Ultrasound Acceleration of rt-PA Thrombolysis Depends on Acoustic Intensity

Yoshikazu Sawaguchi* and Zuojun Wang

* Department of Clinical Pharmaceutics Nihon Pharmaceutical University; 10281 Komuro, Ina-machi, Kitaadachi-gun, Saitama 362–0806, Japan; and Division of Ultrasound Device Development and Application (DOUDA), the Jikei University School of Medicine; 3–25–8 Nishi-Shinbashiki, Minato-ku, Tokyo 105–8461, Japan.

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Regular Article

Recombinant tissue-type plasminogen activator (rt-PA) is effective and widely used in the treatment of acute ischemic stroke (AIS). However, symptomatic intracranial hemorrhage (ICH), an adverse reaction of rt-PA, is known to occur depending on underlying diseases and rt-PA doses, and to occur more frequently with a greater delay from stroke onset until initiation of rt-PA. Therefore, limitations on the use of rt-PA, such as having to be started within 4.5 h of stroke onset, mean that rt-PA is only indicated in some stroke patients. However, the number of patients in whom rt-PA is indicated could increase if symptomatic ICH induced by rt-PA could be reduced. Therefore, we believe that, if the incidence of adverse reactions such as ICH could be reduced by using lower rt-PA doses together with ultrasound (US), the number of patients eligible for rt-PA treatment would increase. In other words, we hypothesized that, if thrombolysis can be accelerated by US, then recanalization rates similar to currently used doses of rt-PA can be achieved at reduced rt-PA doses. Therefore, to investigate to what extent US enhances the thrombolytic efficacy of rt-PA, the relationship between acceleration of rt-PA thrombolysis and US intensity was quantitatively evaluated in an in vitro bovine thrombus model. It was found that, within a range of US output that is noninvasive in humans, the combined use of US can increase thrombolytic activity up to 2.5 times more than with rt-PA alone. These findings suggest that US can greatly reduce the required doses of rt-PA.

Key words noninvasive ultrasound; sonothrombolysis; tissue-plasminogen activator (t-PA); acute ischemic stroke (AIS)

Intravenous administration of the thrombolytic agent recombinant tissue-type plasminogen activator (rt-PA) is effective and widely used in patients with acute ischemic stroke (AIS). However, time restrictions, such as the need to start rt-PA within 4.5 h of stroke onset, and the fact that rt-PA may not be indicated with some underlying disorders, such as hypertension or high blood sugar, means that only some stroke patients are eligible for treatment. The current inclusion criteria for rt-PA are narrow, and when treatment is given without following the rt-PA inclusion criteria, a significantly increased risk versus benefit has been reported. The current rt-PA inclusion criteria are considered appropriate. However, even when the rt-PA inclusion criteria are followed, recanalization rates are only about 40%, and life-threatening adverse reactions such as symptomatic intracranial hemorrhage (ICH) occur in about 10% of cases. These many clinical problems call for urgent development of novel stroke therapies in AIS patients.

As an approach to improve recanalization rates with rt-PA in AIS, transcranial sonothrombolysis (TST), using transcranial ultrasound sonication to the site of blood flow occlusion during rt-PA thrombolysis therapy, has markedly improved recanalization rates. This together with novel rt-PA agent development is actively being investigated. In addition, to prevent ICH as an rt-PA adverse reaction, the use of inhibitors of matrix metalloproteinase (MMP), which plays an important role in blood–brain barrier (BBB) disruption, is being investigated. Data suggesting that the number of rt-PA-eligible patients can be increased and that the inclusion time window from stroke onset can be prolonged have often been reported, but this still has not resulted in an actual increase in indications or a prolonged time window. Symptomatic ICH as a life-threatening adverse reaction is one major reason for the narrow rt-PA indications. Because the incidence of symptomatic ICH seems to increase in an rt-PA dose-dependent manner, maintaining recanalization rates at lower rt-PA doses may increase rt-PA therapy indications and prolong the time window. We have focused our interest on TST for this reason. Ultrasonication during rt-PA therapy has already been shown to significantly improve recanalization rates, thus suggesting that US accelerates rt-PA thrombolysis. Therefore, if thrombolysis can further be accelerated by US, then recanalization rates similar to those obtained with currently used doses of rt-PA can be achieved at reduced rt-PA doses.

However, despite active research on thrombolysis acceleration methods using US, the relationship between acoustic intensity and US-accelerated thrombolysis has not been quantitatively investigated. Therefore, to clarify the relationship between US acoustic intensity and the rt-PA thrombolysis acceleration rate, we calculated the dose by which rt-PA could be reduced based on the rate of US acceleration of thrombolysis. The aim was to obtain basic data on reducing the incidence of symptomatic ICH in order to increase rt-PA indications and prolong the time window.

