Original Article

DOI: 10.5582/ddt.2022.01064

In vitro study about prevention of vascular reocclusion by low intensity ultrasonic irradiation

Yoshikazu Sawaguchi[1,2,*], Zuojun Wang[2], Hiroyuki Yamamoto[3], Norio Nakata[2]

[1] Department of Medical Technology, Faculty of Biomedical Engineering, Toin University of Yokohama, Yokohama, Japan;
[2] Division of Artificial Intelligence in Medicine, the Jikei University School of Medicine, Tokyo, Japan;
[3] Department of Microbiology and Molecular Cell Biology, Nihon Pharmaceutical University, Saitama, Japan.

SUMMARY For the treatment of acute ischemic stroke, the current standard of care is thrombolysis by the administration of intravenous (IV) recombinant tissue-type plasminogen activator (rt-PA). Although this approach is proven to be effective, reocclusion within 24 hours occurs in about 20% of patients who receive recanalization by rt-PA. In addition, the administration of anticoagulants within 24 hours after IV rt-PA increases the risk of intracranial hemorrhage; therefore, treatment with anticoagulants is contraindicated in this population. To address the need for an approach to sustain the effects of thrombolysis prevent blood vessel reocclusion without the use of anticoagulants, this study proposes a novel method using a low-intensity ultrasound (US) irradiation. An *in vitro* thrombus-growth model, in a latex rubber container was developed to study the effect of thrombus-growth suppression by US irradiation at 500 kHz in a 37°C water bath. The US acoustic intensity was set at or below 0.72 W/cm^2, which is the maximum allowed for noninvasive acoustic irradiation. Low-intensity US irradiation of the thrombus-growth model resulted in a remarkable suppression of thrombus growth (100.22 ± 10.1 mg *vs*. 50.22 ± 5.3 mg, $p < 0.0001$), and the clot-growth inhibition depended logarithmically on acoustic intensity. Thrombus growth can be suppressed by low-intensity US irradiation, opening a new way to combat vascular reocclusion after rt-PA treatment of acute ischemic stroke.

Keywords Acute ischemic stroke, reocclusion, prevention, non-invasive ultrasound, recombinant tissue-type plasminogen activator

1. Introduction

As the only approved therapeutic drug for patients with acute ischemic stroke (AIS), intravenous (IV) recombinant tissue-type plasminogen activators (rt-PAs) are widely used (*1,2*), yet still limited by many factors, including a narrow time window for treatment, the risk of systemic hemorrhage, and a high rate of non-recanalization (*3,4*). To enhance the effect of IV rt-PA and decrease its systemic adverse effects, many studies have shown that ultrasonic treatment may be a promising new regimen to either destroy clots directly with high intensity focused ultrasound (US) (*5-9*) or enhance chemical thrombolysis with relatively low intensity US (*10-12*).

Although, recanalization may be achieved successfully by IV rt-PA or other thrombolytic therapy, the development of blood-vessel reocclusion occurs with high probability (14-34% of cases) within 24 hours after IV rt-PA therapy (*10,13*). One of the main reasons for blood-vessel reocclusion is thrombus regrowth. When antiplatelet drugs are administered to prevent reocclusion within 24 hours after IV rt-PA therapy, the rate of symptomatic intracranial hemorrhage significantly (*4,13*). Consequently, the use of drugs such as anticoagulants to prevent reocclusion is prohibited.

In our previous work, we reported the use of low-intensity US to inhibit thrombus-growth. This method, which requires no surgery or medication, is a novel approach to overcome reocclusion after IV rt-PA therapy (*14*). In that study, thrombus-growth was inhibited by US irradiation administered at 0.72 W/cm^2 intensity in an *in vitro* thrombus-growth model, which was prepared using bovine plasma in a cylindrical acrylic container. This *in vitro* thrombus-growth model used clots for growth that included surplus coagulation factors. These coagulation factors were slowly released in plasma, thereby coagulating the surrounding plasma into new clots. The rate of thrombus-growth inhibition was calculated from the experimental results, and the

