Hemostatic mechanism underlying microbubble-enhanced non-focused ultrasound in the treatment of a rabbit liver trauma model

Da-wei Zhao^{1,*}, Meng Tian^{1,*}, Jian-zheng Yang¹, Peng Du¹, Jie Bi¹, Xinjian Zhu² and Tao Li¹

¹Department of Ultrasound, Daping Hospital & Institute of Surgery Research, Third Military Medical University, Chongqing 400042, P.R. China; ²State Key Laboratory of Trauma, Burns and Combined Injury, Daping Hospital & Institute of Surgery Research, Third Military Medical University, Chongqing 400042, P.R. China

*These authors contributed equally to this paper.

Corresponding author: Tao Li. Email: taoli39@163.com

Abstract

The aim of our study was to investigate the hemostatic mechanism underlying microbubble-enhanced non-focused ultrasound treatment of liver trauma. Thirty rabbits with liver trauma were randomly divided into three groups—the microbubble-enhanced ultrasound (MEUS; further subdivided based on exposure intensity into MEUS1 [0.11 W/cm²], MEUS2 [0.55 W/cm²], and MEUS3 [1.1 W/cm²]), ultrasound without microbubbles (US), and microbubbles without ultrasound (MB) groups. The pre- and post-treatment bleeding weight and visual bleeding scores were evaluated. The serum liver enzyme concentrations as well as the blood perfusion level represented by mean peak contrast intensity (PI) ratio in the treatment area were analyzed. The hemostatic mechanism was evaluated by histological and transmission electron microscopic examination of liver tissue samples. The MEUS subgroups 1–3 (grade 0–1, grade 0–2, and grade 1–2, respectively) exhibited significantly lower post-treatment visual bleeding scores than the US and MB groups (both, grade 3–4; all, $P < 0.05$). Subgroups MEUS1 (0.346 ± 0.345 g) and MEUS2 (2.232 ± 2.256 g) exhibited significantly lower post-treatment bleeding weight than the US and MB groups (5.698 ± 1.938 and 5.688 ± 2.317 g, respectively; all, $P < 0.05$). Additionally, MEUS subgroups 1–3 exhibited significantly lower post-treatment blood perfusion levels (PI ratios, 0.64 ± 0.085 , 0.73 ± 0.045 , and 0.84 ± 0.034 , respectively) than the US and MB groups (PI ratios, 1.00 ± 0.005 and 0.99 ± 0.005 , respectively; all, $P < 0.05$). In the MEUS group, hepatic cells became edematous and compressed the hepatic sinus and associated blood vessels. However, the serum liver enzyme levels were not significantly altered. Microbubble-enhanced non-focused ultrasound does not significantly affect blood perfusion and liver function and can be used to induce rapid hemostasis in case of liver trauma.

Keywords: Ultrasound, microbubbles, liver, hemorrhage, hemostasis, therapy

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Introduction

Despite its relatively protected position, the liver is the most frequently traumatically injured solid intra-abdominal organ. Associated injuries to other organs and uncontrolled hemorrhage from the liver are the main causes of death, with mortality rates ranging from 5 to 54% having been reported in literature.^{1,2} While surgery is the conventional treatment for liver trauma, there are major challenges associated with surgery in cases involving severe bleeding, because of the brittle architecture, low tensile strength, and abundant blood supply associated with the liver. In the past two decades, the therapeutic outcome in patients with major liver injuries appears to have improved because

of the increased use of non-operative management and minimally invasive damage control techniques such as therapeutic transcatheter arterial embolism, laparoscopy, and computed tomography-guided percutaneous drainage.^{3–6} These new approaches avoid not only primary surgery, but also any consequent severe postoperative complications such as soft-tissue necrosis, vascular injury, continuous bleeding, and leakage from the intrahepatic bile duct. However, most of the existing minimally invasive methods require complex instruments and application techniques.

Recently, several novel minimally invasive techniques have been developed for stoppage of bleeding, including percutaneous radio-frequency coagulation,^{7,8} microwave

coagulation,^{9,10} and high-intensity focused ultrasound (HIFU).^{11–15} These techniques can significantly promote hemostasis, with minimal invasiveness and rapid recovery. However, these techniques can also cause coagulation necrosis because of their heat coagulating properties. Furthermore, these methods have certain limitations. For instance, temperatures above 70°C often lead to dehydration and atrophy of tissue along with deformation of the adjacent structures.¹⁶ However, if blood flow in the target area is high, heat can be dissipated during the application process (heat sink effect), which can permit prolonged treatment.

Acoustic cavitation is a major physical effect of ultrasound application. Microbubbles can act as cavitation nuclei to enhance the mechanical damage during the bubble oscillation resulting from ultrasound application. Microbubble-enhanced ultrasound (MEUS) has been shown to not only cause intravascular inertial cavitation at a low acoustic intensity, but also greatly increase the potential for cavitation-related defects such as microvascular rupture¹⁷ and petechial hemorrhage.¹⁸ Thus, MEUS could result in severe mechanical damage to the endothelium of capillaries or even small vessels at high amplitudes.^{19–21} Previously, we found that extracorporeal application of MEUS can temporally interrupt regional liver circulation in rabbits at an acoustic intensity of 0.89 W/cm².²² Therefore, we became interested in the possibility of using MEUS to produce hemostasis in the hemorrhagic liver.

