A Quantitative Pressure and Microbubble-Size Dependence Study of Focused Ultrasound-Induced Blood-Brain Barrier Opening Reversibility In Vivo Using MRI

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Focused ultrasound in conjunction with the systemic administration of microbubbles has been shown to open the blood-brain barrier (BBB) selectively, noninvasively and reversibly. In this study, we investigate the dependence of the BBB opening’s reversibility on the peak-rarefractional pressure (0.30–0.60 MPa) as well as the microbubble size (diameters of 1–2, 4–5, or 6–8 μm) in mice using contrast-enhanced T₁-weighted (CE-T₁) MR images (9.4 T). Volumetric measurements of the diffusion of Gd-DTPA-BMA into the brain parenchyma were used for the quantification of the BBB-opened region on the day of sonication and up to 5 days thereafter. The volume of opening was found to increase with both pressure and microbubble diameter. The duration required for closing was found to be proportional to the volume of opening on the day of opening, and ranged from 24 h, for the smaller microbubbles, to 5 days at high peak-rarefractional pressures. Overall, larger bubbles did not show significant differences. Also, the extent of BBB opening decreased radiially towards the focal region until the BBB’s integrity was restored. In the cases where histological damage was detected, it was found to be highly correlated with hyperintensity on the precontrast T₁ images. Magn Reson Med 000:000–000, 2011. © 2011 Wiley-Liss, Inc.

Key words: blood-brain barrier; focused ultrasound; Gd-DTPA-BMA; CE-T₁

The blood-brain barrier (BBB) is a specialized vascular system consisting of endothelial cells lining cerebral microvessels, with highly selective membranes, connected together by tight junctions (TJ) (1). The structure of the brain endothelium together with pericytes and astrocyte feet, tightly regulates the flow of molecules between the blood and the brain parenchyma through the capillary lumen, forcing a transcellular route, rather than a paracellular route as in most endothelia (2). The BBB acts as a physical and metabolic barrier, with a range of passive and active features, which restricts the penetration of molecules, except if they are both lipid soluble and of a molecular weight (MW) under 400 Da (3). The BBB, among the rest of biological membranes, has uniquely restrictive permeability properties and effectively excludes 98% (4) of small-molecule drugs and 100% of all large molecule drugs and neurotherapeutic agents, including recombinant proteins and enzymes, antibodies, etc. (3).

The use of focused ultrasound (FUS) in conjunction with microbubbles has been demonstrated by several groups (5–8) to successfully induce BBB opening noninvasively, locally and reversibly. The FUS–induced BBB opening has been shown by our group to allow molecules of various MWs into the murine brain, such as Gd-DTPA-BMA (MW 574 Da) (5), and fluorescent dextrans (MW 3 and 70 kDa) (9). Several particles within a wide size range have also been shown to pass through the disrupted BBB, e.g., MRI contrast agents such as Magnevist® (MW: 938 Da) and MION (MW: 10,000 Da) (10), trypsin blue (MW: 961), horseradish peroxidase (MW: 40,000 Da) (11) and antibodies (MW: 150,000 Da) (12). In this study, the MRI contrast agent was used as a tracer to depict the area of opening quantitatively.

