Abstract—Focused ultrasound, in the presence of microbubbles, has been used non-invasively to induce reversible blood–brain barrier (BBB) opening in both rodents and non-human primates. This study was aimed at identifying the dependence of BBB opening properties on polydisperse microbubble (all clinically approved microbubbles are polydisperse) type and distribution by using a clinically approved ultrasound contrast agent (Definity microbubbles) and in-house prepared polydisperse (IHP) microbubbles in mice. A total of 18 C57 BL/6 mice (n = 3) were used in this study, and each mouse was injected with either Definity or IHP microbubbles via the tail vein. The concentration and size distribution of activated Definity and IHP microbubbles were measured, and the microbubbles were diluted to $6 \times 10^8$/mL before injection. Immediately after microbubble administration, mice were subjected to focused ultrasound with the following parameters: frequency = 1.5 MHz, pulse repetition frequency = 10 Hz, 1000 cycles, in situ peak rarefractional acoustic pressures = 0.3, 0.45 and 0.6 MPa for a sonication duration of 60 s. Contrast-enhanced magnetic resonance imaging was used to confirm BBB opening and allowed for image-based analysis. Permeability of the treated region and volume of BBB opening did not significantly differ between the two types of microbubbles ($p > 0.05$) at peak rarefractional acoustic pressures of 0.45 and 0.6 MPa, whereas IHP microbubbles had significantly higher permeability and opening volume ($p < 0.05$) at the relatively lower pressure of 0.3 MPa. The results from this study indicate that microbubble type and distribution could have significant effects on focused ultrasound-induced BBB opening at lower pressures, but less important effects at higher pressures, possibly because of the stable cavitation that governs the former. This difference may have become less significant at higher pressures, where inertial cavitation typically occurs. (E-mail: ek2191@columbia.edu) © 2014 World Federation for Ultrasound in Medicine & Biology.

Key Words: Blood-brain barrier opening, Focused ultrasound, Microbubble type, Microbubble distribution.

INTRODUCTION

One of the main obstacles in the treatment of neurodegenerative diseases (e.g., Parkinson’s disease, Alzheimer’s disease) is the blood-brain barrier (BBB). Although the primary function of the BBB is to prevent toxins from entering the brain parenchyma, it also impedes the delivery of therapeutic agents $\geq 400$ Da (Pardridge 2005). Different strategies have been proposed to temporarily disrupt the BBB, including hyper-osmolar solutions (such as mannitol) and focused ultrasound (FUS) in combination with microbubbles. In contrast to the hyper-osmolar methods, FUS in the presence of microbubbles was found to be the only non-invasive approach capable of temporarily opening the BBB in the targeted region (Choi et al. 2007; Hynynen et al. 2001). With the use of carefully selected acoustic parameters, FUS-induced BBB opening was found to be safe in both rodents (Baseri et al. 2010) and non-human primates (Marquet et al. 2011; McDannold et al. 2012; Tung et al. 2011a).

Although the exact mechanism is still not completely understood, the interaction between capillary walls and acoustically driven microbubbles was found to be one of the key factors that lead to disruption of the BBB (Tung et al. 2011b). Until now, most studies have used commercially available and U.S. Food and Drug Administration-approved ultrasound contrast agents (UCAs). These include the protein-coated UCA Optison (Choi et al. 2007; McDannold et al. 2008) and the lipid-coated UCA Definity (McDannold et al. 2012; Tung et al. 2011b). Compared with protein-coated...
microbubbles, lipid-based microbubbles are formed by self-assembled monolayer phospholipids and are more responsive to ultrasound (Sirsii and Borden 2009). Definity microbubbles are highly polydisperse agents with bubble diameters ranging from submicrons to >10 μm. As a result, the resonant frequencies of these bubbles cover a wide range on the spectrum (>10 MHz) (Cheung et al. 2008; Goertz et al. 2007). Using Definity microbubbles, Baseri et al. (2010) evaluated the BBB opening threshold and most tolerable acoustic pressure ranges in mice at 1.525 MHz. The acoustic pressure window of 0.3–0.46 MPa was determined to be tolerable at the parameters used in that study (pulse length = 20 ms, pulse repetition frequency = 10 Hz).

