Microbubble-Mediated Sonothrombolysis Improves Outcome After Thrombotic Microembolism-Induced Acute Ischemic Stroke

Yongkang Lu, MD, PhD*; Junfen Wang, MD, PhD*; Ruizhu Huang, MD; Gangbin Chen, MD; Lintao Zhong, MD; Shuxin Shen, MD, PhD; Chuanxi Zhang, MD; Xinzhong Li, MD; Shiping Cao, MD, PhD; Wangjun Liao, MD, PhD; Yulin Liao, MD, PhD; Jianping Bin, MD, PhD

Background and Purpose—Microthrombi originating from disintegrated clots or formed in situ may account for the poor clinical improvement of acute ischemic stroke after recanalization therapy. We attempted to determine whether microbubble-mediated sonothrombolysis could dissolve platelet-rich and erythrocyte-rich microthrombi, thereby reducing their brain injury-causing potential.

Methods—Platelet- and erythrocyte-rich microthrombosis were induced by periadventitial application of 5% ferric chloride or thrombin to mesenteric microvessels in 75 Sprague–Dawley rats. Acute ischemic stroke was induced by intracarotid injection of platelet- or erythrocyte-rich microthrombi in another 50 rats. Rats were randomly divided into control (CON), ultrasound (US), ultrasound and microbubble (US+MB), recombinant tissue-type plasminogen activator (r-tPA), and US+MB+r-tPA groups. The post-treatment mesenteric microvessel recanalization rates, cerebral infarct volumes, and neurological scores were determined.

Results—The recanalization rates of platelet- and erythrocyte-rich microthrombi in mesenteric microvessels were higher ($P<0.05$), and the cerebral infarct volumes and neurological scores of rats with either microthrombi were lower in the US+MB group than in the CON group ($P<0.01$). The infarct volumes and neurological scores were greater in the r-tPA group than in the US+MB and US+MB+r-tPA groups after treatment of rats with platelet-rich microthrombi ($P<0.05$). In contrast, after treatment of rats with erythrocyte-rich microthrombi, the infarct volumes and neurological scores were similar in the r-tPA and US+MB groups, but smaller in the US+MB+r-tPA group ($P<0.05$).

Conclusions—Microbubble-mediated sonothrombolysis improved the outcomes of microthrombi-induced acute ischemic stroke. Thus, this method may serve as an attractive adjunct to recanalization therapy for acute ischemic stroke. (Stroke. 2016;47:1344-1353. DOI: 10.1161/STROKEAHA.115.012056.)

Key Words: microbubbles ■ thrombolytic therapy ■ thrombus ■ stroke ■ ultrasonics

Although early recanalization of the major occluded brain arteries has become an essential part of therapy after acute ischemic stroke, a subset of patients fails to achieve clinical improvement despite recanalization.1 One important reason for this failure might be microcirculation no-reflow, a phenomenon in which major vessel recanalization does not result in adequate microvascular reperfusion.2–5 Microthrombi, originating from upstream clot fragmentation or formed in situ, have been shown to play pivotal roles in the development of the no-reflow phenomenon.6–8 These microthrombi can exhibit a range of histological patterns and may be platelet or erythrocyte rich.9–11 Until now, efficacious therapies for reducing cerebral microthrombi and improving stroke outcomes have not been well established. Despite the use of the latest thrombectomy devices, microemboli may still be found in cerebral arterioles downstream from the treated vessel.12,13 Intravenous antiplatelet agents may suppress local microthrombus and microembolus formation, but clinical trial results have not demonstrated improved outcomes. Rather, they have highlighted an increased risk of intracranial hemorrhage after the combined use of antiplatelet agents and pharmacological fibrinolysis.14–16

Microbubble-mediated sonothrombolysis is a promising treatment for cerebral microthrombi and is based on ultrasound driven cavitation of microbubbles that accelerate thrombolysis via localized mechanical stress on the thrombi.17