Reversibility of the BBB opening in several animal models has previously been shown, but the timeline varied among different studies, together with the variation of parameters used. Using Optison (mean diameter of 2–4.5 μm) and a 260-kHz transducer at 0.40 MPa, or a 0.69 MHz transducer at pressure amplitudes of 0.8 and 1 MPa (11), contrast-enhanced fast spin-echo T₁-weighted images using intravenously administered Magnevist® revealed that the BBB was closed 5 h after sonication in rabbits (10). Using Sonovue (mean diameter of 2.5 μm) at 1.1 MHz, T₁-weighted fast spin-echo images (1.5 T) showed that the BBB signal enhancement in the opened region, following Magnevist® injection, returned to its initial state 8 h after sonication in rabbits (13), whereas at 1.63 MHz and varying the acoustic pressures from 1 to 4.7 MPa, the BBB closed within 24–48 h (6). In rats, with the use of Optison, at 1.5 MHz and 1.1 MPa, immunoelectron microscopy showed that the BBB was shown permeable to Horseradish Peroxidase Passage (HRP) and Lanthanum Passage up to 6 h after sonication, and remained impermeable 24 h later, while immunoelectron microscopy showed that TJs appeared disintegrated only up to 4 h after FUS and were later restored (14). Using Definity (mean diameter 1.1–3.3 μm) and an unfocused single-element transducer at 2.15 MHz, T₁ spin-echo images (7 T), the
BBB was shown to recover within 4–27 h (15). In Alzheimer’s disease-model mice, using Sonovue at 1.525 MHz, and peak-rarefractional pressure (PRP) of 0.50 MPa, the enhancement due to Gd-DTPA-BMA (Omniscan) in CE-T1 images (9.4 T) revealed that the BBB opening was a transient phenomenon, closing within 24 h (16). In large animals, i.e., pigs, using lipid or albumin-coated microbubbles at 2 MHz transducer and 2.0 W/cm², Evans blue color spectrophotometry as well as contrast-enhanced T1-weighted spin-echo echo planar images (1.5 T) revealed differences between the treated and untreated sides of the brain indicating altered BBB permeability, lasting up to 3 h after sonication (17).

Even though the mechanism of FUS-induced BBB opening is not yet known, overall it has been shown to be affected by acoustic parameters of the ultrasound beam, such as the applied peak rarefractional pressure (5–7,18–20), which we further investigated in this study by longitudinally monitoring the BBB self-repairing characteristics. The interaction of intravenous administered microbubbles with the ultrasound beam generates a range of biological effects (21). At low acoustic pressures, stable cavitation of the gas-filled microbubbles, microstreaming and acoustic radiation force may lead to shear stress exerted by the microbubbles onto the vessel endothelial cells and these effects can activate the mechanosensitive ion channels (22) or deform the vascular endothelium (23) yielding BBB opening. At higher acoustic pressure levels, or when oscillating near a boundary, microbubbles can collapse and produce shock waves and high-velocity jets (24–26), a phenomenon referred to as inertial cavitation. The BBB has been shown to open in the presence of inertial cavitation, but without it being a necessary condition (20,27).

Most studies so far have been utilizing poly-dispersed ultrasound contrast agents (i.e., Definity, Sonovue and Optison). The duration of the microbubble interaction with the vessel wall has been found to increase with the microbubble diameter (28). It has been well established that the microbubble diameter dictates its resonance frequency (29,30), expansion ratio (31), lifetime of stable cavitation (28,32), pressure threshold for inertial cavitation (28,32), and damage to the brain parenchyma (33). Choi et al. (19) used mono-dispersed microbubbles of 1–2 μm and 4–5 μm in diameter with a sorting method developed by Feshitan et al. (34) and 3-kDa fluorescent dextran. They showed that the FUS-induced BBB opening was dependent on both the size distribution in the injected microbubble volume, together with different pressure dependence for the opening threshold at each diameter size. Vlachos et al. (35) also showed that the exchange rate between the blood plasma and the brain tissue is proportional to the microbubble size and PRP, and that it increases and then reaches a plateau, for larger mono-dispersed microbubbles (4–5 μm and 6–8 μm) and higher PRPs, but is significantly lower for smaller bubbles (1–2 μm).

In this study, we investigated the dependence of both the spatial extent and the duration of FUS-induced BBB opening in vivo with different microbubble sizes and PRPs. Gd-DTPA-BMA retention in the brain parenchyma was used as a signature of the area of BBB opening. Volumetric quantification of the region of BBB opening was assessed by measuring the diffusion volume of Gd-DTPA-BMA in the sonicated region of the brain, i.e., the right hippocampus, detected by the longitudinal signal enhancement. Starting with the day of sonication and continuing up to 5 days following sonication, pre and postcontrast enhancement T1-weighted high resolution MR images were consecutively acquired at each time-point. The objective of this study was to provide a more thorough insight into, as well as a control over, the self-repairing characteristic of the BBB after FUS opening and its closing timeline, when different PRPs and microbubble sizes are used. It could also contribute to the optimization of the parameters used, and to a more efficient drug delivery, i.e., repeated drug administration over an extended time period.

