

Original Article

Effects of microbubble-enhanced ultrasound combined with prothrombin on microwave ablation in rabbit VX2 liver tumor

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Abstract: Objective: This study aimed to investigate effects of microbubble-enhanced ultrasound (MEUS) combined with prothrombin on microwave ablation (MWA) on VX2 liver tumors in a rabbit model. Methods: 80 rabbits with VX2 liver tumors were randomly divided into Group A (sham + NS), Group B (sham + NS + P), Group C (MEUS), and Group D (PMEUS). After treatment, targeted liver tumors were ablated with MWA. On 0, 3, 7 and 14 d, volume of coagulated area was measured. Tissues in ablated area, transition area and surrounding area were collected. Results: On 0, 3, 7 and 14 d, coagulated volume in Group D was larger than remaining three groups ($P<0.05$). On 7 d and 14 d, tumor volume in Group D was smaller than remaining three groups ($P<0.05$). Fibrotic band in Group D was wider than in remaining three groups ($P<0.05$). Cellular ultrastructure injury in ablated area on 0 d and mitochondrial injury in transition area on 7 d were more severe in Group D than in remaining three groups. On 0, 3, 7 and 14 d, proliferative cellular nuclear antigen-positive index in transition area in Group C and Group D was lower than Group A and Group B ($P<0.05$). On 0, 3 and 7 d, apoptosis index in transition area in Group D was higher than remaining three groups ($P<0.05$). Conclusions: MEUS combined with prothrombin on MWA can significantly expand ablation volume, enhance the necrosis of ablated tissues, inhibit tumor growth/metastasis and improve therapeutic effect of MWA on rabbit VX2 liver tumors.

Keywords: Microbubble, microwave ablation, prothrombin, VX2 liver tumor, rabbit

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer, with about 500,000 to 1,000,000 new cases reported each year worldwide [1, 2]. Unfortunately, only 20% of HCC patients are amenable to curative therapy (liver transplantation or surgical resection). Several image-guided ablation techniques such as radiofrequency ablation (RFA), microwave ablation (MWA), laser ablation and hepatic cryotherapy, play a key role in the management of HCC [3]. Thermal ablation refers to that localized heating or freezing is used to enable the *in situ* destruction of malignant tumors, without damaging the surrounding parenchyma [4, 5]. The minimally invasive, image-guided ablation tech-

niques have reduced cost and decreased morbidity compared with standard surgical resection, and are suitable for patients with surgically unresectable HCC [6]. However, ablative techniques may potentially cause damage to important vessels and have inadequate ablation of perivascular tissues due to the so-called "heat sink" effect [7]. Therefore, image-guided ablation is unable to consistently produce a necrotic zone large enough to encompass the hepatic tumor with an appropriate margin.

Studies have shown that vascular occlusion via Pringle maneuver combined with RFA can increase the volume of necrotic tissues and create a more spherical lesion [8-10]. However, the above methods for occluding hepatic blood are

mostly invasive. After combined usage of therapeutic arterial embolization (TAE) [11, 12], the tumor can still be supplied by surrounding portal veins and other collateral circulation although the hepatic arteries are embolized via a catheter, resulting in incomplete ablation.

Acoustic cavitation is one of the major physical effects of ultrasound (US). US contrast agents can nucleate inertial cavitation and increase ultrasonic absorption for the noninvasive ultrasound surgery [13]. US agent-induced endothelial damage can be inherently thrombogenic, or aid the sclerotherapeutic thrombogenesis of thrombogenic drugs at subtherapeutic doses [14]. Since newly generated vessels are fragile, leaky, dysfunctional and uniquely sensitive to low-intensity US, they are often the targets in US therapy [15, 16]. Wood et al [17] found the combined usage of microbubbles and low-intensity US can disrupt tumor vasculature of murine melanoma, dilate capillaries and cause hemorrhage histologically.

However, the mechanical disruption of the vasculature secondary to acoustic cavitation is transient and especially it has little influence on the vessels with few blood flow. Some studies have shown that combined use of prothrombin and US can enhance and prolong the vascular effects of MEUS [18, 19]. In the present study, our results showed MEUS combining with prothrombin produced a larger thrombotic area and improved the therapeutic effect of MWA on the rabbit liver tumor [20]. We hypothesized that the combination of MEUS and prothrombin could improve the therapeutic effects of MWA on the rabbit VX2 liver tumor due to the additional circulation blockage secondary to prothrombin induced intravascular thrombosis. In this study, histopathology, transmission electron microscopy, and immunohistochemistry were employed to assess the therapeutic effects of MWA combined with PMEUS on the VX2 liver tumor in a rabbit model.

Materials and methods

Animals

The whole procedures in this study were approved by the Animal Care and Use Committee of the university (No: XJYYLL-2013016). A total of 80 healthy New Zealand White rabbits weighing 2.0-2.5 kg were purchased from the

Laboratory Animal Center of the Fourth Military Medical University. One tumor-bearing rabbit with VX2 tumor in the thigh was purchased from the Second People's Hospital of Lianyungang in Jiangsu, China.

Implantation of VX2 liver tumor

The VX2 tumor in the thigh was collected and cut into blocks (about 1 mm³ in size) under a sterile condition. Then, healthy animals were anesthetized by ear vein injection with 2% pentobarbital at 1.5 ml/kg. After sterilization, laparotomy was performed via a 2-cm midline incision in the epigastric region, and the left liver was exposed. The hepatic parenchyma was lifted and a lesion of about 2 mm in width, 1.5 cm in length and 1 cm in depth was made from the surface. Two VX2 tumor blocks were transplanted to the bottom of the lesion, followed by filling with 1 mm³ gelatin. After light compression on the implantation site to prevent bleeding and shedding of tumor blocks, the liver was placed back to the abdominal cavity, followed by wound closure.

