Supplementary Material

Preparation of Microbubbles (MBs)

Dipalmitoylphosphatidylcholine, polyethylene glycol-40 stearate, and bovine-polyethylene glycol 2000-distearoylphosphatidylethanolamine, purchased from Avanti Polar Lipids, were dissolved in propylene glycol at a molar ratio of 82:10:8 and then heated at 70°C in a water bath until the solution was clear. Glycerol and saline were then added to the solution and mixed by purging nitrogen to obtain a homogenous lipid solution. Two milliliters of the above prepared solution were added to a 3-mL penicillin vial and sealed. The air headspace of each vial was purged with 10 mL of octafluoropropane and then activated using a Sonicator 3000 (Misonix, Farmingdale, New York, United States) to prepare biotinylated MBs (control MBs, C-MBs). Then, the vials were stored overnight at 4°C; then, the underlying clear fluid was removed, and the same volume of 0.9% saline was reintroduced.

To prepare magnetic MBs (M-MBs), superparamagnetic microbeads (mean diameter: 50 nm) conjugated to streptavidin (200 μL, 1 x 10^12/mL, Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the biotinylated MBs suspension (1 x 10^9/mL) and incubated for 30 minutes. Then, the sample was washed 3 times with phosphate-buffered saline (PBS, 3% v/v) to remove unconjugated microbeads. After that, the sample was placed on the magnetic separator and the fraction of MBs that migrated to the magnet was collected while the remaining was discarded.

Acoustic Stability of MBs In Vitro

Briefly, the in vitro acoustic stability of the MBs was determined using a Siemens ultrasonic imaging system (Acuson Sequoia 512) equipped with a 15L8-S probe for contrast pulse sequencing (frequency: 7.0 MHz, frame rate: 16 Hz, mechanical index: 0.18). C-MBs or M-MBs were loaded into a 2% agarose model and diluted 40-fold with physiological saline at 37°C for 1 hour. Contrast-enhanced ultrasound (CEUS) images were collected after 10, 20, 30, 40, and 60 minutes. The video intensity (VI) was analyzed using MCE 2.7 software (University of Virginia, Charlottesville, Virginia, United States) as previously described. The experiments were performed in triplicate.

Preparation and Characterization of Ex Vivo Macrothrombi

Many studies have shown that the thrombolytic effects of MBs or thrombolytic drugs were varied with different types or ages of thrombi. To verify whether the thrombolytic effects of M-MBs would vary with different thrombi, we applied red and white thrombi and two ages of thrombi to research in this study. Since the time window for thrombolytic treatment of acute myocardial infarction is within 12 hours, we used 12-hour clots as older thrombi to investigate the efficacy of sonothrombolysis. Briefly, the customized transparent polyethylene tube was roughened internally to minimize the effect of the liquid flow rate on thrombolysis. For white macrothrombi, whole blood was drawn from Sprague-Dawley (SD) rats, gently mixed with 3.8% sodium citrate, and centrifuged at 1,500 revolutions per minute for 10 minutes to obtain platelet-rich plasma (the supernatant), which was then mixed with the boundary layer between the supernatant and the erythrocyte layer (ratio = 9:1). After adding procoagulant solution (adenosine diphosphate: 80 μmol/L, CaCl2: 80 mmol/L, and thrombin: 0.5 IU/mL), 0.1 mL of the platelet-rich plasma mixture was immediately added to a 120-degree-angle polyethylene tube with an internal diameter of 2 mm. Then, the thrombi were incubated at 37°C for 25 minutes and stored at 4°C for 3 hours or 12 hours before thrombolysis.

For red macrothrombi, 0.1 mL of citrated blood originating from SD rats was immediately added to a 120-degree-angle polyethylene tube after being mixed with the procoagulant solution. The stored method was the same as above.

In Vitro Experimental Setup

As shown in Supplementary Fig. S2, two 120-degree-angle polyethylene tubes (one of which contained a thrombus) formed a diamond that was parallel to the ground and was connected to the main tube by a three-link pipe, forming a bifurcation structure with an angle of 60 degrees to simulate the angle between the two total iliac arteries in vivo. Consistent with the previous study, degassed PBS was used as the liquid medium in the circulating flow system which was submerged in 37°C water. The distance between the origin of the thrombus and the beginning of the bifurcation in the circulation system was ~1 cm. A peristaltic pump was used to maintain the flow rate of the system at 13 mL/min.

