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ORIGINAL ARTICLE

Experiment on enhancing antitumor effect of intravenous epirubicin hydrochloride by acoustic cavitation in situ combined with phospholipid-based microbubbles

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Abstract
Objective An in vivo study on enhancing antitumor effect of intravenous epirubicin hydrochloride (EPI) by acoustic cavitation in situ combined with phospholipid-based microbubbles (PMB) was reported.
Methods Five-week-old male nude mice were used, and HCT-116 cells were *s.c.* (subcutaneous injection) inoculated in axilla of these mice. Five groups were designed and five consecutive treatments were applied to investigate the tumor growth, body weight growth, and normal organ growth.
Results Inhibition effects on tumor growth were observed in all groups treated by EPI. However, the potency of tumor growth retardation caused by EPI depended on the application of PMB and US in situ. An effective retardation on tumor growth and improved mice survival status were observed when intravenous EPI combined with sonicated PMB in situ. Short-term lingering sensitive period for chemotherapeutic drugs after acoustic cavitation was also observed in experiment under asynchronous administration of EPI and sonicated PMB.
Conclusion Intravenous EPI combined with PMB-mediated cavitation in situ might provide a potential application for US-mediated chemotherapy.

Keywords Acoustic cavitation · Chemotherapy · Epirubicin hydrochloride · Microbubble

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Introduction

Acoustic cavitation is a physical technique to enhance transmembrane delivery of various drugs using ultrasound energy. Several physical events accompanied by ultrasound (US) application, including thermal effects, radiation pressure and mechanical impact, can contribute to penetration enhancement.

These physical events can affect cell permeation properties directly and indirectly. Ultrasound-enhanced chemotherapy has been explored by some reports [1, 2]. Enhancing effect of acoustic cavitation induced by high-frequency ultrasound (10 or 16 MHz) may produce strong direct actions of thermal effects [3].

In contrast, tumor permeability enhancement induced by low-frequency sonication (20 to 100 kHz) combined with bubbles can be related to indirect actions, such as mechanical impact. And the structure of lipid bilayer in tumor cell membrane can result in disruption due to the collapse of cavitation bubbles in solution [4, 5].

US-mediated destruction of drug-loaded sonicated microparticles can be used to deliver encapsulated drug for potential targeted release. A number of microparticles suitable for the ultrasonically enhanced drug delivery have been suggested, such as polymeric ultrasonic microbubbles [6, 7], gas-filled liposomes [8, 9], and modified lipospheres [10]. However, drug-loading rate and encapsulation efficiency of these gas-nuclear microparticles are usually low because of the limited cavity on shell for loading drug. Increasing shell width to enhance loading cavity was suggested to solve these problems. But this might increase the preparation complexity and reduce reproducibility in pharmaceutical industry.

In addition, the clinical use of US-mediated chemotherapy in vivo is impaired from a number of biological features and operational conditions [11, 9]. For example, asynchronous effect between US-triggered cavitation and drug entering cell are not avoided because of blood circulation.

In our previous study in vitro [12, 13], it was observed that there was a period of cell membrane sensitivity that persisted after ultrasonic cavitation. Chemotherapeutic drugs can effectively enter cancer cells in the lingering sensitive period after the completion of acoustic cavitation caused by eruption of microbubbles.

In the present study, synchronous and asynchronous acoustic cavitations on chemotherapy in vivo were assessed by applying phospholipid-based microbubbles (PMB) combined with intravenous epirubicin hydrochloride. Related parameters such as US power, duration of US treatment, and microbubble concentration were set in according to our previous experiments in vitro and preliminary experiment in vivo.

The advantage of this technique is that it does not require encapsulating drugs in particles during preparation. Therefore, the complicated preparation for loaded microparticles can be simplified, which may offer convenient application in future US-mediated chemotherapy.

Materials and methods

Preparation of PMB

Blank PMB was prepared by sonication-lyophilization method which was reported in our previous study [14, 15]. The brief description of preparation was described as follows. Hydrogenated phosphatidylcholine (HPC) (HPC >99%, Doosan Corporation Biotech BU, Kyonggi Do, Korea), polyethylene glycol 1,500 (Qingming Chemical Plant, Zhejiang Province, China), and poloxamer 188 (Shenyang Chemical Plant, Liaoning Province, China) were dissolved in normal butanol (analytical grade, Beijing Chemical Plant, Beijing, China) and sonicated at 30°C (JY 92-II ultrasonic processor, KunShan US Instrument Inc., KunShan, China) at frequency of 40 kHz, power of 160 W for 3 min. The solution was stored at 0°C for 30 min and at −20°C for 1 h. Then, the coagulated solution was lyophilized at 5×10^{-4} Pa pressure for 20 h (primary drying at −48°C for 15 h and then the temperature was gradually raised to 10°C within 5 h). Lyophilized powder was put in 10-ml penicillin vials (200 mg/vial) and saturated with perfluoropropane (C_3F_8, electronic grade, Institute of Special Gas, Tianjing, China).

