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Research article

An in vitro comparative study on clot lysis efficiency of urokinase and reteplase with the synergy of ultrasound needle

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ABSTRACT

Objectives: Ultrasound Needle, which is an improved ultrasonic horn device, has shown great potential for promoting the diffusion of thrombolytic drugs within clots and enhancing clot lysis efficiency. However, the clot lysis efficiency of different thrombolytic drugs with the synergy of Ultrasound Needle remains unknown. In this study, we aimed to compare the lysis efficiency of the non-fibrin-specific drug urokinase and fibrin-specific drug reteplase with the synergy of Ultrasound Needle.

Materials and methods: Twenty-five milliliters of human blood was incubated for 1.5 h to form in vitro clots and then received the corresponding treatment protocols: control group (normal saline), US group (10 min of Ultrasound Needle treatment), UK group (30000IU of urokinase), r-PA group (2 mg of reteplase), US + UK group, and US + r-PA group. After treatment, the morphological changes of the clots were analyzed by B-mode ultrasound imaging and hematoxylin and eosin (H&E) staining. Lysis efficiency was evaluated based on the relative end weight (final weight/initial weight). The fibrin density of the different groups after treatment was assessed by immunofluorescence staining.

Results: Morphological examination and relative end weight analysis showed that combination therapies induced a more thorough dissolution of clots compared with single therapies, and the US + r-PA group exhibited higher lysis efficiency than the US + UK group. In addition, immunofluorescence staining showed that the US + r-PA group had fewer remaining thrombus fibrins than the US + UK group after treatment.

Conclusions: The Ultrasound Needle can significantly improve the clot lysis efficiency of both fibrinolytic drugs, and fibrin-specific reteplase exhibited superior lysis efficiency over non-fibrin-specific urokinase with the synergy of the Ultrasound Needle.

1. Introduction

Bleeding from cerebrovascular accidents or craniocerebral trauma can result in intracranial hematoma [1–3], and the volume of the hematoma is a key factor in determining the prognosis of patients [4]. If a large hematoma cannot be evacuated in time, its mass effect

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will cause a dramatic increase in intracranial pressure, which may result in cerebral hernia and even death [5,6]. Craniotomy is considered to be an effective conventional therapy for intracranial hematoma, but results in unwanted additional tissue damage [2,7]. Therefore, minimally invasive methods involving simple operations with minimal trauma, such as drilling and drainage, have been applied to evacuate intracranial hematomas [8–10]. However, a clinical study showed that only patients with sufficient volume reduction (preferably reduced to 15 ml or less) can achieve effective improvement in clinical prognosis [11], which suggested that high clot lysis efficiency is required for minimally invasive strategies to evacuate the hematoma.

Minimally invasive ultrasound techniques have been shown to enhance thrombolysis efficiency in many studies [12–14]. Taking advantage of the widely used catheter-directed therapy, in a thrombo-embolized rabbit inferior vena cava (IVC) model with a simultaneous catheter-directed recombinant tissue plasminogen activator (rt-PA) thrombolysis procedure, guided moderate mechanical index longer pulses from a modified diagnostic ultrasound transducer combined with intraclot infusion of microbubbles significantly accelerated the thrombolysis process [15]. Even though ultrasound-stimulated microbubble cavitation-assisted thrombolysis exhibits enhanced efficacy in vessel thrombosis, the evacuation of a large-volume intracranial hematoma demands stronger thrombolysis efficacy compared with the evacuation of an intravascular thrombus. In a clinical trial [16], a 2-MHz ultrasound catheter was inserted inside an intracranial hematoma through minimally invasive surgery and synergized with rt-PA to evacuate the hematoma. The evacuation efficiency of the hematoma was enhanced compared with that of rt-PA alone, but the treatment lasted up to 12 h. Another ultrasound catheter with a higher frequency of 10 MHz was used to lyse large-volume blood clots in vitro [17,18]. Although the lysis efficiency was increased, continuous ultrasound irradiation for 1 h was not sufficiently rapid as a treatment strategy.