Therapeutic ultrasound device

The therapeutic ultrasound (TUS) transducer comprises an air-backed, spherically concave disk (25 mm in diameter, Kunshan Risheng Electronics, Kunshan, China) with a curvature (160 mm in radius). A wave generator and a specially designed power amplifier (250-350 V peak-to-peak, Mianyang Sonic Electronics, Mianyang, China) drive the transducer. The transducer has an aluminum shell, the front aperture (28 mm in diameter) of which is covered with a polyimide membrane. To provide acoustic coupling, the 10-mm-long front chamber of the disk is filled with degassed water. The geometrical focus of the transducer is exactly 150 mm from the tip, where the US beam is 28 mm in diameter. A needle hydrophone (TNU001A, NTR, Seattle, WA, USA) adjusted by a precision 3-D motion stage is set up to measure the acoustic output of the transducer at a range of 1 cm outside the tip. The transducer is operated at 831 kHz with a 400-cycle pulse length and a pulse repetition frequency of 9 Hz. The acoustic pressure (peak negative pressure) output is 4.6 MPa. The transducer works in an intermittent mode with 6 s on and 6 s off. The actual working duty cycle is approximately 0.22%.

Reagent

Hemocoagulase Atrox for Injection (Nuokang Bio-pharmaceutical Inc., Shandong, China) purified by Bothrops atrox, was in the form of white powder. Other reagents included mannitol, gelatin (hydrolysis) and calcium chloride. The hemocoagulase was used for hemostasis and has no thrombosis risk because it cannot reduce the platelet count.

Grouping

14 days after implantation of VX2 tumors, the animals were randomly assigned to four groups (n=20 per group): Group A, animals received sham US plus normal saline (sham + NS); Group B, animals received sham US, normal saline and prothrombin (sham + NS + P); Group C, animals received therapeutic US plus microbubbles (MEUS); Group D, animals received therapeutic US, microbubbles plus prothrombin (PMEUS).

Therapeutic ultrasonography

Rabbits in each group were fasted for 24 h prior to treatment. After anesthesia, animals underwent laparotomy and the liver tumor was exposed. The left liver lobe was pulled out slightly and then fixed *in situ*. The TUS transducer with gel was placed on the liver tumor. The transducer covered the tumor approximately 28 mm in diameter. Sham TUS exposure lasted 10 min, normal saline (0.5 mL/min) was intravenously injected simultaneously in Group A and Group B, and then 2 U prothrombin was injected into the liver tumor in Group B. The TUS treatment lasted 10 min, the microbubble SonoVue (Bracco, Milan, Italy) saline solution (0.5 mL/min) was intravenously injected simultaneously in Group C and Group D, and then 2 U prothrombin was injected into the liver tumor in Group D. The dose of SonoVue for cavitation was 0.2 mL/kg (diluted in 5-mL saline).

Therapeutic MWA

After above treatment, the treated liver tumors were ablated with microwave therapeutic system equipped with a cooled-shaft antenna (3 mm in diameter) (microwave frequency, 2,450 MHz; ECO-100C, Nanjing ECO Microwave System Co. Nanjing, China). The microwave antenna was inserted into the center of the tumor with US guidance, and the tumor was

ablated at 10 W for 3 min. After ablation, the wound was closed.

Measurement of ablation volume with ultrasound

CEUS imaging was performed on 5 animals in each group at pre-designed time points: 0, 3, 7 and 14 days after MWA. The maximum length (L, millimeters), width (W, millimeters), and depth (D, millimeters) of ablated area were measured in each animal, and the volume (V, cubic millimeters) was calculated according to the following formula [21]: $V = \pi/6 L \times W \times D$.

Pathologic examination

At 0, 3, 7 and 14 d after ablation, animals were sacrificed after CEUS imaging, and the liver was collected for the measurement of tumor volume (cm³) as above mentioned. The liver tissues were divided into ablation area, transition area (within 3 mm away from the ablation area) and surrounding area (at least 3 mm away from the ablation area). The liver tissues were divided into two parts: one was processed for HE staining; the other was cut into blocks (1 mm³) for the observation of cellular ultrastructure by electron microscopy (JEM-1230 electron microscope, JEOL Ltd, Tokyo, Japan).

Immunohistochemistry

Liver tissues were collected, fixed in paraformaldehyde, embedded in paraffin, and sectioned. After treatment with 3% hydrogen peroxide for 5 min, sections were rinsed with PBS thrice and then subjected to antigen retrieval in citric acid for 15 min. Following rinsing in PBS, sections were incubated with primary antibody (Proliferative cellular nuclear antigen, PCNA; 1:100; Cell Signal Technology) at 37°C for 1 h and then with secondary antibody at 37°C for 1 h. After washing in PBS thrice, sections were subjected to visualization with 3,3-diaminobenzidine (DAB). Counterstaining was done with hematoxylin, followed by mounting with neutral gum. In the negative control, the primary antibody was replaced with PBS. Then, sections were observed under a light microscope, and cells with brown nucleus were positive for PCNA. Five sections were used for observed in each group and 5 fields were selected randomly at a magnification of 400 ×. The number of positive cells and number of total cells were determined, and then the proportion of PCNA

