Liposome quercetin enhances the ablation effects of microwave ablation in treating the rabbit VX2 liver tumor model

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ABSTRACT

Objective: This study aimed to investigate whether liposomal quercetin (LQ) could enhance the effects of microwave ablation (MWA) in treating the rabbit VX2 liver tumor model.

Methods: Rabbits with VX2 liver tumors were randomly divided into three groups: intravenous LQ group (LQ group), MWA group and LQ combined with MWA (LQ þ MWA) group. Five rabbits were randomly selected and sacrificed from each group at 12 h and on days 3, 7 and 14 of the operation. The tumor samples were detected and quantified by immunohistochemistry, Western blot, and reverse transcription polymerase chain reaction (RT-PCR).

Results: For up to 7 days, the coagulation necrosis volume (CV) of the LQ þ MWA group was larger than that of MWA and LQ groups (p < 0.05). Fourteen days after the operation, the total tumor volume of the LQ þ MWA group was smaller than that of the LQ group and the MWA group (p < 0.05). The survival time of the LQ þ MWA group was significantly longer than that of the MWA and LQ groups (p < 0.01). Heat shock protein 70 (HSP70), hypoxia inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF), tumor microvessel density (MVD) were lower in the LQ þ MWA group than the MWA and LQ groups at 12 h, on days 3 and 7. At hour 12 and on days 3 and 7, HSP70 mRNA and HIF-1α mRNA expression of MWA group were significantly higher than that of the LQ and LQ þ MWA groups (p < 0.001). At 12 h, and on days 3 and 7, apoptotic rate of tumor cells in LQ þ MWA group was significantly higher than that of the MWA and LQ groups (p < 0.05). At 12 h and on days 3, 7 and 14, the proliferation index of tumor cells in residual tumor in LQ þ MWA group was lower than that in the MWA and LQ groups (p < 0.05).

Conclusion: Preoperative infusion of LQ can significantly enhance the MWA effects of liver VX2 tumor, inhibit the excessive proliferation of residual tumor and angiogenesis, and decrease metastasis and prolong the survival period of experimental animals.

Introduction

Microwave ablation (MWA), as a kind of ablation therapy that uses thermal stimulation to destroy tumors directly, has the advantages such as fast heating, short time, repeatable and less trauma [1–3]. In the treating liver cancer with a diameter less than or equal to 3.0 cm, it can achieve the effect of surgical resection [4]. However, complete ablation of tumor using MWA or RFA may be difficult, and residual tumor leads to local tumor recurrence or even distant metastasis when the diameter of liver cancer is larger than 3 cm [5,6]. Govaert et al. found significant hypoxia zones in the ablation transition zone after tumor received RFA, which might be related to the invasive growth of intrahepatic micrometastases [7]. Ke et al. also detected that the expression levels of proliferation-related and invasion-related proteins, such as proliferating cell nuclear antigen (PCNA) and hepatocyte growth factor-1α (HIF-1α), were increased in the zone surrounding ablation area in rabbits with VX2 liver tumors, which might contribute to the tumor recurrence after RFA [8]. Similarly, Velez et al. used low-power MWA to establish the model of incomplete subcutaneous tumor ablation in mice. They found that the expression of heat shock protein 70 (HSP70), HIF-1α and vascular endothelial growth factor (VEGF) in the residual tumor surrounding the ablation zone was significantly increased, which became the main reason for tumor recurrence and metastasis caused by incomplete MWA ablation [9].

Yuan et al. found that liposomal quercetin (LQ) inhibited tumor growth and prolonged survival time in mice bearing tumor [10]. In our previous study, we found LQ could increase hepatocyte apoptosis and MWA-induced hepatic...
parenchyma destruction through suppressing the expression of HSP70 and HIF-1α expression in the liver surrounding the ablation zone in a normal rabbit liver [11]. The present study was designed to test whether LQ can suppress tumor recurrence and metastasis through suppressing the expression of HSP70 and HIF-1α expression in residual tumor surrounding ablation zone in a rabbit VX2 liver tumor model.