relationship between this rate and the US acoustic intensity was analyzed. Results indicated that the rate of thrombus-growth inhibition was approximately linear in acoustic intensity. However, the relationship exhibited a slightly S-shaped curve, suggesting the possibility that US reflection may have influenced the experimental results. In other words, we posited that our technique of administering US irradiation in a bovine thrombus-growth model in an acrylic cylindrical container may have resulted in the US waves being reflected from the container wall, thereby potentially significantly increasing the acoustic intensity in the center of the container. The main reason for this effect was that the dimension of the ultrasonic beam was enlarged because of the distance between the US transducer and the target, and thus, more reflection was likely to occur. In the previous study, the distance between the US transducer and the surface of the clot was 28 mm; in our subsequent study, the distance was corrected to 15 mm and the data were reacquired. In addition, by expanding the aqueous layer irradiated by US and using more US-absorbing materials than in the previous experiment, we reduced US reflections in the water tank. The overall result was thus less affected by US reflections.

In addition to these modifications, in this study, we used a latex rubber container, which has low US reflectivity, to hold the thrombus-growth model. The US reflectance of acrylic and latex rubber was calculated by the following equation:

$$I_r = (Z_1 - Z_2)^2 / (Z_1 + Z_2)^2,$$

where I_r is the reflectance of acoustic intensity, Z_1 is the acoustic impedance of plasma, and Z_2 is the acoustic impedance of acrylic or latex rubber.

The equation gives a reflectance of about 11.5% for acrylic and about 2% for latex rubber, so we posited that an experimental system fabricated from latex rubber would reduce US reflections. Therefore, by using a thrombus-growth model fabricated from a latex rubber tube, we verified the inhibition of thrombus growth resulting from US irradiation and its dependence on acoustic intensity.

As transmittance increases for lower-frequency US, clinical transcranial US diagnostic equipment often uses US frequencies around 2 MHz, which is a relatively low frequency used to penetrate cranial bone. However, as the transmittance through cranial bone is insufficient even at US frequencies around 2 MHz (*15-17*), we used 500 kHz as the US frequency. This frequency is reported to give superior cranial ultrasonic permeability and is likely safe for brain tissue (*18,19*).

We report herein that clot-growth is suppressed by US irradiation and that, based on an *in vitro* thrombus-growth model, the thrombus-growth rate depends logarithmically on the acoustic intensity within the setting of noninvasive acoustic intensity.

2. Materials and Methods

2.1. Preparation of thrombus-growth model

Citrated and freeze-dried bovine plasma (P4639-10ML, Sigma-Aldrich Japan K. K, Tokyo, Japan) was rehydrated and degassed at −0.04 MPa for 5 minutes. Clots for growth (growth-clots) were prepared from degassed plasma, 1 M calcium dichloride ($CaCl_2$) (FUJIFILM Wako Chemicals, Osaka, Japan), and thrombin (206-18411, FUJIFILM Wako Chemicals), with a final concentration of 50 mM $CaCl_2$ and 1 U/mL thrombin. The clots were incubated at 37°C for 60 min.

2.2. Acrylic plate used in thrombus-growth-suppression study

An acrylic plate cell was prepared as described previously (*11*). The design was a 15-mm discoidal hole drilled in the center of a 3-mm-thick acrylic plate, and a 0.3 mm-thick polycarbonate sheet was affixed to its back face for use as a clot cell (Nissindenki, Tokyo, Japan). A 535-μL discoidal growth-clot that had a 15-mm diameter and 3-mm-thick was prepared in the hole. After being covered with degassed bovine plasma contained in another clot cell, the discoid growth-clot was irradiated with US for 30 minutes. in a 37 °C water bath (Fiure 1a). The anti-thrombus-growth effect was evaluated as detailed in previous study (*11,12*). In brief, the optical density value was recorded before and after ultrasonication, and the clot thickness was calculated from the calibration curve.

2.3. Latex tube used in thrombus-growth-suppression study

A 200-μL growth-clot was prepared in the bottom of a latex tube (Finger Cots Unroll Type S, AS ONE, Osaka, Japan) with a 15-mm-inner-diameter. The latex tube was filled with degassed bovine plasma, which was then irradiated with US for 30 minutes in a 37°C water bath. The anti-thrombus-growth effect was evaluated based on the changes in clot mass on before and after ultrasonication (Figure 1b). The clots mass before ultrasonication was calculated by the following formula: clot with latex tube weight before ultrasonication (without plasma) minus empty latex tube weight. The clots mass after ultrasonication was calculated by the following formula: clot with latex tube weight after ultrasonication (plasma removed by micropipette) minus empty latex tube weight.