**MATERIALS AND METHODS**

**Ultrasound Setup**

The acoustic waves used were generated by a single-element, spherical-segment FUS transducer (center frequency: 1.5 MHz, focal depth: 60 mm, radius: 30 mm; Imasonic, France), which was driven by a function generator (Agilent, Palo Alto, CA) through a 50-dB power amplifier (E&I, Rochester, NY) (Fig. 1a). A central-void (radius: 11.2 mm) of the therapeutic transducer held a pulse-echo ultrasound transducer (center frequency: 10 MHz, focal length: 60 mm), which was used for imaging, with their two foci aligned. The imaging transducer was driven by a pulser-receiver (Olympus, Waltham, MA) connected to a digitizer (Gage Applied Technologies, Lachine, QC, Canada). A cone filled with degassed and distilled water was mounted onto the transducer system and was fitted with a polyurethane membrane (Trojan; Church & Dwight Co., Princeton, NJ). The transducers were attached to a computer-controlled 3D positioning system (Velmax, Lachine, QC, Canada). The targeting procedure has been described elsewhere (5). Briefly, the FUS transducer was moved 3 mm laterally of the sagittal suture and 2 mm anterior of the lambdoid suture (5). A needle hydrophone (Precision Acoustics, Dorchester, Dorset, UK, needle diameter: 0.2 mm) was used to measure the three-dimensional pressure field in a degassed water-tank prior to the in vivo application. The FUS focal spot overlapped with the right hippocampus and the latter portion of the thalamus, since the axial and lateral full-widths at half-maximum intensities of the beam were 7.5 mm and 1 mm, respectively. The left hippocampus fissure was used as a control, and was not sonicated. Pulsed FUS was emitted for 60 s, with a burst rate of 10 Hz, 100 burst cycles, at acoustic pressures adjusted to correspond to 0.30, 0.45, and 0.60 MPa (peak-rarefractional), after accounting for 18% murine skull attenuation. These pressures were obtained experimentally in degassed water (5). The mice were anesthetized using 1.25–2.50% isoflurane (SurgiVet, Smiths Medical PM, Winsconsin) mixed with oxygen during FUS.

**Size Isolated Microbubbles**

The microbubbles used for this study were manufactured in-house, and they were size-isolated from a poly-
dispersed microbubble distribution using differential centrifugation (34). The bubbles had a 1,2-diacyl-sn-glycero-3-phosphocholine (DSPC) and polyoxyethylene-40 stearate (PEG40S) lipid shell and perfluorobutane (PFB) core. After the centrifugation and resuspension processes were repeated several times, three desired ranges of 1–2, 4–5, and 6–8 μm in diameter were isolated. A bolus of 1 μL/g at a concentration of 8 × 10⁷/mL was injected intravenously through the tail vein immediately preceding the sonication.

Magnetic Resonance Imaging

BBB opening in the murine hippocampus was confirmed using a 9.4 T system (Bruker Medical; Boston, MA). All mice were anesthetized orally using 1–2% of isoflurane mixed with oxygen and were placed inside the vertical-bore, having a fixed position in a plastic tube with a 3.0 cm diameter birdcage coil. Vital signals were monitored and respiration rate was approximately 55 breaths/min. Each MRI session included a pre and a postcontrast enhancement, T₁-weighted 2D FLASH acquisition (TR/TE: 230/3.3 ms, flip angle: 70°, NEX: 18, resolution 86μm x 86μm, slice thickness: 500μm, 23 slices, F.O.V.: 22 mm x 16.5 mm, matrix size 256 x 192, receiver bandwidth: 50 kHz), obtained respectively 15 min after sonication and 55 min after injection of the MRI contrast agent (Gd-DTPA-BMA). Based on prior experience, signal enhancement in the area of the sonicated hippocampus reaches a peak approximately 1 h after IP injection. Therefore, the CE-T₁ images were acquired 55 min following the injection. Omniscan™ (Gd-DTPA-BMA) was used to enhance the MR contrast in the murine brain, as a tracer for the BBB opening since it diffuses into the brain parenchyma following BBB’s altered permeability in the targeted area. Gd-DTPA-BMA was administered intraperitoneally (IP) at a dose of 6 mmol/kg. As previously reported, an IP bolus provides more temporally consistent and sustained MR enhancement than an IV bolus and is logistically simpler (15). Preliminary work on the IP bolus dosage indicated that the 6 mmol/kg low enough so as to avoid the signal decrease which is observed when, due to excessive Gd-DTPA-BMA, the T₂ relaxivity dominates the T₁ relaxivity. The same MRI session was repeated daily starting from the day of the BBB opening (day 0) and lasting up to 5 days. In the five cases where significant signal enhancement was detected on day 5, MRI sessions were repeated on day 7.