The aim of this study was to investigate the hemostatic microcosmic mechanism of microbubble-enhanced non-focused ultrasound for the treatment of liver trauma in a rabbit model and analyze its short-term influence on liver function.

Materials and methods

Ethical statement

This study was performed in strict accordance with the recommendations^{23,24} in the Guide for the Care and Use of Laboratory Animals of the Third Military Medical University. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University (Permit Number: SYXK-PLA-2007035). All surgeries were performed under anesthesia with sodium pentobarbital, and all efforts were made to use a minimum number of animals and minimize their suffering.

Animals

A total of 30 New Zealand white rabbits weighing 2.15 ± 0.19 kg each were obtained from the Animal Center of the Daping Hospital, Third Military Medical University, Chongqing, China. The rabbits were housed in a temperature-controlled room with a 12 h light-dark cycle and fed standard rabbit chow and water for 12 h before surgery. The animals were randomly divided into three groups. Rabbits in the MEUS group (MEUS; n=20) received therapeutic ultrasound with microbubble injection, those in the

ultrasound group (US; n=5) received therapeutic ultrasound without microbubble injection, and those in the microbubbles group (MB; n=5) received microbubbles without ultrasound treatment after the induction of liver trauma. Based on the differences in acoustic intensity, the 20 rabbits in the MEUS group were randomly divided into three subgroups: MEUS1 (0.11 W; n=10), MEUS2 (0.55 W; n=5), and MEUS3 (1.1 W; n=5).

Custom-made therapeutic non-focused ultrasound device

The therapeutic, non-focused ultrasound wave was generated by a custom-made ultrasound device (Institute of Ultrasound Imaging, Chongqing Medical University, Chongqing, China) with a 1.6 cm diameter transducer composed of a cylindrical aluminum shell. A hydrophone was set up to measure the acoustic output of the transducer at a distance of 10 cm outside the tip. The transducer was operated at a frequency of 976.6 kHz. The acoustic output could be adjusted to vary between 0.11 and 3.50 W. The pulse duty ratio was 50%, with a frequency of 1 kHz. The acoustic pressure (peak negative pressure) output was 0.19 MPa. The insonation procedure was performed by the same operator for all animals.

Microbubble preparation

Lipid-coated microbubbles (Zhifuxian,²² provided by the Department of Ultrasound, Xianqiao Hospital, Third Military Medical University, Chongqing, China) were composed of a suspension of 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine prepared by lyophilization and stirred with perfluoropropane gas using a high-speed mechanical amalgamator. The average particle diameter was 2 μ m, and the concentration of bubbles in the suspension was 9×10^{10} /mL. The microbubble suspension was used for nucleation of the ultrasound cavitation at a dose of 0.1 mL/kg and contrast-enhanced ultrasound (CEUS) at a dose of 0.01 mL/kg.

Liver trauma model and treatment protocols

The modified liver trauma model was induced as previously described.²² The animals were anesthetized with an intravenous injection of 2.5% pentobarbital sodium (30 mg/kg) and immobilized on a warming pad on the operating platform in the supine position. Insertion of a closed intravenous catheter system (BD Intima II, Becton Dickinson Medical Devices, USA) into the proximal auricular vein allowed blood sampling and microbubble infusion. An abdominal area of approximately 12 cm $\times$ 15 cm was sheared, sterilized with 10% povidone iodine, and draped with sterile towels. Midline laparotomy was performed in order to expose the liver. A portion of the middle liver lobe was pulled out slightly and placed on saline-soaked gauze to fix *in situ*. An incision, 15 mm in length and 3 mm in depth, was made along the central line of the liver with a number 11-scalpel blade. The initial bleeding was collected by placing absorbent paper over the incision for 10 s

Table 1 Visual bleeding score system

Score	Bleeding manifestations
0	Complete bleeding arrest
1	Only slow leakage of blood
2	Slow leakage and minor active bleeding
3	Visually profuse bleeding
4	Significant profuse bleeding

immediately after incision. The initial bleeding weight (g) was determined using a precision scale (BSA 124S, Sartorius AG, Goettingen, Germany). Subsequently, the incision region was slightly contacted to avoid any pressure and immediately insonated using the transducer coupled with blood and saline. The treatment lasted for 5 min. The microbubble suspension (0.1 mL/kg diluted in 3 mL saline) was simultaneously injected at a constant rate of 0.7 mL/min with the aid of a syringe pump. Blood was collected and weighed again for 5 min after treatment as described above. The visual bleeding scores, which were simultaneously evaluated, were graded into five levels according to the scoring system originally described by Zhao *et al.* (Table 1).²²

Following the same treatment protocol, the microbubble suspension was replaced with the same amount of saline in the US group. Procedures in the MB group were performed with the same dose of microbubble injection but without therapeutic ultrasound exposure.

CEUS analysis

CEUS analysis was performed using a Phillips IU22 medical diagnostic ultrasound system (Philips Medical System, Andover, MA, USA) with a 5–12 MHz transducer (L 12-5 50 mm, Philips Medical system, Andover, MA, USA) at a mechanical index of 0.08, followed by intravenous bolus injection of microbubbles at a dose of 0.01 mL/kg. Depth, gain, and other settings were maintained constant during CEUS analysis. In the MEUS and MB groups, the target liver lobe was imaged prior to treatment in order to examine the blood perfusion of the liver lobe. In the US group, however, pretreatment imaging was not performed in order to avoid the introduction of microbubbles into circulation. Immediately after treatment, CEUS was performed at 10 min intervals for an hour in all groups.