In our previous study [19], Ultrasound Needle (an improved ultrasonic horn) was used to dissolve intracranial hematomas in an in vitro model. Ultrasound Needle has been shown to enhance clot lysis efficiency by promoting urokinase penetration within the clots. Ultrasound catheter-based sonothrombolysis relies on microjetting and the radiation force induced by the front-facing transducer to destroy fibrins and increase the contact area between the thrombolytic drugs and fibrins. Unlike ultrasound catheters, ultrasound energy is transmitted from the rear transducer to the Ultrasound Needle tip, and the tip vibrations can mechanically destroy the hematoma directly, in addition to the cavitation effect induced by the tip vibrations. Therefore, the combination of the Ultrasound Needle with urokinase achieved rapid clot weight reduction in 8 min, which was more efficient than the lysis efficacy of the strategies reported in previous studies [20,21]. However, the different types of clinically available thrombolytic drugs exhibit different characteristics [22]. Whether the combination of Ultrasound Needle with different types of thrombolytic drugs will produce different lysis efficiencies has not yet been studied. Therefore, in this study, we compared the lysis efficiency of the non-fibrin-specific drug urokinase and fibrin-specific drug reteplase with the synergy of Ultrasound Needle.

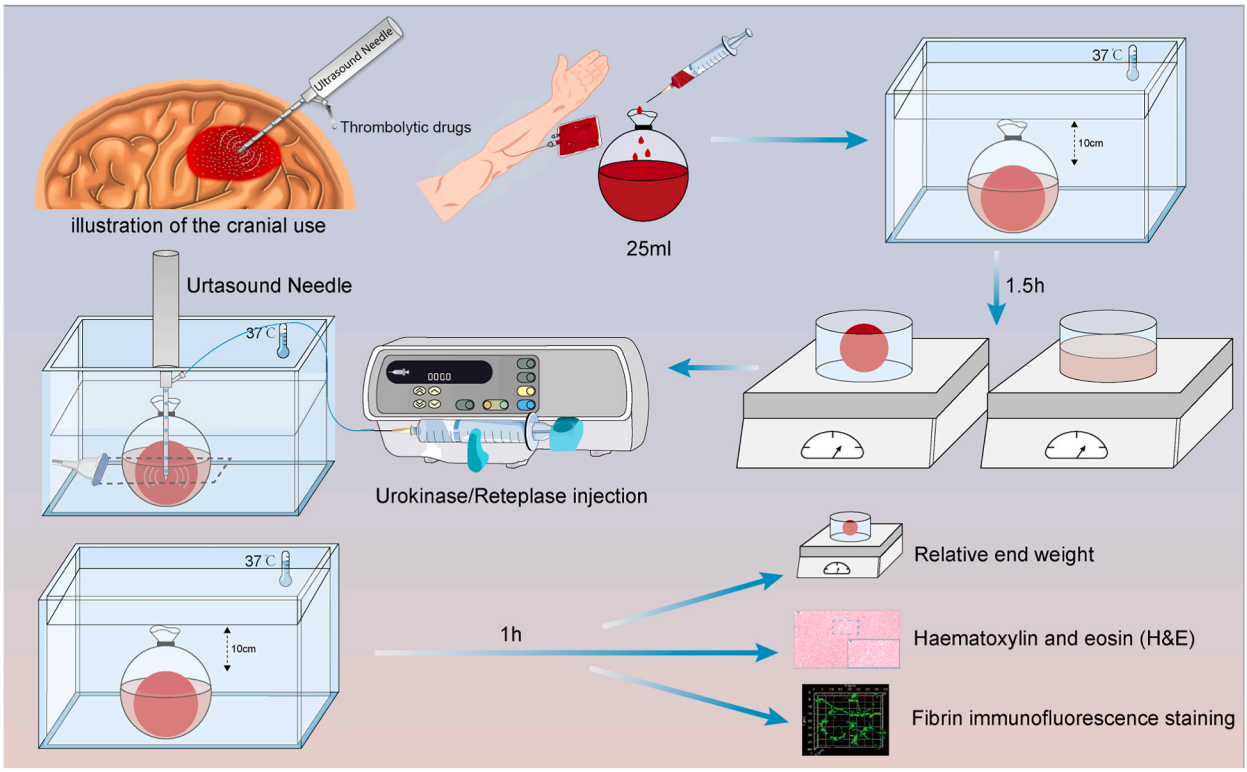

Fig. 1. Illustration of the cranial use and experimental workflow. Twenty five milliliters of blood was collected from volunteers and injected into a balloon for 1.5-h incubation to form clots. The weight and morphology of the clots were examined to evaluate the clot lysis efficiency of different treatment groups.

