Brain Temperature During 340-kHz Pulsed Ultrasound Insonation
A Safety Study for Sonothrombolysis

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Background and Purpose—Because ultrasound is used for improving thrombolysis of cerebral infarction but continuous ultrasound insonation also has significant thermal effects, we evaluated brain temperature increase and tissue destruction during pulsed ultrasound emission.

Methods—We examined 340-kHz pulsed ultrasound effects in male Wistar rats. Ultrasound was applied transcranially for 30 minutes on different power levels (1 to 7 W/cm²). Temperature was measured at different locations (brain, in the focus of ultrasound beam, inner ear, temporalis muscle, and rectum). The cooling time after 30-minute insonation for every power level was recorded, and animals were examined by postmortem brain histology (TUNEL and hematoxylin/eosin).

Results—Brain temperature increased within 2 to 5 minutes of insonation. Brain temperature increase and cooling time were in proportion to power level, and even with the highest intensity of 7 W/cm² for 30 minutes, the maximum elevation of mean brain temperature was 0.9°C, with the highest cooling time of 40 minutes. No deleterious side effects of this treatment could be found in histological examination.

Conclusions—Using a pulsed ultrasound design, only a moderate temperature increase could be observed with no histopathological abnormalities. Deleterious side effects of mid-kilohertz ultrasound (eg, intracerebral hemorrhage) are therefore not a consequence of local brain temperature increase. (Stroke. 2006;37:1883-1887.)

Key Words: animal models ■ temperature ■ thrombolytic therapy ■ ultrasonography

A increasing number of studies have addressed the therapeutic capacities of ultrasound. Ultrasound is used for sonothrombolysis and local drug and gene delivery. Most therapeutic effects need a longer or repetitive application of ultrasound and higher energy levels.

Ultrasound can have chemical (membrane and enzyme dysfunction) as well as thermal effects. In the setting of sonothrombolysis for stroke therapy, thermal effects are important because temperature is linked to brain metabolism. Thus, an increase of brain temperature has to be assessed regarding unwanted effects (like necrotic or apoptotic cell death) in ischemic areas but also regarding direct damage of intact tissue.

We performed the TRUMBI trial, a sonothrombolysis study using 340 kHz, which was stopped because of a higher intracranial hemorrhage rate of ultrasound-treated patients.

Because of the serious adverse effects in this study, we had to go back to animal experiments verifying so far unknown tissue effects of ultrasound. Despite the efficacy of sonothrombolysis using 2 MHz as shown in the CLOTBUST trial, some data suggest a thrombolytic effect of mid-kilohertz ultrasound as well.

Nolle demonstrated a positive correlation of power output and tympanic temperature: up to 1.8° with 12 W using a 20-kHz emitter in a continuous wave insonation mode. Suchkova showed that one half of a continuous wave effect was obtained with the sound only one tenth of the time. A significant enhancement of fibrinolysis was achieved even with a 1% duty cycle. But others still demonstrated that exposure to pulsed ultrasound can significantly elevate intracranial temperature. We therefore investigated the thermal effects of pulsed ultrasound with a duty cycle of 20% on the brain in an in vivo rat model. We used an emitter in the mid-kilohertz range (340 kHz) and increasing electrical power levels (1 to 7 W/cm²) to validate extracranial temperature measurements for monitoring brain temperature and early tissue changes like apoptosis or acute hemorrhage.

Materials and Methods

Animals
Male Wistar rats (n=5; Charles River; Sulzfeld, Germany) were used and weighed between 470 and to 600 g. They were housed under standard conditions with a 12-hour light/dark cycle and food...
and water ad libitum. All experiments were in accordance with the local ethics committee and were performed under stable external temperature and without any attempt to actively cool down brain temperature.

**Surgery**

Animals were initially anesthetized in a small chamber filled with 2% isoflurane and were then placed into a stereotactic frame (Kopf Instruments), breathing independently a mixture of 50% oxygen/air with 1.3% isoflurane. In the stereotactic frame, interfering cooling effects were avoided by keeping the body temperature during the experiment constant (37.0 ± 0.1°C). Therefore, we used a rectal temperature probe connected to a heating pad (CMA 150; CMA Microdialysis AB). The head of the animal had no contact to this pad during the whole experiment.