The built-in QLAB software (Philips Medical System, Bothell, WA, USA) was used to analyze user-defined regions of interest (ROIs) for mean peak acoustic intensity parameters. ROI, 3 mm × 3 mm in size, were placed in each of the treatment regions as well as in a remote normal region, which acted as the control. The QLAB software automatically calculated background-subtracted plots of mean peak contrast intensity (PI). The PI values were defined as PI_t and PI_n for the treatment and normal regions, respectively. For normalization, the PI ratio of each rabbit at each time point was calculated as PI_t/PI_n .

Histopathological examinations

After the experiments, five animals in each group were sacrificed by intravenous injection of an overdose of pentobarbital sodium. Tissue samples of the treated regions were dissected, fixed directly in 4% formalin, dehydrated overnight, and embedded in paraffin wax for sectioning. The next day, the sections were washed with phosphate buffered saline, stained with hematoxylin and eosin (HE), dehydrated, cleared, and mounted. Sections were examined using a light microscope (Olympus BX41, Olympus Company, Tokyo, Japan) and photographed.

The freeware ImageJ v1.49 software (NIH image software, Bethesda, Maryland, USA; <http://rsb.info.nih.gov/ij>) was used to measure the area of hepatic sinusoids and hepatic plate by converting the RGB images into gray-scale images and setting a threshold processing. The area percentages of hepatic sinusoids and hepatic plate in the whole image as well as their area ratio were calculated.

Transmission electron microscopy

During the histopathological examination, liver tissues of the treated regions were simultaneously cut into small pieces (approximately 1 mm × 1 mm × 1 mm) and fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer. The specimens were dehydrated using a graded series of acetone solutions and embedded in epoxy resin. Ultrathin sections were prepared using an ultra-microtome (Leica EM UC7 RT, Leica, Vienna, Austria), stained with uranyl acetate and lead citrate, observed under an electron microscope (JEM 1400PLUS, Jeol, Akishima, Japan), and photographed.

Serum parameters

In order to analyze the changes in hepatic function, blood samples from the peripheral vessels were collected from the remaining five animals in the MEUS1 subgroup until 48 h post-treatment. The blood samples were centrifuged for 10 min at 4°C at 3500 rpm. Serum samples were collected and stored at -80°C. They were later submitted to the clinical laboratory for assays of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) levels and the AST/ALT ratio using a serum multiple biochemical analyzer.

Statistical analyses

All data are presented as mean ± standard deviations. Visual bleeding scores were compared among the treatment groups using the Mann-Whitney test. Differences in bleeding weight and PI ratio at 0 min as well as the serum liver enzyme levels of AST, ALT, AST/ALT, ALP, and GGT were analyzed by one-way analysis of variance; in cases where the total difference was significant, differences between two groups were analyzed using Dunnett's multiple comparison test. The calibers of the hepatic vein and central veins as well as the hepatic plate/hepatic sinusoid area ratio were analyzed using unpaired two-tailed *t*-tests. All statistical

analyses were performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Hemostatic effect analysis

Rabbits of all groups exhibited obvious bleeding due to incision before treatment. After treatment, bleeding in the MEUS1 subgroup was almost completely resolved (Figure 1(A c)), while that in the two control groups (Figure 1(A a,b)) and the other MEUS subgroups (Figure 1(A d,e)) was unabated. The pre- and post-treatment visual bleeding scores, ranging from grade 3 to 4, exhibited no significant differences among the groups ($P > 0.05$; Figure 1(B)). The visual bleeding scores declined significantly after treatment, from grades 3–4 to grades 0–1, 0–2, and 1–2 (all, $P < 0.01$) in the MEUS subgroups 1–3, respectively. However, in the two control groups, the scores remained high even after treatment, ranging between grades 3 and 4 (Figure 1(B)). There were significant differences in the pre- and post-treatment visual bleeding scores between the MEUS subgroups. Comparison of

post-treatment visual bleeding scores of different MEUS subgroups with those of the US and MB groups revealed significant differences (all, $P < 0.05$); however, the differences between the US and MB groups were not statistically significant ($P > 0.05$).

The initial 10 s bleeding weight, ranging from 0.252 ± 0.018 to 0.274 ± 0.034 g, was not significantly different among the groups ($P > 0.05$). The post-treatment values of 5 min bleeding weight in the MEUS1 (0.346 ± 0.345 g) and MEUS2 (2.232 ± 2.256 g) subgroups were significantly lower compared to those in the US (5.698 ± 1.938 g) and MB (5.688 ± 2.317 g) groups (all, $P < 0.05$). Comparison of those between the MEUS3 (3.078 ± 1.181 g) and the US and MB groups, there were not statistically significant (all, $P > 0.05$) (Figure 1(C)).