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Multiple animal studies have demonstrated that ultrasound in combination with microbubbles can recanalize acute intravascular thrombi in large vessels, including the iliofemoral arteries, arteriovenous grafts, central venous catheters, coronary arteries, and intracranial arteries, underlining the potential of the treatment for dissolving microvascular thrombi. However, data on the effects of ultrasound and microbubble (US+MB) treatment on the thrombolysis of microthrombi are lacking, and it is unclear whether US+MB treatment may improve the outcomes of acute ischemic stroke induced by cerebral microthrombi. Only one study has evaluated the efficacy of the treatment for dissolving erythrocyte-rich microthrombi in a rat hindlimb model. Animal studies have not addressed the utility of US+MB treatment for dissolving platelet-rich microthrombi, which account for the largest proportion of thromboemboli in stroke. Furthermore, neither platelet- nor erythrocyte-rich microthrombi have been used to evaluate the stroke outcomes after the treatment.

In this study, we hypothesized that microbubble-mediated sonothrombolysis could dissolve both platelet- and erythrocyte-rich microthrombi, consequently improving the outcomes of microthrombi-induced acute ischemic strokes. To verify this hypothesis, we evaluated the lytic efficiency of US+MB treatment on both types of thrombi in vitro and in rat models of mesenteric microthrombosis. Furthermore, we investigated the therapeutic effect of the treatment in rats with acute ischemic stroke, induced using thrombotic microemboli.

**Materials and Methods**

The animal studies were approved by the Animal Research Committee of Southern Medical University (Guangzhou, China), and the investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised in 1996).

**In Vitro Clot Preparation and Experimental Setup**

Platelet- and erythrocyte-rich thrombi, also known as white and red thrombi, were prepared as previously described and formed in the rubber tubes (diameter, 5 mm; Figure 1A). A closed-loop flow system was filled with phosphate buffered saline (45 mL) and kept at 37°C (Figure 1B). The thrombus was attached to a cotton thread and suspended in the flow system. A peristaltic pump was used to maintain a flow rate of 5 mL/min through the system (Methods in the online-only Data Supplement).

**In Vitro Thrombolysis**

White and red thrombi were randomly divided into 5 treatment groups: control (CON), ultrasound (US), US+MB, recombinant tissue-type plasminogen activator (r-tPA), and US+MB+r-tPA groups (n=5 per group). In the CON group, each thrombus was suspended in the flow system but was left untreated. In the US and US+MB groups, ultrasound treatment was conducted using a Siemens Acuson Sequoia with 4V1c sector-array transducer (frequency range, 1–4.5 MHz; diameter in azimuthal direction, 32 mm; elevation plane, 5 mm) by use of contrast pulse sequencing (Siemens Medical Systems). The transducer was immersed in the water and positioned at a 35-mm distance to the center of clot. Ultrasound insonation was performed at a mechanical index of 1.9 and a frequency of 2 MHz, and lasted for 30 minutes. For US+MB treatment, lipid-shelled perfluoropropane microbubbles were prepared as previously described, added into the flow system (0.008 mL/min), and combined with ultrasound treatment. In the r-tPA group, r-tPA (0.02 mg/mL; Actilyse, Boehringer Ingelheim) was added to the PBS. In the US+MB+r-tPA group, microbubbles (0.008 mL/min) and half-dose r-tPA (0.01 mg/mL) were added into the flow system and combined with ultrasound treatment. The diagram of in vitro thrombolysis is shown in Figure 1B.

After the US+MB treatment, liquid waste from the flow system was collected and the diameters of residues were measured using a Coulter Counter (Multisizer 3, Beckman Coulter).