Animal Preparation

All procedures used in this study involving animals were approved by the Columbia University Institutional...
Animal Care and Use Committee. A total of forty-two (n = 42) wild-type mice, (strain C57BL/6, mass: 20–25g, sex: male, Harlan, Indianapolis, IN) was used for the purposes of this study, separated into ten groups. Each mouse in the first nine groups was sonicated with a different combination of PRP, i.e., 0.30, 0.45, or 0.60 MPa, and a microbubble diameter, i.e., 1–2, 4–5, or 6–8 μm. These mice underwent MRI for several days after FUS. The sham group had three (n = 3) mice, for which the whole procedure was repeated without FUS, i.e., anesthesia, injection of microbubbles, and MRI sessions for up to 5 days.

Histology and Imaging

On day 7, all mice were euthanized and transcardially perfused with 30 mL PBS and 60 mL 4% paraformaldehyde. Brains were soaked in paraformaldehyde for 24 h. Skulls were removed, and the brains were fixed again in 4% paraformaldehyde for 6 days, followed by conventional postfixation procedures. The paraffin-embedded specimens were sectioned horizontally at a thickness of 6 μm. 24 sections were stained and examined for each brain. Sections were stained with hematoxylin and eosin and then examined for red blood cell extravasations into the brain parenchyma as well as cell loss.

Image Processing and Volumetric Measurements

The enhancement in CE-T1 MR images was quantified in elliptic cylindrical VOIs encompassing the hippocampal formation, at two contralateral regions, in the right (sonicated) and the left (control) hemisphere, on each brain for each day. The major diameter of the elliptic cylinders was 4.3 mm, the minor diameter 3.4 mm, and the height 4.5 mm, covering an area of approximately 14,000 voxels from a total of nine consecutive horizontal slices, on each side. Therefore, the total volume on each cylinder was ~52 mm³. In order to quantify the BBB opening volume at the sonicated side, an intensity threshold was determined and the contrast-enhanced pixels in the vessels and ventricles were excluded. To address these issues, the signal intensity was averaged over a small circular region of 1 mm in diameter, centered around the nonsonicated contralateral side close to the hippocampus and used as a reference. The number of voxels in the right (sonicated) and left (control) VOI at an intensity of 2.5 standard deviations (S.D.) or above the reference were counted. The total number of these voxels in the left VOI was then subtracted from the respective total number of voxels in the right VOI, to exclude volume contrast-enhancement in the vessels and ventricles (Fig. 1b). Precontrast images were used for the detection of any hyperintense areas before Gd-DTPA-BMA injection, and were not coregistered with the postcontrast.

Statistical Analysis

The measurements in the sham group were used as the baseline, denoting that the integrity of the BBB was fully restored. Following the calculation of the mean and standard deviation (S.D.) of the volumetric measurements for each group, i.e., mice sonicated at the same PRP and microbubble size, a two-tailed Student’s t-test was performed, and if no statistically significant difference compared to the control baseline was observed (P > 0.05), then the BBB was considered to have closed. In other words, the closing criterion was checked on each day for each group and not for each measurement individually. All groups met the closing criterion by day 5. For the five individual cases where signal enhancement was significant on day 5, MRI was repeated on day 7. Not all of these cases however belonged to the same group, and the groups they belonged to could still meet the closing criterion based on the statistical analysis.

In order to investigate the differences in the timeline for closing between microbubble sizes and pressures, a statistical analysis was performed on the volume of diffusion of Gd-DTPA-BMA on day 0. To evaluate the effect of the microbubble size, a two-tailed Student’s t-test was performed for each PRP, i.e., between 1–2 μm and 4–5 μm, between 1–2 μm and 6–8 μm, as well as between 4–5 μm and 6–8 μm. The purpose was to examine any statistically significant differences between the different groups regarding the volume of BBB opening and the time required for the opened BBB to be reinstated.