Materials and methods
Experimental materials and animal model establishment

The experiment was carried out with the approval of the animal ethics committee of Henan Province. All related experiments were conducted in accordance with the principles set by the Animal Care and Use Committee of our institution. The experiments were performed using Japanese white rabbits weighing 3.0–3.5 kg (aged 4–5 months), which were provided by the animal experiment center of Henan Province (SCXK2016-0011). LQ was prepared in the pharmaceutical laboratory of Zhengzhou University, and the preparation method was the same as described in previous research [11]. The liposomal size was 114 nm ± 12 nm. The zeta-potential was -38.9 ± 3.3 mV and the polydispersity coefficient was 0.356 ± 0.084.

VX2 tumor-bearing rabbit was donated by the animal experimental center of Tongji Medical College, Huazhong University of Science and Technology. The rabbit VX2 liver tumor model was established as described by Duan et al. reported [12]. Then contrast-enhanced computed tomography was used to examine the location and size of the tumor in each rabbit after the rabbits were fed for 16 days to ensure the growth of the VX2 tumors. The eligible tumor-bearing rabbits were divided into three groups according to the random number method: intravenous (IV) LQ 0.5 mg/kg group (LQ group), MWA group alone (40 s, 30 W) and IV LQ combined with MWA (LQ + MWA) group (IV LQ 24 h before MWA treatment). Total 30 rabbits were assigned in each group, 10 rabbits in each group were used to observe the survival period. The humane endpoint was defined by a 50% decrease in food intake and >20% loss in body weight. The animals were then humanely euthanised by an overdose injection of pentobarbital sodium (100 mg/kg) as described by Song et al. [13]. At 12 h and on days 3, 7 and 14 after the operation, five rabbits in each group were randomly selected and sacrificed by intravenous injection of pentobarbital sodium (100 mg/kg). The whole liver and lung tissues were taken out for preservation and the tumor metastasis was examined.

Treatment of each group

The rabbits were fasted for 24 h before the operation, and then anesthetized with 0.2–0.3 ml/kg gluteal muscle of the fast sleeping new II injection (Dunhua Shengda Animal Medicine Co., Ltd, Jilin Province). After anesthesia, the rabbit was laid supine on the operating table. The left lobe of the rabbit’s liver was exposed through the incision of the abdominal wall along the midline under aseptic condition. Twenty-four hours before the operation, 0.5 mg/kg LQ was injected via the ear vein of the rabbit for 5 min in the LQ and LQ + MWA groups. In the LQ group, the abdominal wall of the rabbit was cut and the tumor in the left lobe of the liver was exposed. A 14 G microwave antenna with a 2-cm tip was inserted along the length of the tumor. After 40 s without the ablation treatment, the antenna was pulled out and the puncture channel was blocked with a gelatin sponge strip to prevent bleeding.

The microwave antenna with 2-cm tip was placed at the center of the tumor along the length of the tumor through part of the normal liver tissue. The power output was set to 30 W and lasted for 40 s for each rabbit in the MWA and LQ + MWA groups to ensure that the tumor had residue after the ablation. The MWA was performed with a 2450 ± 50 MHz microwave generator (ECO Microwave System Co., Ltd, Nanjing). The temperature of the microwave antenna was about 120 °C, the temperature of the ablation zone surrounding the microwave antenna was about 75–80 °C, and the temperature of the periphery of the ablation zone was about 45 °C. The MWA procedure for residual VX2 liver tumor was standardized as described by Ke et al. reported [8]. In order to prevent needle-tract bleeding, a thermocoagulation was performed along the needle tract when the ablation finished.

Specimen collection

The total volume of the residual tumor and necrosis was calculated according to the formula: \( V = \frac{a \times b^2}{2} \), where ‘a’ is the longest and ‘b’ is the smallest diameter of the tumor in vivo. In the MWA and LQ + MWA groups, the tumor tissue was cut along the MWA antenna path (this section was the largest ablation section). In the LQ group, the liver tumor was cut along the long axis of the tumor.