**Mesenteric Microvascular Thrombosis Model**

Mesenteric microvascular thrombosis was induced by periadventitial application of 5% ferric chloride (platelet-rich) or thrombin (erythrocyte-rich) to the selected microvessels (diameter, 70–100 μm) in

![Figure 1. In vitro model for thrombolysis and thrombi characteristics. A, Appearance of white and red thrombi formed in rubber tubing. B, Diagram of experimental setup. C, Representative hematoxylin and eosin–stained and scanning electron microscopy images of white and red thrombi.](http://stroke.ahajournals.org/)

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Cerebral Thrombotic Microembolism Model

White and red thrombi were prepared as described above and frozen in liquid nitrogen, followed by mechanical comminution. The resultant microthrombi that were able to pass through a 100-μm mesh, but were retained on a 70-μm mesh, were resuspended in normal saline. Acute ischemic stroke was induced by intracarotid injection of platelet- or erythrocyte-rich microthrombi. Briefly, the right distal external carotid artery was ligated and the right common carotid artery was temporarily clamped. Subsequently, suspended microthrombi (0.1 mL) were gently injected into the external carotid artery. Immediately thereafter, the external carotid artery was ligated proximal to the injection site, and the common carotid artery clamp was released.

Histological Examinations

To confirm the presence of mesenteric and cerebral microvascular thromboses/embolisms, specimens were paraffin embedded and stained with hematoxylin-eosin (H&E), anti-CD41 antibody (Abcam) and anti-fibrinopeptide A antibody (Abcam). Anti-CD41 and anti-fibrinopeptide A immunohistochemistry indicated the presence of platelets and fibrin, respectively. To determine the presence of cerebral infarction and hemorrhage, H&E staining was performed 24 hours after surgery, using a 3.0 T system (3T Trio-Tim; Siemens) and a locally made radiofrequency coil (online-only Data Supplement). The brains were then harvested and sectioned into 1.5-mm-thick coronal slices for 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining. Macroscopic evidence of cerebral infarction was compared with MRI findings for the corresponding sections. Images were measured by observers blinded to the treatment paradigm. To eliminate error introduced by brain edema, infarct volumes were calculated using an indirect method. The infarct volume was calculated as the sum of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere and expressed as a volume percentage of the infarct compared with the contralateral hemisphere.

In Vivo Thrombolysis

The animal grouping and ultrasound parameters were the same as those described for the in vitro thrombolysis. In the mesenteric microvascular thrombosis model, after the selected microves sel occluded, as evidenced by thrombus formation and blood flow decrease, an interval of 5 minutes was required before the treatment to ensure that the thrombus was stable and unlikely to be washed away by the bloodstream. During treatment, the ultrasound probe was positioned 4 cm above the thrombosed microvessel, and the distance was bridged with a gel-filled spacer. In the cerebral thrombotic microembolism model, after intracarotid injection of microthrombi, an interval of 5 minutes was required before the treatment to allow for ligation of external carotid artery and closure of cervical incision. During treatment, the probe was placed 0.5 cm above a dehaired area behind the rat’s eye on the ipsilateral side of the head and was coupled to the scalp with ultrasound gel. The sound beam was aimed to expose the whole hemisphere of the brain by manually swinging the probe. The ultrasound treatment lasted for 30 minutes. In the US+MB group, microbubbles were injected (0.008 mL/min) via a tail vein catheter. In the r-tPA group, r-tPA (10 mg/kg) was administered through the tail vein; 10% was administered as a bolus and the remainder as a 30-minute infusion. In the US+MB+r-tPA group, microbubbles (0.008 mL/min) and half-dose r-tPA (5 mg/kg) were administered through different tail veins.

B-Mode and Doppler Ultrasonic Imaging

Ultrasonic evaluation of in vitro thrombolytic effect was performed using a Sequoia ultrasound system with a linear array ultrasound transducer (15L8-S; mechanical index, 0.17; frequency, 14 MHz; Siemens Medical Systems). The transverse and longitudinal areas of the tubing lumens and the flow velocity, at the points of thrombus suspension, were measured using B-mode and Doppler ultrasonic imaging, respectively. The total duration of ultrasound exposure for imaging in each group was 3 minutes.