US frequency characteristics are such that, at higher frequencies, image resolution is better and energy is more easily attenuated. On the other hand, at lower US frequencies, image resolution is poorer, energy attenuation is more difficult, and penetration of hard tissue (bone) is easier. Therefore, for transcranial ultrasound (TUS) systems used clinically, a US probe with a relatively low frequency range of 1.5–2.5 MHz is used.
to achieve a balance between US penetration and image resolution. However, this frequency range does not satisfy all situations. For example, skull characteristics and thickness differ based on factors such as patient ethnicity, sex, and age; thus in some cases, lower US penetration of the skull makes US diagnosis more difficult.14–16 Therefore, the benefits of TST may not be achieved in patients in whom US penetration is insufficient. For this reason, we selected a frequency of 500 kHz to better adapt TST for a larger number of AIS patients. Our group previously reported that TUS at a frequency of 500 kHz penetrated the skull, was effective in accelerating thrombolysis, and was safe.17–19 Based on accumulated data, we believe that this was an appropriate frequency for the present study. Moreover, the primary objective in this study was thrombolysis acceleration and not imaging diagnosis, so there was no need for concern about image resolution. In regards to US acoustic power, the U.S. Food and Drug Administration (FDA) standards consider an acoustic intensity of $\leq 0.72 \text{ W/cm}^2$ to be noninvasive to humans; therefore, an acoustic intensity range $\leq 0.72 \text{ W/cm}^2$ was used in this study.

**METHODS**

**Clot Preparation** A 15$\phi$ hole was placed in the center of a 3-mm-thick acrylic sheet, and a 0.3-mm polycarbonate sheet was affixed for use as the clot cell (Fig. 1a). Bovine plasma prepared by dissolving lyophilized bovine plasma (Sigma-Aldrich Japan K.K., Tokyo, Japan) in ultrapure water was mixed with a solution of 200 mM CaCl$_2$ (final concentration: 20 mM) in the ratio of 9:1, and 530 $\mu$L of the mixture was poured in the hole. This was allowed to stand in a moist airtight container at 37°C for 1 h, resulting in preparation of a 15-mm-diameter, 3-mm-thick, discoidal clot.

**Thrombolysis** The rt-PA solution was prepared according to the manufacturer’s instructions (GRTPA® inj. 6 million IU, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) at 600000 IU/mL. This was diluted to the desired concentration in degassed saline at the time of use. Then, rt-PA solution 600 $\mu$L adjusted to the desired concentration was carefully poured without any overflow into another clean clot cell. The two clot cells, one with rt-PA solution and the other with a prepared clot, were put together face to face, avoiding mixing of bubbles and secured in a clot cell holder (Fig. 1b).

An ultrasonication device was designed such that the US probe could be aligned over the clot center axis. And this device allowed simultaneous examination of a US-exposed clot and a non-US exposed clot. Ultrasonication was performed in a 37°C water bath with the probe tip about 2 mm below the water surface, and 500-kHz sonication was continued for 30 min. To prevent US reflection, two 5-mm-thick latex sheets and a 14-mm-thick acoustic absorber tile (EUA101A, Eastek Corporation, Tokyo, Japan) were placed on the bottom surface of the water bath (Fig. 1c).

**Ultrasonication Conditions** The US probe beam characteristics were measured using an Acoustic Intensity Measurement System (AIMS: Onda Corporation, Sunnyvale, CA, U.S.A.) (Fig. 2a). Based on the US probe beam characteristics, the distance from the probe surface to the clot cell was set at 22 mm so that only the US main lobe would pass through the clot cell (15 $\phi$). The acoustic near field length limit of the 500kHz 10mm-diameter probe was calculated from the formula $X_0=D^2/4\lambda$ to have an acoustic near field length distance of 8.33 mm, therefore the clot cell was set completely in the acoustic far field. Figure 2b shows the acoustic field distribution at a distance of 22 mm from the probe. There is a single peak intensity distribution within a range of $\pm 7$ mm from the probe center. The drive voltage was adjusted such that the peak value of this distribution, namely, the center axis value, was 0.72 W/cm$^2$, which is the output limit for diagnostic US.
systems specified by the FDA standard.

**Evaluation of Suppression of Thrombus Growth** In a previous report, we described a method to measure the thickness (mm) of a discoidal bovine clot using a spectrophotometer. Clot absorbance (wavelength: 412 nm) was measured before and after ultrasonication (Fig. 3b) to evaluate reduced clot thickness (mm) as an index of thrombolytic activity. To convert clot absorbance to clot thickness (mm), a calibration curve for “thickness” and “absorbance” of a clot prepared in a calibration curve cell was used (Fig. 3c).