RESULTS

An example of the volume quantification is shown in Fig. 2. The VOIs were manually traced to overlap with the right and left hippocampi. On both sides, voxels with an intensity 2.5 S.D. or above the reference intensity are overlaid in red. As shown in these images, when there is no diffusion of Gd-DTPA-BMA from the vasculature to the brain parenchyma, e.g., on the control side, only the vessels and the ventricles have CE-T1 intensity above the threshold. However, when the permeability of the BBB is altered on the right hippocampus as a result of the FUS, Gd-DTPA-BMA diffuses in that region and the area of opening is overlaid in red. The example in Fig. 2 shows the same brain in multiple 2D slices where 6–8-μm bubbles at a PRP of 0.30 MPa were used. The volume of opening was reduced radially towards the focal region over several subsequent days, until no trans-BBB diffusion was detected on day 3, signifying that the BBB was successfully reinstated.

For all cases of microbubble sizes and pressures studied, the BBB was found to be reinstated by day 5, and the duration of opening depended on the microbubble diameter and PRP used. In Fig. 3, reconstructions of the horizontal planes, sliced coronally at the level of the hippocampal formation, at all pressures and microbubble sizes are shown. In Fig. 3, it is also shown that the area of Gd-DTPA-BMA diffusion in the brain and its spatial characteristics depend on the microbubble size and pressure used. The BBB permeability to Gd-DTPA-BMA appears to occur dorsally in the brain only in the cases when larger microbubbles were used, i.e., not in the 1–2 μm case, where diffusion of the contrast agent Gd-DTPA-BMA was observed mainly ventrally in the brain near
the vasculature. This effect becomes more apparent at higher PRPs, i.e., 0.60 MPa.

Volumetric measurements are shown in Fig. 4a–c for the 1–2, 4–5, and 6–8 μm microbubble cases. At 0.30 MPa with the 1–2 μm microbubbles no BBB opening was detected. Depending on the microbubble and pressure used, the BBB closing occurred within 24 h and 5 days after sonication. More specifically, with the 1–2 μm microbubbles (Fig. 4a), closing was found to be closed on day 1 and 2 at 0.45 and 0.60 MPa, respectively. With the 4–5 μm and 6–8 μm microbubbles, the BBB was found to be closed on day 2 at 0.30 MPa, day 3 at 0.45 MPa, and day 5 at 0.60 MPa. A proportional relationship between the volume and the duration of the BBB opening regardless of the PPR and microbubble size used to induce the opening was thus established (Fig. 5). Linear regression showed a correlation of $R^2 = 0.72$. Excluding day 0 a logarithmic fit indicated a correlation of $R^2 = 0.78$.

The results of the statistical analysis on day 0 are shown in Table 1. At 0.30 MPa, opening was induced only in the 4–5 and 6–8 μm cases, with no statistically significant difference ($P > 0.05$) between these two diameter ranges (Table 1), and the BBB was restored by day 2 in both cases. At 0.45 MPa, the volume of BBB opening induced with 1–2 μm microbubbles, was statistically different ($P < 0.01$) than that with larger microbubbles (Table 1), and occurred within 24 h, compared to 2–3 days needed in the cases of the larger microbubbles. Finally, in Table 1, it is shown that at 0.60 MPa, there was a statistically significant difference between the 1–2 μm and 6–8-μm bubbles ($P < 0.05$), and at least 2 days were required for the BBB to be reinstated in the 1–2 μm case, as opposed to 4–5 days required for the larger microbubbles.

Good correlation was also found between all histological damage cases and hyperintensity in the precontrast $T_1$-w images. An example is shown in Fig. 6, where hyperintensity was detected at the sonicated region in the precontrast image, and cell loss was detected on the H&E stained slices at the level of the hippocampus. Damage was observed upon histological examination only in five animals, approximately 13% of the total number of animals used, all of which were at higher PRPs.