PMB solution was obtained by adding 2 ml 0.9% NaCl solution in lyophilized PMB. Measured by coulter counter (Coulter Corporation, Hialeah, FL, USA), PMB concentration was about 2×10^9 bubble/ml, with average diameter 3.4 μm.

Chemotherapeutic drug

Epirubicin hydrochloride (EPI) was purchased from Haizheng Company (Zhejiang province, China). EPI is an anthracycline antitumor antibiotic. The administration route of EPI is *i.v.* injection. In this experiment, EPI solution for *i.v.* injection was sterilized by filtration through 0.22 μm filters.

Animals and cell line

Five-week-old male nude mice (23–26 g body weight, BALB/c/nu, Beijing, China) were bought from animal center of Wenzhou Medical College. Colon cancer HCT-116 cells (2×10^7 cells per mouse) were axilla-inoculated subcutaneously, and tumors were allowed to develop. Treatments were initiated when the tumor volume reached 100 mm^3. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by Beijing Laboratory Animal Research Center.

Sonication

Unfocused ultrasound was generated by sonicator (CZT-8A, Xinzhen Company, ShenYang, China) equipped with a 1-cm^2 transducer head. In designed experiments, 1-MHz ultrasound and 3 W/cm^2 output power density were applied through aquasonic coupling gel for 30 s to the tumor region of a mouse.

Experiment design

The mice were randomly assigned to the following five groups. In each group, five mice were used.

Blank control—tumor without any treatment;
Group 1— *s.c.* injected PMB solution in tumor combined with sonication in situ (*s.c.* PMB);
Group 2— *i.v.* injected EPI solution combined with sonication in situ (*i.v.* EPI);
Group 3—*s.c.* injected PMB solution in tumor combined with sonication in situ, at the same time *i.v.* injected EPI solution (*s.c.* PMB + *i.v.* EPI);
Group 4— *i.v.* injected EPI solution at 10 min after *s.c.* injected PMB solution in tumor combined with sonication in situ (*s.c.* PMB + delayed *i.v.* EPI).

For the test groups, five consecutive treatments were applied on days 1, 3, 5, 7, and 9. The experiment ended at 10 day after first treatment. Injected PMB dose was 5 ml/kg. According to tumor sensitivity to drugs, injected EPI dose was 10 mg/kg for HCT-116 cells. EPI solution (2 mg/ml) was prepared by dissolved EPI power in 0.9% NaCl. Ultrasound of 1 MHz was applied locally to the tumor for 30 s at a power density of 3 W/cm^2.

Inhibition on tumor growth study

Tumor volume was measured by a caliper 1 day after each treatment. Tumor volume was calculated based on the equation: $V = (l_1 + l_2) \times (w_1 + w_2)^2/16$, where ($w_1$, w_2) and (l_1, l_2) were width and length of the tumor, as measured twice by a caliper. The tumor volume relative growth rate at n day was calculated based on the equation:

$$\text{Tumor volume relative growth rate} = \left[(V_n - V_{\text{initial}})/(V'_n - V'_{\text{initial}})\right] \times 100\%,$$

where (V_n, V_{initial}) and (V'_n, V'_{initial}) were the tumor volume of test group and blank control measured respectively at n day and start of the treatment.

After the last treatment, tumor volume total growth rate was calculated according to the equation:

$$\text{Tumor volume total growth rate} = \left[(V_{\text{final}} - V_{\text{initial}})/(V'_{\text{final}} - V'_{\text{initial}})\right] \times 100\%.$$

After last measurement, animals of blank control and experiment groups were killed. Then tumors were dissected and weighed to determine tumor weight growth rate, which was based on the equation:

$$\text{Tumor weight growth rate} = \left(W_{\text{tumor,experiment}}/W'_{\text{tumor,blank}}\right) \times 100\%,$$

where $W_{\text{tumor, experiment}}$ and $W'_{\text{tumor, blank}}$ were the tumor weight of test groups and blank control, respectively.

