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● *Original Contribution*

MEASURING ABSOLUTE BLOOD PERFUSION IN MICE USING DYNAMIC CONTRAST-ENHANCED ULTRASOUND

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Abstract—We investigated the feasibility of estimating absolute tissue blood perfusion using dynamic contrast-enhanced ultrasound (CEUS) imaging in mice. We developed a novel method of microbubble administration and a model-free approach to estimate absolute kidney perfusion, and explored the kidney as a reference organ to estimate absolute perfusion of a neuroblastoma tumor. We performed CEUS on the kidneys of CD1 nude mice using the VisualSonics VEVO 2100 imaging system. We estimated individual kidney blood perfusion using the burst–replenishment (BR) technique. We repeated the kidney imaging on the mice after a week. We performed CEUS imaging of a neuroblastoma mouse xenograft tumor along with its right kidney using two sets of microbubble administration parameters to estimate absolute tumor blood perfusion. We performed statistical tests at a significance level of 0.05. Our estimated absolute kidney perfusion (425 ± 123 mL/min/100 g) was within the range of previously reported values. There was no statistical difference between the estimated absolute kidney blood perfusions from the 2 wk of imaging (paired *t*-test, $p = 0.09$). We estimated the absolute blood perfusion in the neuroblastoma tumor to be 16.49 and 16.9 mL/min/100 g for the two sets of microbubble administration parameters (Wilcoxon rank-sum test, $p = 0.6$). We have established the kidney as a reliable reference organ in which to estimate absolute perfusion of other tissues. Using a neuroblastoma tumor, we have determined the feasibility of estimating absolute blood perfusion in tissues using contrast-enhanced ultrasound imaging. (E-mail: andras.sablauer@stjude.org) **© 2017 The Authors. Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).**

Key Words: **Contrast-enhanced ultrasound, Absolute blood perfusion, Burst–replenishment.**

INTRODUCTION

With the advent of personalized therapies, the need for longitudinal monitoring of pathologic tissues in clinical and translational settings to compare the efficacy of therapies has gained prominence. Contrast-enhanced ultrasound (CEUS) is a relatively recent, cost-effective technique compared with more traditional imaging modalities like magnetic resonance imaging (MRI) and computed tomography (CT). CEUS imaging has many applications in cardiology (Kaufmann et al. 2007; Kaul 2008) and radiology (Chung and Kim 2015; Wilson and Burns 2010; Wilson et al. 2009), with a focus on cancer and peripheral vascular disease, where the estimation of microvascular density (blood volume per unit mass) and blood perfusion (blood flow per unit mass) is particularly important.

Contrast-enhanced ultrasound imaging involves the injection of gas-filled micron-sized bubbles (microbubbles) that do not extravasate. This property makes them ideal contrast agents for imaging vascularity and blood perfusion (Walday et al. 1994; Yanagisawa et al. 2007), allowing longitudinal studies of drug therapies, for example, anti-angiogenic treatment (Guibal et al. 2010; Zhou et al. 2011). Non-linear CEUS uses multiple US pulses with varying amplitude or phases to subtract linear harmonic response from tissue and capture the non-linear subharmonic (Needles et al. 2010) response of the microbubbles in blood, effectively creating a blood-to-tissue contrast. This non-linear signal is proportional to the density of microbubbles in blood (Greis 2011; Lampaskis and Averkiou 2010), allowing relative estimation of blood density and vascular volume in the tissue. The linear

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relationship between signal intensity and microbubble concentration is limited by the biophysical properties of the microbubbles (*e.g.*, size) and the post-processing of the raw ultrasound signal by the ultrasound equipment manufacturer.

There are two widely established methods for blood perfusion estimation using non-linear CEUS imaging: (i) the bolus perfusion (BP) method and (ii) the burst–replenishment (BR) method. In the BP method, imaging occurs after the intra-venous administration of microbubbles as a bolus. The linearized signal from a region of interest (ROI) on the resulting image is then fit using a model to estimate blood perfusion parameters. The estimated blood perfusion parameters are sensitive to the total volume of microbubbles administered and the rate of injection (Needles et al. 2010). The BP method is useful for comparative studies and does not provide absolute blood perfusion of the tissues (Feingold et al. 2010; Mahoney et al. 2014; Needles et al. 2010).