Half of the sample was wrapped and immediately put into the liquid nitrogen tank for immunohistochemistry, Western blot (WB) and reverse transcription-polymerase chain reaction (RT-PCR) analyses. The other half was sectioned at 3- to 5-mm intervals along the MWA needle path. One of the two pieces with the largest cross-sectional area was put into 2% 2,3,5-triphenyl tetrazolium chloride (TTC) at room temperature for 15 min to detect the extent of the white coagulated zones. The tumor necrosis zones or ablation necrosis zones can be stained white by TTC, while the incomplete ablation area was stained patchy red by TTC due to the expression of mitochondrial enzyme activity in the tissue. The measurement end point of each treatment group was the midpoint of white coagulation without TTC staining in the zone of coagulation and reddish tissue change with patchy areas of TTC staining in the region of the infarction zone (Figure 1). The coagulation volume (CV) was calculated according to the following formula: \( V = \frac{A \times B^2}{2} \), where ‘A’ and ‘B’ are axes of the CV, respectively.
Tissue samples were fixed with 4% paraformaldehyde for 3 days; they were paraffin-embedded and cut into sections with thickness of 4 \( \mu \)m. The pathological changes were observed by hematoxylin and eosin staining. The expression of HSP70, HIF-1 \( \alpha \), VEGF, platelet endothelial cell adhesion molecule (CD31) and PCNA was determined by using the two-step method of Supervision. The relevant experimental steps were followed from studies by Duan et al. [11,12] and Liang et al. [14]. All sections stained with HE and immunohistochemistry were observed by two pathologists with 8 and 15 years’ working experience.

The positive staining of HSP70 mainly showed that the tumor cells and their nuclei were brownish yellow. The integral optical density (IOD) of HSP70 was calculated by Image-Pro Plus software (version 6.0, Media Cybernetics, MD, USA) to evaluate the expression level of HSP70 at different time points in each group. The expression of HIF-1 \( \alpha \) was mixed brownish yellow staining of the cytoplasm and/or nucleus. Microimaging software (Westover scientific, Inc, WA, USA) was used to analyze HIF-1 \( \alpha \) staining to determine the positive rate of HIF-1 \( \alpha \) staining of tumor cells [15]. The intensity score (IS) was used to calculate the expression intensity of HIF-1 \( \alpha \). The staining intensity of cells was divided into four grades: 0 for negative, no cell coloration; 1 for weak positive, light yellow cell coloration; 2 for medium positive, brownish yellow cell coloration; 3 for strong positive, brown cell coloration. The following formula was used for calculation: IS = \( \sum \left[ \frac{(0 \times F0)}{C2} \right] + \left[ \frac{(1 \times F1)}{C2} \right] + \left[ \frac{(2 \times F2)}{C2} \right] + \left[ \frac{(3 \times F3)}{C2} \right] \), where F is the percentage of the number of cells with staining intensity of each level in the total number of cells in the field of vision, and the number represents the staining intensity level.

Tumor microvessel density (MVD) was evaluated by CD31, which was linear brown or brownish yellow. The distribution of blood vessels in the whole section was observed using a low power microscope. The single endothelial cells or clusters of endothelial cells with CD31 positive were counted as one microvascular as long as they were separated from the adjacent microvasculature, tumor cells or other connective tissue, no matter whether there was a vascular cavity or not. The number of blood vessels in 5 visual fields was counted under 200 times visual field, and the average value was the MVD value of each specimen [16]. The TUNEL method (Roche, Mannheim, Germany) was used to detect the apoptosis of tumor cells. Five areas at \( \times 200 \) magnifications were taken from each sample, and the results were analyzed by

![Figure 1. The survival time of each group and largest cross-sectional tumor necrosis in each group after the operation. (A) The survival time of each group. The largest cross-sectional tumor necrosis in the LQ (B), MWA (C) and LQ + MWA (D) groups on day 7.](image-url)
calculating the positive percentage of apoptotic positive cells. PCNA positive cells were stained with brown granules in the nucleus. A total of 200 cells from 5 fields were randomly selected under 400 times field of vision. According to the formula, the PCNA-positive cell proliferation index (PI) was calculated according to the following formula: the number of PCNA-positive cells/tumor cells.

