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● *Original Contribution*

MICROVASCULAR INJURY AND PERFUSION CHANGES INDUCED BY ULTRASOUND AND MICROBUBBLES IN A MACHINE-PERFUSED PIG LIVER

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Abstract—Localized drug delivery and uptake can benefit from the combined action of ultrasound and microbubbles at a specific site. Some of the possible mechanisms suggested are vessel poration and/or cell poration, but the exact acoustic parameters that trigger those phenomena remain unknown. *Ex vivo* machine perfusion of human-sized organs is a technique that provides an ideal environment for pre-clinical investigations with high physiologic relevance not possible with *in vitro* experiments. In this work, *ex vivo* machine-perfused pig livers were combined with an image-guided therapy system to investigate microvascular flow changes caused by the interaction of ultrasound-driven microbubbles with the vasculature. The effects of acoustic pressure (1.7–4 MPa peak negative pressures) and number of cycles (1000 or 20 cycles) were examined. Perfusion changes caused by the action of ultrasound on microbubbles in the microcirculation were qualitatively and quantitatively assessed with contrast-enhanced ultrasound and used as a metric of the extent of vessel perforation, thus, extravasation. Areas that were exposed to peak negative pressures above 1.7 MPa underwent a detectable and irreversible perfusion change. Complete devascularization of the area exposed to ultrasound was observed at much larger acoustic pressures (∼4 MPa). Shorter acoustic pulses (20 cycles) produced markedly fewer perfusion changes than longer pulses (1000 cycles) under the same acoustic amplitude exposure. (E-mail: maverk@uw.edu) **© 2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Sonoporation, Extravasation, Machine-perfused pig liver, Drug delivery, Vessel poration.**

INTRODUCTION

Microbubbles are currently considered one of the most promising and powerful theranostic tools used in localized drug delivery research. Several published studies report that the physical interactions of microbubbles with ultrasound have the potential to enhance drug delivery by altering cell membrane permeability, a process referred to as *sonoporation*, allowing the uptake of normally impermeable molecules into the cells (Böhmer et al. 2009; Lentacker et al. 2013). Sonoporation has been extensively studied both *in vitro* (using multiple types of cell cultures) (Forbes et al. 2008; Lammertink et al. 2015; van Wamel et al. 2006) and *in vivo*, mainly in small animal tumor models (Couture et al. 2012; Kotopoulis et al. 2014). It was found that compounds <1 kDa (Keyhani et al. 2001), macromolecules (Guzmán et al. 2002) and genes (Shen et al. 2008; Song et al. 2011) were successfully delivered into tumor cells. However, despite more than 15 y of research, only one small pilot clinical study has been published that involved ultrasound and microbubble-enhanced drug delivery in pancreatic cancer (Kotopoulis et al. 2013).

The translation of pre-clinical research into widely accepted clinical practice is still a great challenge. This is partially because contrary to *in vitro* studies, in which tumor cells are directly exposed to therapeutics and microbubbles, in *in vivo* applications, the microbubbles and drugs have to escape the systemic circulation and extravasate into the interstitial space to reach the tumor cells (Böhmer et al. 2010). To achieve a significant amount of extravasation, oscillating microbubbles need to modify the structure of the endothelial cells in the capillaries feeding the tumor. Increasing the endothelial

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openings allows therapeutics to enter the tumor interstitial space. However oscillating microbubble activity in capillaries may also affect local perfusion. It was previously reported that cavitation of microbubbles exposed to moderate intensities (peak negative pressure $<$1.7 MPa and $<$100 cycles) is capable of locally increasing perfusion (Belcik et al. 2015), whereas inertial cavitation in capillaries induces microvascular damage, leading to perfusion shutdown (Goertz et al. 2012; Hwang et al. 2006). The fine balance between significant extravasation *in vivo* and extensive vascular damage is yet to be explored.

Research performed using small animal tumor models also has some disadvantages resulting mainly from animal size. In small animals (usually mice), the penetration depth is limited, and the acoustic field distribution (that eventually triggers microbubbles) significantly differs from ultrasound diffraction and attenuation patterns in the human body. Additional considerations are the establishment of microbubble treatment dosages because of the small blood volume of the animals and establishment of the proper injection protocol. The need to investigate basic drug delivery mechanisms in an environment that closely resembles the *in vivo* conditions is therefore essential before clinical studies.