2.4. Ultrasound conditions

The US conditions were established as detailed in a previous study (*20*). In brief, a 10-mm-diameter US transducer (Honda Electronics, Aichi, Japan) was

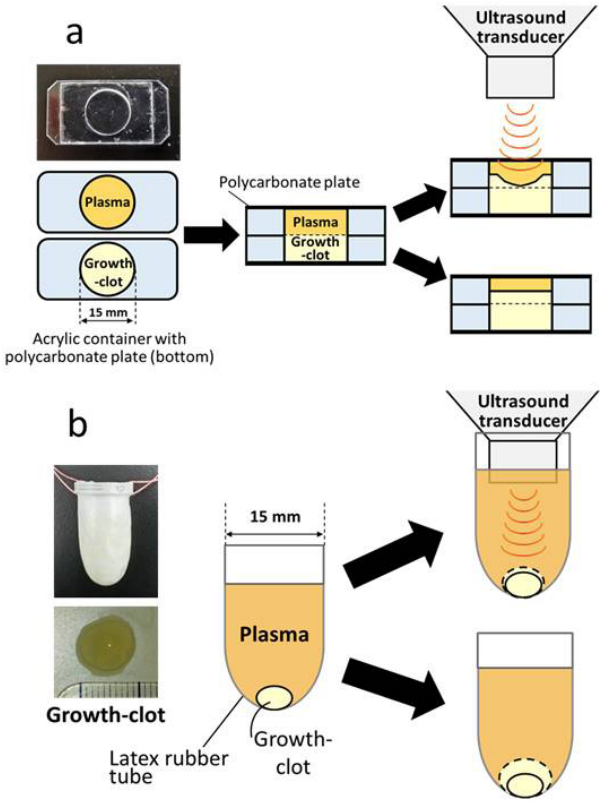
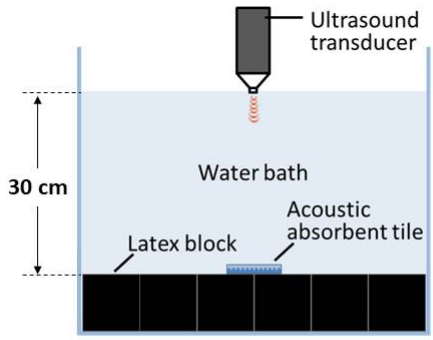


Figure 1. Schema of experimental apparatus for the anti-clot-growth model. (a) Fresh plasma and growth clot were prepared in two acrylic containers and were overlapped in such a way that no air was trapped between them. The clot was irradiated by US from the plasma side for 30 minutes. in a 37°C water bath. **(b)** Fresh plasma was poured around the growth clot prepared in a rubber latex tube, and the clot was irradiated by US in plasma for 30 minutes. in a 37°C water bath. The growth-clot in the rubber latex tube was about 9 mm in diameter, which is the same size as the acoustic-field distribution skirt of the US main robe. One scale of the measure in the picture of Growth-clot is 1mm. In both experimental conditions, the distance from the transducer to the surface of the growth-clot was adjusted to 15 mm.

operated at 500 kHz in the continuous-wave mode and at a maximum acoustic intensity of 0.72 W/cm^2. Measurements were made in a clear water tank using the Acoustic Intensity Measurement System (AIMS, Onda, Sunnyvale, CA, USA) with a 0.2-mm-diameter hydrophone probe (HNP-0200, Onda). The average acoustic intensity was measured to be 0.25 W/cm^2 at the maximum intensity 0.72 W/cm^2 by using a radiation method with the Ultrasound Power Meter (UPM-DT-1AV, Onda). An acoustic intensity of 0.72 W/cm^2 is the maximum average intensity allowed by the U.S. Food and Drug Administration (FDA) for diagnostic US equipment.

Rubber blocks with a side length of 10 cm were place on the bottom of the water bath, and an acoustic-absorbing tile (EPI_EUA101A, Onda) was placed under the transducer and on the rubber blocks. The distance between the US transducer and the acoustic-absorbing tile was about 30 cm (Figure 2).