In the study described here, we aimed to compare Definity and in-house prepared polydisperse (IHP) microbubbles, both of which are formed by high shear gas dispersion in an aqueous lipid-shell mixture. Although these two microbubbles have similar compositions, their behavior in the application of FUS-induced BBB opening has not been studied. The two main goals of this study were: (i) to evaluate whether Definity and IHP microbubbles have similar effects on BBB opening properties, and (ii) to determine whether IHP can serve as a surrogate for the commercially available Definity microbubbles. The efficiency of BBB opening using these microbubbles was evaluated by analyzing the increase in brain tissue permeability and the total volume of BBB opening. Microbubble type dependence was evaluated at different in situ acoustic pressures, ranging from 0.3 to 0.6 MPa. Tung et al. (2010a) reported (using Definity microbubbles) that inertial cavitation occurred at 0.45 and 0.6 MPa, but not at 0.3 MPa, in mice. Therefore, the FUS parameters selected in this study covered both stable and inertial cavitation regimes for Definity microbubbles. Finally, BBB reversibility was monitored and histologic observations of the brains were performed for evaluation of tolerability.

**METHODS**

**Microbubbles**

As indicated previously, two types of microbubbles were used in this study: Definity (Lantheus Medical Imaging, North Billerica, MA, USA) and IHP microbubbles. Definity vials, which are composed primarily of an aqueous solution of lipids and octafluoropropane (C₈ F₈) gas, were stored at 4°C before use. Immediately before sonication, Definity vials were activated (at an initial temperature of 4°C) via mechanical agitation using a VialMix (Lantheus Medical Imaging) shaker for a pre-set time of 45 s. The IHP microbubbles were manufactured according to a previously published protocol (Feshitan et al. 2009). Briefly, 1, 2-distearoyl-sn-glycerol-3-phosphocholine and polyethylene glycol 2000 were mixed at a 9:1 ratio. Ten milligrams of the mixture was dissolved in a 10-mL solution consisting of filtered phosphate-buffered saline/glycerol (10% volume)/propylene glycol (10% volume) using a sonicator (Model 1510, Branson Ultrasonics, Danbury, CT, USA). Each IHP microbubble vial (total volume = 5 mL) contained 2 mL of lipid solution, and the vial was sealed and stored at 4°C. Before activation, the air in the IHP vial was vacuumed out via a 26-gauge needle and the head space of the vial was filled with decafluorobutane (C₄ F₁₀) gas. This vacuuming-filling procedure was repeated five times for each vial to ensure high C₄ F₁₀ concentration. The vial was then activated via a VialMix shaker for 45 s.

Immediately after activation, the concentration and size distribution of each microbubble vial were measured with a Coulter Counter Multisizer (Beckman Coulter, Fullerton, CA, USA), which measures microbubbles in the range 0.6–18 μm. The microbubbles were then diluted in sterile saline (Vedo, Saint Joseph, MO, USA), yielding a concentration of approximately 6 × 10⁸ bubbles/mL.

**Preparation of animals**

A total of 18 mice (C57BL/6, Harlan, Indianapolis, IN, USA) were used in this study. Each mouse was anesthetized with a mixture of oxygen and 1%–2% isoflurane (SurgiVet, Smiths Medical PM, Dublin, OH, USA) and placed prone with its head immobilized by a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The hair on the mouse head was removed with an electric trimmer and depilatory cream to minimize impedance mismatch. All procedures involving animals were approved by the Columbia University Institutional Animal Care and Use Committee.