Magnetic Resonance Imaging and Triphenyltetrazolium Chloride Staining

Cerebral magnetic resonance imaging (MRI) was performed in rats before and 24 hours after surgery, using a 3.0 T system (3T Trio-Tim; Siemens) and a locally made radiofrequency coil (online-only Data Supplement). The brains were then harvested and sectioned into 1.5-mm-thick coronal slices for 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining. Macroscopic evidence of cerebral infarction was compared with MRI findings for the corresponding sections. Images were measured by observers blinded to the treatment paradigm. To eliminate error introduced by brain edema, infarct volumes were calculated using an indirect method. The infarct volume was calculated as the sum of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere and expressed as a volume percentage of the infarct compared with the contralateral hemisphere.

Neurological Score

Neurological functions, at baseline and 24 hours after embolization, were studied by an observer blinded to the treatment paradigm using a Bederson behavior test. The neurological scores ranged from 0 to 3, with higher scores indicating more serious neurological injury (Movies III–VI in the online-only Data Supplement).

Results

In Vitro Thrombolytic Effects of Microbubble-Mediated Sonothrombolysis

As shown in Figure 1A, in vitro thrombi were 0.37±0.09 cm in diameter and 2.85±0.47 cm in length. H&E staining and scanning electron microscopy showed that platelet-rich thrombi (white thrombi) exhibited a compact fibrin–platelet mesh pattern, interspersed with limited deposits of nucleated cells and erythrocytes (Figure 1C). In contrast, erythrocyte-rich thrombi (red thrombi) were composed of a mass of erythrocytes trapped in the fibrin network, with nucleated cells dispersed throughout.

After treatment of the white and red thrombi, the increase in both the transverse and the longitudinal areas of the tubing lumens, at the points of thrombus suspension, was greater in the US+MB group than in the CON group (P<0.05; Figure 2A, B). Similarly, the reduction of flow velocities at the sites of the thrombi and the loss of clot masses were also greater in the US+MB group than in the CON group (P<0.05; Figure 2B and 2C). After treatment of the white thrombi, the changes in lumen areas, flow velocities, and clot masses were significantly smaller in the r-tPA group than in the US+MB group (P<0.05), whereas they were similar in the US+MB and US+MB+r-tPA groups. In contrast, after treatment of the red thrombi, the changes in lumen areas, flow velocities, and clot masses were similar in the r-tPA and US+MB groups, but significantly larger in the US+MB+r-tPA group (P<0.05). The changes in lumen areas, flow velocities, and
Clot masses of the white thrombi were significantly smaller than those of the red thrombi in the r-tPA and US+MB+r-tPA groups ($P<0.05$).

Coulter particle analysis showed that the white and red thrombi debris, after US+MB treatment, was $<8\ \mu m$ in diameter (Figure 2D), suggesting that they were unlikely to cause distal embolization.

**In Vivo Thrombolytic Effects of Microbubble-Mediated Sonothrombolysis**

Intravital microscopy showed that thromboses were successfully induced in mesenteric microvessels (Figure 3A). H&E staining showed that the white microthrombi in the mesenteric microvessels contained a fibrin–platelet network, with a few nucleated cells and erythrocytes dispersed within it.
(Figure 3B). In contrast, the red microthrombi were mainly composed of clustered erythrocytes. Immunohistochemical staining showed that both CD41 (platelets) and fibrinopeptide A (fibrin) were expressed in the white and red microthrombi.

In the CON group, the white and red microthrombi grew larger and blood flow slowed within a few minutes of establishing the thrombosis models (Figure 3A). After US+MB treatment, both microthrombi types dissolved, blood flow was restored, and occlusion did not recur within 30 minutes. For both types of mesenteric microvessel microthrombi, the recanalization rates were higher in the US+MB and US+MB+r-tPA groups than in the CON group (P<0.05; Figure 3C). The recanalization rate was higher in the r-tPA group than in the CON group after treatment of the red microthrombi (P<0.01), whereas there was no significant difference between the groups after treatment of the white microthrombi.