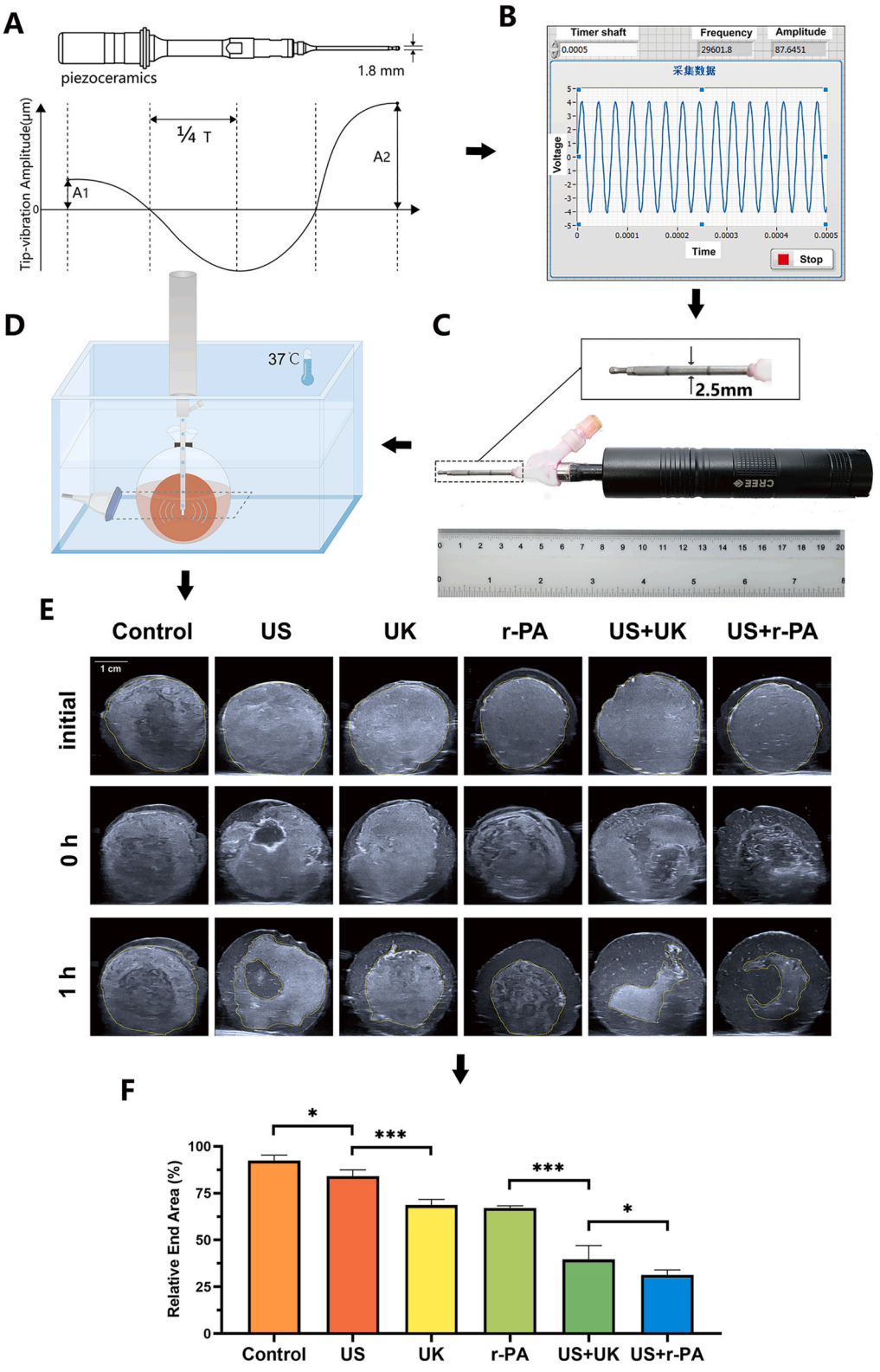

Fig. 2. Schematic illustration of the working mode of the Ultrasound Needle and B-mode ultrasound evaluation of clot lysis. A, Schematic of the amplitude amplification process of the Ultrasound Needle. T, a complete sinusoidal period for vibration. B, Frequency and amplitude of the Ultrasound Needle tip. C, Photograph of the Ultrasound Needle. D, Illustration of B-mode ultrasound examination. E, B-mode images for different treatment groups at three time points: before treatment (initial), after treatment (0 h), and 1 h after treatment (1 h). The boundary of the clot was

outlined by ImageJ software, as indicated by the yellow curve. F, Statistical histograms of the relative end area for different treatment groups. (n = 3). *, P < 0.05; ***, P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