A custom-ordered automated spectroscopy program (JASCO) was used to measure the clot absorbance distribution. Namely, an XY stage on which the clot cell was placed was moved horizontally relative to the optical axis, and absorbance (wavelength: 412 nm) was automatically measured at 13 points (±6.0 mm from the center at 1-mm intervals) along the diameter through the center of the clot. Next, the absorbance distribution was similarly automatically measured along the diameter in 3 different directions, each with a 45° tilt relative to the nearby diameter (Fig. 3a). In other words, the absorbance distribution was measured in 8 directions along the radius from near the clot center. The mean values for
each clot were calculated from measurements at 8 equidistant points from the clot center axis. However, only 4 points were measured for the center, so the mean value of 4 points for the center was calculated.

**Statistical Analysis** Five sets of two each of the discoid clots (total of 10) were prepared for this study. Differences in clot thickness between the US-exposed clot and the non US-exposed clot of each two-clot set were examined by Student's *t*-test. The level of statistical significance was *p* < 0.05.

**RESULTS**

The risk of ICH with rt-PA therapy for AIS is known to differ among ethnic groups, and compared to Western countries where ICH tends to occur more often. In other words, rt-PA at a dose of 0.9 mg/kg is used in Western countries, whereas a dose of 0.6 mg/kg is used in Asian countries. In addition, TUS penetration in Asians is lower than in Westerners. Therefore, in this study focusing on Asian patients, for whom TST is less likely to be indicated, TST was investigated at a standard 0.6 mg/kg dose of rt-PA. The rt-PA concentration used in this study was 1000 IU/mL, which corresponds to the serum concentration at a dose of 0.6 mg/kg based on the package insert for alteplase (GRTPA®). Estimated serum concentrations of 667 IU/mL with a 0.4 mg/kg dose and 333 IU/mL with a 0.2 mg/kg dose were also evaluated at the same time. US-accelerated thrombolysis was compared, and the thrombolysis acceleration rates at each acoustic intensity were examined. Thrombolytic activity increased with ultrasonication in all 3 groups at different rt-PA concentrations. Thrombolytic activity was also greater closer to the clot center, namely, at a higher acoustic intensity. In the rt-PA 333 and 1000 IU/mL groups, exposure to an acoustic intensity of ≥0.28 W/cm² within 4 mm of the clot center markedly increased thrombolytic activity. Moreover, in the rt-PA 667 IU/mL group, exposure to an acoustic intensity of ≥0.16 W/cm² within 5 mm of the clot center markedly increased thrombolytic activity. On the other hand, as expected, differences in thrombolytic activity were observed in the non-US group at different rt-PA concentrations, but no large differences in thrombolytic activity that were associated with distance from the clot center were seen (Fig. 4).

To examine the relationship between acoustic intensity and thrombolytic activity, the Fig. 4 results are converted to acoustic intensity on the X-axis, and the relationship between acoustic intensity and thrombolytic activity at rt-PA concentrations of (●) 333 IU/mL, (■) 667 IU/mL, and (▲) 1000 IU/mL is analyzed. In addition, (-----) shows thrombolytic activity with rt-PA 1000 IU/mL alone.
other words, with US acoustic intensity of 0.17 W/cm² using rt-PA 667 and US acoustic intensity of 0.41 W/cm² using rt-PA 333 IU/mL, thrombolytic activity was about the same as when using rt-PA 1000 IU/mL alone (Fig. 5).

To examine to what extent rt-PA thrombolysis was accelerated by US, the thrombolysis acceleration ratio was evaluated at each acoustic intensity ([ΔUS (mm)−Δnon-US (mm)]/Δnon-US (mm)×100] in the 3 groups of rt-PA concentrations. In addition, how the thrombolysis acceleration ratio varied with differences in rt-PA concentration was also analyzed (Fig. 6). The relationship between the thrombolysis acceleration ratio and acoustic intensity for each rt-PA concentration was plotted nearly along the same line in almost all cases. In all groups using noninvasive US acoustic intensity, thrombolysis was accelerated up to about 2.5 times greater than thrombolytic activity with rt-PA alone. The correlation coefficients obtained from the regression lines for each rt-PA concentration were: rt-PA 333 IU/mL, 0.994; rt-PA 667 IU/mL, 0.993; and rt-PA 1000 IU/mL, 0.994. Each regression line yielded high values ≥0.993.