**Figure 3.** Effects of microbubble (MB) mediated sonothrombolysis on mesenteric microvascular microthrombi. A, Representative intravital microscopy images of mesenteric microvessels in different conditions. Arrows denote the locations of intravascular microthrombi. B, Representative images of white and red microthrombi in mesenteric microvessels as visualized by hematoxylin and eosin (H&E) staining, or labeled with antibodies against CD41 and fibrinopeptide A (FPA). C, Quantification of the recanalization rates of thrombosed mesenteric microvessels. *P<0.01, #P<0.05 vs control group. r-tPA indicates recombinant tissue-type plasminogen activator; and US, ultrasound.

**Stroke Outcomes**

Microthrombi prepared for embolization exhibited a homogeneous morphology, with diameters of 70 to 100 μm (Figure 4A). Their components and structures were similar to those of the large in vitro thrombus. Thirty minutes after microemboli injection, the rats were euthanized and histological evidence of intravascular microthrombi in the brain was obtained. H&E and immunohistochemical staining showed that the cerebral arteriole lumens (diameter, ≈100 μm) were obstructed by white or red microthrombi, which contained platelets (CD41) and fibrin (fibrinopeptide A; Figure 4B). Twenty-four hours after embolization, H&E–stained coronal sections of the brains showed multiple small infarctions in the form of poorly stained regions involving the cerebral cortex, hippocampus, caudate-putamen, and thalamus, predominantly in the cortex (Figure 4C). In vivo ultrasound treatment was performed as shown in Figure 4D.
MRI showed that the rats used in the experiment did not demonstrate any signs of cerebral infarction or hemorrhage before microemboli injection (data not shown). Twenty-four hours after the rats were embolized with the white or red microthrombi, MRI and TTC-stained sections showed that the infarcts were multiple with most distributed in the cortex and few in the subcortical regions, in consistent with histological findings. The cerebral infarct volumes were smaller in the US+MB group than in the CON group (white microthrombi: 9.52±2.99% versus 26.88±3.10% for MRI, 8.46±2.92% versus 25.68±3.38% for TTC; red microthrombi: 8.64±2.82% versus 24.60±3.13% for MRI,

Figure 4. Thrombotic microembolism-induced acute ischemic stroke models. A, Representative hematoxylin and eosin (H&E) stained and scanning electron microscopy images of in vitro white and red microthrombi. B, Representative images of white and red microthrombi in cerebral arterioles as visualized by H&E staining, or labeled with antibodies against CD41 and fibrinopeptide A (FPA). C, H&E staining of coronal brain sections 24 h after embolization showing multiple poorly stained small infarcts. D, Diagram of in vivo sonothrombolysis.
8.02±2.83% versus 22.98±3.92% for TTC; all P<0.01; Figures 5A and 5B and 6A and 6B). After treatment of rats with white microthrombi, the infarct volumes were larger in the r-tPA group than in the US+MB group (17.12±2.98% versus 9.52±2.99% for MRI, 16.08±3.20% versus 8.46±2.92% for TTC; P<0.01), whereas they were similar in the US+MB and US+MB+r-tPA groups. After treatment of rats with red microthrombi, the infarct volumes were similar in the r-tPA and US+MB groups, but smaller in the US+MB+r-tPA group than in the r-tPA group (3.96±1.43% versus 9.46±2.94% for MRI; P<0.05). H&E staining showed no intracranial hemorrhage in rats with microthrombi after US+MB or US+MB+r-tPA treatment (Figure II in the online-only Data Supplement).

In addition, there was also no evidence of intraparenchymal or perivascular hemorrhage, as well as degeneration or necrosis of cells in the brain and scalp of healthy rats after US or US+MB treatment (Figure III in the online-only Data Supplement).

Before microemboli injection, the neurological scores of all rats were 0 according to Bederson behavior test. Twenty-four hours after embolization with the white or red microthrombi, the neurological scores of rats were 2 or 3 in the CON group, but were lower in the US+MB group (both P<0.01; Figures 5C and 6C). After treatment of rats with white microthrombi, neurological scores were higher in the r-tPA group than in the US+MB group (P<0.05), whereas they were similar in the US+MB and US+MB+r-tPA groups. In contrast, after treatment of rats with red microthrombi, there was no significant difference of neurological scores among the r-tPA, US+MB, and US+MB+r-tPA groups.