2.1. Ultrasound device

The Ultrasound Needle is an improved ultrasonic horn device with a frequency of 29.6 kHz. The horn is made of solid titanium alloy with a tip diameter of 1.8 mm and a metallic sheath (diameter: 2.5 mm) is attached outside the tip for drug injection (Fig. 2A–C). The working parameters for clot lysis were set as follows: (1) duty cycle: 20%, (2) pulse repetition frequency: 100 Hz, (3) input power: 6.7 W, and (4) tip vibration amplitude: 87.6 μm. The settings of these parameters were based on our previous study [19]. We measured the temperature of the blood clot center immediately after the operation of the Ultrasound Needle (39.8 ± 0.9 °C), and the temperature of the clot area further away from the center was lower.

2.2. In vitro hematoma model

This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Army Medical University (APPROVAL NUMBER:2021-Research No. 099-01). All blood samples were obtained from healthy adult volunteers after obtaining their written informed consent.

For each clot, 25 ml of blood was injected into a balloon. Following this, 500 μL of 5% $CaCl_2$ solution and 500 μL of thrombin solution (20IU/500 μL, Yge Pharmaceutical Co., Ltd., Hunan, China) were added. After mixing, the balloon was placed in a water bath set at a constant temperature of 37 °C. After incubation for 1.5 h, the liquid was filtered, and the remaining clot was weighed and recorded as the initial clot weight (Fig. 1). This method is based on the method described in other references. [17,18].

3. Treatment protocols of clot lysis

The clots were randomly divided into different groups: (1) control group, (2) ultrasound (US) group, (3) urokinase (UK) group, (4) reteplase (r-PA) group, (5) US + UK group, and (6) US + r-PA group. For the control group, the Ultrasound Needle was vertically inserted into the center of the spherical clot with an average diameter of 3 cm (sham radiation) under the guidance of the diagnostic probe, which was perpendicular to the Ultrasound Needle, and the needle tip was positioned based on the two-dimensional diagnostic ultrasound image. Following this, 5 mL of saline (0.9% NaCl) was injected through the needle sheath using a microinjection pump at a rate of 30 ml/h. For the US group, the Ultrasound Needle was inserted into the clot, and the clot was irradiated for 10 min without liquid injection. For the UK group, the Ultrasound Needle was inserted into the clot and 5 mL of urokinase solution (30000IU/5 mL, Humanwell Pharmaceutical Co., Ltd., Wuhan, China) and was injected through the needle sheath using a microinjection pump at a rate of 30 ml/h. The treatment protocol of the r-PA group was the same as that of the UK group, except that the UK solution was replaced with 5 ml reteplase solution (2 mg/5 mL, China Resources Biotech Pharmaceutical Co., Ltd., Shandong, China). For the US + UK group, the treatment protocol was the same as that of the UK group, and the Ultrasound Needle was used for 10 min. For the US + r-PA group, the treatment protocol was the same as that of the r-PA group, and the Ultrasound Needle was used for 10 min. Finally, the liquid was filtered and the remaining solid components were weighed and recorded as the final clot weight. The clot lysis efficiency was assessed by calculating the relative end weight (final clot weight/initial clot weight) (Fig. 1).

3.1. Evaluation of clot lysis by B-mode ultrasound imaging

Eighteen clots were used in this experiment, with three clots for each group. B-mode ultrasound imaging was performed using an X4-12L linear array probe with a frequency range of 4.0–12.0 MHz (VINNO70, VINNO Technology Co., Ltd., Suzhou, China). The probe was placed in a water tank parallel to the cross section of the central part of the balloon. Two-dimensional images of the blood clots were acquired for each group: before treatment, immediately after treatment, and 1 h after treatment (Fig. 2D and E). The blood clot area before treatment and 1 h after treatment was outlined using ImageJ software, and the relative end area (n = 3) was calculated using the following formula: (relative end area = area 1 h after treatment/area before treatment).

