Effective and Safe Conditions of Low-Frequency Transcranial Ultrasonic Thrombolysis for Acute Ischemic Stroke

Neurologic and Histologic Evaluation in a Rat Middle Cerebral Artery Stroke Model

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Background and Purpose—Transcranial ultrasound (TUS) enhances thrombolysis and is expected to be useful for the treatment of ischemic stroke. However, neither its effectiveness in improving neurologic outcome nor its safety in living tissue has been fully established. We examined the efficacy and safety of low-frequency TUS under appropriate conditions of ultrasound for thrombolytic treatment in a rat middle cerebral artery stroke model.

Methods—Sixty-five male Wistar rats were used. Rats with right middle cerebral artery stroke exhibiting left hemiparesis were blindly selected and randomly assigned to 1 of 3 groups: (1) control, no therapy; (2) tPA, intravenous administration of tissue plasminogen activator 3 hours after middle cerebral artery stroke, or (3) TUS, tPA administration and application of TUS (490 kHz, continuous wave, at an intensity of 0.8 W/cm²). Twenty-four hours after the onset of stroke, neurologic improvement was evaluated and brains were then removed. Thrombolysis at the origin of the right middle cerebral artery was examined. Thrombolysis ratio, cerebral infarct ratio, and rate of histologic evidence of hemorrhage were compared in the 3 groups.

Results—Significantly better neurologic improvement (P=0.008), a higher thrombolysis ratio (P=0.041), and a reduction of cerebral infarct volume (P=0.047) were obtained in the TUS group compared with the tPA group, without an increase in hemorrhagic transformation.

Conclusions—Our findings suggest that thrombolytic treatment with low-frequency TUS under appropriate conditions could be an effective and safe method of treatment for ischemic stroke. (Stroke. 2008;39:1007-1011.)

Key Words: neurologic improvement ■ rats ■ safety ■ thrombolysis ■ ultrasound

The thrombolytic efficacy of transcranial ultrasound (TUS) in stroke treatment has attracted a great deal of attention among stroke investigators. Use of low-frequency ultrasound has been studied to enhance the thrombolytic effect and has yielded an improvement of the thrombolytic effect, as well as good skull penetration in vitro. Moreover, a reduction of cerebral infarct volume by means of low-frequency TUS has been reported in a rat middle cerebral artery (MCA) stroke model. However, unexpected hemorrhagic complications occurred in the TRUMBI trial. Some studies were subsequently performed to investigate the reasons for these complications. Certain factors (intensity-dependent effects in the brain, blood-brain barrier disruption, etc) have been considered the major reasons for hemorrhage after low-frequency TUS in stroke treatment in humans, based on speculations regarding the reasons for complications in the TRUMBI trial.

It is likely that the appearance of side effects of low-frequency ultrasound depends on the ultrasound settings used. We previously reported that low-frequency (490 kHz), low-intensity (0.8 W/cm²) ultrasound can penetrate the skull well and facilitate thrombolysis in a rabbit model of femoral artery thrombosis. However, whether our low-frequency, low-intensity ultrasound conditions are effective in enhancing neurologic improvement and are safe for the brain have not yet been assessed in an animal stroke model. One of the major objectives of this study was therefore to evaluate the efficacy of TUS in enhancing neurologic improvement after stroke under the same ultrasound conditions in a rat MCA stroke model. Another important objective of this study was to verify the absence of adverse effects (eg, hemorrhage) of TUS on the brain. The efficacy and safety of low-frequency, low-intensity TUS with appropriate ultrasound conditions for stroke treatment were thus evaluated.
Materials and Methods

All animal procedures were performed under the guidance of the animal research committee (Jikei University School of Medicine, Tokyo, Japan). Male Wistar rats (n=65) weighing 339±32 g (mean±SD) were used in this study. During all surgical procedures, they were anesthetized via inhalation of 2% isoflurane in 100% O₂ delivered via face mask during surgery. Rectal temperature was measured and maintained at 37±0.5°C throughout the surgical procedure with intermittent use of a heating plate (UP-101, Kainuma Sangyo Co, Ltd, Nagoya, Japan).