Discussion
In this study, we demonstrated the efficacy of US+MB treatment in dissolving platelet- and erythrocyte-rich microthrombi in vivo. We found that microbubble-mediated sonothrombolysis significantly reduced cerebral infarction and improved neurological deficits in microemboli-induced acute ischemic strokes through dissolution of the microthrombi, including the platelet-rich ones that are resistant to r-tPA–induced thrombolysis.

To investigate the therapeutic effect of US+MB treatment for acute ischemic strokes induced by thrombotic

Figure 5. Effects of microbubble (MB) mediated sonothrombolysis on cerebral infarction and neurological deficits induced by white microthrombi. A, Magnetic resonance imaging (MRI) and 2,3,5-triphenyltetrazolium chloride (TTC) staining of coronal brain sections 24 h after embolization. B, Quantification of infarct volumes determined using MRI and TTC staining. The infarct volumes were expressed as a volume percentage of the infarct compared with the contralateral hemispheres (see text for details). C, Neurological scores 24 h after embolization. *P<0.01 vs control group. †P<0.01 vs recombinant tissue-type plasminogen activator (r-tPA) group. n=5 per group. US indicates ultrasound.
microemboli, we established a rat stroke model by intracarotid injection of either platelet- or erythrocyte-rich microemboli. This model mimics the clinical situation in which microemboli originate from a disintegrating thrombus, migrate downstream, and obstruct the distal arterioles. The model possesses several characteristics of clinical cerebral microembolism, including (1) microemboli (diameter, ≈100 μm) found in the arterioles of the leptomeningeal space or cerebral cortex, similar to autopsy findings in patients with stroke;11 (2) microemboli comprising fibrin/platelets, erythrocytes, and nucleated cells arranged in a platelet-rich or erythrocyte-rich pattern; (3) small cerebral infarcts and neurological deficits noted 24 hours after embolization; and (4) multiple microinfarcts with most distributed in the cortex and few in the subcortical regions, findings consistent with those of autopsy cases11 and possibly associated with the size, distribution, and hemodynamics of cerebral vessels. Our results indicate that US+MB treatment, which decreased infarct volumes and improved neurological deficits in rats with either platelet- or erythrocyte-rich microemboli, would provide a potential adjunct to recanalization therapy for improving outcomes of acute ischemic stroke, particularly in the case that microvascular flow was not restored after reopening of occluded cerebral artery, which is usually associated with poor clinical outcomes.32,33 We also found that the thrombolytic effect of r-tPA was augmented in combination with US+MB treatment, whereas the incidence of intracerebral hemorrhage was not increased. Hence, US+MB treatment could be performed along with r-tPA administration.

Consistent with the brain results, US+MB treatment was demonstrated to dissolve both the platelet- and erythrocyte-rich microthrombi in mesenteric microvessels, whereas r-tPA was only efficient in dissolving erythrocyte-rich microthrombi. It should be noted that the penetration of ultrasound waves through the skull was less efficient than in the mesenteric thrombosis model. However, in this study, ultrasound treatment was performed at a frequency of 2 MHz, which is widely used in transcranial Doppler devices for the evaluation of cerebral hemodynamics. A wealth of clinical data has indicated that 2-MHz ultrasound penetrates the skull adequately to augment r-tPA–induced thrombolysis and provides sufficient power for microbubble-mediated sonothrombolysis.34–36 Furthermore, we demonstrated that the diameter, histological components, and patterns of the microthrombi in the mesenteric microvessels were similar to those injected into the brain.
These findings, therefore, indicate that dissolution of cerebral microthrombi by US+MB treatment contributed to the improved outcomes of microemboli-induced acute ischemic strokes. We noticed that there was a discrepancy in the thrombolytic efficacy in vivo and in vitro in each treatment group, which may be attributed to the different size of thrombi used in the experiments. The lack of plasminogen in the in vitro flow system may also account for the discrepancy of r-tPA–induced clot lysis, but this effect should be minor because r-tPA preferentially activated plasminogen on the fibrin surface.