An alternative and versatile test platform for performing pre-clinical research is machine-perfused organs, developed primarily to benefit organ transplantation. Specifically, machine perfusion (MP) is a method of sustaining an isolated organ alive outside the body (*ex vivo*) by supplying it with oxygen and nutrients. Izamis et al. (2014) developed and evaluated a sub-normothermic human-sized machine perfusion system suitable for preserving slaughterhouse pig livers. Using biochemical and hemodynamic measurements and contrast-enhanced ultrasound (CEUS), they found that the developed model is a simple, cost-effective approach for at least 3 h of stable, *ex vivo* whole-organ preservation. Because MP enables several hours of controlled and stable experimentation with the organ of interest, while maintaining high physiologic relevance that is lacking in *in vitro* experiments, it is an ideal environment for pre-clinical diagnostic, imaging and therapeutic investigations.

We hypothesized that the combined effects of ultrasound and microbubbles cause vessel poration, which leads to local perfusion changes. Thus, the objective of this work was to measure relative perfusion changes with CEUS quantification in a functioning *ex vivo* machine-perfused pig liver injected with microbubbles and exposed to different acoustic conditions. An experimental setup was developed that allowed real-time monitoring of ultrasound exposure at the area of interest and imaging of the cavitation activity. The effects of acoustic pressure amplitude and pulse length on liver perfusion changes were evaluated with qualitative and quantitative CEUS and were correlated with the degree of vessel perforation and extravasation.

METHODS

Ex vivo *machine perfusion of porcine livers*

The study was approved by the Cyprus National Bioethics Committee and the Cyprus National Veterinary Services. Healthy porcine livers were procured from a local slaughterhouse. Details on liver procurement can be found elsewhere (Izamis et al. 2014). Briefly, immediately after death of the animals, the livers were isolated from the rest of the thoracic and abdominal organs at the slaughterhouse and were flushed with 8 L of lactated Ringers (6 L room temperature and 2 L ice cold) through the portal vein (PV) and hepatic artery (HA).

The organs were then stored on ice and transported to the laboratory (the duration of static cold storage was $<$1 h), where they were connected to the developed sub-normothermic machine perfusion system (Izamis et al. 2014) (Fig. 1a) and sustained for up to 4 h. The perfusion system comprised a 6-L perfusate reservoir (Powdered Williams Medium E [10.8 g/L W4125, Sigma-Aldrich, St Louis, MO, USA] to which had been added 2.2 g/L sodium bicarbonate [S5761, Sigma-Aldrich, Medisell, Cyprus], 1000 IU/L heparin, 2 U/L insulin [Actrapid Penfill Novo Nordisk, Novo Alle, Denmark] and 0.4 mg/L dexamethasone [Dexamed, Medochemie, Limassol, Cyprus]), in which the organ was suspended. A pump (Masterflex L/S Digital Drive 600 rpm, Cole Parmer) circulated the perfusate through an oxygenator (Affinity NT, Medtronic, Minneapolis, MN, USA) supplied with 95% O_2, 5% CO_2 (Tenaris, Bergamo, Italy) before being split and passed through two flowmeters (EW-32461-44 and EW-32460-40, Cole Parmer) that enable pressure-regulated flow to each of the PV and HA, respectively. Effluent was then allowed to flow freely from the liver's vena cava back into the reservoir, closing the circuit. Flow rate was set to 800 mL/min for the PV and 400 mL/min for the HA. The selected flow rates ensured that PV pressure was $\leq$10 cm H_2O and HA pressure was between 60 and 100 cm H_2O. Hepatic stability was ascertained with 1-h interval measurements of bile production, oxygen consumption and sustained vascular perfusion as described in Izamis et al. (2014). The perfusion was monitored *via* CEUS imaging (Fig. 1b, c). The circuit provided individual syringe port access to each of the hepatic vessels so that ultrasound contrast agents could be administered to either the PV or HA.