3.2. Hematoxylin and eosin staining of the clots

Thirty clots were used in this experiment, with five clots for each group. Blood clots from each group were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde fixative, embedded in paraffin, and then sectioned. Slides were stained with hematoxylin and eosin (H&E) for histomorphological analysis.

3.3. Immunofluorescence staining for the detection of blood clot fibrins

Eighteen clots were used in this experiment, with three clots for each group. The blood clots were embedded in paraffin and then

sectioned. Fibrins were stained with an anti-fibrinogen alpha chain antibody (Abcam), and images were captured using a laser scanning confocal microscope (LSM880, ZEISS, Germany). Fibrin immunofluorescence areas were analyzed using ImageJ software in a random field of view at 600 × magnification, and the relative end fluorescence area (n = 5) was calculated using the following formula: relative end fluorescence area = fibrin immunofluorescence area/total image area.

3.4. Statistical analysis

The data obtained in this study are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS Statistics 23.0 software, and one-way analysis of variance (ANOVA) was applied for comparative testing. Pairwise multiple comparisons were conducted using least significant difference (LSD) and Dunnett's T3 methods. The results were considered statistically significant when $P < 0.05$.

4. Results

4.1. Clot lysis examination by B-mode ultrasound imaging

After incubation for 1.5 h, the blood clots in the balloon showed a round, slightly high echo, and a clear boundary (Fig. 2E, initial). After treatment, obvious damage and defects were detected in the clots (Figs. 2E and 0 h, 1 h). Statistical analysis showed that the relative end areas of the US + UK group (39.62 ± 7.45%) and US + r-PA group (31.29 ± 2.75%) were significantly lower than those of other groups ($P < 0.05$). There was no statistical difference between the relative end area of the UK and r-PA groups ($P > 0.05$).