Blood perfusion analysis using CEUS

There were no significant differences in pretreatment contrast agent perfusion to any of the target regions among the experimental groups. The microbubbles rapidly perfused the target regions of the liver lobes and uniformly enhanced the target tissue, without any deficiency. In order to avoid the impact of microbubbles, CEUS examination was

Figure 1 Comparison of post-treatment hemostatic effect among the treatment groups. (A) Representative image of differences in post-treatment bleeding in the ultrasound without microbubbles (US; a), microbubbles without ultrasound (MB; b), microbubble-enhanced ultrasound (MEUS)1 (c), MEUS2 (d), and MEUS3 (e) groups. (B) Comparison of pre- and post-treatment visual bleeding scores among the groups. (C) Comparison of pre- and post-treatment bleeding weight at 10 s and 5 min among the groups. #Significant difference between pre- and post-treatment values; *significant difference in comparison to the US and MB groups. * P and # $P < 0.05$, ** P and ## $P < 0.01$, *** P and ### $P < 0.001$. (A color version of this figure is available in the online journal.)

performed only after treatment in the US group. There were no significant differences in liver perfusion between 0 and 10 min post-treatment between the US and MB groups. In the MEUS subgroups, liver tissues revealed areas with significant filling defects due to poor perfusion. These defects were most obvious in the MEUS1 subgroup. Restoration of blood perfusion in the target areas began 10 min post-treatment, and the levels of blood perfusion were nearly restored to the normal pretreatment levels (Figure 2).

The PI ratio-time curve demonstrated the level of blood perfusion with time. The differences in pretreatment PI ratio among the groups were not significant ($P > 0.05$). The PI ratios of the MEUS1-3 subgroups decreased significantly immediately after treatment, from 1.00 ± 0.027 (calculated from the pretreatment data of all groups) to 0.64 ± 0.085 , 0.73 ± 0.045 , and 0.84 ± 0.034 , respectively. These values were remarkably lower compared to those of the US (1.00 ± 0.005) and MB (0.99 ± 0.005) groups (all, $P < 0.05$) at 0 min post-treatment. At 10 min post-treatment, the PI ratios of all of the MEUS subgroups recovered

to values close to 1 and remained relatively stable. Nevertheless, their PI ratios were consistently lower than those of the US and MB groups, which always fluctuated around 1 throughout the whole process (Figure 3).

Histological examination

Histological findings revealed no significant evidence of histological damage in the liver tissue samples of the US (Figure 4(a) to (c)) and MB (Figure 4(d) to (f)) groups. In the two control groups, HE-stained slices exhibited normal hepatic architecture, including intact hepatic lobule (Figure 4(a) and (d)), central vein (Figure 4(e)), portal area (Figure 4(b)), and hepatic plate (Figure 4(c) and (f)). These specific hepatic structures were visible quite obviously. Furthermore, the hepatic sinusoids between hepatic plates were clear (Figure 4(c) and (f)).

On the other hand, liver tissue samples of the MEUS1 subgroup exhibited several histological changes, which might facilitate hemostasis. First, the hepatocytes were swollen and cloudy because of severe edema (Figure 4(g))

Figure 2 Contrast-enhanced ultrasound images demonstrating the pre- and post-treatment blood perfusion levels in all groups. In contrast to the ultrasound without microbubbles (US) and microbubbles without ultrasound (MB) groups, the microbubble-enhanced ultrasound (MEUS) subgroups exhibit obvious filling defects (arrow) at the target treatment areas at 0 min post-treatment

Figure 3 Comparison of pre- and post-treatment peak contrast intensity (PI) ratio-time curves among the groups. In contrast to the ultrasound without microbubbles (US) and microbubbles without ultrasound (MB) groups, the microbubble-enhanced ultrasound (MEUS)1–3 subgroups exhibited a significant decrease in PI ratios immediately after treatment. *Significant difference in comparison to the US group; #significant difference in comparison to the MB group. * P and # $P < 0.05$, ** P and ## $P < 0.01$, *** P and ### $P < 0.001$

and exhibited a typical pathological ballooning degeneration at high magnification (Figure 4(i)). Second, the swollen hepatocytes were crowded together, rendering the calibers of the interlobular (Figure 4(h)) and central (Figure 4(j)) veins obviously lower in comparison with those of the US and MB groups. Similarly, the swollen hepatocytes compressed and shrank the sinusoids, resulting in their near-complete disappearance (Figure 4(i)). Similar to the above pathological variations, the incisional periphery was also encircled by the swelling hepatocytes, such that the sinusoids had nearly evanesced (Figure 4(l)). Notably, there were clear boundaries between the affected and normal hepatic tissues at the periphery of the incision (Figure 4(l), black dotted line). Third, because of the slow blood flow resulting from the narrowing of blood vessels, blood coagulation could be observed in the interlobular (Figure 4(h)) and central (Figure 4(j)) veins. Fourth, obvious hepatocyte edema was noted at the periphery of the large vessels, which was visible as a light zone upon HE staining (Figure 4(k)).