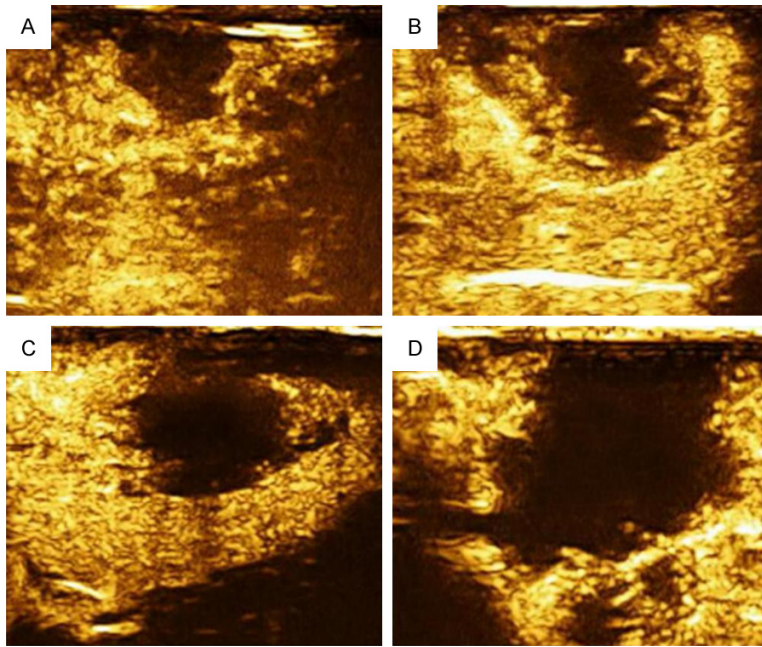


Figure 1. Ablated tissues on ultrasonography at 7 days after ablation. A. Group A; B. Group B; C. Group C; D. Group D.

positive cells was calculated: PCNA positive index (%): (number of PCNA positive cells/number of total cells) $\times$ 100%.

TUNEL staining

TUNEL staining was done with In Situ Cell Death Detection Kit (POD; Roche Diagnostics GmbH, Germany). In brief, after deparaffinization, sections were rinsed in PBS thrice and then incubated with Proteinase K (20 μ g/mL, diluted in Tris/HCl, PH 7.4~8.0) at room temperature for 30 min. After rising in PBS thrice, sections were treated with 50 μ L of TUNEL reaction mixture at 37°C for 60 min, followed by washing in PBS thrice. Then, sections were incubated with 50 μ L of POD reagent in a humidified environment at 37°C for 20 min. After rising thrice, visualization was done by addition of DAB (50 μ L). Counterstaining was performed with haematoxylin, followed by mounting. Cells with brown nucleus were positive for apoptosis. Five sections were used for observed in each group and 5 fields were selected randomly at a magnification of 400 $\times$. The number of apoptosis positive cells and number of total cells were determined, and then the proportion of apoptosis positive cells was calculated: apoptosis index (%): (number of apoptosis positive cells/number of total cells) $\times$ 100%.

Tumor metastasis and grading

At 7 and 14 days after ablation, the liver, kidney, abdominal cavity and pelvic cavity were examined by ultrasonography on 5 rabbits, especially lymph node metastasis. Metastasis was confirmed by anatomic examination. Once metastasis was identified, the metastasis was visually graded as follows: 0, the organ has normal structure and there is no newly generated mass; 1, there are 1-2 newly generated masses, and metastatic VX2 tumor is confirmed in the pelvic lymph nodes by pathological examination (1-2 cm in diameter); 2, there are 3-10 newly generated masses, and metastatic VX2 tumor is confirmed in the pelvic lymph nodes by pathological examination (2-5 cm in diameter); 3, there are more than 10 newly generated masses, and metastatic VX2 tumor is confirmed in the pelvic lymph nodes by pathological examination (>5 cm in diameter).

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Statistical analysis

Statistical analysis was performed with SPSS version 13.0 and data are expressed as mean $\pm$ standard deviation (SD). Data at different time points were compared with one-way analysis of variance (ANOVA), followed by paired comparison with Student's t test or Chi square test. A value of $P < 0.05$ was considered statistically significant.

Results

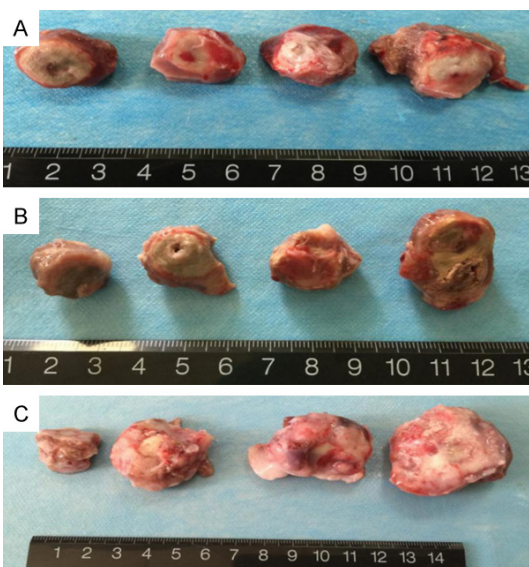
Volume of ablated tissues

At 0, 3, 7 and 14 d, the volume of ablated tissues in the Group D was significantly larger than in the Group A, Group B and Group C (all $P = 0.000$). The volume of ablated tissues in the Group C was markedly larger than in Group A and Group B (0 d: A vs C, $P = 0.006$; B vs C, $P = 0.007$); (3 d: A vs C, $P = 0.007$; B vs C, $P = 0.001$); (7 d: A vs C, $P = 0.007$; B vs C, $P = 0.001$); (14 d: A vs C, $P = 0.000$; B vs C, $P = 0.000$); there was

Table 1. Volume of ablated tissues at different time points in 4 groups (cm³)

Group	0 d	3 d	7 d	14 d	F	P
A	0.447±0.180 ^{*,#}	0.454±0.161 ^{*,#}	0.472±0.154 ^{*,#}	0.765±0.204 ^{*,#Δ}	3.825	0.031
B	0.467±0.168 ^{*,#}	0.290±0.075 ^{*,#}	0.304±0.080 ^{*,#}	0.652±0.151 ^{*,#Δ}	9.129	0.001
C	1.086±0.337 [*]	0.919±0.174 [*]	1.087±0.354 [*]	3.707±0.850 ^{*,Δ}	36.247	0.000
D	3.059±0.476 [#]	2.734±0.407 [#]	2.796±0.482 [#]	10.159±1.444 ^{#Δ}	98.367	0.000
F value	75.933	110.701	66.894	138.257		
P value	0.000	0.000	0.000	0.000		

Note: Data are expressed as mean ± SD. ^{*}P<0.05 vs Group D at the same time point; [#]P<0.05 vs Group C at the same time point; ^ΔP<0.05 vs other time points.