Radial Distance from US transducer centerline (mm)	0	|0.5|	|1.0|	|1.5|	|2.0|	|2.5|	|3.0|	|3.5|	|4.0|	|4.5|
Acoustic intensity at 15 mm form US transducer (W/cm^2)	0.72	0.69	0.63	0.52	0.39	0.25	0.11	0.07	0.03	0.01

Figure 2. Experiment environment and acoustic-field distribution schema showing US irradiation environment. The water layer irradiated by US was designed to be less susceptible to the influence of US reflections. The acoustic-field distribution was measured by the AIMS at 15 mm from the US transducer, which is the distance from the US transducer to the clot surface. The acoustic intensity is shown in 0.5 mm intervals from the center of the US transducer. The average acoustic intensity was 0.2 W/cm^2.

2.5. Calculation of anti-clot-growth ratio

To evaluate the effect of US irradiation on the clot-growth rate, we calculated the anti-clot-growth ratio (R) at each acoustic intensity level using the following equation:

$$R = (\Delta \text{ non-US} - \Delta \text{ US}) / \Delta \text{ non-US} \times 100\%$$

where, Δ non-US (Δ US) is the clot size change in thickness (mm) or weight (mg) when not exposed to US irradiation for the experimental system with the acrylic container or the experimental system with the latex rubber container.

2.6. Statistical analyses

Five sets of two clots (for a total of 10 clots) were prepared for each intensity level. The differences in clot thickness or mass between the clots exposed and not exposed to US irradiation were examined using the paired Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Thrombus-growth-suppression effect by ultrasound in the thrombus growth model in acrylic container

Results of low-intensity US irradiation in a thrombus-growth model prepared in an acrylic container revealed that thrombus-growth was significantly suppressed at all measurement points, with an acoustic intensity of

0.01-0.72 W/cm^2 (Figure 3a). The rate of clot-growth suppression was about 50% at 0.72 W/cm^2, which is the maximum acoustic intensity of the ultrasonic diagnostic equipment allowed by the FDA. The rate of clot-growth suppression was found to depend logarithmically on the acoustic intensity, as indicated by the high correlation with the logarithmic formula (Figure 3b). Furthermore, based on its approximate expression, the acoustic intensity threshold above which clot-growth is suppressed by US irradiation was calculated to be close to 0 W/cm^2.

3.2. Thrombus-growth-suppression effect by ultrasound in the thrombus growth model in latex rubber container

US irradiation of the thrombus-growth model prepared in a rubber latex tube reveals that the clot-growth was remarkably suppressed at peak acoustic intensities of 0.18, 0.36, and 0.72 W/cm^2, representing average intensities of 0.06, 0.13, and 0.25 W/cm^2, respectively (Figures 4a and 4b).

The rate of clot-growth suppression was about 50% at an acoustic intensity of 0.72 W/cm^2. The acoustic intensity was not adjusted to be the average acoustic intensity of 0.25 W/cm^2, but instead to the maximum acoustic intensity of 0.72 W/cm^2 because use of the average acoustic intensity would lead to a maximum acoustic intensity exceeding the threshold allowed for noninvasive acoustic intensity. Analysis of the relationship between the rate of clot-growth suppression and acoustic intensity showed a high correlation with a logarithmic expression, as evident when using an acrylic container (Figure 4c). Furthermore, based on this approximate expression, the acoustic intensity threshold

