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● *Original Contribution*

HEMOCOAGULASE COMBINED WITH MICROBUBBLE-ENHANCED ULTRASOUND CAVITATION FOR AUGMENTED ABLATION OF MICROVASCULATURE IN RABBIT VX2 LIVER TUMORS

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Abstract—We investigated a new method for combining microbubble-enhanced ultrasound cavitation (MEUC) with hemocoagulase (HC) atrox. Our goal was to induce embolic effects in the vasculature and combine these with an anti-angiogenic treatment strategy. Fourteen days after being implanted with a single slice of the liver VX2 tumor, rabbits were randomly divided into five groups: (i) a control group injected intra-venously with saline using a micropump; (ii) a group given only an injection of HC; (iii) a group treated only with ultrasound cavitation; (iv) a group treated with MEUC; (v) a group treated with MEUC + HC. Contrast-enhanced ultrasound was performed before treatment and 1 h and 7 d post-treatment to measure tumor size, enhancement and necrosis range. QontraXt software was used to determine the time–intensity curve of tumor blood perfusion and microvascular changes. At 1 h and 7 d after treatment with MEUC + HC, the parameters of the time–intensity curve, which included peak value, regional blood volume, regional blood flow and area under the curve value and which were measured using contrast-enhanced ultrasound, were significantly lower than those of the other treatment groups. The MEUC + HC treatment group exhibited significant growth inhibition relative to the ultrasound cavitation only, HC and MEUC treatment groups. No damage was observed in the surrounding normal tissues. These results support the feasibility of reducing the blood perfusion of rabbit VX2 liver tumors using a new method that combines MEUC and HC. (E-mail: 1091251425@qq.com) **© 2017 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Microbubble-enhanced ultrasound cavitation, Hemocoagulase, Microvascular, Rabbit VX2 liver tumor.**

INTRODUCTION

The vasculature associated with tumors serves as the source of oxygen for the tumor. It is well established that the aberrant characteristics of tumor vasculature contribute to tumor hypoxia (Dewhirst et al. 2008). Structural and functional alterations caused by radiation damage may further contribute to changes in tumor oxygenation (Dewhirst et al. 1990, 2008). Hence, cutting or blocking the tumor vasculature could inhibit tumor growth.

Sonoporation is the use of sound, usually at ultrasonic frequencies, to disrupt membranes. In combination with microbubbles, it has been used to permeate the cell membrane and the vascular wall. Membrane disruption and microvascular mechanical damage caused by sonoporation represent important biological effects of microbubble activation or cavitation, which relies on the magnitude of the expansion and collapse during bubble oscillation. This process can also generate significant light emissions, high pressure, fluid flow, shear stress, shock waves and microjets (Hwang et al. 2005; Marmottant and Hilgenfeldt 2003; Miller 2007; Wu 2007), which result in hemorrhage, edema and thrombus formation in various tissues. Cavitation includes either rapid growth and collapse of bubbles (inertial cavitation) or sustained oscillatory motion of bubbles (stable cavitation), both of which have strong physical, chemical and biological effects in tissues (Deckers and Moonen 2010; Treat et al. 2007). These

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effects are enhanced by the addition of microbubbles. Microbubbles can induce nucleate cavitation, increase ultrasonic absorption (Brayman and Miller 1999; Dalecki et al. 1997; Miller and Gies 1998; Wu 1998), reduce the cavitation threshold and enhance the effect of ultrasound cavitation (Li et al. 2003; Miller 2004; Miller and Gies 2000; Miller and Quddus 2000). Microbubble-enhanced ultrasound (MEUS) causes the so-called ultrasonic drilling effect (Forbes and O'Brien 2012; Karshafian et al. 2009; Sheikov et al. 2004), which has been recognized as a significant factor in the transient disruption of cell membrane permeability and allows easier transport of extracellular compounds into the cytoplasm of viable cells. MEUS, at a high-pressure amplitude, can also cause severe mechanically induced hemorrhage and damage to the endothelium of capillaries or small vessels (Chen et al. 2010; Li et al. 2004; Sheikov et al. 2004) in the liver, kidneys and brain. MEUS can also cause subsequent thrombosis, with acute shutdown of blood flow that induces necrosis and apoptosis of cancer cells and inhibits tumor growth (Burke et al. 2011; Chen et al. 2010; Li et al. 2004; Samuel et al. 2009; Sheikov et al. 2004; Shi et al. 2000; Tachibana et al. 2008; Wood et al. 2008, 2010). Selective blockage of regional blood flow is desirable and of clinical relevance because it can potentially be used therapeutically to block the circulation of a tumor.