**Figure 2.** Macroscopic liver VX2 tumor at 3, 7 and 14 days in 4 groups. A. 3 d; B. 7 d; C. 14 d.

no significant difference between Group A and Group B (0 d: A vs B, $P=0.924$; 3 d: A vs B, $P=0.293$; 7 d: A vs B, $P=0.407$; 14 d: A vs B, $P=0.836$) (**Figure 1**). In addition, the volume of ablated tissues at 14 d was markedly larger than that at other time points (Group A: 14 d vs 0 d, $P=0.012$; 14 d vs 3 d, $P=0.013$; 14 d vs 7 d, $P=0.018$); (Group B: 14 d vs 0 d, $P=0.033$; 14 d vs 3 d, $P=0.000$; 14 d vs 7 d, $P=0.000$); (Group C: 14 d vs 0 d, $P=0.000$; 14 d vs 3 d, $P=0.000$; 14 d vs 7 d, $P=0.000$); (Group D: 14 d vs 0 d, $P=0.000$; 14 d vs 3 d, $P=0.000$; 14 d vs 7 d, $P=0.000$) (**Table 1**).

Tumor volume

At 0 and 3 d, there was no significant difference in the tumor volume among 4 groups (0 d: $P=0.894$; 3 d: $P=0.990$) (**Figure 2A**). At 7 and 14 d, the tumor volume in the Group D was sig-

nificantly smaller than in other groups (7 d: A vs D, $P=0.000$; B vs D, $P=0.000$; C vs D, $P=0.039$) (14 d: A vs D, $P=0.000$; B vs D, $P=0.000$, C vs D, $P=0.036$), and the tumor volume in the Group C was markedly smaller than in Group A and Group B (7 d: A vs C, $P=0.000$; B vs C, $P=0.000$) (14 d: A vs C, $P=0.009$, B vs C, $P=0.002$), but there was no significant difference between Group A and Group B (7 d: A vs B, $P=0.511$; A vs B, 14 d: $P=0.532$) (**Figure 2B, 2C**). In addition, the tumor volume in the Group D remained unchanged over time ($P=0.534$), but it increased significantly over time in remaining groups ($P=0.000$) (**Table 2**).

Microscopic findings

Fibrotic band at different time points: At 3 d, fibrotic band was found under a light microscope (**Figure 3A**), and a large amount of tumor cells and a small amount of hyperplastic fibrous tissues were found at a high magnification (**Figure 3B**). At 7 and 14 d, the fibrotic band was widened gradually (**Figure 3C**), and necrotic tumor cells were identified at a high magnification, and a large amount of hyperplastic fibrous tissues and a few red blood cells were noted in the ablated tissues (**Figure 3D**). In 4 groups, the fibrotic band was widened over time (**Table 3**).

Transmission electron microscopic findings

At 0 d: Chromatin condensation and margination, disappearance of nucleolus and absence of several organelles were found in the tumor cells of four groups. The cell structure was almost complete, the nuclear membrane and cell membrane were observable, and the in the cell junctions were identified in the Group A and Group B (**Figure 4A**). In the Group C and Group D, the tumor cells had unclear structure, the majority of tumor cells had disrupted nuclear

Table 2. Tumor volume at 0, 3, 7 and 14 d in 4 groups (cm³)

Group	0 d	3 d	7 d	14 d	F	P
A	0.984±0.204	1.134±0.223 ^Δ	6.859±1.126 ^{*,#Δ}	10.698±4.910 ^{*,#Δ}	17.530	0.000
B	1.056±0.350	1.112±0.301	6.475±1.068 ^{*,#Δ}	11.821±1.885 ^{*,#Δ}	107.813	0.000
C	0.975±0.201	1.074±0.460	2.447±0.793 [*]	5.477±1.703 ^{*,Δ}	23.317	0.000
D	1.088±0.310	1.074±0.347	1.166±0.468 [#]	1.445±0.593 [#]	0.757	0.534
F value	0.201	0.038	50.215	15.004		
P value	0.894	0.990	0.000	0.000		

Note: Data are expressed as mean ± SD. ^{*}P<0.05 vs Group D at the same time point; [#]P<0.05 vs Group C at the same time point; ^ΔP<0.05 vs other time points.