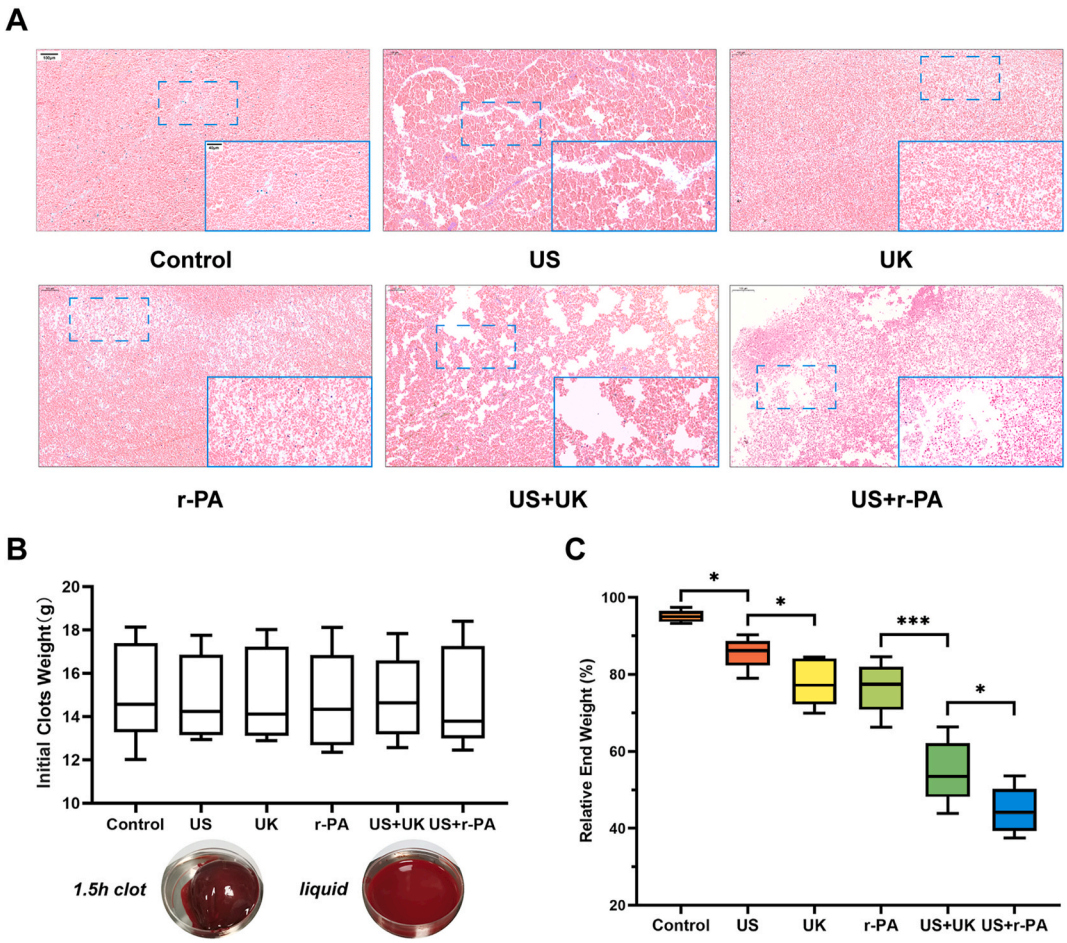

Fig. 3. H&E staining and clot weight analysis of different treatment groups. A, H&E staining evaluation. The images in the blue solid line box (40 × magnification) in the lower right corner are the magnified view of the dotted line box (10 × magnification). B, The initial clot weights of different groups before treatment and photographs of a well-formed 1.5-h clot and filtered fluid. C, The relative end weights of different groups after treatment. (n = 5) *, $P < 0.05$; ***, $P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

However, the relative end area of the US + r-PA group was lower than that of the US + UK group (31.29 ± 2.75% vs 39.62 ± 7.45%; P < 0.05) (Fig. 2E and F).

4.1.1. Hematoxylin and eosin evaluation of clot lysis

Hematoxylin and eosin staining revealed that the structural organization of the clots in the control group was intact and compact. The clots in the US group showed irregular interspaces induced by mechanical destruction. The clots in the fibrinolytic drug-alone groups showed scattered and sparse density areas. The fibrinolytic drug combined with ultrasound groups showed wider sparse density areas and larger interspaces (Fig. 3A).

4.1.2. Relative end weight of different treatment groups

There were no significant differences in the initial clot weight among all groups (P > 0.05) (Fig. 3B). After treatment, the relative end weight of the US group was significantly lower than that of the control group (85.70 ± 4.14% vs 95.07 ± 1.58%; P < 0.05). There was no statistical difference between the UK and r-PA groups (77.99 ± 6.20% vs 76.63 ± 6.72%; P > 0.05). However, the relative end weight of the US + r-PA group was significantly lower than that of US + UK group (44.54 ± 6.11% vs 54.84 ± 8.21%; P < 0.05) (Fig. 3C).