Body weight change study

Growth rates of animals' body weight and normal organs' weight can be used to evaluate animals' survival status. Therefore, related data were noted during all the experiment. Growth rates of animals' body weight and normal organs' weight were calculated as following formulas.

$$\text{Body weight growth rate} = \left[(W_{\text{body, final}} - W_{\text{body, initial}})/W_{\text{body, initial}}\right] \times 100\%,$$

where $W_{\text{body, final}}$ and $W_{\text{body, initial}}$ were the animals' weight at the start and the end of treatment, respectively.

$$\text{Growth rate of normal organs} = \left[(W_{\text{body, final}} - W_{\text{tumor, final}}) - (W_{\text{body, initial}} - W_{\text{tumor, initial}})\right]/W_{\text{body, initial}} \times 100\%,$$

where $W_{\text{body, initial}}$ and $W_{\text{body, final}}$ were the animals' weight at the start and the end of treatment, respectively. $W_{\text{tumor, initial}}$ and $W_{\text{tumor, final}}$ were the tumor weight at the start and the end of treatment, respectively.

Statistical analysis

Statistically significant difference for multiple groups was determined using a one-way ANOVA with a Newman–Keuls post test. Statistical significance between individual groups was determined using a Student's *t* test. All testing was done using the SAS 8.01 (1999–2000, SAS Institute Inc, Cary, NC, USA). The data difference was considered to be statistically significant when the *P* value was less than 0.05.

Results and discussion

HCT-116 tumor model mice

HCT-116 cells were *s.c.* inoculated in axilla of male nude mice. HCT-116 tumor easily grew in the axilla cavity of male nude mice. By day 35 upon the *s.c.* inoculation, HCT-116 internal tumors were clearly visible in test mice (Fig. 1). The xenograft tumor in the axilla cavity of male nude mice had almost spherical shape and well-defined boundary, which was important for the purposes of this study.

Result of tumor volume growth during consecutive treatments

Tumor relative growth rate under five consecutive treatments was shown in Fig. 2. There was little significance

Fig. 1 Photograph of HCT-116 tumor model mice **a** before inoculation; **b** 35 day after inoculation

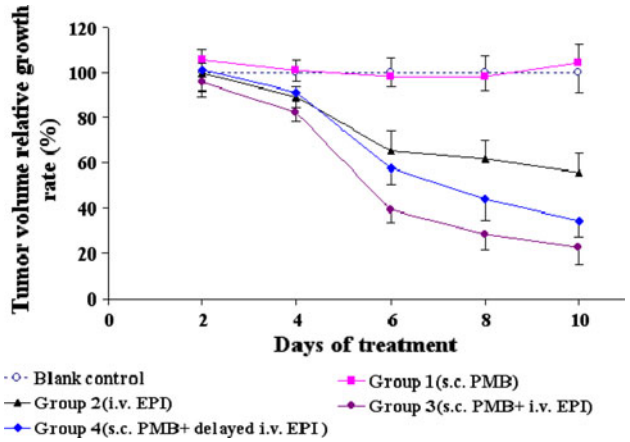

Fig. 2 Growth curves of HCT-116 tumors inoculated in male mice. EPI (10 mg/kg) was dissolved in 0.9% NaCl. Five consecutive treatments were applied on days 1, 3, 5, 7, and 9. Tumor volume was measured 1 day after each treatment. Ultrasound parameters: frequency—1 MHz; power density—3 W/cm^2, US duration—30 s

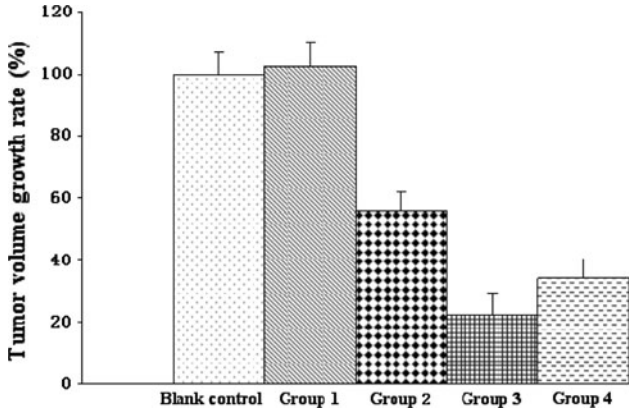

Fig. 3 *Vertical bar* chart of HCT-116 tumor volume growth inoculated in male mice. EPI (10 mg/kg) was dissolved in 0.9% NaCl. Ultrasound parameters: frequency—1 MHz; power density—3 W/cm^2, US duration—30 s

between blank control and Group 1 (*s.c.* PMB) ($P > 0.05$). Compared with blank control, however, Group 1 showed a decreasing trend in the first 3 days of treatment and an increasing trend in last 2 days.

