Applied Acoustics 138 (2018) 216–225

Contents lists available at ScienceDirect

Applied Acoustics

journal homepage: www.elsevier.com/locate/apacoust

Ultrasound-mediated microbubbles destruction for treatment of rabbit VX2 orthotopic hepatic tumors

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ARTICLE INFO

Keywords:
Low frequency focused ultrasound
VX2 orthotopic hepatic tumor model
Ultrasound-mediated microbubble destruction
Contrast-enhanced CT
Autophagy

ABSTRACT

The aim of this study was to determine whether ultrasound (US) mediated microbubbles (MBs) destruction (UMMD) could inhibit VX2 orthotopic hepatic tumor growth in rabbit models. Twenty-four VX2 orthotopic hepatic tumor rabbit models were randomly divided into four groups (n = 6 each): saline group, SonoVue alone group, US alone group, US + SonoVue group. Tumor volume (TV), peak intensity (PI) of contrast enhanced ultrasound (CEUS) in US + SonoVue group were significantly lower than that in other groups after treatment ($P < 0.05$, for all). Immunohistochemical analysis of tumor tissue showed that microvascular density (MVD) in US + SonoVue group was significantly lower than that in other groups ($P < 0.05$, for all). Extensive interstitial hemorrhage, intravascular thrombosis, destroyed nucleus membrane, mitochondria vacuolation and chromatin condensation were observed in US + SonoVue group, whereas these changes were rarely appeared in other groups. Our study indicated that UMMD could inhibit the growth of VX2 hepatic tumors in rabbits by irreversible destroying tumor microvessel and tumor cells.

1. Introduction

Hepatic carcinoma ranks as the fifth most frequent cancer worldwide [1]. Because of the increasing incidence and high morbidity and mortality, it is still one of the most important disease for health care systems [2,3]. The main curative therapies for hepatic carcinoma are partial hepatectomy and liver transplantation. Both methods involve major complicated surgery and the recurrence rate are very high [1]. Meantime, a lifetime of immuno-suppressant drugs is essential to prevent from rejecting the new organ for patients who get a transplantation, which greatly reduces the quality of life.

The growth and development of tumor can be inhibited via blocking its angiogenesis which are closely related to cancer progression and metastasis. Compared with normal blood vessels, neovascularization within tumor has congenital developmental defects, such as incomplete endothelium, lack of an elastic fiber layer, high permeability, abnormal blood flow and delayed maturity [4], which provides an excellent opportunity for physical therapy of tumor. Based on this background, therapeutic ultrasound using microbubble-mediated ultrasonic cavitation has great potential for inhibit tumor growth through the biological and physical effects [5].

The anti-tumor drugs, delivered by ultrasound-mediated microbubbles destruction (UMMD) to penetrate the tumor parenchyma, have been studied recent years [6]. This approach makes it easy to not only reduce the systemic side effects of chemotherapeutic drugs but also improve the local accumulation of drugs, therefore enhanced therapeutic effects. In the recent decade, more and more researches [7–12] indicated that ultrasound mediated microbubble stable cavitation, as an anti-tumor drug delivery system, played a supplementary role in tumor therapy and its own mechanical effect to tumor was temporary and reversible, whereas the drugs played a key role in suppressing tumor growth. But some kind of tumor cells are known to be drug resistant and ultrasound mediated drug-carrying microbubbles have difficulties in hypoxic tumor therapy [13]. Though several studies had reported that UMMD alone could not only inhibit tumor growth in subcutaneous tumor mouse models [11,14–16], but also strengthen the therapeutic effect of tumor with drug-loaded or gene-loaded microbubbles [8,17,18]. The aim of this study was to assess whether low frequency focused ultrasound mediated microbubble destruction (UMMD) alone can cause permanent and irreversible damage to VX2 orthotopic hepatic tumor and inhibit tumor growth in rabbit models.

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https://doi.org/10.1016/j.apacoust.2018.04.007
Received 25 November 2017; Received in revised form 24 March 2018; Accepted 5 April 2018
Available online 14 April 2018
0003-682X/

2. Materials and methods

2.1. Preparation of VX2 orthotopic hepatic tumor model rabbit

Animal experiments were approved by the Ethics Committee of Laboratory Animal Welfare of Zhejiang University, Zhejiang, China. The experiment procedure met the criteria outlined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were designed to minimize the animals' suffering.

One VX2 tumor-bearing rabbit was obtained from Experimental Animal Center of Nanjing Medical University (Jiangsu, China). Thirty adult New Zealand white rabbits of clean grade (2.0–3.0 kg body weight, male) were purchased from Zhejiang Provence Academy of Medical Sciences (Zhejiang, China). The age of rabbit ranged from 12 months to 16 months. During the experiment, all rabbits were fed with standard fodder.

Before the experiment, 35 mg/kg 3% sodium pentobarbital (Sigma) were slowly injected through auricular vein to each rabbit, then rabbits were sustained a surgical plane. VX2 tumor tissue blocks were taken from VX2 tumor-bearing rabbit, washed with sterile PBS, removed the necrotic tissue and then subdivided viable tumor tissue into small pieces of 3–5 mm^3 for hepatic implantation. The recipient rabbits were prepared and draped the abdomen in standard surgical position. The implantation site was shaved and cleaned with an alcohol- or iodine-based agent. A vertically oriented subxyphoid mini-laparotomy incision was made with a blade. The peritoneum was exposed after blunt dissection through the avascular linea alba. The liver was visualized after carefully divided the peritoneum. One vertical stab point at the hepatic lobe was made with a trocar, roughly 2 cm deep. A single tumor fragment was then pushed into the parenchyma with the stylet of trocar. A small piece of gelatin sponge was subsequently covered the needle track to decrease bleeding. After implantation was finished, the abdominal incision was closed in fascial and cutaneous.