Hepatic lobules receive dual blood supply from interlobular hepatic veins as well as arteries, and the blood flows through the hepatic sinusoids and empties into the central vein. Finally, the central veins coalesce into the hepatic vein and drain into the inferior vena cava. After treatment, the swollen hepatocytes significantly compressed the interlobular hepatic veins and arteries in the portal area, as well as the central vein, causing these vessels to become thinner and even blocked. Interlobular hepatic arteries are so thin in normal tissues, that they are nearly invisible in affected tissues constricted by swollen hepatocytes. The calibers of the interlobular hepatic ($24.42 \pm 17.54 \mu\text{m}$ versus $79.94 \pm 25.29 \mu\text{m}$; $P < 0.05$; Figure 5(a)) and central veins ($61.35 \pm 21.46 \mu\text{m}$ versus $138.10 \pm 21.27 \mu\text{m}$; $P < 0.05$; Figure 5(b)) in the MEUS1 subgroup were significantly lower compared to those in the US group.

Similarly, the swollen hepatocytes occupied the space of the hepatic sinusoids, resulting in the near-complete

disappearance of the latter. The average percentages of the hepatic plate versus sinusoids in the US and MB groups were 66.41% versus 33.59% and 66.04% versus 33.96%, respectively, while the corresponding values in the MEUS1 subgroup were 97.38% versus 2.62% (Figure 5(c)). The area ratio of hepatic plate/sinus in the US group (1.982 ± 0.139) and the MB group (1.969 ± 0.323) was significantly lower compared to that in the MEUS1 subgroup (48.75 ± 24.28 ; $P < 0.05$; Figure 5(d)).

Transmission electron microscopy

In the US group, transmission electron microscopy findings revealed no ultrastructural damage in the hepatocytes or associated vessels in the portal region. The cells exhibited nuclei with dense chromatin, well-structured cytoplasm without vacuolation, undilated rough endoplasmic reticulum, and mitochondria with well-conserved crests. Glycogen granules were evenly dispersed in the cytoplasm (Figure 6(a)). However, in the MEUS group, the hepatocytes exhibited balloon degeneration and damaged cellular organelles after treatment (Figure 6(b)). In the US group, endothelial cells of the interlobular hepatic arteries (Figure 6(c)) and veins (Figure 6(d)) and the bile canaliculi (Figure 6(e)) exhibited no evident post-treatment damage. In contrast, in the MEUS group, endothelial cells of the interlobular hepatic arteries (Figure 6(f)) and veins (Figure 6(g)) as well as the bile canaliculi (Figure 6(h)) exhibited structural damages, and there was an obvious obstruction of red blood cells in the interlobular hepatic veins (Figure 6(g)).

Serum enzyme analysis

The serum AST, ALT, AST/ALT, and ALP levels were stable until 48 h after MEUS treatment and exhibited no significant differences in their pretreatment and 48 h post-treatment values ($P > 0.05$; Figure 7).

Discussion

Recent studies have shown that microbubble contrast agents, which can improve diagnostic sonographic images by enhancing backscattered signals because of their acoustic property, play an important role in enhancing the therapeutic efficiency of MEUS for hemorrhage resulting from liver trauma. HIFU was initially investigated for its efficacy in the control of liver hemorrhage.¹¹ Hemorrhage control relies on both the thermal and mechanical effects of HIFU, which can be improved by the infusion of microbubble contrast agents.¹⁴ However, the thermal effects of HIFU can result in tissue temperatures exceeding 70°C within seconds of application, which could cause tissue burns upon encountering significant acoustic impedance. In addition, the extracorporeal application of HIFU requires precise image-guided navigation and accurate focusing and is a time-consuming procedure for the treatment of liver hemorrhage. Moreover, because of its precise focus, HIFU is considered suitable only for the treatment of point bleeding or limited areas of hemorrhage and not widespread areas.

Figure 4 Histological findings of hematoxylin and eosin (HE) staining demonstrating the hemostatic mechanism underlying microbubble-enhanced ultrasound (MEUS) treatment. Microscopic evaluation of liver tissue samples of the ultrasound without microbubbles (US; a–c) and microbubbles without ultrasound (MB; d–f) groups at different degrees of magnification (scale bars: a, d, 200 μm ; b, e, 50 μm ; c, f, 20 μm) revealed normal intact hepatic lobules (a, d); portal area including interlobular artery, vein, and bile duct (b); central vein (e); and hepatic plate and sinusoids (c, f). In contrast, microscopic evaluation of tissue samples of the MEUS group (g–i) at different levels of magnification (scale bars: g, i, 200 μm ; h, j, k, 50 μm ; i, 20 μm) revealed various tissue changes. Blood coagulation can be observed in the interlobular (h) and central (j) veins. The swollen hepatocytes crowded together effectively, rendering the interlobular (h) and central (j) veins obviously smaller in diameter than those in the US and MB groups. Similarly, the sinusoids almost vanished because of compression due to the swollen hepatocytes (i). Obvious hepatocyte edema was observed at the periphery of large vessels, which was visible as a light zone upon HE staining (k). There were clear boundaries between the affected and normal hepatic tissues at the periphery of the incision (l)

Furthermore, because of their large size, HIFU systems cannot be used in on-site first-aid situations.