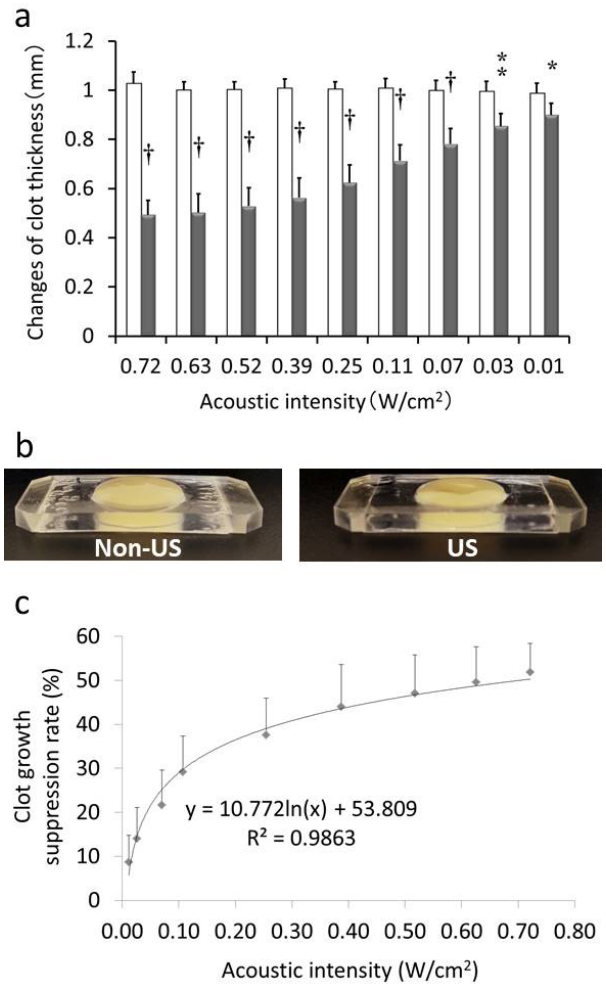


Figure 3. Clot-growth suppression in an acrylic plate. (a) The suppression of clot growth by US irradiation was evaluated in one dimension by calculating changes in clot thicknesses before and after US irradiation. **(b)** Post-experimental growth-clot photograph. **(c)** The relationship between clot-growth-suppression rate and acoustic intensity is plotted and fit with a logarithm. (■) Group irradiated by US, (□) group not irradiated by US. *$p < 0.05$, **$p < 0.001$, $^{\dagger}p < 0.0001$ vs non-US (Student's *t*-test), mean ± SD ($n = 5$ for each group).

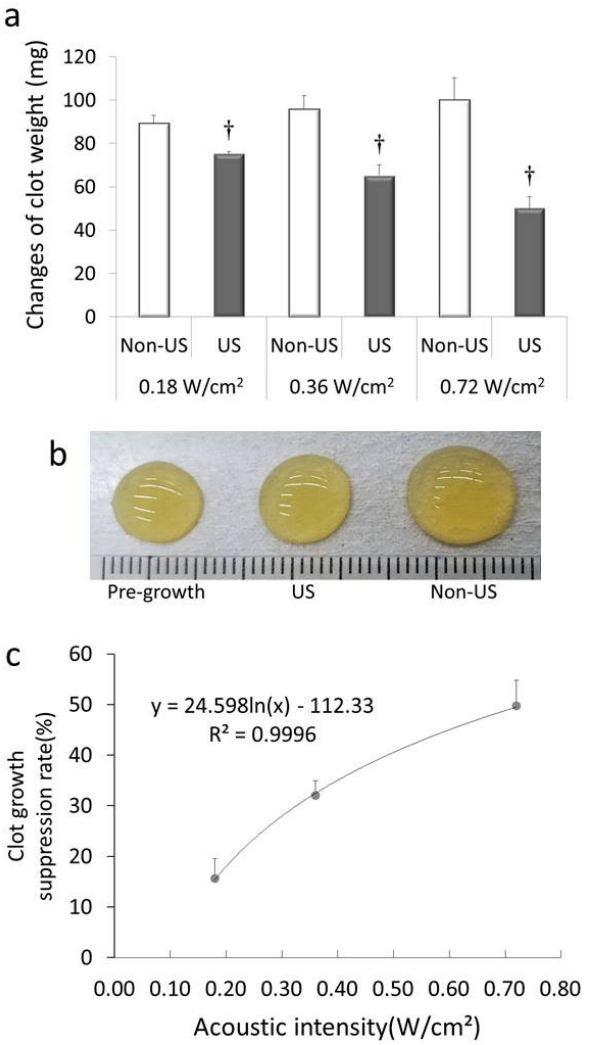


Figure 4. Clot-growth suppression in latex tube. (a) The suppression of clot-growth by US was evaluated in three dimensions by calculating the increase in thrombus mass. **(b)** Growth-clot photograph before and after the experiment with an acoustic intensity 0.72 W/cm^2. One scale of the measure is 1 mm. **(c)** The relationship between clot-growth-suppression rate and acoustic intensity is plotted and fit with a logarithm. $^{\dagger}p < 0.0005$ *vs.* non-US (Student's *t*-test), mean ± SD ($n = 5$ for each group).

above which clot-growth suppression occurs as a result of peak US irradiation was calculated to be about 0.1 W/cm^2 or an average acoustic intensity about 0.035 W/cm^2.