Assessment of MB Responsiveness to a Magnetic Field

The MBs were fluorescently labeled by the addition of dioctadecyl tetramethyl indocarbocyanine perchlorate (Dil, 1 mg/mL; Molecular Probes, Carlsbad, California, United States). The magnetic responsiveness of MBs was tested in three situations as follows:

First, 0.2 mL of the prepared MBs was placed in an Eppendorf tube (2 mL) and diluted to 1.8 mL with PBS. Then, a permanent magnet (20 x 10 x 5 mm, 5,000 Gauss; Tianlihe Magnetics, Shenzhen, China) was placed on the side of the Eppendorf tube, and the movement of the MBs was observed after 5 minutes.

Second, the responsiveness of the MBs to a magnetic field was assessed by fluorescence microscopy (BX51; Olympus, Tokyo, Japan). After being diluted 10-fold, the MB suspension
was pipetted onto a glass slide and covered with a coverslip. A magnet (20 × 10 × 5 mm, 5,000 Gauss) was placed on one side of the slide beyond the visual field to determine the responsiveness of the MBs to the magnetic field.

Third, in the flow visualization experiment, fluorescently labeled MBs were used to assess whether magnetic guidance could increase the number of MBs locally (i.e., in the lumen between the origin of the thrombus and the beginning of the bifurcation in the flow system). Before the experiment, B-mode US and pulse-wave (PW) Doppler US confirmed the lack of blood flow in the lumen where the thrombus was positioned. For better imaging results, we increased the concentration of MBs injected. C-MBs or M-MBs at a concentration of 6 × 10⁶ MBs/ml were injected into the flow system at a rate of 0.01 ml/min. A magnet (20 × 10 × 5 mm, 5,000 Gauss) was fixed behind the thrombus. The video of the region in the anterior segment tube of thrombus was captured by fluorescence microscopy with a 4× magnification objective at excitation and emission wavelengths of 549 and 565 nm, respectively.

**In Vitro Thrombolysis of White and Red Macrothrombi**

In the US + M-MB group, M-MBs at a concentration of 2 × 10⁸ MBs/ml were infused into the flow system at 0.01 ml/min. Before the treatment, B-mode US and PW Doppler US confirmed the lack of blood flow in the lumen where the thrombus was placed. The total treatment time was 30 minutes. Each treatment cycle was 10 seconds, repeating 6 times per minute. During each treatment cycle, magnet was always fixed behind the thrombus, while the US probe was first stationary and then scanned uniformly for 2 seconds from the front end of the thrombus to the back end of the thrombus at the end of eighth second. After that, US probe was reset to the front end of the thrombus (Supplementary Fig. S3). As our previous studies and other researchers both have demonstrated that 2-MHz US was safe and effective in sonothrombolysis in vivo and in vivo, as well as in human, this study thus chose 2-MHz US for thrombolytic therapy. The US insonation treatment was performed using a Siemens ultrasonic imaging system (Acuson Sequoia 512) and a 4V1c sector-array transducer (frequency: 2 MHz; mechanical index: 1.9; azimuthal plane: 32 mm; elevation plane: 5 mm) that was submerged in the water and placed 3.5 cm away from the thrombus. In the US + M-MB + recombinant tissue-type plasminogen activator (r-tPA) group, the same amount of M-MBs and 0.01 mg/ml r-tPA were injected into the flow system and combined with US treatment under the magnetic field. In the US + C-MB group, C-MBs were infused into the system at the same amount as M-MBs in the absence of magnet guidance; the US insonation protocol was also the same as that applied in the US + M-MB group. In the control group, the thrombus was suspended in the flow system but was left untreated. After the treatment, relevant US imaging measurements were obtained.

**Establishment of Left Iliac Arterial Thrombosis Model in Rats**

For the in vivo white thrombus model, male SD rats weighing approximately 250 to 300 g were anesthetized with an intraperitoneal injection of 3% pentobarbital (50 mg/kg). Each rat was fixed in the supine position by applying tape to the legs. Before the establishment of the thrombus model, the basal flow of the left iliac artery was measured using a US transducer with coupling gel applied to the epilated groin region. Then, a lateral incision was made in the left lower quadrant of the abdomen to expose the left iliac artery which was dissected away from the surrounding tissues (~1 cm) with care to avoid damaging the nerve running close to the artery. The dissected artery was dried properly to avoid dilution of the ferric chloride (FeCl₃) solution. A small piece of plastic film was placed under the artery to prevent damage to the surrounding tissues, and a piece of filter paper (8 × 4 mm) soaked with 5% (w/v) FeCl₃ was placed around the artery. After 5 minutes of exposure, the filter paper was removed, and the artery was washed with PBS and dried with filter paper. B-mode US and PW Doppler US confirmed the successful establishment of the model indicated by the existence of a completely occlusive thrombus in the left iliac artery that remained stable for 1 hour. If the model was not successfully established, 5% FeCl₃ was applied again for 5 minutes. Therapeutic US (frequency: 2 MHz; mechanical index: 1.9; azimuthal plane: 32 mm; elevation plane: 5 mm) was then used for thrombolysis.