Thrombus Preparation and Surgical Procedures

We prepared a rat model of MCA stroke and prepared the thrombus according to the method previously reported by Zhang et al. In brief, blood was drawn from the same rat on the day before the experiment. After a 2-hour incubation (37°C), the blood was stored for 22 hours (4°C). Then, a 25-mm-long, fibrin-rich thrombus was prepared. The right internal carotid artery of the rat was exposed under a surgical microscope. The prepared thrombus was injected with saline through an external carotid artery stump with a modified PE-50 catheter (outer diameter of the tip, 0.3 mm) connected to a 100-μL Hamilton syringe.

Neurologic Evaluation

In all animals (including those of the control group), an initial neurologic evaluation was performed 3 hours after the surgical procedure and after the animal awakened from anesthesia. On a 5-point scale (0=no neurologic deficit, 1=right Horner syndrome, 2=failure to extend the left forepaw fully, 3=turning to the left, and 4=circling to the left), neurologic evaluation was blindly performed 3 hours (after awakening from anesthesia) and 24 hours after production of the right MCA stroke. Animals with scores of 3 or 4 were entered into this study (n=58). Seven rats with scores of 0, 1, or 2 were excluded from evaluation. The animals were then reanesthetized for treatment. In the control group, rats were reanesthetized without treatment.

Classification of Animal Groups

The animals included in this study (n=58) were randomly allocated to the following 3 groups: (1) control group (n=15), no therapy; (2) tPA group (n=21), intravenous administration of tissue plasminogen activator (monteplase; Cleactor, Eisai Co, Ltd, Tokyo, Japan) in 0.6-mL saline via the tail vein over 2 minutes 3 hours after the onset of stroke; or (3) TUS group (n=22), tPA administration and application of TUS. The usual human dose of monteplase (0.22 mg/kg) differs from that of alteplase (0.9 mg/kg). The strength of effect of human tPA in the rat is only 10% of that in humans. In this study, a dose sufficient for rats (1.2 mg/animal) was administered in consideration of drug efficacy. The mean body weights of animals in the treatment groups were as follows: control group, 333±45 g; tPA group, 345±28 g; and TUS group, 338±23 g (mean±SD). No significant differences were noted among the 3 groups. The mean durations of anesthesia during surgery for animal model production were as follows: control group, 47.6±15.8 minutes; tPA group, 51.5±8.7 minutes; and TUS group, 47.2±6.8 minutes (mean±SD). No significant differences were noted among the 3 groups. When operative time was <1 hour, general anesthesia was continued until 1 hour had elapsed to avoid differences in anesthesia time among animals. An additional 1 hour of general anesthesia for treatment was performed 3 hours after MCA stroke in each group. No significant difference in total anesthesia time was noted among the 3 groups. TUS without tPA administration was not performed, because ultrasound alone does not activate the fibrinolytic cascade.

Ultrasound System and Method of TUS

The ultrasound system used was described in our previous study. The ultrasound transducer used in this study was designed and custom-made for animal studies and was a ceramic type with a diameter of 5 mm. The transducer was connected to an amplifier (Honda Electronics Co, Ltd, Toyohashi, Japan), and an ultrasound generator (Wave Factory 1941, NF Corp, Japan) yielded 490-kHz continuous-wave irradiation. The transducer was set on the right coronal suture of the skull after a scalp incision had been made to ensure that ultrasound irradiation from the transducer anatomically covered the origin of the right MCA. To minimize the harmful effects of and to maximize skull penetration by TUS, ultrasound conditions, including a frequency of 490 kHz and an intensity of 0.8 W/cm², were selected for this study. Ultrasound intensity was confirmed with an ultrasound power meter (UPM-DT-1, Ohmic Instruments, Inc).

To avoid tissue heating, TUS was applied intermittently. After 2 minutes of continuous TUS, irradiation was interrupted for 30 seconds. This pattern of TUS application was repeated 4 times during a 10-minute period, after which TUS was not performed for 5 minutes to enable cooling. These 10-minute cycles of TUS, each followed by a 5-minute break, were repeated 4 times over a period of 60 minutes. The actual duration of TUS was thus 32 minutes. Rectal temperature was measured and maintained at 37±0.5°C during the experiment.