**WB analysis and RT-PCR**

Tissue samples were taken from an area of 0.5 cm area surrounding the ablation necrosis area in the MWA and LQ + MWA groups, and 0.5 cm surrounding the tumor necrosis area in the LQ group as described by Duan et al. [11] reported. The protein extraction and determination of HSP70 and HIF-1α in residual tumor cells were the same as described in our previous study [11,12]. The expression of HSP70 mRNA and HIF-1α mRNA were detected by RT-PCR according to previous studies [11,12,16]. PCR primers were designed as follows: HSP70: Forward primer 5'-AAGCCAGACGACAATCAGGA-3'; Reverse primer 5'-CATGGCTGCAAGACCTCTGT-3'; HIF-1α: Forward primer 5'-GCATCTCCGTCTCCTAACCA-3'; Reverse primer 5'-ACACGTTAGGGCTTCTTGGA-3'; β-actin: Forward primer 5'-TGGCTCTAACCAGTCCCC-3'; Reverse primer 5'-AGTGCACGGTGACATCCG-3'.

**Statistical analysis**

SPSS 24.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Continuous data were expressed as mean ± standard deviation. Repeated measurement analysis of variance was used to compare the measurement data between groups and within groups. Least significant difference was used to compare between groups and within groups. Least significant difference was used to compare the rate, and Kaplan Meier survival analysis was used to evaluate the survival period. A P value < 0.05 was considered statistically significant.

**Results**

**Comparison of curative effect, metastasis and survival time of liver tumor in each group after the operation**

Ten rabbits in each group were taken to observe the survival period, and all were sacrificed within 75 days after the operation. The median survival time of LQ, MWA and LQ + MWA groups was 23, 39 and 53 days, respectively. The survival time of the LQ + MWA group was significantly longer than that of the other two groups (p < 0.01). No statistical difference existed between the LQ group and the MWA group (Figure 1). On day 7, the CV of the LQ, MWA and LQ + MWA groups were 1.34 ± 0.35 cm³, 3.85 ± 0.72 cm³, 5.69 ± 1.96 cm³, respectively. The CV of the LQ + MWA group was larger than that of the MWA group and LQ group (p < 0.05), and the CV of MWA group was larger than that of the LQ group.

Before treatment, the volume of tumor in the LQ, MWA and LQ + MWA groups was 1.66 ± 0.46 cm³, 1.70 ± 0.57 cm³, 1.81 ± 0.49 cm³, respectively. Fourteen days after the operation, the total volume of residual tumor and necrosis in the LQ, MWA and LQ + MWA groups was 7.45 ± 3.75 cm³, 8.47 ± 2.69 cm³ and 5.70 ± 1.59 cm³, respectively. The total volume of the MWA group was not significantly different from that of the LQ group (p > 0.05). The LQ + MWA group was smaller than the LQ and MWA groups (p < 0.05). The metastasis of each group on 14 days after the operation is shown in Table 1. Liver, lung, abdominal wall and omentum metastases were small than those of the LQ and MWA groups.

| Group (n) | Liver | Lung | Abdominal wall | Omentum | Ascites |
|----------|-------|------|---------------|---------|--------|
| LQ (5)   | 4     | 3    | 3             | 4       | 3      |
| MWA (5)  | 4     | 3    | 3             | 5       | 3      |
| LQ + MWA (5) | 2     | 1    | 2             | 2       | 2      |