Microbubble-enhanced ultrasound is also under investigation as a method for delivering anti-cancer agents to tumor tissues (Deckers and Moonen 2010). Hemocoagulase (HC) atrox (Nuokang Bio-Pharmaceutical, Shenyang, China), which is distilled from the Brazilian spearhead agkistrodon snake, is a tolerable and effective method of hemostasis. HC atrox is a serine protease that clots fibrinogen and can successfully stop local blood loss (Lv et al. 2008). It has been widely used and plays an important role in plastic surgery, abdominal surgery and human vitrectomy. Patients were given 1 kU of HC intra-venously or intra-muscularly 30 min before the operation (Kudo et al. 2009; Miller et al. 1996). The anti-tumor effects of snake venom are expressed in two ways: by killing tumor cells directly or by penetrating and interfering with the operating mechanisms of the cell membrane *via* the enzyme in the cytotoxic venom. The Premzl research group isolated the secreted phospholipase ammodytoxin from the venom. Ammodytoxin killed tumor cells directly by binding to protein B, which is found on the surface of tumor cells (Premzl et al. 2008). However, because the rim of the tumor is less affected by the treatment and acts as a site for revascularization and regrowth of the tumor in large part because of the vascular rebound effect, HC as a monotherapy has exhibited only limited effectiveness in achieving sustained anti-tumor effects.

The Li research group reported that the non-invasive diagnosis and treatment of hemorrhage can increase the destruction caused by microbubble-enhanced ultrasound cavitation coupled with the hemocoagulase liposome complex (Li et al. 2014).

In the study described here, our goal was to find a tolerable, effective way to combine ultrasound cavitation with HC using the microvascular-blocking effects of microbubbles. To our knowledge, no similar study has been reported in the literature.

METHODS

Experimental design

We performed the study on 60 New Zealand rabbits weighing 2.5 to 3.0 kg, regardless of gender. They were purchased from the Experimental Animal Center of the Fourth Military Medical University (FMMU) and fed a standard laboratory diet and tap water *ad libitum*. The experiments on laboratory animals were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the FMMU, Xi'an, China. Fourteen days after tumor implantation, 60 rabbits with palpable tumors (approximately 1–1.5 cm) were randomly divided into five groups (12 rabbits/group): (i) control group; (ii) HC injection-only group; (iii) ultrasound cavitation (UC) treatment group; (iv) microbubble-enhanced ultrasound cavitation (MEUC) treatment group; and (v) MEUC + HC treatment group. Conventional gray-scale ultrasound and contrast-enhanced ultrasound (CEUS) were performed before treatment and 1 h, 1 d and 7 d post-treatment. Liver specimens were evaluated histologically. Twelve rabbits in each group were euthanized 1 d (6 rabbits) and 7 d (6 rabbits) after the different treatments.

Rabbit VX2 liver tumor model

As described previously (Luo et al. 2009), we established the rabbit VX2 tumor liver model: We removed the VX2 tumor tissue from the thigh of a tumor-bearing rabbit and cut it into 3- to 4-mm^3 cubes under sterile conditions. The 60 recipient animals were anesthetized with an intramuscular injection of 2% pentobarbital at 0.2 mL/kg, and their upper abdomens were shaved in preparation for a preliminary ultrasound (MyLab 40, Esaote, Genoa, Italy) examination performed using an LA532 liner array transducer, to determine the target implantation site within the liver. Next, the tumor that had been placed in normal saline was sliced into 1- to 2-mm^3 fragments. We used a 17-gauge, 7.8-cm co-axial introducer, which has a hollow core and a sharp, blunt inner stylet (Fig. 1a). The sharp inner stylet was removed, and a small tumor fragment (1–2 mm^3) together with a gelatin sponge of the same

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