Neurologic Improvement Ratio

Neurologic evaluation was performed blindly again 24 hours after the onset of stroke. Animals with an improvement in score to level 2, 1, or 0 were judged to have exhibited neurologic improvement. The neurologic improvement ratio in each group was determined as the ratio (in percentage) of the number of animals with neurologic improvement to the total number of animals in that group. The mean difference in neurologic score before versus after treatment was also determined. The actual mean pretreatment and posttreatment neurologic scores are listed in Table 1.

Thrombolysis Ratio

After neurologic evaluation, the rats were deeply anesthetized with 5% isoflurane inhalation and transcardially perfused with 50 mL heparinized saline (5 U/mL) and 50 mL 10% buffered formalin. The brain was carefully removed and then examined to determine whether thrombus was present in the origin of the right MCA. The thrombolysis ratio was defined as the ratio (in percentage) of the number of animals with dissolution of thrombus to the total number of animals in each group.

Cerebral Infarct Ratio

Each brain was cut into 2-mm-thick coronal sections. Seven coronal sections were obtained from 1 brain. Then 5-μm thinly sliced specimens were prepared and stained with hematoxylin and eosin. Stained coronal specimens were scanned with a flatbed scanner (ES-8500, Seiko Epson Corp, Japan) at a resolution of 1600 dots per inch, and the scanned images were saved on a personal computer. The images were analyzed with Photoshop image-processing software. The outlines of the area of cerebral infarct and the contralateral cerebral hemisphere on each scanned image were traced with a graphics tablet system (model i-400, Intuos, Wacom Co, Ltd, Japan). The image of the enclosed area was saved in a compressed tagged image file format. The areas of cerebral infarct and of the contralateral cerebral hemisphere in the tagged image file format were

<table>
<thead>
<tr>
<th>Table 1. Mean Pretreatment and Posttreatment Neurologic Scores</th>
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<tr>
<td>Pretreatment*</td>
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<td>Posttreatment†</td>
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</table>

*No significant difference was noted among the 3 groups in pretreatment score.
†The difference between before vs after treatment in mean neurologic score was statistically significant in the TUS group (P=0.025, Mann–Whitney U test).
measured with ImageJ version 1.31 software. The total volume of cerebral infarct was determined by integration of the areas from the sections. The cerebral infarct ratio was determined as the ratio (in percentage, mean $\pm$SD) of the infarct volume to the volume of the contralateral hemisphere in each group.

**Histologic Examination for Hemorrhage**

Histologic examination was performed to determine the incidence of hemorrhage (Figures 1A and 1B). The incidence of hemorrhage was evaluated according to the methods described by Niessen et al.\textsuperscript{21} In brief, scoring for histologic hemorrhage was performed as follows: 0=no hemorrhage, 1=single, microscopically visible hemorrhage, 2=multiple microscopically visible hemorrhages, 3=microscopically visible, non–space-occupying hemorrhage, and 4=microscopically visible, space-occupying hemorrhage.

**Statistical Analysis**

Logistic-regression models were applied to determine the effects of TUS on the thrombolysis ratio and neurologic improvement ratio in the 3 groups, with odds ratios (ORs) and 95% CIs calculated. The mean differences from pretreatment neurologic score, mean body weight, and mean time of surgery for animal model production in the 3 groups were statistically examined with 1-factor ANOVA. The mean difference in neurologic score before versus after treatment was statistically analyzed with the Mann–Whitney $U$ test. Cerebral infarct volume ratios were also statistically analyzed with the Mann–Whitney $U$ test. Statistical analyses were performed with the STATA 8.0 statistical analysis program (STATA Corp, College Station, Tex).

**Results**

**Neurologic Improvement Ratio**

One animal (7.14%) in the control group, 5 (25%) in the tPA group, and 14 (66.7%) in the TUS group exhibited neurologic improvement. A significant difference was observed between the tPA and TUS groups ($P=0.008$, Figure 2). The OR was 0.17 (95% CI, 0.04 to 0.65). No significant difference was observed between the control and tPA groups.