10 days after tumor implantation, contrast-enhancement computed tomography (CE-CT) was performed in each VX2 rabbit tumor model. Rabbits with tumor size about 1.0 cm were enrolled in this study (a total of 24 rabbit models), which were randomly divided into four groups: Saline group: intravenous saline only; SonoVue alone group: intravenous microbubbles only; US alone group: intravenous saline + low frequency focused ultrasound exposure; US + SonoVue group: intravenous microbubbles + low frequency focused ultrasound exposure.

2.2. Instruments and reagents

Low frequency focused ultrasound therapy apparatus, which was designed by the Institute of Technology Innovation Company of Zhejiang University (Zhejiang, China), composed of a multidimensional movement structural, a concave disk ultrasonic generator, degassed water balloon and an ultrasonic positioning probe. CT scanner was a 32-slice spiral CT (SOMATOM Definition Flash, Siemens, Forchheim, Germany). The parameters of CT were as following: 120 kVp; 200 mAs; slice thickness, 3 mm; matrix, 512 × 512; field of view, 15 cm. The ultrasound (US) apparatus was Esaote Mylab90 color Doppler ultrasonic diagnostic instrument (Italy) equipped with a LA322 linear array probe (frequency: 3–11 MHz), which had built-in real-time contrast-enhanced ultrasound (CEUS) imaging technology with low mechanical index (MI) of 0.04. The entire movie sequence was stored on magnetic disks for analysis.

The US contrast agent SonoVue (Bracco Company, Italy) was supplied with lyophilized powder; it was suspended by injecting 5 ml sterile saline and vigorously shaking the vial by hand to constitute a homogeneous microbubble suspension, which include about 2×10^8 sulfur hexafluoride-filled microbubbles per milliliter. The diameter of each microbubble was typically between 2–8 μm. Microbubbles (MBs) were reconstituted by shaking the vial completely before administered as a bolus injection of 0.6 ml through auricular veins to each rabbit.

All the rabbits were anesthetized with 3% sodium pentobarbital (Sigma) by slowly intravenous injection of 35 mg/kg during the performance of each US examination and treatment. The rabbit VX2 tumor models were sacrificed using an intravenous injection dose of sodium pentobarbital exceeding 390 mg/kg.

2.3. CE-CT, US and CEUS

VX2 rabbit models were anesthetized with pentobarbital before imaging procedure. CE-CT was performed (1) to determine whether tumor models were successfully established, (2) to display the tumor location, number, and size before treatment, (3) and also to assess the tumor perfusion before and at 7 days after treatment. The contrast medium Iopamiro (Bracco Company, Italy) was injected through auricular vein, 1 ml/s (2.0 ml/kg body weight).

US was used to assess tumor location, size, boundary, echogenicity (Fig. 1). When the tumor was displayed clearly, the ultrasound probe was held steady and remained at the same position, CEUS was performed after the injection of 0.6 ml of SonoVue (Bracco SpA, Milan, Italy) as a bolus via auricular vein using the CnTI imaging mode. A timer on the sonographic unit was activated at the beginning of the injection, and the entire movie sequence was stored on magnetic optical disks for analysis.

CEUS was performed to assess the enhancement pattern and enhanced intensity of each tumor before and at 1, 7, 14, 21 days after the treatment to monitor the tumor growth, tumor perfusion and evaluate the therapeutic efficiency of UMMD.

2.4. Experimental procedures

Rabbits in US alone group and US + SonoVue group received three consecutive daily cavitation treatments with the same acoustic conditions. The ultrasonic generator equipped with a degassed water balloon was placed over the tumor and coupled with coupling gel. The acoustic parameters which we used were based on our previous study [16] (Table. 1). Nine treatment points were selected at the tumor different sections which were located using ultrasonic positioning probe. Each point irradiation was performed fifteen times, a total of 150 s with a 10% duty cycle. As soon as the irradiation was activated, 0.6 ml 0.9% saline was injected intravenously to US alone group and 0.6 ml MBs was injected intravenously to US + SonoVue group at 0 s, 50 s, 100 s of each target point respectively. The rabbit in SonoVue alone group received three consecutive daily administrations of MBs with the same dose of US + SonoVue group and fake cavitation, in which the probe was placed over the tumor with coupling gel but not powered on. Saline group only received three consecutive daily administrations of 0.9% saline. The time line of this study was showed in Fig. 2.

2.5. Acoustic quantitative analysis

Tumor volume (TV) was calculated based on the formula: $TV = 1/2(a \times b^2)$, where a and b represent length and width of the tumor, respectively [19]. One board-certified abdominal radiologist with 5 years of CEUS experience reviewed cine loops off-line using TOMTEC Sono-Liver 1.1 software. A region of interest (ROI) was drawn around the margin of the contrast-enhanced tumor using an electronic-cursor, trying to avoid the normal liver parenchyma. The peak intensity (PI) in the tumor was calculated automatically by the system from the time-intensity-curve (TIC).

2.6. Histological examination

The tumor tissues were surgically resected for histological examination, and were cut along the maximum longitudinal plane. Tumor halves were fixed in 10% neutral formalin for more than 24 h, then they were washed with tap water. On the next day, they were dehydrated