To establish the in vivo red thrombus model, the protocol was performed as in a previous study with some modifications. Briefly, after the rat was anesthetized and the iliac artery was exposed, deep external crush damage to the left iliac artery was induced using a circular hemostatic clamp applied uniformly around the vessel. The damage was induced 1 cm away from the bifurcation of the aorta and left iliac artery over a length of 0.4 cm. Serial vessel damage was repeated for 3 minutes, after which the flow was reestablished. Then, a region of the vessel distal to the damage was narrowed with a 4–0 silk suture to reduce the flow by 80% and induce red thrombus formation. B-mode US and PW Doppler US were subsequently used to determine whether a completely occlusive thrombus had formed. If a thrombus had failed to form after 30 minutes, then the US was applied again to check the model at a time interval of 5 minutes. After a thrombus had formed, the silk suture was removed. The thrombus was required to be stable for 1 hour before a treatment was applied.

In both thrombus models, the origin of the epigastric artery was ligated through a midline abdominal incision.

**Thrombolysis of Left Iliac Arterial Thrombi in Rat Models**

A polyethylene tube (PE 50) was inserted into the right internal jugular vein for injection of the MBs. In the US + M-MB group, M-MBs (2 × 10⁶/mL) were infused through the
jugular vein at a flow rate of 0.01 mL/min. The treatment protocol was the same as described in vitro and the coupling gel was used between the US probe and iliac artery. In the US + M-MB + r-tPA group, the same amount of M-MBs and 5 mg/kg r-tPA were infused, the other treatment was the same as above. In the US + C-MB group, C-MBs were infused through the jugular vein at the same flow rate as the M-MBs, and the US insonation protocol was the same as that applied in the US + M-MB group. For the control group, 0.9% (w/v) saline was infused at the same flow rate, but the thrombus was left untreated. After the treatment, relevant US imaging measurements were obtained.

**The Safety of Thrombolysis of Magnetic Microbubbles In Vivo**

To evaluate whether M-MB explosion causes damage to tissues or blood vessels under the magnetic field, the following studies were performed.

For evaluating tissue damage, we divided the animals into four groups (n = 3 per group) as described above, exposed the left iliac artery but did not administer any embolic injury (to exclude the influence of left iliac artery blood flow blockage on muscle injury); the experimental treatments of the four groups were the same as described above. After 30 minutes of treatments, the gluteus sufericidis muscle near the left iliac artery was taken for hematoxylin and eosin (HE) staining to evaluate whether the muscle microvasculature was damaged and whether red cells oozed from the blood vessels.

For evaluating damage to the artery, the left iliac artery was ligated with 4–0 silk at the distance of 1 cm from the bifurcation of the aortic artery without any damage treatment (to avoid damaging to the iliac artery and interrupting the assessment of vascular endothelial injury after relevant treatments). The experimental treatments were the same as described above (n = 3 per group). After 30 minutes of treatments, the part of the artery at the front end of the ligation was taken for HE staining to assess the injury to the vascular endothelium.

In addition, various organs (brain, heart, liver, spleen, lungs, and kidneys) were collected at 12 hours after treatment from 5 randomly selected rats in the control and US + M-MB groups for detecting the safety of the M-MBs, and the organs were fixed with 4% formaldehyde and embedded in paraffin. HE staining was used to estimate injury.

**Supplementary Table S1** Size distribution and concentration of microbubbles

<table>
<thead>
<tr>
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<th>Concentration (10^9/mL)</th>
<th>Mean diameter (μm)</th>
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<tbody>
<tr>
<td>C-MB</td>
<td>1.05 ± 0.04</td>
<td>2.86 ± 1.13</td>
</tr>
<tr>
<td>M-MB</td>
<td>1.07 ± 0.02</td>
<td>2.89 ± 1.11</td>
</tr>
</tbody>
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Abbreviations: C-MB, control microbubble; M-MB, magnetic microbubble.
Supplementary Fig. S1 Physical characteristics of microbubbles. (A, B) Graph of the size distribution and concentration of control microbubbles (C-MBs) and magnetic microbubbles (M-MBs). (C) Contrast-enhanced ultrasound (CEUS) images of C-MBs and M-MBs loaded in an agarose phantom at 37°C at various intervals over 1 hour. (D) Quantitative analysis of the video intensity of CEUS images. n = 3. (E) Magnetic responsiveness of C-MBs and M-MBs in vitro under a magnetic field. Abbreviation: IU, intensity of contrast-enhanced ultrasound.
Supplementary Fig. S2 In vitro experimental operation diagram. The upper figure is a detailed assembly diagram of diamond structure; the lower figure is a general illustration.