**Sonication protocol and MRI**

A single-element FUS transducer (focal length = 60 mm, radius = 30 mm, Imasonic, Voray/l’Ognon, France) with a center frequency of 1.5 MHz was used for all sonications. A pulse-echo transducer (radius = 11.2 mm, focal length = 60 mm, center frequency = 10 MHz; Olympus NDT, Waltham, MA, USA) was confocally mounted at the center opening (diameter = 11.2 mm) of the FUS transducer (Vlachos et al. 2010). A piece of polyurethane membrane (Trojan, Church & Dwight, Princeton, NJ, USA) was used to seal the transducer cone, which was filled with de-ionized and de-gassed water. The transducer system was attached to a computer-controlled 3-D positioner (Velmeq, Lachine, QC, Canada). The FUS transducer was connected to a matching circuit and driven by a computer-controlled function generator (Agilent, Palo Alto, CA, USA) and a 50-dB power amplifier.
The pulse-echo transducer was driven by a pulse-receiver (Olympus), which was connected to a digitizer (Gage Applied Technologies, Lachine, QC, Canada) for data acquisition. The –6-dB focal zone of the FUS transducer was measured with a needle hydrophone (Precision Acoustics, Dorchester, UK) in de-gassed water to be $7.5 \times 1 \times 1 \text{ mm}^3$.

In this study, the FUS beam was targeted at the right hippocampus; the left side served as control. Targeting was achieved using a grid; the procedure has been described in great detail elsewhere (Choi et al. 2007). The FUS focus was placed 3 mm below the skull, and 18% attenuation was accounted for acoustic pressure loss through the skull (Choi et al. 2008). With each type of microbubble (Definity or IHP), a bolus of 1 $\mu$L/g diluted microbubbles ($6 \times 10^8$ number/mL) was injected intravenously through the tail vein immediately before sonication. Each vial of microbubbles was used for three mice after activation. In situ peak rarefactual acoustic pressures (PRPs) used in this study were estimated to be 0.30, 0.45 and 0.60 MPa, and three mice were sonicated at each PRP. The pulsed FUS was applied with a pulse repetition frequency of 10 Hz, 1000 cycles and total duration of 60 s.

Upon completion of the FUS sonication (with an intervening delay of 15 min for animal transfer), the BBB opening was confirmed with a 9.4-T MRI system (Bruker Medical, Boston, MA, USA). The mice were placed in a birdcage coil (diameter 3 cm) while being anesthetized with 1%–2% isoflurane; vital signs were monitored throughout the imaging sessions. On day 0, dynamic contrast-enhanced (DCE) MR images were collected before and after an intraperitoneal injection of 0.3 mL gadodiamide (GD-DTPA) (Omniscan, GE Healthcare, Princeton, NJ, USA). Approximately 55 min after the injection, an additional post-contrast T1-weighted 2-D FLASH acquisition was performed (Vlachos et al. 2010). The T1-weighted images had a resolution of $86 \times 86 \mu$m² and slice thickness of 500 $\mu$m (23 slices per scan, no inter-slice gap). The BBB opening region was segmented (intensity > 2.5 standard deviations of the background) with a manually positioned elliptic cylinder (major diameter = 4.3 mm, minor diameter = 3.4 mm, height = 4.5 mm) over the right hippocampus following the shape of the focal spot in that plane. An additional elliptic cylinder of the same size was placed on the unsonicated (left) side of the brain. Voxels above the segmentation threshold on the unsonicated side were subtracted from those on the sonicated side to exclude the vessels and ventricles.

**Statistics**

Statistical analyses were carried out to compare the dependence of BBB opening on microbubble type at all three PRPs. Student’s $t$-tests were performed to compare the permeability and volume of BBB opening between the two types of microbubbles used in this study. The BBB closing timeline was compared with one-way analysis of variance followed by a post hoc Tukey honestly significant difference test. A $p$-value < 0.05 was considered to indicate statistical significance.