Compared with blank control and Group 1, positive retardation effects on tumor volume growth rate were observed in test groups treated by EPI. This implied that retardation effect on tumor growth was resulted from the cytotoxic death of EPI rather than mechanical action of ultrasound and PMB.

As shown in Fig. 2, Group 3 (*s.c.* PMB + *i.v.* EPI) had the strongest inhibition on tumor volume growth, followed with Group 4 (*s.c.* PMB + delayed *i.v.* EPI). Group 2 (*i.v.* EPI) had the least effect on tumor volume growth among test groups treated by EPI. The difference between Group 3 and Group 2 was statistically significant ($P < 0.05$). These results indicated that PMB could enhance the acoustic cavitation and therefore induce drug transmembrane delivery, even under asynchronous administration of EPI and sonicated PMB.

From the profile slope of groups treated by EPI, Group 3 and Group 4 also showed advantages over Group 2. From data of profile slope, it suggested that acoustic cavitation induced by sonication in situ combined with PMB was not only strong but also very stable in facilitating the retardation effect of intravenous EPI on tumor growth.

Result of tumor volume total growth

Figure 3 showed the accumulated retardation effect of different groups on tumor volume growth. After five consecutive treatments, Group 3 (*s.c.* PMB + *i.v.* EPI) showed the strongest retardation effect on tumor volume growth among the test and followed with Group 4 (*s.c.* PMB + delayed *i.v.* EPI). The difference between Group 3 and Group 4 was not statistically significant ($P > 0.05$).

From the total growth rate profile of tumor volume, the order of inhibition on tumor volume growth was: Group 3

(*s.c.* PMB + *i.v.* EPI) > Group 4 (*s.c.* PMB + delayed *i.v.* EPI) > Group 2 (*i.v.* EPI) > Group 1 (*s.c.* PMB) ≈ blank control.

In our previous study [12], chemotherapeutic drugs can effectively enter cancer cells within a relatively long session even after the completion of acoustic cavitation caused by PMB eruption. There was a short-term lingering sensitive period on cell membrane for chemotherapeutic drugs. In this study, the promotion of Group 4 (*s.c.* PMB + delayed *i.v.* EPI) in tumor retardation validated the application of cavitation lingering sensitive period in enhancing the susceptibility of tumor to chemotherapeutic drugs.

The lingering sensitive period after acoustic cavitation might provide a potential application for chemotherapy under certain situation where sonication treatment and intravenous administration are not synchronously allowed.

Result of tumor weight growth

To reflect US-mediated inhibition on tumor weight growth, tumor weight growth rate was also evaluated. As shown in Fig. 4, the order of inhibition on tumor weight growth was similar to that of inhibition on tumor volume growth. Compared with blank control, Group 1 (*s.c.* PMB) showed little inhibition effect on tumor weight growth ($P > 0.05$). Group 3 (*s.c.* PMB + *i.v.* EPI) showed the strongest inhibition on tumor weight growth among the test and followed with Group 4 (*s.c.* PMB + delayed *i.v.* EPI). The difference between Group 3 and Group 4 was not statistically significant ($P > 0.05$).

From the result of tumor weight growth rate, it confirmed the practicality that using acoustic cavitation induced by PMB could enhance intravenous chemotherapeutic drugs entering tumor effectively, even under asynchronous administration of chemotherapeutic drugs and sonicated PMB.

Result of mice body weight growth

Growth rates of animals' body weight and normal organs' weight were shown in Figs. 5 and 6 respectively. From the data, especially from the growth rate of normal organs, animal survival status could be assessed.

Blank control and Group 1(*s.c.* PMB) showed positive effects in body weight growth and normal organs' weight growth, while EPI addition groups showed negative effects. Blank control and Group 1 (PMB + US) did not show statistical differences in animals' body weight growth and normal organs growth ($P > 0.05$). These results indicated that PMB did not change animal survival status.

For the three groups treated by EPI, the order of negative effect on animal body was Group 2 (*i.v.* EPI) > Group 4 (*s.c.* PMB + delayed *i.v.* EPI) > Group 3 (*s.c.* PMB + *i.v.* EPI).