Intracranial hemorrhage, one of the most common and devastating treatment side effects, was encountered in patients with stroke after treatment with 300-kHz ultrasound and normal doses of r-tPA. However, we did not find any evidence of intracranial hemorrhage after US+MB treatment in the present study. This discrepancy may be ascribed to the administration of r-tPA and the different ultrasound parameters applied. We used a 2-MHz ultrasound beam emitted by a diagnostic ultrasound device, which has been demonstrated to not increase bleeding. Arterial reocclusion and distal embolization are also potential treatment side effects. Ultrasound application (6.3 W/cm²) was shown to increase the rate of arterial reocclusion after initial recanalization due to platelet activation. Meanwhile, US+MB treatment exerts its thrombolytic effects by mechanically disintegrating clots, the fragments of which may result in downstream capillary embolization. However, we did not observe post-treatment mesenteric microvascular recanalization, possibly because of the low ultrasound intensity applied in this study. In addition, our in vitro experiments showed that the post-treatment clot debris was <8 μm in diameter and unlikely to cause distal embolization. In this study, we also found that ultrasound insonation at a mechanical index of 1.9 did not cause healthy brain or scalp damage. Given these observations, microbubble-mediated sonothrombolysis might avoid the adverse effects exerted on an ischemic brain by setting appropriate ultrasound parameters.

Because platelet-rich microthrombi demonstrated a more complete cross-linking fibrin network, they were supposed to be less sensitive to ultrasonic disruption than erythrocyte-rich ones. However, in this study, microbubble-mediated sonothrombolysis was equally efficient at dissolving both platelet- and erythrocyte-rich microthrombi. This interesting result might be attributed to the ultrastructure of the platelet-rich microthrombi and the mechanism of sonothrombolysis. Our scanning electron microscopy results showed abundant pores in the fibrin network of the platelet-rich microthrombi, which may facilitate deep microbubble penetration into the interior of the fibrin network by virtue of acoustic radiation forces and microbubble oscillations. The acoustic cavitation of these microbubbles, in turn, might create larger holes within the thrombi, ultimately contributing to the fragmentation of the platelet-rich microthrombi. Because platelet-rich thrombi account for a large proportion of the thromboemboli in acute ischemic stroke victims, and are usually resistant to fibrinolytic therapy, microbubble-mediated sonothrombolysis might provide an attractive adjunct to current pharmacological and interventional recanalizations in acute ischemic stroke victims. There are several limitations to our study. First, both the in vitro and in vivo thrombi were relatively fresh, and fresh thrombi are known to be more vulnerable to sonothrombolysis than older ones. Thus, the treatment efficiency against older thrombi is uncertain. However, the present results provide reference information for clinical acute stroke patients who are hospitalized within several hours of the ictus. Second, we did not evaluate the progression of intracranial hemorrhage beyond 24 hours. The risk of hemorrhage in stroke patients is known to persist for several days or weeks and increase again during the healing phase. Nevertheless, late-onset intracranial hemorrhage is considered to be more related to the degree of ischemia and the development of collaterals rather than to the treatment methods applied. Therefore, an evaluation of the hemorrhagic complications of microbubble-mediated sonothrombolysis within 24 hours is reasonable.

