Ultrasound in Med. & Biol., Vol. 42, No. 7, pp. 1706–1713, 2016

0301-5629/$ - see front matter

ELSEVIER

● *Letter to the Editor*

ENHANCEMENT OF FLUORESCENT PROBE PENETRATION INTO TUMORS *IN VIVO* USING UNSEEDED INERTIAL CAVITATION

(*Received* 18 *June* 2015, *revised* 18 *January* 2016, *in final form* 27 *January* 2016)

Abstract—Ultrasound-induced cavitation has found many applications in the field of cancer therapy. One of its beneficial effects is the enhancement of drug intake by tumor cells. Our group has developed a device that can create and control unseeded cavitation in tissue using ultrasound. We conducted experiments on tumor-bearing mice using our device to assess the impact of sonication on the penetration of fluorescent probes into tumor cells. We studied the influence of pressure level, timing of sonication and sonication duration on treatment efficiency. Our results indicate that fluorescent probes penetrate better into tumors exposed to ultrasound. The best results revealed an increase in penetration of 61% and were obtained when sonicating the tumor in presence of the probes with a peak negative pressure at focus of 19 MPa. At this pressure level, the treatment generated only minor skin damage. Treatments could be significantly accelerated as equivalent enhanced penetration of probes was achieved when multiplying the initial raster scan speed by a factor of four. © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Cavitation, Sonoporation, Probe, Fluorescence, Melanoma.**

INTRODUCTION

Ultrasound cavitation is an essential mechanism involved in the therapeutic local enhancement of drug delivery by ultrasound for cancer treatment (Mo et al. 2012). Two types of cavitation can be defined. Stable cavitation involves stable oscillations of the bubble radius as the pressure wave passes through the medium. It generates shear forces and microstreaming. Inertial cavitation appears as the ultrasonic intensity increases. It makes the bubbles implode and generate shock waves and large pressures and temperatures (Pitt et al. 2004; Wu and Nyborg, 2008). Both types of cavitation have been reported to have an effect on drug or gene delivery (Miller et al. 1996; Pitt et al. 2004).

A prerequisite to cavitation is the presence of bubbles. Many methods involve ultrasound contrast agents (UCAs) used in diagnostic ultrasound (Mo et al. 2012; Tran et al. 2007). These microbubbles can be targeted to specific sites by adding ligands on the bubble shell. Drug-loaded nanoparticles can also be attached to the bubbles (Unger et al. 2002), allowing targeted and local delivery of therapeutic agents (Zhou, 2013).

Injection of micron-sized contrast agents not only facilitates the inception of cavitation using low-pressure amplitude but also allows a visualization of the treatment using conventional ultrasound contrast imaging. However, UCAs have some limitations. They have a limited lifetime because of their limited stability after injection. Their micron-order size also limits their extravasation and prevents them from reaching the center of the tumor (Wagstaffe et al. 2012). Alternatives to this limitation have been proposed in the form of smaller nanoparticles as small as 200 nm (Wagstaffe et al. 2012). Both technologies, however, present a limitation in their penetration into the tumor. Their use also adds an extra stage and cost to the treatment. Finally, the use of bubbles originally designed for imaging purposes implies regulatory issues and long-term evaluation for clinical application and marketing in a therapeutic purpose.

Our approach considers the inception of inertial cavitation without using external cavitation-enhancing nuclei, so-called "unseeded cavitation." This was made possible by using confocal transducers that allow a tight spatial control of the volume where cavitation can appear (Prieur et al. 2015b). The setup is also able to generate very high pressure amplitudes at focus, while keeping low pressure levels outside the focal region. Because our method does not require the injection of cavitation-enhancing particles, it is simpler and less dependent on the volume or vascularization of the tumor.

In this article we describe *in vivo* experiments on tumor-bearing mice. The goal was to estimate the effect of ultrasound exposure on the penetration of molecules into tumor cells. In the context of ultrasound-enhanced drug delivery, we used fluorescent probes in place of therapeutic molecules and compared their penetration into tumor cells with and without ultrasound exposure. We studied the influence of the pressure level, the timing of the sonication, and the exposure duration as previous studies using contrast agents have indicated that these factors could influence the uptake of molecules (Afadzi et al. 2013; Lentacker et al. 2014; Qiu et al. 2010).

METHODS

Ultrasound system

The ultrasound system consists of two confocal transducers placed with an angular separation of 66° (Caviskills, Vaulx-en-Velin, France). Each transducer is a spherical cap 50 mm in diameter, with a radius of curvature of 50 mm. They are truncated in one direction for better accessibility, reducing their dimension to 40 mm in this direction (Fig. 1). Their center frequency is 1 MHz.