**DISCUSSION**

The results of the present study suggest that the ultrasound intensity and the rt-PA concentration had an obvious significant synergistic effect. As shown in Figs. 4 and 5, the thrombolysis was accelerated more at a higher rt-PA concentration with the same ultrasound intensity. But when we used the thrombolysis acceleration ratio as an index of the US-acceleration effect, it was dependent only on acoustic intensity, with minimal influence of the rt-PA concentrations as shown in Fig. 6. Therefore we adopted the thrombolysis acceleration ratio, which may express the effect of US more concisely and stably, as the main index for US-accelerated thrombolysis in this study. The thrombolysis acceleration ratio was up to about 2.5 times greater with noninvasive US, thus demonstrating that combined US can reduce required rt-PA doses by up to about 60%. The present study assumed future use for stroke treatment, so a bovine fibrin clot was used as a model of a white thrombus that easily forms in arteries. However, whether similar effects occur with a red thrombus using fresh blood and tenecteplase in clinical studies of acute myocardial infarction.11,12) Lower rt-PA doses may be an effective approach because rt-PA can induce MMP-3 and MMP-9, reducing induction of MMP-3 and MMP-9 in the brain by rt-PA has been reported in a stroke animal model, and this has received attention as a key factor related to why rt-PA can cause ICH. Therefore, MMP inhibitors can reduce the incidence of ICH.22) Because rt-PA can induce MMP-3 and MMP-9, reducing rt-PA doses is also important in reducing ICH. Moreover, an increase in dose-dependent adverse neurological events such as ICH has been reported with increased doses of alteplase and tenecteplase in clinical studies of acute myocardial infarction.11,12) Lower rt-PA doses may be an effective approach in reducing these events. The European Cooperative Acute Stroke Study (ECASS) II and III studies showed that the time window for rt-PA can be extended from 3 to 4.5 h, and the main reason was no change in the incidence of ICH.23,24) Therefore, if the incidence of ICH can be reduced or kept the same at times later than 4.5 h, the rt-PA time window of 4.5 h can be prolonged.

In regards to US safety, Alexandrov et al. used 2-MHz transcranial Doppler (TCD) in AIS patients for a total of 2 h, 1 h during IV rt-PA therapy and 1 h after rt-PA was completed, and reported that recanalization rates with US increased from
29 to about 39% with no increase in adverse events, thus showing that diagnostic US approximately 2 MHz frequency was very safe in rt-PA treated-AIS patient. However, in the phase II TRUMBI trial using 300kHz US to increase skull penetration, ICH was reported in up to 93% of patients, and the clinical study was discontinued. Therefore, when using a frequency range lower than 1 MHz, appropriate US conditions must be selected.

Azuma et al. investigated the possible cause of the ICH in phase II TRUMBI trial using human cranium. With US in burst mode at ≤1 MHz, significant standing waves occurred due to reflections and the standing waves caused bubbles generation in the skull. This means standing waves and cavitation may underlie the incidence of ICH. Azuma et al. also reported that shortening the pulse width was effective in reducing bubbles generation.

Furuhata and Saito in our group previously investigated the ways of reducing standing waves using a Schlieren system. They reported that using amplitude modulation (AM) and frequency modulation (FM) during US generation can reduce standing waves by as much as 80%. On the other hand, the present study was conducted using 500-kHz continuous-wave US. The continuous wave may also be an efficient way to reduce standing waves and cavitation generation. Compared to the burst wave or the pulsed wave, the continuous wave can obtain the same temporal average intensity with a much lower instantaneous intensity. It was noted that the bubbles generation was confirmed with an instantaneous intensity of 2.5W/cm² in the study of Azuma et al. and that an instantaneous intensity of 14W/cm² was used in TRUMBI trial. Therefore, with a much lower instantaneous intensity (≤0.72 W/cm²), the 500-kHz continuous wave US used in this study may have a much larger possibility to be safe in rt-PA treated AIS patient. Further investigation to demonstrate its ability in reducing standing waves and avoiding cavitation generation may lead to development of a TST that is safe and effective in a large number of AIS patients.

CONCLUSION

This study evaluated thrombolysis rates with different concentrations of the thrombolytic agent alteplase (rt-PA) and quantitatively examined the relationship between US acceleration of thrombolysis and acoustic intensity. It was found that the ultrasound intensity and the rt-PA concentration had an obvious synergistic effect, and that the thrombolysis acceleration ratio was dependent only on US acoustic intensity. This relationship was directly proportional at acoustic intensities ≤0.72 W/cm². Combined use of US at a noninvasive acoustic intensity enabled current rt-PA doses of 0.6mg/kg to be reduced by 1/3 and 2/3 to 0.4 and 0.2mg/kg, respectively, while still providing similar thrombolysis efficacy as with 0.6mg/kg of rt-PA alone. These findings suggest that combined use of US can reduce required rt-PA doses, thus decreasing the incidence of ICH. These study data support the effectiveness of noninvasive US and can provide a future basis for selecting appropriate transcranial US conditions.

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Conflict of Interest The authors declare no conflict of interest.

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