A large body of evidence from *in vitro* studies indicates that inertial acoustic cavitation is associated with the production of the ultrasound-induced mechanical bioeffects. Because of the lack of endogenous gas bodies, blood and tissues are resistant to the effects of inertial cavitation unless exposed to extremely high acoustic intensities and negative pressures, such as those produced by HIFU. Microbubbles, which are regarded as exogenous effective gas body nuclei, decrease the cavitation threshold and improve the cavitation effects in low-intensity MEUS. Moreover, *in vivo* studies have demonstrated that low-intensity MEUS can produce vascular effects such as capillary rupture and

endothelial cell damage¹⁷ as well as increased capillary permeability²⁵ due to its inertial cavitation and mechanical bioeffects. An *in vivo* experiment reported that the vascular effects induced by the application of pulsed, focused MEUS at an acoustic intensity of 0.89 W/cm² can temporarily shut down blood perfusion of the targeted liver.¹⁹ Recently, studies have reported achieving hepatic hemostatic effects with different types of microbubbles under different MEUS exposure conditions such as duration and acoustic power. Zhao *et al.* demonstrated the potential and efficacy of intraoperative acoustic hemostasis using MEUS at a very low acoustic intensity (0.4 W/cm²) in the treatment of liver hemorrhage in a rabbit liver trauma model.²² Additional *in vitro* studies have demonstrated that the application of

Figure 5 Post-treatment variations in anatomical parameters in the central vein, hepatic plate, and hepatic sinusoids. (a, b) The calibers of the interlobular and central hepatic veins in the microbubble-enhanced ultrasound (MEUS1) group were significantly lower compared to those in the ultrasound without microbubbles (US) and microbubble without ultrasound (MB) groups after treatment. (c) The area percentages of the hepatic plate and sinus in the MEUS1 group were obviously different from those in the US and MB groups. (d) The post-treatment area ratio of the hepatic plate divided by hepatic sinusoids in the US group (1.98 ± 0.069) and the MB group (1.969 ± 0.323) was significantly lower compared to that of the MEUS1 subgroup (48.75 ± 9.912 ; all $P < 0.05$). *Significant difference in comparison to the US group; #significant difference in comparison to the MB group. * P and # $P < 0.05$, ** P and ## $P < 0.01$, *** P and ### $P < 0.001$.

pulsed MEUS causes hepatocyte swelling, sinusoid thinning, periportal connective tissue hemorrhage, and necrosis.²⁶

In the present study, we achieved hemostasis in a liver trauma model using non-focused MEUS at a very low acoustic intensity of 0.11 W/cm^2 . Hemostatic efficacy was evaluated based on both bleeding visual scores and 5 min bleeding weight. Our results indicated that MEUS treatment resulted in the complete stoppage of liver hemorrhage. Moreover, in contrast to HIFU, there was no evidence of tissue burns in the target areas upon MEUS application in the present study.^{11,13,15,27}

We also investigated the hemostatic effect of ultrasound at different power levels in the treatment of hepatic trauma. Our results indicated that the hemostatic effect did not increase with the increase in power, but rather decreased correspondingly. The radius of microbubbles increases with the increase in acoustic pressure. As the pressure continues

to increase, the radius of microbubbles increases to a certain extent in the positive pressure phase, after which, the microbubbles cannot be compressed to a smaller radius in the negative pressure phase, which leads to a decrease in the cavitation effect.²⁸

Our method resulted in the temporary occlusion of hepatic blood flow to the liver. At 10 min post-treatment, blood perfusion was close to the normal level; it recovered to the normal level within 1 h post-treatment. During the CEUS procedure, we found no signs of bleeding in the target region, indicating that effective blood reperfusion provided sufficient and necessary conditions for the recovery of the liver. However, it should be noted that, in previous studies, the time span of blood flow perfusion was relatively large, and the time points of post-treatment reperfusion were not intensively observed.²² By determining the PI ratios of treated areas to normal regions, we were able to clearly establish that blood flow in the treated regions was worse than that in the normal regions. These results indicate that the application of MEUS resulted in certain histological variations in the target areas.

Pathological findings revealed that the cavitation effect induced by MEUS led to severe liver cell edema and balloon degeneration. Tissue sections stained with HE exhibited large whitened or lightly stained regions due to the balloon degeneration of hepatocytes. Swollen hepatocytes compressed the sinusoids, central vein, and interlobular vessels of the portal area, resulting in the narrowing or even disappearance of these structures. The area ratio of hepatic plate/sinus in the US group was significantly lower compared to that in the MEUS1 subgroup. The calibers of the interlobular and central veins in the MEUS1 subgroup were significantly lower compared to those in the US group. Additionally, the mechanical effects of MEUS might have caused platelet activation²⁹ as well as the release of tissue coagulation factors,¹⁵ leading to faster blood coagulation. Interestingly, we also noted obvious blood coagulation in the interlobular hepatic arteries and central veins. These histological changes efficiently decreased blood flow to the damaged tissue. We hypothesize that blood flow to target tissues cannot be restored to normal levels, and this might be related to the acoustic cavitation-induced changes that influence the microcirculation.