In the BR method, imaging occurs after achieving a constant concentration of microbubbles (steady state) in blood in the tissue of interest after the continuous intravenous administration of microbubbles at a constant rate. A destructive US pulse (called the burst) is applied to the imaging plane, which destroys the microbubbles in the US beam path. Subsequently, blood in the US beam path is replenished with microbubbles in blood from adjacent tissue regions. This replenishment kinetic is then fit using a model to estimate blood perfusion parameters. The mono-exponential BR model is a widely used empirical model. More detailed mathematical BR models have been developed that take into account the shape of the ultrasound beam and blood vessel network architecture to provide more realistic blood perfusion estimates (Arditi et al. 2006; Hudson et al. 2009; Krix et al. 2003; Meyer-Wiethe et al. 2005; Potdevin et al. 2004). The BR method has been used to measure relative perfusion parameters *in vivo* (Schlosser et al. 2001; Yankeelov et al. 2006) and absolute flow parameters *in vitro* (phantoms) (Chen et al. 2009).

The success of the BR method is contingent on maintaining a steady-state microbubble concentration in blood in the tissue of interest during image acquisition. This requirement is critical for studies that use the microbubble concentration to estimate blood density in the tissue using the microbubble concentration of a reference organ or an artery, especially when the tissue of interest and the reference are imaged at different times. The current recommendation for the tolerable maximum volume of fluid injections in mice is 12 mL/kg over 5–10 min (Hau and Schapiro 2010; Morton et al. 2001; Turner et al. 2011). This limits the time available to achieve steady state and acquire images to 5 min using the recommended rate of constant infusion (VisualSonics). Some studies have been done that have relaxed the requirement for steady state to overcome this limitation (Jirik et al. 2013; Loveless et al. 2008; Paprottka et al. 2015; Yankeelov et al. 2006). The duration of imaging in these studies is limited by the clearance rate of microbubbles.

In general, non-linear CEUS imaging is limited to detecting changes in perfusion parameters compared with a baseline (Lucidarme et al. 2003; Mahoney et al. 2014; Sullivan et al. 2009; Yankeelov et al. 2006). Despite the wide use of the BR method in large animal models (dogs, sheep, rabbits) (Schneider et al. 2014), BR methods appropriate for mice have not been established (Hyvelin et al. 2013). Doppler imaging is used for measuring velocity of blood in large vessels ($>$1 mm), and it is not sensitive to slower blood flows in tissues at the capillary scale and has angular dependence (Evans et al. 2011). Techniques used for measuring tissue perfusion (invasive flowmeter, microsphere technique) in rats and larger laboratory animals are challenging in mice because of their smaller size (Prinzen and Bassingthwaighte 2000; Sullivan et al. 2009; Yankeelov et al. 2006).

In this study, we investigated the possibility of using CEUS to measure absolute blood perfusion of tissues in mice. Our approach is to characterize blood perfusion of individual kidneys and use the kidney as the reference organ to normalize perfusion measurements of other tissues (Fig. 1). We have illustrated our approach by measuring blood perfusion in a subcutaneously implanted neuroblastoma tumor. Accurate estimation of tumor blood perfusion is important as recent studies have indicated the beneficial effect of normalizing tumor vasculature and blood perfusion on pediatric tumor response to therapy (Dickson et al. 2007a, 2007b; McGee et al. 2010). We imaged multiple image planes sampling the 3-D tissue volume following the recommendations of recent studies (Feingold et al. 2010; Mahoney et al. 2014).

METHODS

Study design

Kidney blood perfusion. We imaged the kidneys of 10 10-wk-old CD-1 nude mice (Charles River Laboratories, Wilmington, MA, USA) using the Vevo 2100 system (FujiFilm VisualSonics, Toronto, ON, Canada) to estimate absolute kidney blood perfusion. We performed non-linear CEUS imaging (Needles et al. 2010) on both kidneys of each mouse twice, a week apart, to test the repeatability of the kidney perfusion measurements. We used the VEVO MS-250 transducer (FujiFilm VisualSonics) at 18 MHz with the abdominal pre-set. We anesthetized the mice inside a chamber using 3% isoflurane in oxygen before imaging and maintained anesthesia during imaging with