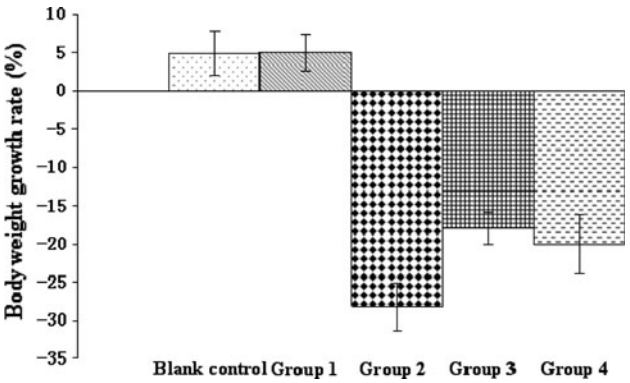

Fig. 5 *Vertical bar* chart of mice body weight growth. EPI (10 mg/kg) was dissolved in 0.9% NaCl. Ultrasound parameters: frequency—1 MHz; power density—3 W/cm^2, US duration—30 s

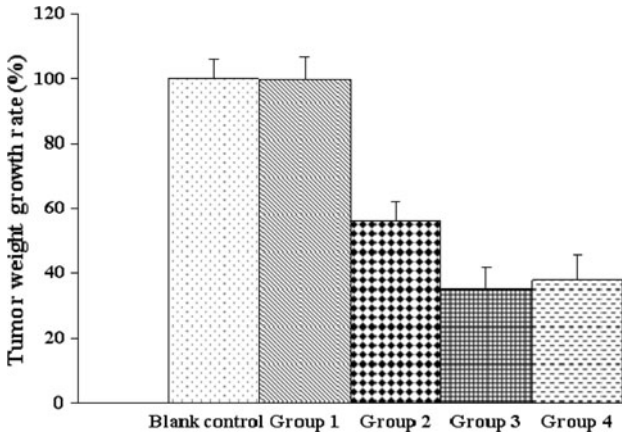

Fig. 4 *Vertical bar* chart of HCT-116 tumor weight growth inoculated in male mice. EPI (10 mg/kg) was dissolved in 0.9% NaCl. Ultrasound parameters: frequency—1 MHz; power density—3 W/cm^2, US duration—30 s

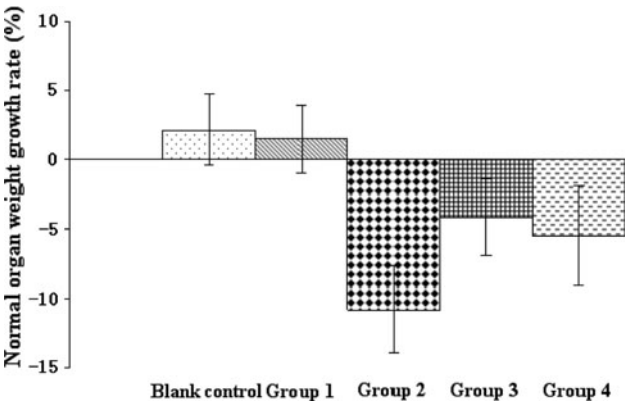

Fig. 6 *Vertical bar* chart of normal organs' weight growth. EPI (10 mg/kg) was dissolved in 0.9% NaCl. Ultrasound parameters: frequency—1 MHz; power density—3 W/cm^2, US duration—30 s

As shown in Fig. 5, the order of adverse effect on normal organs' weight was similar to the order of negative effect on animal body.

The decreased animal body weight for EPI administration groups might be resulted from two sides—tumor inhibition effect and adverse effects on normal organs of EPI. The side effects of EPI on normal organs include diarrhea, stomach pain, irregular heartbeat, and loss of appetite. All these side effects of EPI could cause decreased normal organ weight.

Considering both tumor inhibition and adverse effects on normal organs, the order of animal survival status for EPI administration groups was Group 3 (*s.c.* PMB + *i.v.* EPI) > Group 4 (*s.c.* PMB + delayed *i.v.* EPI) > Group 2 (*i.v.* EPI). From these results, acoustic cavitation induced by PMB could increase the tumor inhibition of chemotherapeutic drugs and reduce their adverse effect on normal organs.

Conclusion

Inhibition effects on tumor growth were observed in all groups treated by EPI. However, the potency of cavitation-enhanced drug uptake depended on the application of PMB in situ. An effective retardation on tumor growth and improved mice survival status were observed when intravenous EPI combined with sonicated PMB in situ. In this paper, short-term lingering sensitive period for chemotherapeutic drugs after acoustic cavitation was also observed, which corroborated our previous experiment in vitro. And this technique might provide a potential application for US-mediated chemotherapy under asynchronous administration of chemotherapeutic drugs and sonicated PMB. Further investigation with different chemotherapeutic drugs should be done to develop the lingering sensitive period in enhancing chemotherapy, especially sonication treatment and drug administration are not synchronously allowed.

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Conflict of interest The authors report no conflicts of interest.

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