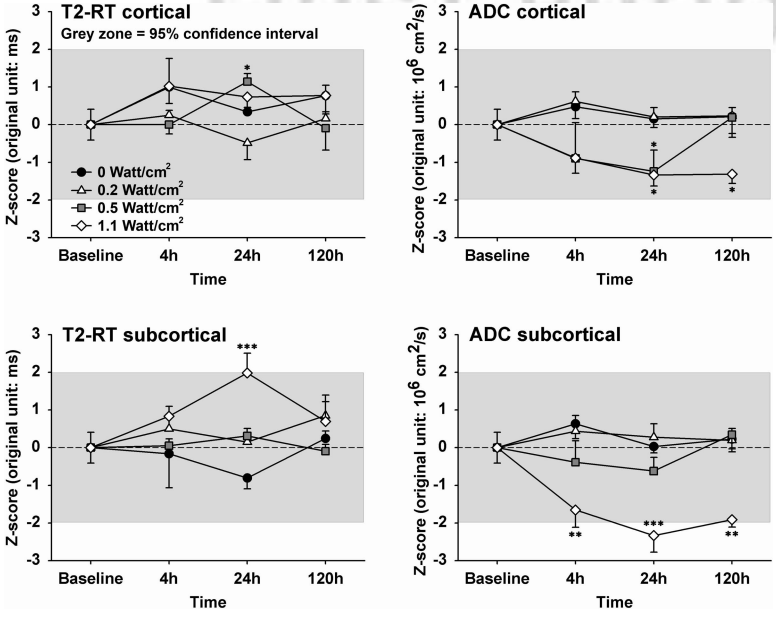

Figure 3. Subgroup analysis of T2-RT and ADC values derived from cortical and subcortical ROIs. The gray zone gives the 95% CI (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

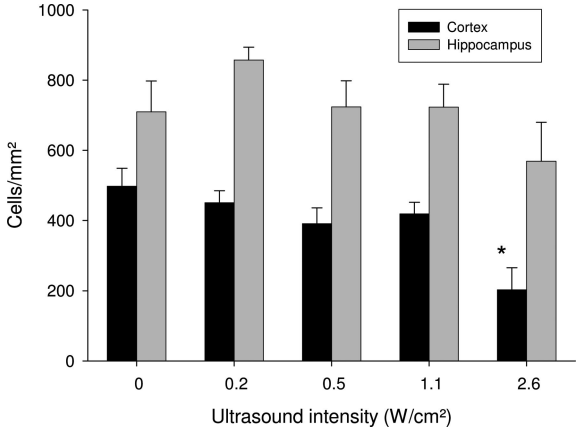

Figure 4. Histological evaluation of cortical and hippocampal neurons 7 days after insonation with different ultrasound intensities. Values are cells/mm^2 ±SEM (*P=0.03).

stated above, the histological pattern of necrosis that was found in our study is not typical for thermally induced damage.

Another concern with low frequencies may be heating and structural damage of the skin exposed to ultrasound. To prevent skin damage, we used a previously described cooling system[15] shown to operate without loss of efficacy.

Transcranial application of therapeutic ultrasound, which may be understood as a potential extension or alternative to established treatment strategies in acute cerebral vessel occlusion, has become an interesting field of research. Several studies point toward the possibility that diagnostic ultrasound equipment may enhance thrombolysis with rtPA. In a small randomized study, a higher rate of recanalization and a favorable clinical outcome were described after continuous monitoring of MCA occlusion with transcranial color-coded duplex sonography.[3] The CLOTBUST trial found a significant effect on recanalization rates after continuous monitoring using a diagnostic Doppler equipment,[2] a finding consistent with a previous phase 1 trial.[16] Most important, therapeutic application of conventional Doppler ultrasound appeared to be safe with no increase in the rate of cerebral hemorrhage.[17] A small observational study described an increased rate of recanalization of MCA occlusion even in the absence of a thrombolytic drug.[18] The authors attributed this effect to enhancement of endogenous enzymatic fibrinolysis.

However, the extent of efficacy of diagnostic (MHz) ultrasound is still under debate. A recent in vitro study using 1.8-MHz diagnostic Doppler equipment found significant enhancement of thrombolysis only without interposed temporal bone. The effect was completely neutralized when sonification was performed transcranially.[19] The observation was attributed to the low-power transmission of high-frequency ultrasound through the skull.

The question about an optimal frequency of therapeutic ultrasound applications is of special interest. Tissue and bone penetration is much better with low frequencies, allowing higher ultrasound energy levels at the site of the vessel occlusion.[7,20] Furthermore, there is in vitro evidence that the therapeutic effect of ultrasound is frequency dependent, with best results in the low-frequency (kHz) range. Suchkova et al found markedly greater fibrinolytic enhancement at 40 kHz than at 1 MHz.[7] In an in vitro transcranial model, Behrens et al found superior efficacy of therapeutic 185-kHz ultrasound compared with diagnostic 1-MHz ultrasound.[9] Best results on clot disruption may be obtained in the very low–frequency range, favoring a frequency between 20 and 40 kHz.[8,10]

However, in contrast to MHz applications of therapeutic ultrasound that rely on the use of diagnostic transcranial ultrasound equipment, only very little information on safety issues of low-frequency transcranial ultrasound is available. The recently published TRUMBI trial demonstrated an increased rate of cerebral hemorrhage in patients treated with rtPA in combination with noninvasive 300-kHz ultrasound.[12] In contrast to these results, the main aspects of structural damage found in our study related to tissue necrosis. These differences in the pathologic pattern may possibly be explained by differences in the biological interactions of the examined ultrasound frequencies (20 versus 300 kHz) and ultrasound intensities.

These results show the need of careful evaluation of low-frequency ultrasound. However, it is emphasized that the damaging effects were clearly dose dependent. An intensity range was delineated (0.2 W/cm^2) that did not show any tissue interactions and that should be evaluated in further animal models on stroke therapy. This intensity has proven therapeutically efficacious in previous in vitro experiments,[6,8] compares to the intensity that was used in the CLOTBUST trial, and is considerately lower than the intensity used in the TRUMBI trial.

There are limitations to this study. Interaction of low-frequency ultrasound with brain tissue depends on various factors, including the total volume exposed to ultrasound. Therefore, data gathered in a rat model will not be directly transferable to the conditions in the human skull. Experiments were performed in healthy animals. Knowledge about interactions of ultrasound with healthy brain tissue is very important because in case of therapeutic sonification of intracranial vessel occlusion, healthy tissue is always exposed to the sound field. However, mechanisms of damage may be different in ischemic tissue. This may be attributable to increased vulnerability of tissue and vessels and to reduced perfusion, which may lead to more pronounced heating effects. Studies of low-frequency ultrasound in intracranial vessel occlusion models will have to bear this in mind.

In summary, we have shown that low-frequency ultrasound may be harmful to healthy brain tissue. Knowledge of these mechanisms of interaction may lead to an increased understanding of comprehensive and safe application of low-frequency ultrasound as a therapeutic tool in acute cerebral vessel occlusion.

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