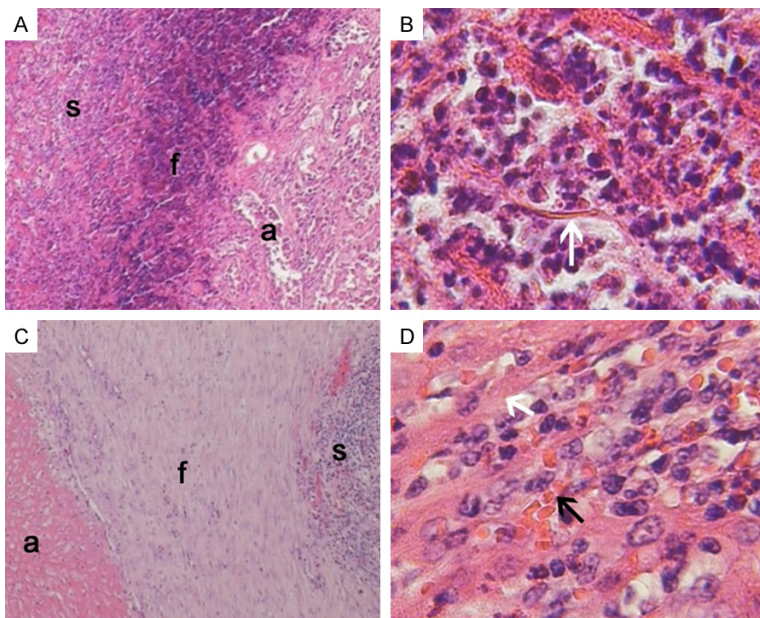


Figure 3. Microscopy of ablated tissues after HE staining. A. At 3 d, fibrotic band was found in the transition area; 40 ×; B. At 3 d, tumor cells with complete nucleus were found in the fibrous band; C. At 14 d, the fibrotic band was wide; 40 ×; D. At 14 d, tumor cells with nuclei were also noted in the fibrotic band at a high magnification, intranuclear cavitation, necrotic cells, a large amount of fibrous tissues (white arrow) and a few red blood cells (black arrow) were found; 400 ×. Note: a: ablation area; s: surrounding area; f: fibrotic band.

membrane and cell membrane, the intercellular space was widened, and especially the vacuoles of different sizes were found in the cytoplasm of tumor cells in the Group D (**Figure 4B**).

At 7 d and 14 d: Cell swelling and mild mitochondrial and endoplasmic reticulum swelling were observed in the transition area of the Group A and Group B (**Figure 4C**). In the Group C and Group D, the nuclear membrane space in the Group C and Group D was wide, the cell membrane was blurry and incomplete, the heterochromatin increased, and the mitochondria and endoplasmic reticulum were expanded.

These findings were more severe in the Group D, and mitochondrial cristae fractured in some cells (**Figure 4D**).

PCNA expression

There were a few PCNA positive cells at 0 d, and the number of PCNA positive cells peaked at 7 d in the transition area. At 3 and 14 d, the PCNA positive index of the transition area in the Group D was significantly lower than in other groups ($P<0.05$); the index of the transition area in the Group C was markedly lower than in Group A and Group B ($P<0.05$). At 0 and 7 d, the index of the transition area in the Group C and Group D was significantly lower than in other groups ($P<0.05$), but there was no marked difference between Group C and Group D ($P>0.05$). At 0, 3, 7 and 14 d, there was no significant

difference in the PCNA positive index between Group A and Group B ($P>0.05$) (**Figure 5**).

TUNEL staining

There were no evident apoptotic cells in the ablation area and surrounding area of four groups. In the transition area, the apoptotic cells were present at 0 d and peaked at 3 d.

At 0, 3 and 7 d, the number of apoptotic cells in the transition area of Group D was significantly larger than in other groups ($P<0.05$). At 14 d,

Table 3. Fibrotic band at 0, 3, 7 and 14 d in 4 groups (μm)

Group	0 d	3 d	7 d	14 d	F	P
A	0 ^Δ	196.6±17.1 ^{*Δ}	291.6±66.1 ^{*.#Δ}	379.2±96.5 ^{#Δ}	37.892	0.000
B	0 ^Δ	185.2±20.0 ^{*Δ}	312.6±61.1 ^{*.#}	369.8±113.9 ^{*.#}	31.401	0.000
C	0 ^Δ	209.0±39.6 ^Δ	551.2±47.2 ^Δ	740.0±136.4 ^{*Δ}	99.001	0.000
D	0 ^Δ	236.0±30.6 ^Δ	616.0±65.9 ^Δ	1054.2±150.1 ^{#Δ}	152.933	0.000
F value		2.977	37.057	33.910		
P value		0.063	0.000	0.000		

Note: Data are expressed as mean $\pm$ SD. ^{*} $P < 0.05$ vs Group D at the same time point; [#] $P < 0.05$ vs Group C at the same time point, ^Δ $P < 0.05$ vs other time points. (3 d: A vs B, $P = 0.533$; A vs C, $P = 0.498$; A vs D, $P = 0.043$; B vs C, $P = 0.202$; B vs D, $P = 0.012$; C vs D, $P = 0.151$) (7 d: A vs B, $P = 0.591$; A vs C, $P = 0.000$; A vs D, $P = 0.000$; B vs C, $P = 0.000$; B vs D, $P = 0.000$; C vs D, $P = 0.110$) (14 d: A vs B, $P = 0.907$; A vs C, $P = 0.000$; A vs D, $P = 0.000$; B vs C, $P = 0.000$; B vs D, $P = 0.000$; C vs D, $P = 0.001$).