**Postoperative expression of HSP70 and HSP70 mRNA in each group**

WB (Figure 2(A)) and RT-PCR (Figure 2(B)) analyses of HSP70 and HSP70 mRNA in the LQ group showed little change on the four observation points. The WB showed that the expression of HSP70 in the MWA and LQ + MWA groups increased at 12 h, peaked on day 3, and decreased on days 7 and 14. The RT-PCR analysis showed that the expression HSP70 mRNA was peaked at 12 h in the MWA group and LQ + MWA groups, and gradually decreased on days 3, 7 and 14. The HSP70 mRNA expression in MWA group was higher than that of the LQ and LQ + MWA groups, and in the LQ + MWA group was higher compared with the LQ group. No statistical difference existed among the three groups on day 14.

The IOD of HSP70 expression in residual tumor cells in each group on the four observation points and typical immunohistochemical manifestations at peak stage are shown in Figure 3. At 12 h and on day 3, the HSP70 expression was found in some tumor cells around the tumor necrosis area of the LQ group, with no statistical difference at the four time points. The expression of HSP70 in residual tumor in the surrounding zone of MWA and LQ + MWA groups increased 12 h after the ablation, reached the peak on day 3, and significantly decreased on days 7 and 14. At 12 h and on days 3 and 7 after the operation, the HSP70 expression in the MWA group was higher than that in the LQ and LQ + MWA groups, while that in the LQ + MWA group was higher compared with the LQ group.
Figure 2. Expression of HSP70 and HSP70 mRNA in each group. WB (A) and RT-PCR (B) analyses of HSP70 and HSP70 mRNA in each group. At 12 h and on day 3, 7 and 14 after the operation, WB analysis (A) showed significant HSP70 expression in the MWA and LQ + MWA groups, but rarely in the LQ group. At 12 h and on days 3 and 7, the HSP70 mRNA expression of the MWA group was significantly higher than that of the LQ and LQ + MWA groups ($p < 0.001$); LQ + MWA group was significantly higher compared with the LQ group ($p < 0.05$).

Figure 3. HSP70 expression in residual tumor in each group at the four observation times. (A) IOD of HSP70 expression in the residual tumor of each group at the four time points. On days 1, 3 and 7, the HSP70 expression of the MWA group was significantly higher than that of the LQ and LQ + MWA groups ($p < 0.001$), and that of the LQ + MWA group was significantly higher compared with the LQ group ($p < 0.05$). Representative immunohistochemical analysis ($\times$200 magnification) showed peak HSP70 expression in residual tumors in LQ (B), MWA (C) and LQ + MWA (D) groups on day 3.
Figure 4. HIF-1α and HIF-1α mRNA expression in the residual tumor. WB (A) and RT-PCR (B) analyses of HIF-1α and HIF-1α mRNA in each group. At 12 h and on days 3, 7 and 14, WB analysis showed significant HIF-1α expression in the MWA and LQ + MWA groups, but rarely in the LQ group. At 12 h and on days 3 and 7, the HIF-1α mRNA expression of the MWA group was significantly higher than that of the LQ and LQ + MWA groups ($p < 0.001$), and that of the LQ + MWA group was significantly higher compared with the LQ group ($p < 0.05$).

Figure 5. HIF-1α expression in residual tumor in each group at different time points. (A) IOD of the HIF-1α expression in residual tumor cells of each group at different time points. On days 1, 3, 7 and 14, the HIF-1α expression in the MWA group was significantly higher than that in the LQ and LQ + MWA groups ($p < 0.05$), and that in the LQ + MWA group was significantly higher compared with the LQ group ($p < 0.05$). Representative immunohistochemical staining ($\times 200$ magnification) showed peak HIF-1α expression in the LQ (B), MWA (C) and LQ + MWA (D) groups on day 3.
HIF-1α and HIF-1α mRNA expression in the residual tumor

HIF-1α (Figure 4(A)) and HIF-1α mRNA (Figure 4(B)) in the LQ group had no significant difference at the four observation points. The WB analysis showed that the expression of HIF-1α in MWA and LQ+MWA groups increased at 12 h, peaked on day 3, and decreased on days 7 and 14. The RT-PCR analysis showed that: HIF-1α mRNA in the MWA group and LQ+MWA group increased at 12 h, peaked on day 3, and gradually decreased on days 7 and 14. The expression of HIF-1α mRNA in the MWA group was higher than that in the LQ and LQ+MWA groups, and that in the LQ+MWA group was higher compared with the LQ group. No significant difference existed among the three groups on day 14.