4. Discussion

This research shows that clot-growth can be suppressed by approximately 50% at most in a thrombus-growth model simply by exposing it to US irradiation at 500 kHz. These results were confirmed by using methods with noninvasive low acoustic intensity in two different experimental systems with acrylic and latex rubber containers.

Our results on clot-growth volume are similar to those obtained in the previous study on clot-growth suppression using acrylic containers. However, the present regression line that shows the relationship between the rate of clot-growth suppression and acoustic intensity differs from the line obtained previously. In the previous study, the relationship was linear, although with a slight S-shape, whereas in this study, the relationship is better approximated as logarithmic. This difference is tentatively attributed to the lower US reflectivity in this study, which minimized the influence of US reflection than that in the previous study. It should also be noted that in the previous experiment with the acrylic container, clot growth was evaluated based on clot length (mm), whereas in this study clot-growth was evaluated based on clot mass (mg) using the rubber latex tube. This difference is analogous to the difference between a one-dimensional and a three-dimensional evaluation, which may contribute to the difference between the results of these two models. Calculating the threshold value of the acoustic intensity and the thrombus-growth-suppression effect using the equations presented in Figure 3c and Figure 4c shows different values of about 0 and 0.1 W/cm^2, one-dimensionally and three-dimensionally, respectively. Therefore, it may be possible to suppress the growth of thrombus with a slight acoustic intensity over 0 W/cm^2, but to suppress reocclusion, it is necessary to suppress the thrombus-growth three-dimensionally, so the threshold value may be about 0.1 W/cm^2. In the experiment to measure clot thickness, an acrylic container with polycarbonate as the bottom material, which has a relatively close acoustic impedance to water among hard materials, was used to maintain the shape of the clots. In the experiment to measure the clot weight, latex rubber was used, which is a material with acoustic impedance closer to that of water. In addition, a large acrylic water bath was prepared to prevent the reflection of US, and a sound-absorbing material was placed on the bottom. However, this technique cannot completely prevent the reflection of US. We calculated that at least about 11.5% reflection occurred in the experiment using acrylic and about 2% in the experiment using latex rubber. However, most of the ultrasonic reflections may have been influenced by the bottom polycarbonate surface of the acrylic container by reducing the distance between the US transducer and the clot to suppress the reflection from the acrylic surface. In that case, US causes almost no diffuse reflection from the acrylic surface, and polycarbonate has an acoustic impedance closer to water than acrylic, so the reflection may have been suppressed even to a smaller degree. In addition, the experimental results using latex rubber with extremely low ultrasonic reflection (Figure 4c) and the experimental results using an acrylic plate (Figure 3b) showed that the thrombus-growth rate depends logarithmically on the acoustic intensity. These findings suggest that the experimental results using the acrylic plate did not cause diffuse reflection as in the previous experiment.

Reproducing the *in vivo* environment with the *in vitro* thrombus-growth model used in this study has some limitations. For example, in a living body, changes occur in the balance between the fibrinolytic system and the coagulation system and with the presence of endothelial cells, blood flow, pulsation, and blood pressure, among other factors, thereby preventing the *in vivo* environment from being completely reproduced in this *in vitro* study. It is unknown whether these factors affect thrombus-growth and the thrombus-growth-suppression effect achieved by US. Therefore, in future work, we will evaluate the preventive effect of US on vascular reocclusion in an animal model. Furthermore, we plan to use a thrombus-growth model with human plasma, fabricated in the same way as for the thrombus-growth model using bovine plasma discussed herein. We will then examine the preventive effect of US on vascular occlusion by studying clot-growth suppression as a function of various US parameters, such as frequency and continuous or pulsed wave, among other variables.

Many studies have been reported on the treatment of thrombosis with US, with or without rt-PA. In particular, magnetic resonance imaging guided high intensity focused US (MRg-HIFU) is expected to serve as a noninvasive method that can accurately target only the thrombus (*5*).