In conclusion, microbubble-mediated sonothrombolysis was demonstrated to be capable of dissolving both platelet- and erythrocyte-rich microthrombi, resulting in recanalization of occluded cerebral arterioles and, consequently, alleviating brain injury. Therefore, microbubble-mediated sonothrombolysis, as an adjunct to recanalization therapy, may offer a new approach to improve outcomes of acute ischemic stroke.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Supplemental Methods

**In Vitro Clot Preparation**
Whole blood samples (5 mL) were drawn from the abdominal aorta of adult male Sprague-Dawley rats and gently mixed with 3.8% sodium citrate for anticoagulation. White thrombi were prepared using platelet-rich plasma (PRP, 2.5 mL), which was obtained from centrifuged blood, and mixed with 0.25 mL of the boundary layer between the supernatant and erythrocyte layers to add additional blood components. After adding ADP (adenosine diphosphate, 80 μmol/L), CaCl\(_2\) (80 mmol/L), and thrombin (0.5 IU/mL), 0.5 mL of either the mixed PRP (white thrombi) or whole blood (red thrombi) was immediately transferred to rubber tubes (diameter, 5 mm) to form thrombi. Small glass needles (diameter, 1.5 mm) were placed through the thrombi and removed after thrombi formation to simulate partially occluded condition. The thrombi were incubated at 37°C for 25 min, and stored at 4°C for 3 h before use.

**Mesenteric Microvascular Thrombosis Model**
Male Sprague-Dawley rats (200–250 g) were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and immobilized in the lateral decubitus position. During the experiment, the mesentery and an intestine segment were pulled through an abdominal incision and observed using intravital microscopy (BX51, Olympus Optical). To induce platelet-rich thrombosis, a small filter paper was saturated with 5% ferric chloride and placed upon the selected mesenteric microvessel (diameter, 70–100 μm) for 3 min to create an endothelial injury. Likewise, a thrombin-saturated filter paper was placed over another mesenteric microvessel for 3 min to induce erythrocyte-rich thrombosis. About 5 min after the filter papers were removed, the microvessels demonstrated occlusion as evidenced by a decreased blood flow velocity, indicating the establishment of a microvascular thrombosis model (Figure I).

**Magnetic Resonance Imaging**
Cerebral magnetic resonance imaging (MRI) was performed using a 3.0 T system (3T Trio-Tim, Siemens) and a locally made radio-frequency coil. Cerebral infarction was assessed using a T2 mapping sequence (field of view, 60 × 42.4 mm; slice thickness, 1.5 mm; repetition time, 4000 ms; echo time, 82 ms; transversal orientation; flip angle, 120°; image matrix, 181 × 320; voxel size, 0.2 × 0.2 × 1.5 mm).

**Histological examination of healthy brain and scalp after ultrasound insonation**
Sprague-Dawley rats were divided into control (CON), ultrasound (US), and ultrasound+microbubble (US+MB) groups (n=5, respectively). Brain and scalp samples were obtained immediately and at 6 h after different treatments. Hematoxylin and eosin staining was carried out for evaluation of cell or tissue damage.

Supplemental Figure Legends

Supplemental Figure I. Preparation of mesenteric microvascular thrombosis models. Representative images of mesenteric microvessels at normal condition, modeling process, and after establishment of platelet-rich (A) or erythrocyte-rich (B)
thrombosis model.

**Supplemental Figure II.** Histopathological sections of the ischemic brain showing no evidence of hemorrhage. Representative hematoxylin and eosin-stained images of ischemic brain at 24 h after US+MB and US+MB+rtPA treatments.

**Supplemental Figure III.** Effects of ultrasound insonation on healthy brain and scalp. Representative hematoxylin and eosin-stained images of brain and scalp tissue at 0 and 6 h after US and US+MB treatments.

**Supplemental Video Legends**

**Supplemental Video I.** Platelet-rich mesenteric microvascular thrombosis model.

**Supplemental Video II.** Erythrocyte-rich mesenteric microvascular thrombosis model.

**Supplemental Video III.** Neurological score, grade 0: no observable neurological deficits (forelimb flexion, decreased resistance to lateral push, or circling).

**Supplemental Video IV.** Neurological score, grade 1: forelimb flexion.

**Supplemental Video V.** Neurological score, grade 2: decreased resistance to lateral push without circling.

**Supplemental Video VI.** Neurological score, grade 3: circling.
Supplemental Figure I

A

White microthrombi

<table>
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<th>Thrombosis</th>
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B

Red microthrombi

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