The HIF-1α expression in residual tumor in each group on the four observation points and typical immunohistochemical manifestations at the peak stage are shown in Figure 5. At 12 h and on day 3, the HIF-1α expression was found in some tumor cells around the tumor necrosis area in the LQ group, but rarely on days 7 and 14. No statistical difference existed at the four time points. In the MWA and LQ+MWA groups, the expression of HIF-1α in the residual tumor increased gradually at 12 h, peaked on day 3, and decreased gradually on days 7 and 14. The HIF-1α expression in the MWA group was significantly higher than that in the LQ and LQ+MWA groups at 12 h and on day 3, and no statistical difference existed on days 7 and 14.

Expression of VEGF and MVD in the residual tumor area

The expression of VEGF in the residual tumor cells on the four observation points and typical immunohistochemical manifestations at the peak stage are shown in Figure 6. No significant difference existed in the VEGF expression among the three groups at 12 h. In the MWA and LQ+MWA groups, the VEGF expression in the residual tumor increased gradually at 12 h, increased significantly on day 3 and decreased on days 7 and 14. The VEGF expression in the MWA group
and LQ + MWA groups was significantly higher than that in the LQ group on days 3, 7 and 14. The VEGF expression in the MWA group was higher than that in the LQ + MWA group on days 3, 7 and 14.

The MVD of each group at different time points and typical immunohistochemical manifestations at the peak stage are shown in Figure 7. No statistical MVD difference existed among the three groups at 12 h. The MVD in the LQ + MWA group was lower than that in the other two groups (p < 0.05) on days 3, 7 and 14. The MVD in the MWA group was higher than that in the LQ + MWA group on days 3, 7 and 14.

The MVD of each group at different time points and typical immunohistochemical manifestations at the peak stage are shown in Figure 7. No statistical MVD difference existed among the three groups at 12 h. The MVD in the LQ + MWA group was lower than that in the other two groups (p < 0.05) on days 3, 7 and 14. The MVD in the MWA group was higher than that in the LQ + MWA group on days 3, 7 and 14.

The MVD in the LQ + MWA group was significantly lower than that in the LQ group (p < 0.05), and that in the MWA group was significantly higher compared with the LQ group (p < 0.05). Representative immunohistochemical staining (×200 magnification) showed peak CD31 expression in the LQ (B), MWA (C) and LQ + MWA (D) groups on day 7.

Apoptosis and PI index of residual tumor cells

The TUNEL analysis showed no statistical difference at all time points in the LQ group (Figure 8). In the MWA and LQ + MWA groups, the peak value reached at 12 h, and gradually decreased on days 3 and 7, and decreased to the baseline level on day 14. The apoptosis rate in the LQ + MWA group was higher than that in the other two groups at 12 h and on days 3 and 7. The apoptosis rate in the MWA group was higher than that in the LQ group at 12 h and on days 3 and 7. No difference existed the three groups on day 14.

The PI index of residual tumor in each group and typical immunohistochemical manifestations at the peak stage are shown in Figure 9. No statistical difference existed at each time point in the LQ group. In the MWA and LQ + MWA groups, the PI index gradually increased at 12 h and on day 3, peaked on day 7 and decreased on day 14. No statistical difference existed at the four time points in the LQ group.