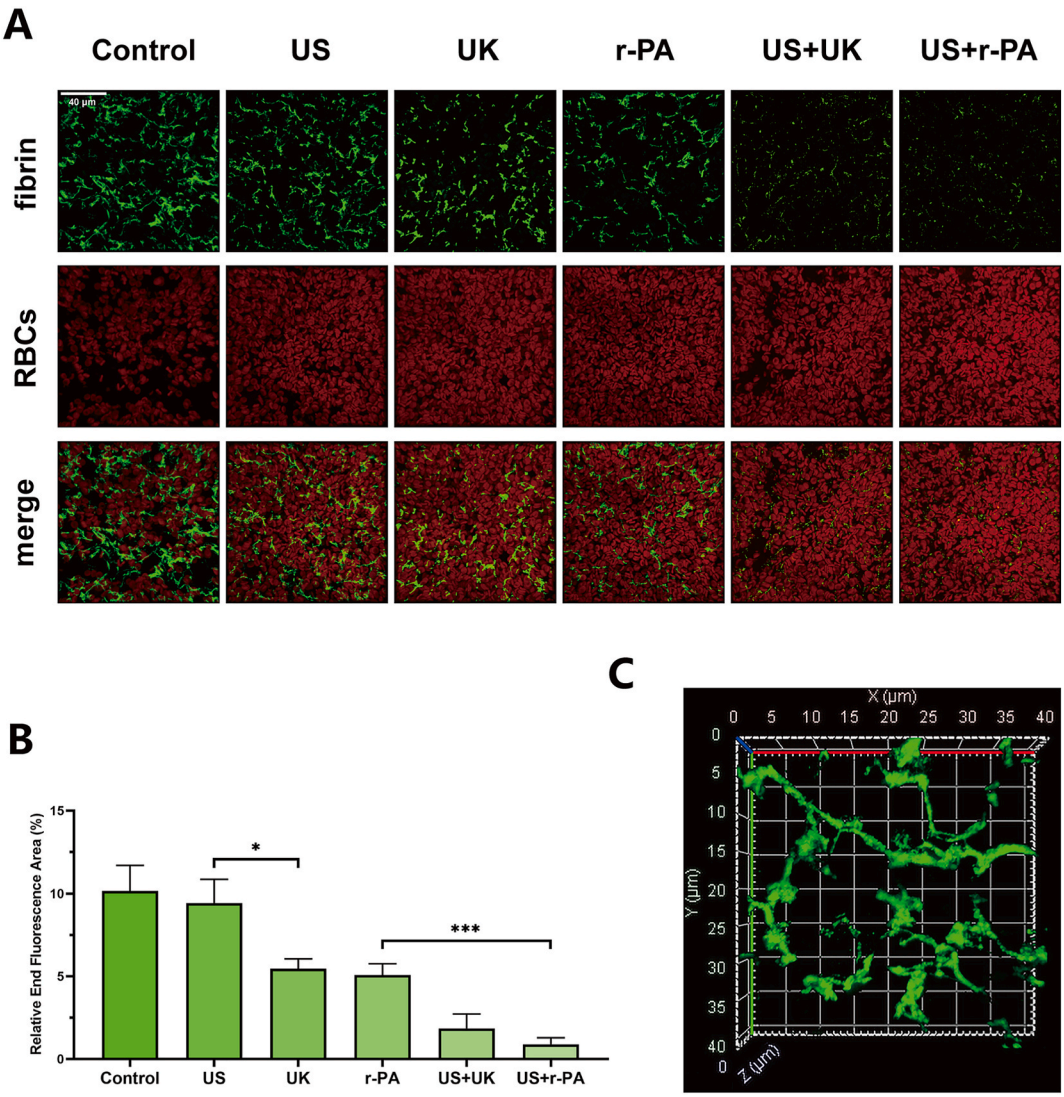

Fig. 4. Microscopic examination of clots for different groups. A, Laser scanning confocal microscopy images (600 × magnification) of clots for different treatment groups. Fibrin is illustrated in green and red blood cell (RBC) is illustrated in red. B, Quantitative analysis of the relative end fluorescence area of different treatment groups. C, Three-dimensional reconstruction image (1000 × magnification) of fibrin immunofluorescence for an untreated clot. Fibrin is illustrated in green. (n = 5) *, P < 0.05; ***, P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.2. Blood clot fibrin detection by immunofluorescence staining

The fibrin distribution of the US group was visually less dense compared with that of the control group, but with no statistical difference in quantitation (9.44 ± 1.42% vs 10.15 ± 1.55%; $P > 0.05$). The green fluorescence fibrin areas of the UK group (5.46 ± 0.59%) and r-PA group (5.07 ± 0.69%) were significantly lower than those for the control and US groups ($P < 0.05$), and there was no difference between the two fibrinolytic drug-alone groups. There was a more obvious decrease in the green fluorescence fibrin area of the combination groups (US + UK group: 1.86 ± 0.87%; US + r-PA group: 0.89 ± 0.39%), and the remaining fibrins showed weak fluorescence and sparse filament-like distribution (Fig. 4A–C).

5. Discussion

The effectiveness of sonothrombolysis has been verified in many in vitro and in vivo studies over the years [13]. In this study, the combination therapy of Ultrasound Needle with either urokinase or r-PA for 10 min showed improved blood clot lysis efficiency compared with the fibrinolytic drug-alone groups. With the assistance of the Ultrasound Needle, the r-PA combination group achieved better clot lysis efficiency than the urokinase combination group. Specifically, the relative end weight of the US + r-PA group was reduced to less than 50% after 10 min of treatment, which showed great potential as an effective minimally invasive strategy for rapid intracranial hematoma evacuation.