Focus US is used for clot breakdown and histotripsy thrombolytic therapy because it requires high acoustic intensity of several hundred watts, and cavitation is involved in the mechanism (*6-9*). Maxwell *et al*. reported that peak negative pressure of approximately 4 MPa is required for US histotripsy thrombolysis of whole blood clots in a model using dogs (*7*). In this study, the US conditions were 500 kHz, continuous-wave, maximum acoustic intensity 0.72 W/cm^2 (peak negative pressure 0.2 MPa), and it is unlikely that cavitation would be induced in degassed plasma. There was no difference in thrombus-growth weight (mg) that 0.72 W/cm^2 US was irradiated to the growth thrombus

using bovine serum instead of bovine plasma (non-US *vs*. US, 2.10 ± 0.557 mg *vs*. 2.07 ± 1.33 mg, mean ± SD, $p = 0.970$, $n = 3$ for each group, data not shown). The slight increase of thrombus weight (about 2 mg for each group) in this experiment seemed to be caused by serum that could not be removed with a micropipette. Therefore, it is thought that thrombus growth is suppressed by a mechanism different from those for clot breakdown and histotripsy thrombolytic models. This mechanism is currently under consideration, but is still unclear.

The use of 0.72 W/cm^2 US is an extremely noninvasive approach, but may require safety considerations when used intracranially. In a clinical study by Daffertshofer *et al.*, significant intracranial hemorrhage was caused when rt-PA was used in combination with US at 300 kHz and 0.7 W/cm^2 (*21*). It is believed that one of the causes of this finding is that cavitation was induced by hot spots caused by standing waves generated in the skull. Therefore, we consider it an important task to verify the inhibitory effect on thrombus-growth in the skull in a future study. However, according to the report by Shimizu *et al*., the safety of transcranial US at 500 kHz and 0.72 W/cm^2 was evaluated with the cynomolgus monkey, and no neurologic deficits were found on histologic evaluation. Furthermore, no neurologic deficits were observed when 500 kHz US and rt-PA were used in combination in a model using rhesus monkeys (*18*). This report suggests that low-intensity 500 kHz transcranial US has the potential to be safely applied to thrombus-growth suppression as well.

Thrombus-growth was suppressed by low-intensity US irradiation in an experimental setting with almost no reflection, and it was possible to measure the threshold acoustic intensity above which clot-growth is suppressed. We expect that this technology will continue to be developed until it can be used to prevent various thromboses, including reocclusion of blood vessels after rt-PA treatment of AIS.