Apoptosis and PI index of residual tumor cells

The TUNEL analysis showed no statistical difference at all time points in the LQ group (Figure 8). In the MWA and LQ + MWA groups, the peak value reached at 12 h, and gradually decreased on days 3 and 7, and decreased to the baseline level on day 14. The apoptosis rate in the LQ + MWA group was higher than that in the other two groups at 12 h and on days 3 and 7. The apoptosis rate in the MWA group was higher than that in the LQ group at 12 h and on days 3 and 7. No difference existed the three groups on day 14.

The PI index of residual tumor in each group and typical immunohistochemical manifestations at the peak stage are shown in Figure 9. No statistical difference existed at each time point in the LQ group. In the MWA and LQ + MWA groups, the PI index gradually increased at 12 h and on day 3, peaked on day 7 and bottomed on day 14. The PI of the LQ + MWA group was lower than that of the LQ group and groups at 12 h and on days 3, 7 and 14, and that of the MWA group was significantly higher than that of the LQ group at 12 h and on days 3 and 7. No statistical difference existed among the three groups on day 14.

Discussion

Quercetin (3,3,4,5,7-pentahydroxflavone), a kind of natural nontoxic flavonoid compound, has the functions of anti-
proliferation and antitumour, inhibiting the synthesis of biomacromolecule, affecting the activity of many enzymes, and promoting apoptosis [17,18]. Quercetin is difficult to dissolve in water. As a common cosolvent of quercetin, dimethyl sulfoxide can cause hemolysis and damage to liver and kidney functions which limited its clinical application [19]. Yuan et al. had confirmed that liposomes can change the water solubility of quercetin and prolong the half-life of quercetin in vivo, reduce drug toxicity and significantly enhance the solubility and bioavailability of quercetin. Thus, liposomes could effectively play the role of inhibiting the expression of HSP70 in tumor cells, inhibiting tumor microvascular formation and promoting tumor cell apoptosis [10].

When tumor cells are stimulated by hyperthermia, HSP70 is highly expressed causing a the risk period after hyperthermia [7]. LQ can increase the effect of tumor ablation by inhibiting the high expression of HSP70 and inducing apoptosis of tumor cells after RFA [10,20]. In this study, HSP70 was highly expressed in the residual tumor surrounding the ablation zone in the MWA group. However, high expression of HSP70 could be effectively inhibited by LQ, which was injected via the ear vein 24 h before operation in the LQ + MWA group. At the same time, the apoptosis index of the LQ + MWA group was significantly higher than that in the other two groups. Therefore, IV LQ at 24 h before MWA also has the ability to inhibit the high expression of HSP70 in the residual tumor after MWA, which can induce apoptosis in the sub lethal tumor cells in this area, thus enhancing the efficacy of MWA in the treatment of rabbit VX2 liver tumor.

Kong et al. used MWA combined with intranasal administration of sorafenib to treat rabbit VX2 liver tumor model, and used MRI to measure the length, width and height of the tumor on days 3, 7 and 14 after the operation, so as to evaluate the effect of various ablation methods on tumor control [21]. In this study, we found that the shape of residual tumor in the MWA and LQ + MWA groups was irregular and the volume of residual tumor could not be measured accurately. The results showed that the LQ + MWA group was better than LQ and MWA groups in tumor damage. With the the disappearance of the antitumour effect of the MWA and LQ and the rapid growth of residual tumors, the tumor necrosis area shrunk 14 days after operation. Therefore, the total volume of residual tumor and necrosis area was used to evaluate the curative effect of three...
treatments. The total volume of the LQ + MWA group is significantly smaller than that of the other two groups. Thus, we confirmed that LQ + MWA treatment can achieve a long-term tumor control effect compared with MWA alone.