The cavitation activity generated by the high-amplitude vibrations of the Ultrasound Needle tip can affect the pressure amplitude of the surrounding fluid and form bubble clouds around the tip, which provides abundant cavitation nuclei [23,24]. Meanwhile, the cavitation process of bubble expansion, contraction, and implosion, as well as the irregular interacting dynamics of bubbles, induce dramatic mechanical effects, including shock waves, light emission, and microjetting [25–27]. In this study, the fissures in the B-mode and H&E images of the US group indicated that the clot tissues were mechanically destroyed (Fig. 2E; Fig. 3A).The increase in sparse areas observed for the US + UK and US + r-PA groups compared with those for the fibrinolytic drug-alone groups indicated that more clot areas were lysed by the fibrinolytic drugs because of the enhanced drug diffusion induced by the Ultrasound Needle.

Urokinase is a classic fibrinolytic drug that directly activates endogenous fibrinogen to initiate thrombolysis [22]. Although it has no specificity for thrombus fibrins, its lysis efficiency is comparable to that of fibrin-specific drugs such as rt-PA and r-PA [28–31]. Masomi-Bornwasse et al. [18] showed that there was no significant difference in the lysis efficiency between urokinase and tenecteplase (another variant of rt-PA) in 1.5-h clots even when combined with an ultrasound catheter. In our study, there was no significant difference in the relative end weight of blood clots between the r-PA and UK groups (76.63 ± 6.72% vs 77.99 ± 6.20%; $P > 0.05$). However, with the assistance of the Ultrasound Needle, the US + r-PA group achieved higher lysis efficiency compared with the US + UK group (relative end weight, 44.54 ± 6.11% vs 54.84 ± 8.21%; $P < 0.05$) (Fig. 3C). The difference in the results between the two studies may be due to the different mechanical effects generated by the ultrasound catheter and Ultrasound Needle. Both types of ultrasound instrument can increase the permeability of the clot tissues for enhanced drug diffusion by microscale destruction induced by ultrasound cavitation. However, compared with ultrasound catheters, the Ultrasound Needle can produce more powerful mechanical destruction in the clots, which can form fissures within the clots and even break down the clots into smaller fragments at the macroscale level (Fig. 2E). Therefore, more biochemical binding sites on the thrombus fibrins are exposed for the specific fibrinolytic drug reteplase to take effect. In view of this, with the synergy of the Ultrasound Needle, the advantage of fibrinolytic drugs with specificity can be fully exerted to lyse the blood clot, thus exhibiting superior efficacy over non-specific fibrinolytic drugs (Fig. 3B and C).

The limitations of this study are as follows. Even though the Ultrasound Needle could create fissures and fragments of the in vitro clots and exhibited excellent clot lysis efficacy, the mechanical destruction of the hematoma in the brain may release large chunks, which may increase intracranial pressure or cause secondary damage to brain tissue. Injection of fluid (thrombolytic drugs) may also increase the intracranial pressure. Injections of a high dose of thrombolytic drug can induce severe hemorrhage in the brain. Therefore, the dose and injection speed should be optimized when the treatment strategy is used in vivo. Several improvements can be made to improve the current sonothrombolysis strategy for in vivo applications. First, adding a channel that can communicate with a suction device to the sheath of the Ultrasound Needle can discharge excess fluid at the treatment site, which may help balance the intracranial pressure. Second, the clots in our in vitro tests showed a spherical shape; however, the intracranial hematoma in the actual case showed different shapes depending on the hemorrhage site. Therefore, moving the needle tip regularly and appropriately during treatment may help increase the contact area of the needle tip with the hematoma, consequently improving the dissolution efficiency.

In conclusion, the combination of Ultrasound Needle with fibrinolytic drugs showed enhanced thrombolytic efficiency compared with single therapies, and fibrin-specific reteplase exhibited superior clot lysis efficiency over non-fibrin specific urokinase with the assistance of the Ultrasound Needle.

Written informed consent

This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Army Medical University (APPROVAL NUMBER:2021-Research No. 099-01) and written informed consent was obtained from all participants. Written informed consent for publication of this paper was obtained from the Second Affiliated Hospital of Army Medical University and all authors.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Junhui Tang: Writing – original draft, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Jiawei Tang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Yiyi Liao:** Investigation. **Luhua Bai:** Investigation. **Tingting Luo:** Investigation. **Yali Xu:** Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zheng Liu:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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