**Temperature Probes**

In addition to the rectal temperature probe, a second probe was inserted into the left temporalis muscle (MT 23/3; Physitemp Instruments) and a third one in the left meatus acusticus externus just in front of the eardrum (IT-18; Physitemp Instruments). After removing the fur overlying the cranial vault using an electric razor, a sagittal incision along the center line of the skull was made and the skin retracted from the top of the skull. A 1-mm-diameter burr hole was drilled in the frontal skull bone in accordance with Paxinos and Watson at 3 mm lateral from the center line and 1 mm anterior of the bregma. The fourth temperature needle probe (MT 23/3; Physitemp Instruments) was then introduced into the hole 5 mm deep, directing the tip of the needle to end under the bregma, resulting in a position in the ultrasonic beam center. All thermocouples were connected to a temperature monitor (BAT-12; Physitemp Instruments).

**Ultrasound Settings**

Transducer diameter (Walnut Tech) was 1 cm and was placed midline on the animal’s head while the animal was fixed in the stereotactic frame (Figure 1). We used a power amplifier AR 40AD1 (Amplifier Research), signal generator HP33120A (Hewlett Packard) with a transducer (Ultran Laboratories, Inc), and a storage oscilloscope TDS 210 (Tektronix Corp) to produce the ultrasonic beam (frequency 340 kHz, duty cycle 20%, amplitude 50 mV peak-to-peak, burst rate 10 Hz, burst count 6800). Electrical intensity to the transducer was increased in steps of 1 W/cm² from 1 W/cm² to 7 W/cm², resulting in a mechanical index 0.08 to 0.2. Ultrasound gel (Aquasonics 100; Parker Labs) was administered between the transducer and skull bone. Ultrasound insonation was started after body temperature had reached a steady state.

**Temperature Monitoring**

The temperature was measured at all locations every 5 minutes for 30 minutes. After the ultrasound beam was turned off, a cooling period was allowed until the brain temperature returned to its baseline temperature. Then the intensity level was increased by 1 W/cm²; ultrasound was turned on, and the temperature was measured again for 7 time points up to 30 minutes. This sequence was repeated 7 times for a total ultrasound time of 3.5 hours.

**Histology**

The animals were euthanized one half hour after ultrasound application and fixation by transcardial perfusion of 4% formalin. Brains were removed and stored in 4% formalin until paraffin embedding and staining. Formalin-fixed brains were cut in 7 to 8 coronal slices per animal and were evaluated every 2 mm for apoptosis using TUNEL staining according to the standard protocol from the producer (NeuroTACS In Situ Apoptosis Detection Kit, R No. TA900XXX), and standard hematoxylin/eosin staining was also performed.

**Results**

**Temperature Probe Location**

Measurements of the different temperature probes were highly correlated (Figure 2). However, there was some type of offset between the temperature probes. The highest temperatures were measured by the thermocouple implanted in the brain tissue. All other sensors showed lower temperatures. The monitoring sites that came closest to the brain temperature were: tympanic (n=2), rectal (n=2), and temporal (n=1) locations. Temperature difference between brain tissue and the closest matching site was up to 0.9°C.

**Temperature Rise**

All animals showed an increase of brain temperature during ultrasound exposure. The highest temperature measured was 38.8°C, which was also the highest relative temperature increase (2.7°C from 36.1°C). On average, the maximum
temperature increase from baseline was <1°C. The maximum temperature was reached after the first 3 to 5 minutes and then slowly fell down without reaching starting temperature as long as ultrasound was turned on. All animals showed an increase of temperature rise correlating to the initial power increases (Figure 3). After turning ultrasound off, different cooling time (time to reach temperature levels at 0 W/cm²) was observed according to power levels (Figure 4).

**Histology**

Neither intracranial hemorrhage nor necrosis or other pathological changes in brain morphology could be observed. Additionally, no animals showed apoptotic cell death in TUNEL staining (Figure 5a and 5b).