**Thrombolysis Ratio**

Three animals (21.4%) in the control group, 9 (45.0%) in the tPA group, and 16 (76.2%) in the TUS group exhibited complete thrombolysis at the origin of the right MCA. A statistically significant difference was observed between the tPA and TUS groups ($P=0.040$, Figure 3). The OR was 0.26 (95% CI, 0.07 to 0.97). No significant difference was observed between the control and tPA groups.

**Cerebral Infarct Ratio**

The mean cerebral infarct ratios were 20.9$\pm$14.1%, 15.1$\pm$13.7%, and 10.6$\pm$14.6% in the control, tPA, and TUS groups, respectively (mean $\pm$SD). A statistically significant difference in cerebral infarct volume was noted between the tPA and TUS groups ($P=0.047$, Figure 4). No significant difference was observed between the control and tPA groups.

**Histologic Examination for Hemorrhage**

Macroscopically visible hemorrhage was not observed in any of the animals. All cases of microscopically small hemorrhage were confined to ischemic areas. The incidence of microscopic hemorrhage was evaluated (Table 2). No statistically
tically significant differences in rates of microscopic hemorrhage were noted among the 3 groups.

Mortality Rate
One animal died in each group after treatment (from 4 to 24 hours after the onset of stroke). Mortality rates were as follows: control group, 6.67%; tPA group, 4.76%; and TUS group, 4.55%. No significant differences were noted among the 3 groups in this parameter. Because we did not know when the animals died, neurologic improvement ratio, thrombolysis ratio, cerebral infarct ratio, and histologic examination for hemorrhage were evaluated after excluding the data for the dead animals.

Discussion
This study revealed that neurologic improvement and safety could be achieved by means of TUS in a rat MCA stroke model. The results of this study clearly indicate that TUS can enhance the thrombolytic effect of systemic tPA administration and thereby significantly reduce cerebral infarct volume and improve neurologic status. Our findings also suggest that TUS does not have major adverse effects on the brain, including induction of hemorrhagic complications.

![Figure 3](https://stroke.ahajournals.org/)

**Figure 3.** Comparison of thrombolysis ratios. The thrombolysis ratio in the TUS group was significantly higher ($P=0.041$) than in the tPA group. NS=not significant.

![Figure 4](https://stroke.ahajournals.org/)

**Figure 4.** Comparison of cerebral infarct ratios. The cerebral infarct ratio (% mean±SD) was significantly lower ($P=0.047$) in the TUS group than in the tPA group. NS=not significant.

Table 2. Incidence of Hemorrhage

<table>
<thead>
<tr>
<th>Animal group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (n=14)</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>tPA (n=20)</td>
<td>11</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TUS (n=21)</td>
<td>12</td>
<td>2</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Scoring of histologic hemorrhage was performed as follows: 0=no hemorrhage, 1=single, microscopically visible hemorrhage, 2=multiple microscopically visible hemorrhages, 3=microscopically visible, non-space-occupying hemorrhage, and 4=macroscopically visible, space-occupying hemorrhage. No statistically significant difference was observed among the 3 groups (Mann–Whitney $U$ test).

In previous studies, cerebral infarct volume reduction was not always achieved when thrombolysis was begun 2 hours after MCA stroke or when MCA reperfusion was performed 3 hours after MCA stroke in rat models of MCA stroke. We found no significant differences in cerebral infarct ratio between the control and tPA groups. Our results were compatible with the above-noted findings. On the other hand, significant differences in neurologic improvement ratio, thrombolytic ratio, and cerebral infarct ratio were noted between the TUS and tPA groups and between the TUS and control groups. Although tPA was given 3 hours after the onset of stroke in the present study, it is noteworthy that thrombolysis combined with TUS yielded significantly better neurologic improvement. The significantly higher neurologic improvement appeared to yield a significantly higher thrombolysis ratio and a significantly reduced infarct volume in the TUS group. It should thus be emphasized that the effects of TUS have the potential to widen the therapeutic time window of thrombolysis after the onset of stroke. Another important finding of this study is that TUS did not increase the incidence of space-occupying hemorrhagic complications. This complication was not observed in any animal, and all hemorrhages were microscopically confined to ischemic areas.