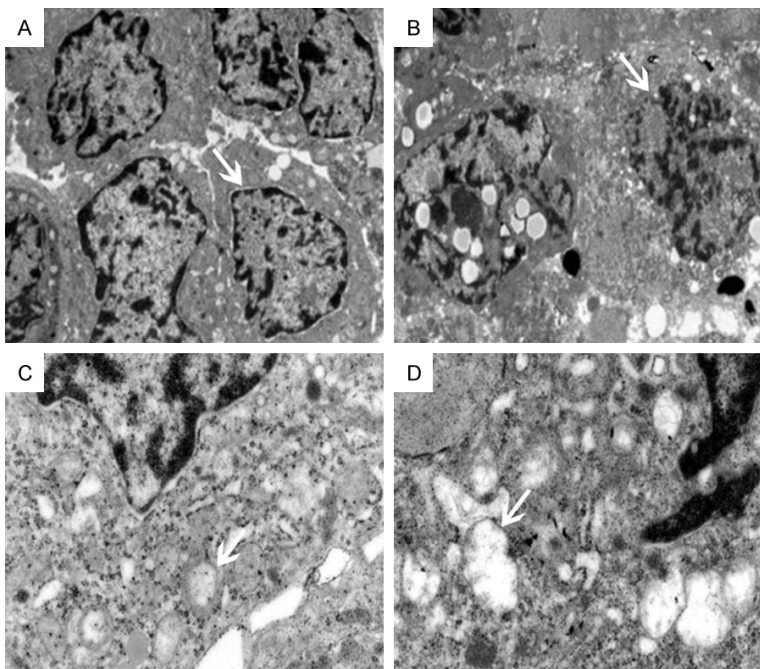


Figure 4. Ultrastructure of tumor cells under a transmission electron microscope. A. Immediately after ablation, some organelles were disrupted in tumor cells, nuclear membrane (white arrow) and cell membrane were observable, and cell junction was identified in the Group A and Group B (scale: 2 μm); B. Immediately after ablation, the tumor cells had unclear structure, some vacuoles were found in the nucleus, the nuclear membrane (white arrow) and cell membrane fractured, and cell junctions could not be identified in some cells of Group D (scale: 2 μm); C. At 7 d, mild mitochondrial swelling was found in the transition area (white arrow) of the Group A and Group B (scale: 0.5 μm); D. At 7 d, the mitochondria were expanded and there were some vacuoles (white arrow) in tumor cells of transition area in the Group D (scale: 0.5 μm).

the apoptosis index in the Group C and Group D was markedly higher than in other groups ($P < 0.05$), but there was no significant difference between Group C and Group D ($P > 0.05$). At 0, 3, 7 and 14 d, the apoptosis index of the transition area was comparable between Group A and Group B ($P > 0.05$) (**Figure 6**).

Tumor metastasis and grade

At 7 d: Tumor metastasis and ascites were found in only Group D. Metastasis was found in several animals of the Group A, Group B and Group C. In the Group A and Group B, most animals developed grade 1-2 metastasis, and the metastasis was mild in the Group C (1 had grade 1 metastasis). At 14 d: Metastasis was found in four groups. Metastasis was the most mild in the Group D, and only 1 had grade 1 metastasis. Most animals in the Group C developed grade 1-2 metastasis, but grade 3 metastasis was not observed. In the Group A and Group B, grade 2-3 metastasis was found in the majority of animals, and metastasis was characterized by abdominal wall metastasis, abdominal or pelvic lymph node metastases and massive ascites (**Table 4**).

Discussion

In recent years, ultrasound-guided thermal ablation has become one of the non-surgical treatments for liver cancer and widely used in clinical practices. However, the tumor structure possesses the non-linear characteristics and the abundant blood supply in the liver and liver tumor may cause the loss of the heat in thermal ablation, which may cause the incomplete ablation

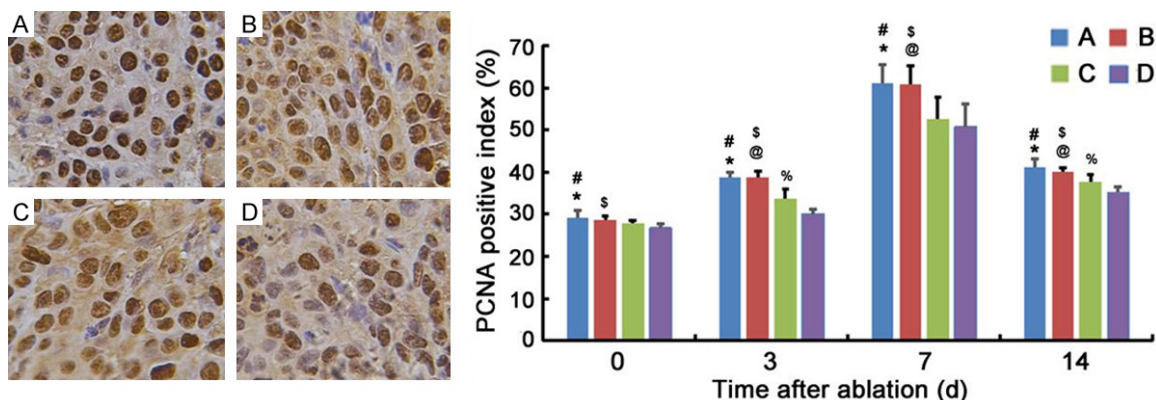


Figure 5. PCNA expression in the transition area at 7 d after MWA (400 ×; positive cells: brown) (left) and PCNA positive index at 0, 3, 7 and 14 d after ablation in 4 groups (right) * $P < 0.05$ vs Group C; # $P < 0.05$ vs Group D; @ $P < 0.05$ vs Group C; \$ $P < 0.05$ vs Group D; % $P < 0.05$ vs Group D. A. Group A; B. Group B; C. Group C; D. Group D. (0 d: A vs B, $P = 0.205$; A vs C, $P = 0.010$; A vs D, $P = 0.000$; B vs C, $P = 0.159$; B vs D, $P = 0.002$; C vs D, $P = 0.073$) (3 d: A vs B, $P = 0.930$; A vs C, $P = 0.000$; A vs D, $P = 0.000$; B vs C, $P = 0.000$; B vs D, $P = 0.000$; C vs D, $P = 0.000$) (7 d: A vs B, $P = 0.836$; A vs C, $P = 0.000$; A vs D, $P = 0.000$; B vs C, $P = 0.001$; B vs D, $P = 0.000$; C vs D, $P = 0.437$) (14 d: A vs B, $P = 0.100$; A vs C, $P = 0.000$; A vs D, $P = 0.000$; B vs C, $P = 0.001$; B vs D, $P = 0.000$; C vs D, $P = 0.001$).