**RESULTS**

The size distributions of Definity and IHP microbubbles (n = 3) are illustrated in Figure 1. The majority (more than 99%) of both types of microbubbles had diameters ≤ 8 $\mu$m (Fig. 1a). Nonetheless, the volume percentage (Fig. 1b) of Definity microbubbles slightly peaked around 3 $\mu$m, whereas IHP microbubbles gradually reached a plateau around 5 $\mu$m.
The BBB opening was revealed as the contrast-enhanced region (hippocampus on the right side) in the post-contrast MR images (Fig. 2). Permeability ($K_{\text{trans}}$) values, ranging from 0 to 0.04 min$^{-1}$, were overlaid on the MR images. Sample permeability maps of brains subjected to FUS using IHP and Definity microbubbles are illustrated in Figure 2. The permeability averaged over the BBB opened region was compared between the two microbubble types across the PRPs used in this study (Fig. 3). No statistically significant difference ($p > 0.05$) was found between groups injected with Definity and IHP microbubbles when sonicated with 0.45 and 0.6 MPa. Nevertheless, the permeability of BBB opening regions in the group injected with IHP microbubbles was...
significantly higher ($p < 0.01$) than that of the Definity injected group at 0.3 MPa.

A similar trend was observed on the BBB opening volume comparisons, as illustrated in Figure 4. Although the mean opening volumes for the groups injected with IHP microbubbles are larger than those of the corresponding groups injected with Definity, only the difference at 0.3 MPa was statistically significant ($p = 0.027$).

The BBB closing (i.e., BBB opening volume on each subsequent day) timelines of all the groups tested are plotted in Figure 5. With the sonication parameters used in this study, BBBs of all mice closed within 3 d of treatment. Comparisons were made between groups using the two types of microbubbles at each PRP. At 0.45 and 0.6 MPa, no significant difference (Tukey’s HSD test, $p > 0.05$) was observed between groups using Definity (black) and IHP (red) microbubbles groups on day 0 as well as the next day. BBB opening volumes were found to be significantly different (Tukey’s HSD test, $p < 0.05$) for the groups sonicated at 0.3 MPa on day 0, but not thereafter.

The safety of BBB opening was evaluated by detecting red blood cell extravasation and dark neurons in histologic images. Hematoxylin-and-eosin-stained brain slides (Fig. 6) revealed that there was no damage in the Definity-injected groups at all ultrasound exposures used in this study. Among all the groups injected with IHP microbubbles, several dark neurons were detected in only one mouse (sonicated at 0.45 MPa). No damage was observed in any other mice injected with IHP microbubbles, including those sonicated at 0.6 MPa.

**DISCUSSION**

Focused ultrasound, in the presence of microbubbles, has been found to be capable of disrupting the targeted BBB in mice and non-human primates (Choi et al. 2007; McDannold et al. 2012; Tung et al. 2011a). Most recently, trans-cranial FUS was applied in clinical trials for the treatment of essential tremor in the United States (Elias 2011) and Canada (Lipsman et al. 2013). These results suggest that FUS has an important role in future treatments of neurodegenerative diseases such as Parkinson’s and Alzheimer’s. The interaction between FUS-driven microbubbles and surrounding blood vessels is a complicated phenomenon, which still...
requires extensive investigations from all perspectives (Wiedemair et al. 2012). In the present study, we focused primarily on the microbubble type dependence of BBB opening. Mice injected with either Definity or IHP microbubbles, to represent the polydispersity of microbubbles currently approved in the clinic, were sonicated with distinct PRPs of 0.3, 0.45 and 0.6 MPa. Despite the differences in lipid-shell material and gas core content, it was concluded from MRI-based analysis that there were no significant differences in permeability and volume of opening using these two types of microbubbles in sonication at relatively high PRPs (0.45 and 0.6 MPa). Interestingly, there were significant differences in both parameters when the FUS PRP was lowered to 0.3 MPa (Figs. 3 and 4). Histologic examinations also indicated that neither microbubble type induced damage in mice sonicated at 0.3 and 0.6 MPa. A minor injury (several dark neurons) was detected in one of nine mice from the IHP group (sonicated at 0.45 MPa).