In the present study, electron microscopy findings revealed no damages in the liver tissue samples of the two control groups, which were treated with either ultrasound exposure without microbubble infusion (US) or microbubble infusion without ultrasound exposure (MB). The appearance of target tissues treated with both microbubble infusion and ultrasound exposure (MEUS subgroups) was significantly different compared to those of the control groups. Electron microscopic findings revealed ultrastructural alterations of the organelles and blood vessels in rabbit liver tissues in the MEUS subgroups. Moreover, endothelial cells of the interlobular hepatic arteries and veins exhibited damages of relatively high severity in the microbubble-treated groups, which corresponds to the findings of previous studies.^{30,31} However, electron microscopy findings revealed no necrosis in target tissues treated with MEUS. It is noteworthy that, while

Figure 6 Comparison of ultrastructural characteristics of rabbit hepatocytes and affiliated vessels with/without microbubble-enhanced ultrasound (MEUS) treatment. Transmission electron microscopy images demonstrated the polygonal morphology of normal hepatocytes and sinusoids in the ultrasound without microbubbles (US) group. The rough endoplasmic reticulum, mitochondria, scattered glycogen particles, and sinusoidal endothelial cells (SECs) were normal and exhibited no damage (a). The hepatocytes were swollen and had become round in shape, and the cellular organelles exhibited severe structural damage after treatment by MEUS (b). In the US group, neither the endothelial cells of the interlobular hepatic arteries (c) or veins (d) nor the bile canaliculi (e) exhibited evident post-treatment damages. In contrast, the endothelial cells of the interlobular hepatic arteries (f) and veins (g) as well as the bile canaliculi (h) exhibited damages after treatment by MEUS; in addition, the interlobular hepatic veins exhibited obvious obstruction of red blood cells (g). Scale bars: a–c, 2 μm ; d–h, 5 μm

Figure 7 Comparison of serological parameters before and until 48 h post-treatment in the microbubble-enhanced ultrasound (MEUS) group. The concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT, and alkaline phosphatase (ALP) were evaluated

Zhao *et al.*²² and Feng *et al.*²⁶ performed morphological analysis by light microscopy, we performed TEM examination, which allowed us to investigate treatment-induced damages at the ultrastructural level.

Finally, we investigated the influence of MEUS on hepatic function by assaying the concentrations of serum metabolic enzymes such as AST, ALT, AST/ALT, ALP, and GGT. The differences in pre- and post-treatment concentrations were found to be insignificant, which indicated the safety of MEUS as a therapeutic technique for the treatment of liver trauma.

In summary, we believe that, owing to the induction of cavitation effects, microbubble-enhanced non-focused ultrasound might represent a potentially new technique for achieving rapid liver hemostasis by inducing certain histological changes to decrease blood flow to damaged tissues in cases of liver trauma. In addition, application of this technique enables the restoration of blood reperfusion within a short duration, thereby avoiding any hindrance to the blood supply to healthy distal liver tissues. However, its long-term effects require further evaluation. Nevertheless, we believe that microbubble-enhanced non-focused ultrasound will prove to be a valuable technique for clinical application in the future. In particular, it will play an important role in achieving rapid on-site hemostasis in cases of emergency.

Authors' contributions: DWZ and MT contributed equally to this paper. DWZ, MT, and TL reviewed the literature and participated in study design; DWZ, MT, JZY, PD, and JB conducted the experiments; DWZ, MT, XJZ, and TL

performed data analysis; DWZ, MT, and XJZ prepared the figures; DWZ and MT wrote the manuscript; and TL edited the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