This setup generates a small focal spot at the intersection of both acoustical axes (approximately $2 \times 2 \times 2$ mm) where the cavitation activity is created. The interaction of the pressure fields generated by each transducer also creates an interference pattern that "traps" the bubbles at the nodes and antinodes. These features provide a way to tightly control where the cavitation cloud appears

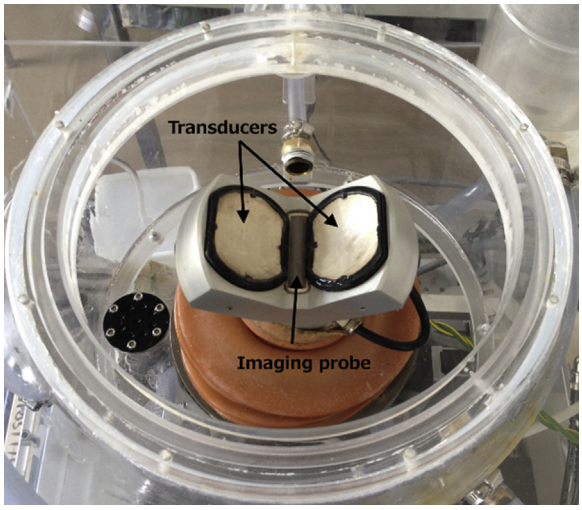

Fig. 1. Confocal transducers and ultrasound imaging probe inside the water tank.

and to keep it in a confined volume. Between both transducers, an HL9.0/40/128Z imaging probe (Telemed, Vilnius, Lithuania) is fixed. Its imaging plane contains the focal spot and is parallel to the truncation direction (Fig. 1).

The assembly containing the transducers and the imaging probe is immersed in a tank filled with degassed water and can be positioned with motors ensuring translation in the three directions. Defined volumes can therefore be sonicated by moving the transducer assembly while the animal lies still.

On top of the water tank, an animal can be placed with the area to be treated facing down toward the transducers (Fig. 2). With an Echo Blaster ultrasound machine (Telemed, Vilnius, Lithuania), the volume to be treated can be visualized and the range of movement in each direction defined. The treatment itself consists of continuous raster scans in horizontal planes. Successive planes with the correct depth separation ensure uniform and complete coverage of the volume to be treated. The treatments are controlled by a computer that synchronizes the transmission of ultrasound with the mechanical movements and ensures a constant speed for the uninterrupted raster scans. The raster scan speed was set to 1 mm/s except in the last test series, for which it was varied. The signal applied to the transducers is a 40-μs-long sinusoidal pulse with a pulse repetition frequency of 250 Hz corresponding to a 1% duty cycle. The raster scan speed and the signal amplitudes are two adjustable parameters that were varied in our experiments.

Fig. 2. Mouse in place under isoflurane anesthesia and being sonicated.

Animals, tumor model, and fluorescent probes

Homozygous female athymic nude mice of the Swiss nu/nu strain (Charles River, Saint-Germain-sur-l'Arbresle, France) were used for a subcutaneous implantation of WM-266-4 human metastatic melanoma xenografts. Tumors were exposed to ultrasound about 20 days after cell implantation, when solid tumors had reached a volume about 500–1000 mm^3 (measured by calipers and estimated as $= 0.5$ [length $\times$ width^2]). All cavitation experiments were conducted under isoflurane gas anesthesia. The animals were handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* and European Directive EEC/86/609, under the supervision of the authorized investigators.

We used the fluorescent probe AngioSense 680 EX (PerkinElmer, Waltham, MA). This probe is a blood pool imaging agent primarily used to study angiogenesis in tumor models but due to vasculature leakage and enhanced permeability at tumor sites it has been shown to extravasate into tumor tissue (Peterson, 2011). It comes in a powder that was dissolved in 600 μL of sterile phosphate-buffered saline to get a final concentration of 4 nmol/100 μL. Injections were done intravenously following the manufacturer's procedure with volumes of 100 μL which corresponded to 4 nmol of probe. The probe weight is about 70,000 g/mol, and the quoted excitation and emission wavelength are 670 and 690 nm, respectively.

A minimum of three animals were included in all groups. A larger number of animals per group would have been beneficial to strengthen the statistics but this was not possible because of the prohibitive cost of the fluorescent probes.

Ultrasound exposure conditions

The timing of the injection, the ultrasound exposure and the sacrifice varied between the two protocols that were defined. In protocol 1, the tumors were sonicated 30 min after injection and the animals were sacrificed 10 min after exposure to ultrasound. In protocol 2, the tumors were sonicated 10 min before injection, and the animals were sacrificed 30 min after injection.

According to Peterson (Peterson, 2011), the recommended timing for tissue imaging when probes have accumulated in tissue is 24 hours post-injection, whereas the time required for the probes to be present in the vasculature after intravenous injection is 30 min. In our tests, we want to minimize the passive penetration of probes to better show the effect of ultrasound cavitation on probe penetration. We chose therefore to wait no more than 30 min after injection for tissue analysis—long enough for the probes to be in the vasculature, but not for them to accumulate in tissue passively.

Three series of tests were conducted. In the first and last series, protocol 1 was used, whereas the second series of tests compared both protocols. The exposure conditions are summarized in Table 1.

The first series of tests aimed at estimating the effect of ultrasound exposure on the penetration of AngioSense 680 EX when varying the peak negative pressure $p-$ created at focus. Local toxicity of the treatment at each pressure level was also estimated. The quoted peak negative pressures were estimated in water using a FOPH 2000 optical hydrophone (RP Acoustics, Leutenbach, Germany). In the article we focus on the negative pressure and refer to its level as a positive value for simplicity. A greater value refers to a larger rarefactional pressure.

In the second series of tests, the influence of injection timing was estimated by comparing the two protocols. Previous works had reported that the permeabilization of the endothelium or of the plasma membrane induced by ultrasound cavitation could last several hours (Choi et al. 2007; Hynynen et al. 2005; Yudina et al. 2011). We wanted to see if our ultrasound conditions allow