The discrepancy at 0.3 MPa can be explained, in part, by analysis of the microbubble size distributions (Fig. 1). Vlachos et al. (2011) investigated the effect of microbubble diameter on BBB opening, where monodispers microbubbles (diameters: 1–2 μm, 4–5 μm and 6–8 μm) were used during FUS sonications. They found that microbubbles with diameters of 1–2 μm resulted in significantly lower permeability enhancement compared with the relatively larger microbubbles (4–5 μm and 6–8 μm). In addition, Samiotaki et al. (2012) reported that BBB opening recovery time with 1- to 2-μm microbubbles was shorter than that with relatively larger microbubbles. As illustrated in Figure 1b, although both types of microbubbles exhibited polydispersity, the volumetric size distribution varied across different diameter ranges. Definity contained a slightly higher volume of 2- to 4-μm, whereas IHP had a relatively higher volume of 4-μm to 8-μm microbubbles (Fig. 1). Although no significant difference was concluded for use of these two types of microbubbles at higher pressures (0.45 and 0.6 MPa), this size distribution difference, albeit small, might have become important at lower pressure levels. Luan et al. (2012) reported that at a resonant frequency of 1.5 MHz, the corresponding microbubble diameter is approximately 5–6 μm. Therefore, even though the number fraction of 2- to 4-μm microbubbles was higher than that in IHP, the 5- to 8-μm microbubbles (higher number fraction in IHP) might have a better chance of interacting with capillary walls at 0.3 MPa. At relatively higher driving pressures, microbubbles with a much wider diameter range contributed to BBB opening, resulting in the insignificant permeability and opening volume at 0.45 and 0.6 MPa.
Passive cavitation detection has been used to study microbubble response under sonication both in vitro and in vivo (Arvanitis et al. 2012; Tung et al. 2010b, 2011a). In particular, Tung et al. (2010b) reported the cavitation effects in mice using Definity microbubbles and PRPs ranging from 0.3 to 0.9 MPa. They observed that only stable cavitation occurred at 0.3 MPa for the mice with opened BBB. Once the PRP reached 0.45 MPa or higher, a broadband response (inertial cavitation) was detected (Tung et al. 2010b). Therefore, the similar permeability and volume of BBB opening findings at 0.45 and 0.6 MPa were presumed to be caused by the dominating inertial cavitation effects for both Definity and IHP microbubbles. On the other hand, the BBB opening at 0.3 MPa was attributed mainly to the stable cavitation (i.e., microbubble oscillations) effects. At this pressure, the relatively large (4–8 μm) microbubbles had a greater probability of interacting with capillaries (diameter around 7 μm) while oscillating. Therefore, the slightly higher 4-μm to 8-μm components presented in IHP microbubbles might have contributed to the significantly different permeability and volume of opening at 0.3 MPa. Therefore, the IHP microbubbles may be considered more efficient for FUS-induced BBB opening compared with Definity microbubbles, especially when only stable cavitation or maximal safety is required. There are several limitations to the present study. There were three animals per group, which might have induced some uncertainties in the results. Another limitation is that no cavitation detection was performed in this study, and the stable and inertial cavitation thresholds for IHP microbubbles remain unknown. Therefore, the interpretation of our results relied on previously published work (Tung et al. 2010b, 2011b), which was performed with Definity microbubbles and monodisperse microbubbles, respectively. In addition to the effect of size distribution difference between the two types of microbubbles used in this study, the different lipid-shell materials or gas contents may also contribute to the BBB opening differences (Tung et al. 2012). Furthermore, pre-activation temperature was recently reported to affect the Definity distribution (Helfield et al. 2012). In this study, Definity was activated at approximately 4°C, whereas the IHP microbubbles underwent C4F10 gas charge before activation (which might have brought the vial temperature up by approximately 5°C). The effect of pre-activation temperatures of microbubble vials on BBB opening is being investigated in our ongoing studies.

CONCLUSIONS

This study evaluated FUS-induced BBB opening in the presence of Definity or in-house polydisperse microbubbles. Microbubble type dependence was investigated by comparing the permeability of sonicated brain and total volume of opening between the two microbubble types. No significant difference was observed at PRPs at or above 0.45 MPa, whereas at 0.3 MPa, BBB opening volume and permeability were significantly higher with IHP microbubbles. The study suggests that IHP microbubbles may be more efficient than Definity microbubbles in FUS-induced BBB opening.

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