**Discussion**

Our group has demonstrated the efficacy of ultrasound in an in vitro thrombolytic model using mid-kilohertz ranges⁶,⁷ and also showing the efficacy in an in vivo embolic stroke model.⁸ In the human ultrasound thrombolysis study (TRUMBI), we used a frequency of 340 kHz; however, higher intracranial bleeding rate led to abortion of this study.¹ Higher frequencies such as 2 MHz used in the CLOTBUST study, did not show this serious adverse effects⁹ but reasons for this difference are still unclear. The balance between thrombolytic efficacy and harmful effects such as tissue heating depends mainly on the frequency used. Effects <40°C are negligible (National Council on Radiation Protection and Measurements, 1992). This is important because Morikawa et al showed that high brain temperature worsens cerebral infarction.¹⁰⁻¹³ Heating of tissue triggers other changes, such as blood perfusion increase, cell activation, or influencing molecular effects such as protein synthesis or membrane integrity. The circulating blood and cerebrospinal fluid may distribute these effects.¹⁴ Barnett demonstrated that the best cooling effect of blood flow is received if a wide focused beam is used.⁴ The authors postulated irreversible damage in the developing embryo if there was a temperature increase of 4°C for 5 minutes. Madio¹⁵ showed changes in gene expression after ultrasound exposure. Induction of heat shock (or stress) proteins suggests that the changes in gene
expression may protect cells from death. However, changes in gene expression may also initiate apoptosis. We could not observe any tissue damage, neither cerebral hemorrhage, necrosis, nor apoptosis. To find these effects, especially apoptosis, in such an early stage of tissue damage is not easy, but in areas like caudate putamen and other areas, early changes could be seen within a few hours. Later histological examinations might have missed acute effects of ultrasound insonation.

Lower frequencies shift their effect from a temperature to a more mechanical effect. It is postulated that 1 MHz ultrasound of 1.6 W/cm² potentiates the inflammatory response by facilitating the adhesion of leukocytes to the endothelium. In our experiments, animals were exposed to pulsed ultrasound with a duty cycle of 20% (resulting at the 7-W setting in an average electrical power of 1.4 W). Even at the highest levels of ultrasound exposure, the observed heating of brain tissue was moderate. Average brain temperature increase in all animals was <1°C. It should be noted that all of these exposures were conducted without any active cooling mechanism. The initial increase in temperature at 0 W/cm² could be explained by warming up of the animal after replacement into the stereotaxic frame with the heating pad from first placing it into the small chamber filled with 2% isoflurane. Therefore, using pulsed ultrasound for brain insonation prevents tissue heating as seen in other experimental designs. The transient increase of temperature during a specific intensity observed in our experiment might be explained by the autoregulation of blood vessels reacting to the increased temperature, especially because ultrasound transducer and position is able to insonate nearly the whole brain of the animal. It was a limitation of our study that we have not measured the cerebral blood flow during and after ultrasound.

But our experiments also showed that there is no good extracranial surrogate measurement for brain temperature in this animal model. Differences in temperature between brain tissues and the surrogate site were up to 0.9°C. Neither tympanic nor temporal measurements could reliably identify the true temperature increase in the brain tissue. Therefore, intracerebral monitoring should be conducted whenever possible in future animal experiments requiring monitoring of brain tissue temperature. We measured brain temperature invasively using a needle probe and in ≥5 mm distance from the ultrasound transducer and not directly under the skull, which was shown as the most sensitive point of tissue heating, to avoid tissue destruction by the temperature probe interfering with our histological evaluation. Nevertheless, we measured temperature on skull surface and ultrasound gel by a needle probe that was always lower than intracranial brain temperature.

Summary
This study demonstrates a good correlation of tissue heating and transcranially transmitted ultrasound of mid-kilohertz range (340 kHz). By using a duty cycle of 20% even with the highest intensity of 7 W/cm² for 30 minutes, only a maximum elevation of brain temperature of 0.9°C was observed. Temperature rise reached its maximum in the first 2 to 5 minutes of insonation and returned to baseline levels according to the power level up to 45 minutes after turning off the ultrasound beamer. No intracranial hemorrhage or apoptotic cell death could be observed and no good surrogate marker of brain temperature measurement could be identified in this experimental setting. The brain itself seems to be the best location for monitoring animal experiments.

On the basis of these data and on the background of the successful CLOTBUST trial, a dose escalation study for mid-kilohertz ultrasound is needed to balance out thrombolytic effects and harmful side effects as well as to compare efficacy and safety with clinical, already successful 2-MHz ultrasound.

Acknowledgments
We are grateful to Mrs A. Braun-Kornmueller and Mrs A. Roedl for their technical assistance, as well as Mrs Garcia-Knapp for her secretarial help.

Disclosures
None.

References


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*Stroke*. 2006;37:1883-1887; originally published online June 1, 2006;
doi: 10.1161/01.STR.0000226737.47319.aa
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/37/7/1883

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