- Prichayudh S, Sirinawin C, Sriussadaporn S, Pak-art R, Kritayakirana K, Samorn P, Sriussadaporn S. Management of liver injuries: predictors for the need of operation and damage control surgery. *Injury* 2014;**45**:1373–7
- Slotta JE, Justinger C, Kollmar O, Kollmar C, Schäfer T, Schilling MK. Liver injury following blunt abdominal trauma: a new mechanism-driven classification. *Surg Today* 2014;**44**:241–6
- Gourgiotis S, Vougas V, Germanos S, Dimopoulos N, Bolanis I, Drakopoulos S, Alfaras P, Baratsis S. Operative and nonoperative management of blunt hepatic trauma in adults: a single-center report. *J Hepatobiliary Pancreat Surg* 2007;**14**:387–91
- van der Wilden GM, Velmahos GC, Emhoff T, Brancato S, Adams C, Georgakis G, Jacobs L, Gross R, Agarwal S, Burke P, Maung AA, Johnson DC, Winchell R, Gates J, Cholewczynski W, Rosenblatt M, Chang Y. Successful nonoperative management of the most severe blunt liver injuries: a multicenter study of the research consortium of new england centers for trauma. *Arch Surg* 2012;**147**:423–8
- Chien L-C, Lo S-S, Yeh S-Y. Incidence of liver trauma and relative risk factors for mortality: a population-based study. *J Chin Med Assoc* 2013;**76**:576–82
- Lin B-C, Fang J-F, Chen R-J, Wong Y-C, Hsu Y-P. Surgical management and outcome of blunt major liver injuries: experience of damage control laparotomy with perihepatic packing in one trauma centre. *Injury* 2014;**45**:122–7
- Felekouras E, Kontos M, Pissanou T, Drakos E, Pikoulis E, Papalois A, Bramis J, Diamantis T, Georgopoulos S, Nikolaos N, Sigala F, Papalambros E, Pappas P, Bastounis E. Radio-frequency tissue ablation in liver trauma: an experimental study. *Am Surg* 2004;**70**:989–93
- Maroulis I, Spyropoulos C, Kalogeropoulou C, Karavias D, Maroulis I, Karavias D. Use of radiofrequency ablation for controlling liver hemorrhage in the emergency setting; report of two cases and review of the literature. *Ulus Trauma Acil Cerrahi Derg* 2013;**19**:167–72
- Takasu A, Norio H, Sakamoto T, Okada Y. Surgical treatment of liver injury with microwave tissue coagulation: an experimental study. *J Trauma* 2004;**56**:984–90
- Tang J, Wang Y, Mei X, An L, Luo Y, Lin Q. Contrast-enhanced ultrasound-guided microwave tissue coagulation therapy for hepatic trauma: an experimental study. *J Trauma* 2008;**64**:1079–84
- Vaezy S, Martin R, Schmiedl U, Caps M, Taylor S, Beach K, Carter S, Kaczowski P, Keilman G, Helton S, Chandler W, Mourad P, Rice M, Roy R, Crum L. Liver hemostasis using high-intensity focused ultrasound. *Ultrasound Med Biol* 1997;**23**:1413–20
- Vaezy S, Martin R, Mourad P, Crum L. Hemostasis using high intensity focused ultrasound. *Eur J Ultrasound* 1999;**9**:79–87
- Vaezy S, Noble ML, Keshavarzi A, Paun M, Prokop AF, Cornejo C, Sharar S, Chi EY, Crum LA, Martin RW. Liver hemostasis with high-intensity ultrasound: repair and healing. *J Ultrasound Med* 2004;**23**:217–25
- Zderic V, Brayman AA, Sharar SR, Crum LA, Vaezy S. Microbubble-enhanced hemorrhage control using high intensity focused ultrasound. *Ultrasonics* 2006;**45**:113–20
- Burgess S, Zderic V, Vaezy S. Image-guided acoustic hemostasis for hemorrhage in the posterior liver. *Ultrasound Med Biol* 2007;**33**:113–9
- Dubinsky TJ, Cuevas C, Dighe MK, Kolokythas O, Hwang JH. High-intensity focused ultrasound: current potential and oncologic applications. *Am J Roentgenol* 2008;**190**:191–9
- Hwang JH, Brayman AA, Reidy MA, Matula TJ, Kimmey MB, Crum LA. Vascular effects induced by combined 1-MHz ultrasound and microbubble contrast agent treatments in vivo. *Ultrasound Med Biol* 2005;**31**:553–64
- Hwang JH. Correlation between inertial cavitation dose and endothelial cell damage in vivo. *Ultrasound Med Biol* 2006;**32**:1611–9
- Gao Y, Gao S, Zhao B, Zhao Y, Hua X, Tan K, Liu Z. Vascular effects of microbubble-enhanced, pulsed, focused ultrasound on liver blood perfusion. *Ultrasound Med Biol* 2012;**38**:91–8
- Yang D, Tan K-B, Gao Y-H, Liu H, Yang W-X. Effects of diagnostic ultrasound-targeted microbubble destruction on permeability of normal liver in rats. *Ultrasonics* 2012;**52**:1065–71
- Wang G, Zhuo Z, Xia H, Zhang Y, He Y, Tan W, Gao Y. Investigation into the impact of diagnostic ultrasound with microbubbles on the capillary permeability of rat hepatomas. *Ultrasound Med Biol* 2013;**39**:628–37
- Zhao X, Li L, Zhao H, Li T, Wu S, Zhong Y, Zhao Y, Liu Z. Liver haemostasis using microbubble-enhanced ultrasound at a low acoustic intensity. *Eur Radiol* 2012;**22**:379–86
- Regulations on the management of experimental animals. China: Ministry of Science and Technology (MOST), 1988
- Guideline on humane treatment of laboratory animals. China: Ministry of Science and Technology (MOST), 2006
- Bekeredjian R, Kroll RD, Fein E, Tinkov S, Coester C, Winter G, Katus HA, Kulaksiz H. Ultrasound targeted microbubble destruction increases capillary permeability in hepatomas. *Ultrasound Med Biol* 2007;**33**:1592–8
- Feng G, Liu J, Zhao X, Wei J, Ou W, Xiao S, Hu Z, Wei H, Liu Z. Hemostatic effects of microbubble-enhanced low-intensity ultrasound in a liver avulsion injury model. *PLoS One* 2014;**9**:e95589
- Zderic V, Keshavarzi A, Noble ML, Paun M, Sharar SR, Crum LA, Martin RW, Vaezy S. Hemorrhage control in arteries using high-intensity focused ultrasound: a survival study. *Ultrasonics* 2006;**44**:46–53
- Ashokkumar M. The characterization of acoustic cavitation bubbles – an overview. *Ultrason Sonochem* 2011;**18**:864–72
- Poliachik SL, Chandler WL, Mourad PD, Ollos RJ, Crum LA. Activation, aggregation and adhesion of platelets exposed to high-intensity focused ultrasound. *Ultrasound Med Biol* 2001;**27**:1567–76
- Ay T, Havaux X, Van Camp G, Campanelli B, Gisellu G, Pasquet A, Denef J-F, Melin JA, Vanoverschelde J-LJ. Destruction of contrast microbubbles by ultrasound: effects on myocardial function, coronary perfusion pressure, and microvascular integrity. *Circulation* 2001;**104**:461–6
- Caruso G, Valentino B, Salvaggio G, Lipari EF, Lipari D, Brancatelli G, Lagalla R, Cardinale AE. Ultrastructural biologic effects of sonography with pulse inversion and microbubble contrast in rabbit liver. *J Clin Ultrasound* 2005;**33**:106–11

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