Funding: This work was supported by Grant-in-Aid for Young Scientists (B) Grant Number 15K19208, in part by the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan, and Nihon Pharmaceutical University Research Grant.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Group. TNIoNDaSr-PSS. Tissue plasminogen activator for acute ischemic stroke. N Engl J Med. 1995; 333:1581-1587.
2. Hacke W, Kaste M, Bluhmki E, Brozman M, Dávalos A, Guidetti D, Larrue V, Lees KR, Medeghri Z, Machnig T, Schneider D, von Kummer R, Wahlgren N, Toni D. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med. 2008; 359:1317-1329.
3. Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Grotta JC, Albers GW, Kaste M, Marler JR, Hamilton SA, Tilley BC, Davis SM, Donnan GA, Hacke W. Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. Lancet. 2010; 375:1695-1703.
4. Graham GD. Tissue plasminogen activator for acute ischemic stroke in clinical practice: a meta-analysis of safety data. Stroke. 2003; 34:2847-2850.
5. Zafar A, Quadri SA, Farooqui M, Ortega-Gutierrez S, Hariri OR, Zulfiqar M, Ikram A, Khan MA, Suriya SS, Nunez-Gonzalez JR, Posse S, Mortazavi MM, Yonas H. MRI-guided high-intensity focused ultrasound as an emerging therapy for stroke: A review. J Neuroimaging. 2019; 29:5-13.
6. Westermark S, Wiksell H, Elmqvist H, Hultenby K, Berglund H. Effect of externally applied focused acoustic energy on clot disruption *in vitro*. Clin Sci. 1999; 97:67-71.
7. Maxwell AD, Cain CA, Duryea AP, Yuan L, Gurm HS, Xu Z. Noninvasive thrombolysis using pulsed ultrasound cavitation therapy - histotripsy. Ultrasound Med Biol. 2009; 35:1982-1994.
8. Hölscher T FD, Raman R. Noninvasive transcranial clot lysis using high intensity focused ultrasound. J Neurol Neurophysiol. 2011; S1. doi:10.4172/2155-9562.S1-002
9. Harnof S, Zibly Z, Hananel A, Monteith S, Grinfeld J, Schiff G, Kulbatski I, Kassell N. Potential of magnetic resonance-guided focused ultrasound for intracranial hemorrhage: an *in vivo* feasibility study. J Stroke Cerebrovasc Dis. 2014; 23:1585-1591.
10. Molina CA, Ribo M, Rubiera M, Montaner J, Santamarina E, Delgado-Mederos R, Arenillas JF, Huertas R, Purroy F, Delgado P, Alvarez-Sabin J. Microbubble administration accelerates clot lysis during continuous 2-MHz ultrasound monitoring in stroke patients treated with intravenous tissue plasminogen activator. Stroke. 2006; 37:425-429.
11. Sawaguchi Y, Wang Z. Ultrasound acceleration of rt-PA thrombolysis depends on acoustic intensity. Biol Pharm Bull. 2017; 40:97-103.
12. Wang Z, Sawaguchi Y, Hirose H, Ohara K, Sakamoto S, Mitsumura H, Ogawa T, Iguchi Y, Yokoyama M. An *in vitro* assay for sonothrombolysis based on the spectrophotometric measurement of clot thickness. J Ultrasound Med. 2017; 36:681-698.
13. Zinkstok SM, Roos YB. Early administration of aspirin in patients treated with alteplase for acute ischaemic stroke: a randomised controlled trial. The Lancet. 2012; 380:731-737.
14. Sawaguchi Y, Wang Z, Furuhata H. Ultrasound control of the growth of thrombus - Potential for the embolus growth suppression & the reocclusion prevention. Jpn J Med Ultrason. 2011; 38:549-555.
15. Itoh T, Matsumoto M, Handa N, Maeda H, Hougaku H, Hashimoto H, Etani H, Tsukamoto Y, Kamada T. Rate of successful recording of blood flow signals in the middle cerebral artery using transcranial Doppler sonography. Stroke. 1993; 24:1192-1195.
16. Hashimoto H, Etani H, Naka M, Kinoshita N, Nukada T. Assessment of the rate of successful transcranial Doppler recording through the temporal windows in Japanese with

special reference to aging and sex. Nihon Ronen Igakkai Zasshi. 1992; 29:119-122.
17. Caplan LR, Brass LM, DeWitt LD, Adams RJ, Gomez C, Otis S, Weschler LR, von Reutern GM. Transcranial Doppler ultrasound: present status. Neurology. 1990; 40:696-700.
18. Shimizu J, Fukuda T, Abe T, Ogihara M, Kubota J, Sasaki A, Azuma T, Sasaki K, Shimizu K, Oishi T, Umemura S, Furuhata H. Ultrasound safety with midfrequency transcranial sonothrombolysis: preliminary study on normal macaca monkey brain. Ultrasound Med Biol. 2012; 38:1040-1050.
19. White PJ, Clement GT, Hynynen K. Local frequency dependence in transcranial ultrasound transmission. Phys Med Biol. 2006; 51:2293-2305.
20. Sawaguchi Y, Wang Z, Itou S, Kikuchi R, Yamamoto H, Tachibana K, Nakajima T, Nakata N. Basic research for development of ultrasonic prevention of vascular occlusion. Neurosonology. 2017; 30:1-4.
21. Daffertshofer M, Gass A, Ringleb P, Sitzer M, Sliwka U, Els T, Sedlaczek O, Koroshetz WJ, Hennerici MG. Transcranial low-frequency ultrasound-mediated thrombolysis in brain ischemia: increased risk of hemorrhage with combined ultrasound and tissue plasminogen activator: results of a phase II clinical trial. Stroke. 2005; 36:1441-1446.

Received August 11, 2022; Revised September 18, 2022; Accepted October 3, 2022.

*Address correspondence to:
Yoshikazu Sawaguchi, Faculty of Biomedical Engineering, Toin University of Yokohama, 1614 Kurogane-cho, Aoba-ku, Yokohama, 225-8503 Japan.
E-mail: sawaguti@toin.ac.jp

Released online in J-STAGE as advance publication October 9, 2022