Daffertshofer et al reported on the TRUMBI clinical trial of thrombolysis, which was performed with low-frequency ultrasound (300 kHz, pulsed wave, with a 0.7 W/cm² spatial-peak temporal-average intensity [SPTA]), which was, however, associated with an unexpectedly high rate of hemorrhage. Many basic studies were subsequently performed to examine the safety of low-frequency TUS. Schneider et al reported a study on the safety of very-low-frequency (20 kHz, continuous wave) TUS in normal rat brains. In that study, no histologic damage was detected by magnetic resonance imaging after irradiation with TUS at 20 kHz with an intensity of 0.2 W/cm². However, because the ultrasound intensity was increased to 0.5 and 1.1 W/cm², the apparent diffusion coefficients decreased significantly. At an ultrasound intensity of 2.6 W/cm², significant loss of neurons was noted on histologic examination. Furthermore, a recent study demonstrated that TUS (20 kHz, continuous wave), even at low intensity (0.2 W/cm²), resulted in a significantly higher death rate when applied to a rat MCA stroke model. These results indicate that low-frequency TUS has the potential for brain damage, depending on the ultrasound conditions used. However, it should be pointed out that the mechanical index (MI)
is an important indicator that can be used to estimate the potential for mechanical effects arising from ultrasound. Several methods for determination of MI have been reported,\textsuperscript{25,26} and the formula used to calculate MI is as follows:

\[ MI = \left(10 \rho_{\text{water}} C_{\text{water}}^{\frac{2}{3}} f \right) \\left(\frac{I_{\text{max}}}{I_{\text{PPA}}}\right) \]

where \( I_{\text{max}} \) is the intensity (W/m\(^2\)), \( \rho_{\text{water}} \) is the density of water (\( \rho_{\text{water}} = 10^3 \) kg/m\(^3\)), \( C_{\text{water}} \) is the speed of sound in water (\( 1.54 \times 10^3 \) m/s), and \( f \) is the frequency (MHz). The MI of ultrasound in Schneider's study, for 0.2, 0.5, 1.1, and 2.6 W/cm\(^2\), can be calculated as 0.55, 0.88, 1.33, and 2, respectively. The MI of ultrasound used in our study was much lower (MI = 0.22) than in the above-noted studies. Because a biologic effect can occur at an MI >0.5,\textsuperscript{25} the results of our study (low hemorrhage rate, strong thrombolytic effect, and significant neurologic improvement) suggest that TUS performed at 490 kHz (continuous wave) with an intensity of 0.8 W/cm\(^2\) meets the requirements for effectiveness and safety of low-frequency TUS. In the TRUMBI trial, although the ultrasound intensity was 0.7 W/cm\(^2\) (I\(_{\text{PPA}}\)) and MI was <0.2, ultrasound was emitted in a pulsed fashion with a 5% duty cycle. I\(_{\text{PPA}}\) is the average intensity between each of the pulsed waves; however, the spatial-peak pulse-average intensity (I\(_{\text{PPA}}\)) should be considered the actual intensity of ultrasound during pulsed-wave irradiation. I\(_{\text{PPA}}\) is defined by the formula

\[ I_{\text{PPA}} = I_{\text{PPA}} \times (1/\text{duty cycle \text{[\%]}}) \times 100. \]

Ultrasound intensity (I\(_{\text{PPA}}\)) could thus theoretically be instantaneously increased 20-fold. The MI for that ultrasound intensity (I\(_{\text{PPA}}\)) can be calculated to be a higher value (MI = 1.19). Because a high MI (MI = 1.19) could have adverse effects, this could have played a role in the hemorrhagic complications in the TRUMBI trial. Although low-frequency TUS has the potential for inducing brain damage, it appears likely that this unfavorable outcome can be avoided with use of appropriate ultrasound conditions. In addition, because differences between humans and animals exist in the reflection of ultrasound in the cranium, further investigations in appropriate animal models, ultrasound conditions, and clinical simulations will be required before clinical trials are performed.

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Disclosures

None.

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