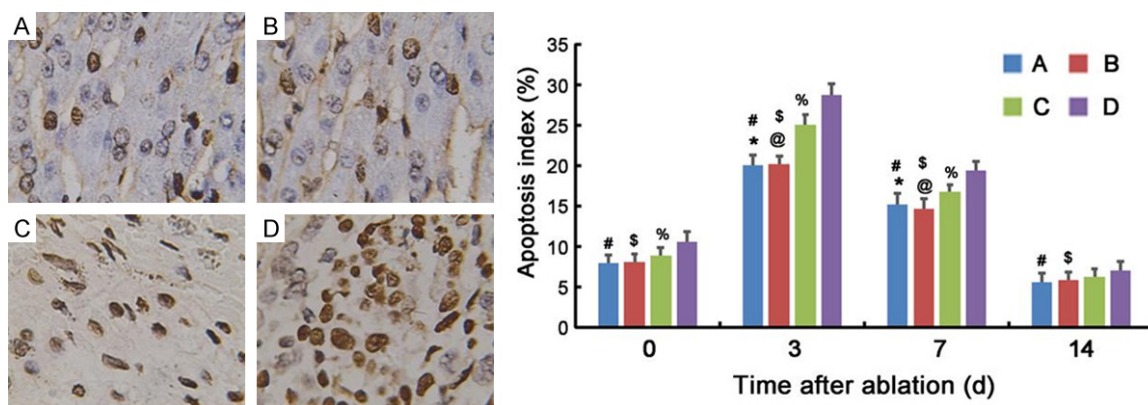


Figure 6. Apoptotic cells in the transition area at 3 d after MWA (400 ×; apoptotic cells: brown) (left) and Apoptosis index of the transition area at 0, 3, 7 and 14 d in 4 groups (right) * $P < 0.05$ vs Group C; # $P < 0.05$ vs Group D; @ $P < 0.05$ vs Group C; \$ $P < 0.05$ vs Group D; % $P < 0.05$ vs Group D. [(0 d: A vs B, $P = 0.870$; A vs C, $P = 0.072$; A vs D, $P = 0.000$; B vs C, $P = 0.100$; B vs D, $P = 0.000$; C vs D, $P = 0.001$) (3 d: A vs B, $P = 0.858$; A vs C, $P = 0.000$; A vs D, $P = 0.000$; B vs C, $P = 0.000$; B vs D, $P = 0.000$; C vs D, $P = 0.000$); (7 d: A vs B, $P = 0.402$; A vs C, $P = 0.005$; A vs D, $P = 0.000$; B vs C, $P = 0.000$; B vs D, $P = 0.000$; C vs D, $P = 0.000$) (14 d: A vs B, $P = 0.492$; A vs C, $P = 0.156$; A vs D, $P = 0.020$; B vs C, $P = 0.455$; B vs D, $P = 0.014$; C vs D, $P = 0.074$)].

Table 4. Tumor metastasis and grade at 7 and 14 d after ablation in 4 groups

Group	7 d				14 d			
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 0	Grade 1	Grade 2	Grade 3
A	2	2	1	0	0	1	2	2
B	1	4	0	0	0	0	3	2
C	4	1	0	0	1	2	2	0
D	5	0	0	0	4	1	0	0

of tumor cells and the residual tumor cells at the edge of tumors. In order to improve the efficacy of ablation, microbubble-induced ultra-

sonic cavitation as a non-invasive method that can temporarily block the blood flow to the tumor has become focus in the treatment of

liver cancer with ablation. In the present study, the pathology, cell proliferation, apoptosis, and outcome of the ablation zone and surrounding tissues were examined at four time points after MWA, aiming to explore the effects of microbubble-induced ultrasonic cavitation combined with prothrombin on MWA in a rabbit model of liver tumor and the potential biological effects were investigated.

Combination treatment increases ablation volume

Ultrasonic cavitation (UC) refers to a biological effect of ultrasound in which microbubbles in a liquid resonate and release energy under the action of ultrasound. Studies have shown that the use of ultrasound contrast microbubbles can significantly reduce the sound pressure threshold for cavitation, and the use of low-intensity ultrasound combined with microbubbles to produce cavitation effects can cause damage to the microvascular endothelium and vascular rupture [22], and can reduce the blood supply to target organs [23]. The combined use of coagulants or prothrombin may further enhance and prolong the destruction of local microvasculature and reduce the blood flow [18, 19, 24]. Chen et al [25] found that the ablation volume of the liver after microbubble-enhanced ultrasound cavitation combined with RFA was 2.8 times that of RFA alone. Our previous study showed microbubble-induced ultrasound cavitation combined with prothrombin significantly increased the ablation volume of low-energy microwave and enhance the ablation efficacy [20]. The results of this study indicated the ablation volume in the Group D was about 7 times that in the Group A and Group B and the ablation volume in the Group C was about 2.5 times that in the Group A and Group B immediately after ablation, indicating that microbubble-induced ultrasonic cavitation is effective to reduce the blood supply to the liver cancer and decrease the “heat sink” effect of MWA, thereby increasing the ablation volume. The microbubble-induced ultrasound cavitation combined with prothrombin enhances the blockage of blood flow to the liver cancer and further expands the ablation volume. At 14 days after ablation, the ablation volume in four groups increased compared with that at other time points, indicating that the necrosis area gradually increases after ablation, and this increase is more obvious in the Group D. This

may be due to the widening of the fibrotic band around the ablation area and the disruption of the fragile microvascular wall in the liver cancer by microbubble-induced ultrasonic cavitation which cause ischemia in the liver tissues surrounding the ablation zone, resulting in secondary cell apoptosis and necrosis and the expansion of ablation volume.