Wan et al. found that the overexpression of HSP70 and HIF-1 α in the residual tumor cells surrounding the ablation zone accelerated the proliferation of the residual tumor cells and increased the expression of VEGF, thus significantly increased MVD [22]. Inhibition of HSP70/HIF-1 α expression pathway can improve the therapeutic effect of incomplete ablation model [22]. Tikhonova et al. [23] found that HSP70 can interfere with the cell signal transduction pathway and reduce cell response to hypoxia stress. At the same time, Tikhonova et al. [23] found that HSP70 could form a long-term complex with HIF-1 α and increase the stability of HIF-1 α. In this study, we found that the expression trend of HSP70 and HIF-1 α in the residual tumor of the ablation groups was similar: increased at 12 h, peaked on day 3, and decreased gradually on days 7 and 14. However, HSP70 and HIF-1 α in the LQ + MWA group was significantly lower than that of the LQ + MWA group at 12 h and on day 3. Therefore, we confirmed that the infusion of LQ before MWA could simultaneously inhibit the high expression of HSP70 and HIF-1 α in the residual tumor.

Angiogenesis is an important mechanism of tumor proliferation, metastasis and recurrence [24]. Kong et al. found that incomplete RFA could promote the expression of HIF-1 α in residual tumor tissues, and HIF-1 α can promote the overexpression of VEGF, promote the angiogenesis of residual tumor tissues, and accelerate the progress of residual tumor tissues, which was the main reason for the accelerated growth of tumor lesions after incomplete ablation [25]. In this study, we found that the expression of HIF-1 α and VEGF in the residual tumor cells of the MWA group was significantly higher than that of the LQ + MWA and LQ groups at 12 h, on days 3 and 7 because the tumor was not completely ablated. On days 7 and 14, the MVD and PI index of the residual tumor in MWA group were significantly higher than that of the LQ and LQ + MWA groups. Therefore, it was inferred that the angiogenesis pathway of LQ + MWA group was inhibited, and then the proliferation of tumor cells was inhibited; therefore, the tumor growth was

![Figure 9. PI index in each group. (A) Percentage of PCNA-positive cells in residual tumor cells of each group at different time points. At 12 h and on days 3, 7 and 14, the percentage of PCNA-positive cells in residual tumor cells in the LQ + MWA group was significantly higher than that in the LQ and LQ + MWA groups (#p < 0.05). At 12 h and on days 3 and 7, the percentage of PCNA-positive cells in residual tumor cells in the MWA group was significantly higher than that in the LQ group (&p < 0.05). Representative immunohistochemical staining (×400 magnification) showed peak percentage of PCNA-positive cell in the LQ (B), MWA (C) and LQ + MWA (D) groups on day 7.](image)
obviously inhibited. However, the MVD of residual tumor in the MWA group was higher than that in the other two groups due to the higher expression of HIF-1α and VEGF. Although the tumor necrosis of MWA group was significantly larger than that of the LQ group on day 7 after the operation, the total volume of the MWA group was similar as that of the LQ group on day 14. Hence, we speculated incomplete ablation may lead to more rapid growth of residual tumor during days 7–14 after the operation.

The present study had several limitations. First, no significant difference was found in the tumor necrosis rate, growth rate and the expression of HSP70, HIF-1α, VEGF expression between the control group and the LQ group in the pre-experiment stage. Then, no control group was established in our study. Second, the levels of HSP70, HIF-1α, VEGFmRNA and HIF-1α mRNA were still high on day 3. Whether an additional dose of LQ injection in the next few hours or days for keeping higher drug concentration can achieve a better inhibition effect on the expression of HSP70 and HIF-1α need to be explored. Additional studies are under way to evaluate whether increasing the sample size, detection items, observation time point, and the dosage and times of LQ infusion can strengthen the effect of LQ on tumor recurrence and metastasis after MWA.

In conclusion, preoperative infusion of LQ can significantly improve the effects of MWA on VX2 liver tumors by suppressing the expression of HSP70 and HIF-1α in the residual tumor, inhibiting the excessive proliferation of residual tumor and angiogenesis, decreasing metastasis and prolonging the survival period of rabbits.

Disclosure statement

The authors have no conflicts of interest to declare.

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