Supplementary Fig. S3 Detailed sketch of ultrasound thrombolysis in vitro.
Supplementary Fig. S4 Characteristics of 3- and 12-hour thrombi before treatment. (A, B) Quantification of the length (A) and mass (B) of the 3-hour white macrothrombi. (C, D) Quantification of the length (C) and mass (D) of the 3-hour red macrothrombi. (E, F) Quantification of the length (E) and mass (F) of the 12-hour white macrothrombi. (G, H) Quantification of the length (G) and mass (H) of the 12-hour red macrothrombi. n = 20 per group. Abbreviations: C-MB, control microbubble; M-MB, magnetic microbubble; r-tPA, recombinant tissue-type plasminogen activator; US, ultrasound.
Supplementary Fig. S5 Characteristics of 12-hour thrombi and thrombolytic effect of microbubbles in vitro. (A) Representative images of thrombi by hematoxylin and eosin (HE) staining and scanning electron microscopy. (B) Representative ultrasound images of macrothrombi (white thrombi for the upper two lines of images, red thrombi for the lower two lines of images) in the longitudinal view before and after treatment. The yellow direction line roughly represents the direction of the magnetic field. (C–F) Statistical results of thrombolytic effect of the white and red thrombi after treatment: longitudinal area of thrombus changes (C), clot mass losses (D), blood flow velocity (E), and recanalization rate (F). *p < 0.05 vs. control group, #p < 0.05 US + M-MB vs. US + C-MB group, $p < 0.05 US + M-MB + rtPA vs. US + C-MB group, &p < 0.05 US + M-MB + rtPA vs. US + M-MB group, Σp < 0.05 internal group, n = 20 per group. Abbreviations: C-MB, control microbubble; M-MB, magnetic microbubble; Pre, pretreatment; Post, posttreatment; r-tPA: recombinant tissue-type plasminogen activator; US, ultrasound.
Supplementary Fig. S6 Comparison of 3- and 12-hour thrombi after treatment. (A, B) Comparative analysis of thrombus area changes (A) and clot mass losses (B) in 3- and 12-hour white thrombi. (C, D) Comparative analysis of thrombus area changes (C) and clot mass losses (D) in 3- and 12-hour red thrombi. *p < 0.05 internal group, n = 20 per group. Abbreviations: C-MB, control microbubble; M-MB, magnetic microbubble; r-tPA, recombinant tissue-type plasminogen activator; US, ultrasound.

Supplementary Fig. S7 Quantification of video intensity of contrast-enhanced ultrasound images in the occluded iliac artery in front of thrombus. *p < 0.05, n = 3 per group. Abbreviations: C-MB, control microbubble; M-MB, magnetic microbubble.
Supplementary Fig. S8 Representative contrast-enhanced ultrasound (CEUS) images of the left hindlimbs of rats after treatment. (A, B) Example CEUS time-intensity curves from hindlimbs in white and red thrombi models, respectively. (C, D) CEUS images obtained from select intervals of the curves in A and B, respectively (0 second immediately postdestruction). Abbreviations: C-MB, control microbubble; IU, intensity of contrast-enhanced ultrasound; M-MB, magnetic microbubble; Post, posttreatment; r-tPA, recombinant tissue-type plasminogen activator; US, ultrasound.
Supplementary Fig. S9 Effects of microbubbles blasting on muscles and vessels under magnetic field. (A) Hematoxylin and eosin (HE) staining of muscle around the left iliac artery. The black arrow indicates microvessel. (B) HE staining of the anterior vessel at ligation site of the left iliac artery. The top left inset is a corresponding magnified view of the yellow box. Abbreviations: C-MB, control microbubble; M-MB, magnetic microbubble; r-tPA, recombinant tissue-type plasminogen activator; US, ultrasound.
Supplementary Fig. S10 Representative hematoxylin and eosin staining of organ samples from rats after treatment with control or US + M-MB group. The stained images show no obvious injury or necrosis (i.e., few inflammatory cells, such as neutrophils, and apoptotic cells and little swelling) in the white or red thrombi models. Abbreviations: M-MB, magnetic microbubble; US, ultrasound.

References