Combination treatment improves thermal ablation

Transmission electron microscopy showed more cells had nuclear membrane damage in the Group D compared with other groups at 0 d. This may be due to the reduced “heat sink” effect after microbubble-induced ultrasonic cavitation which increases the heat at the ablation site and more severe coagulative necrosis in the ablation area. At 7 days after ablation, compared with other groups, the damage to the organelles in the transition area was the most severe in the Group D. This may be related to microvascular destruction in the transition zone by the microbubble-induced ultrasound cavitation combined with prothrombin, which causes ischemia and relevant cell degeneration and necrosis.

Mechanism underlying the inhibitory effect on tumor growth

The newly generated vessels have weak wall, incomplete basement membrane and poor elasticity, and thus they are more sensitive to the sonoporation induced by ultrasonic cavitation. Wood et al [16, 26, 27] conducted several studies on mouse melanoma, and found intravenous injection of microbubbles combined with low-intensity ultrasound treatment for 1-3 min could produce significant antivascular effect. Histological examination showed the pathology after ultrasound treatment was characterized by the expansion of capillaries and intercellular edema in the tumor [26]. After treatment with ultrasound alone for 3 min, the tumor growth was inhibited and the survival time was prolonged in 28% of animals. It is mainly attributed to the destruction of blood vessels in the tumor, which leads to the necrosis of tumor cells, the subsequent inhibition of tumor growth and prolongation of survival time. Lin et al [28] also found the microbubble oscillation and destructive cavitation effect could cause the vascular wall rupture in the tumor

and inhibit the tumor growth. The present study confirmed these findings. Our study showed the tumor volume remained unchanged in the Group D but the tumor volume in other groups increased over time after ablation. We speculate this is as a result of two factors. Firstly, the microbubble-induced ultrasound cavitation combined with thrombin increases the ablation volume, which reduces the volume of tumor tissues remaining around the ablation area after ablation or even abolish the tumor tissues, which improves the ablation effect, and thus the tumor cells at the ablation area, especially those at the edge of the ablation area, are completely killed and the residual survived tumor cells reduce; secondly, the microbubble-induced ultrasound combined with thrombin may cause the rupture of microvascular endothelium in the tumor, which induces the formation of blood clots and thereafter reduces the blood supply to the tumor, leading to the ischemia in the tumor parenchyma. Both are effective to inhibit the tumor growth.

Pathophysiology of repair and capsulation, and outcomes

Our results showed that the number of metastatic tumors and the degree of tumor metastasis in the Group D were significantly improved as compared to other three groups at the same time point; the time to metastasis in the Group D was also later than in other groups. This may be ascribed to the fact that the ablation volume in the Group D is larger than in other groups and there are less residual tumor tissues after ablation in the Group D. In addition, the larger fibrotic band in the transition area of Group D may be another reason for the improvement of metastasis. Under a light microscope, tumor cells with intact nucleus and some fibrous tissues were noted in the fibrotic band of surrounding area in the early stage after ablation. At 14 days after ablation, the nuclear cavitation degeneration and necrosis of tumor cells were observed, which was accompanied by the formation of a large amount of fibrous tissues. Electron microscopy also confirmed the necrosis in the ablation area of the Group D was more complete and the cell injury in the transition area was more serious. These findings confirm that the formation of fibrotic band is a pathological process of tissue repair in the transitional area after ablation

induced degeneration and necrosis. Moreover, the fibrotic band in the transition area was widen over time, indicating that the fibrotic tissues increase in the transition zone after ablation, which is beneficial to the tissue repair. The fibrotic band envelops the ablation area, which may limit the spread of tumor cells after incomplete ablation and delay the metastasis. This gains opportunities and time for further thermal ablation and treatment. However, the specific mechanism and the reliability should be further studied.

Multiple factors promote cell apoptosis in the surround area of thermal ablation

He et al [29] and Luo et al [30] used microbubbles in the HIFU of rabbit VX2 liver cancer, and they found the apoptotic index of tissues around the ablation area in the microbubble-enhanced HIFU group was significantly higher than in the HIFU alone group. This was consistent with our findings. Our results showed apoptotic cells in the transition area of four groups. At 3 and 7 days, the apoptotic index in the Group D was significantly higher than in other groups. This may be related to the microwave ablation induced apoptosis [31, 32]. In microbubble-induced ultrasound cavitation, more heat accumulates in the ablation area of the VX2 liver cancer, which causes a more significant thermal stress [32-34] in the transition area. In addition, in the Group D, microvascular destruction caused by ultrasound cavitation may also promote cell apoptosis [35], which may be a factor enhancing the apoptosis.

Inhibitory effects on cell proliferation in the surrounding area of thermal ablation

PCNA is an accessory protein for DNA polymerase δ , synthesized in the nucleus, and involved in the division, synthesis and proliferation of DNA. PCNA has been used as a marker of cell proliferation [36]. Our results showed the PCNA-positive cells in the transition area of four groups after thermal ablation, and the proportion of positive cells increased over time and peaked at 7 days. At 0, 3, 7 and 14 d, the proportion of PCNA-positive cells in the Group C and Group D was significantly higher than in other two groups. At 3 and 14 days, there was a significant difference between Group C and Group D. This indicates that microbubble-induced ultrasound cavitation can inhibit tumor

proliferation in the surrounding area of ablation area. It may be related to the destruction of tumor microvessels due to ischemic necrosis and the subsequent inhibition of tumor cell proliferation.

Conclusion

This study shows that microbubble-enhanced ultrasound cavitation combined with prothrombin can increase the ablation effects on the rabbit liver VX2 tumor, which is characterized by the larger ablation volume, more severe tissue destruction, inhibition of tumor growth and metastasis, and increased cell apoptosis in the transition area. In addition, it may suppress the proliferation of tumor cells, the fibrotic band formed in the transition area may promote the organization and repair of injured tissues surrounding the ablation area, limit the spread of tumor cells and delay the tumor metastasis, which are clinically important for the treatment of liver cancer.

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Disclosure of conflict of interest

None.

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