**DISCUSSION**

In this longitudinal study, the reversibility of BBB opening was investigated using Gd-DTPA-BMA that cannot cross the BBB when the BBB is reinstated or closed. Spatial (i.e., volume) and temporal (i.e., duration) characteristics of the intact or BBB’s altered function were studied, while opening was induced using three different microbubble sizes and three different PRPs. BBB opening induced by FUS has been shown in several studies to be transient but with differing reports on the duration which has indicated the dependence on the acoustic parameters and bubbles used. Also, several studies have shown that the BBB opening is dependent on the acoustic pressure used as well as the microbubble size. The features of the BBB self-repairing characteristic were studied for the first time under a combination of different acoustic parameters, and a range of mono-dispersed microbubbles. Our study indicated a proportional relationship between the BBB opening volume and the time required for closing. Therefore, both the BBB opening volume and duration were shown to be dependent on the acoustic pressure and the microbubble size used.

The spatial characteristics of the BBB opening and its reversibility were unveiled as follows. Firstly, as seen in

<table>
<thead>
<tr>
<th>PRP</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
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<tr>
<td>0.30 MPa</td>
<td>4-5 μm</td>
<td>6-8 μm</td>
<td>1-2 μm</td>
<td>4-5 μm</td>
<td>6-8 μm</td>
</tr>
<tr>
<td>0.45 MPa</td>
<td>4-5 μm</td>
<td>6-8 μm</td>
<td>1-2 μm</td>
<td>4-5 μm</td>
<td>6-8 μm</td>
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<tr>
<td>0.60 MPa</td>
<td>4-5 μm</td>
<td>6-8 μm</td>
<td>1-2 μm</td>
<td>4-5 μm</td>
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FIG. 3. Coronal reconstructions from horizontal CE-$T_1$ images with one example provided in each case of PRP and microbubble size.
the examples of Figs. 2 and 3, the BBB function was reinstated in a reverse direction to that of the diffusion after opening, i.e., closing starts from the outer opened regions and ends at the focal region while also being dependent on the hippocampal vasculature. It has also been shown (34) that the permeability values towards the center of the focal region were higher. A first explanation is that the peak of the acoustic pressure distribution lies at the center of the focal spot which may result in larger BBB openings, hence taking longer to close. Another explanation could be that where the vasculature is denser, there is a higher number of opening sites, requiring thus longer timelines for closing to be completed.

Overall, even though the volume of opening induced by the 6–8-μm bubbles was greater than the volume induced by the 4–5-μm microbubbles, these two microbubble sizes did not differ significantly in this study (Table 1) and the days required for closing were also similar (Fig. 4b,c). According to these observations, several conclusions can be drawn. First, it could be assumed that their differences were not significant because both 4–5-μm and 6–8-μm bubbles are within the diameter size range of the capillary, i.e., 4–8 μm, and therefore they are both in contact with the capillary wall, exerting forces on it while being acoustically driven. However, the mechanical stress on the capillary walls due to the acoustically driven microbubbles for a specific pressure when induced by the 6–8-μm is larger than when induced by the 4–5-μm bubbles, because the latter would require higher PRP to reach the same size expansion as the 6–8-μm and have the same effect (28), hence the BBB opening volume slightly increased.

Table 1
Statistical Significance of the Volume of Diffusion of Gd-DTPA-BMA on Day 0, Comparing Different Cases of Microbubble Diameters per PRP

<table>
<thead>
<tr>
<th>PRP</th>
<th>6–8 μm</th>
<th>4–5 μm</th>
<th>1–2 μm</th>
<th>4–5 μm</th>
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<tr>
<td>0.30 MPa</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
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<tr>
<td>0.45 MPa</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>0.60 MPa</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
<td>$P &lt; 0.05$</td>
</tr>
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Another interesting observation is that the BBB opening volume using 1–2-μm bubbles was significantly lower than for the larger microbubbles (Table 1) and the BBB closed significantly faster. Therefore, for more efficient BBB recovery at high pressures, smaller microbubbles may be preferable. The relative expansion of a microbubble is inversely proportional to its resting diameter (31). 1–2-μm bubbles induced no opening at 0.30 MPa, where only stable cavitation is expected (20). This is probably due to the fact that stable oscillations of smaller microbubbles may not be sufficient to induce the repeated stress against the capillary wall at 0.30 MPa, but are adequate at 0.45 MPa, and even more so at 0.60 MPa. It has been also shown that fragmentation occurs more frequently for microbubbles with a small rather than a large resting diameter at a threshold size of 2.5 μm. Below that level, fragmentation occurs (31) and bubbles smaller than 2 μm can be fragmented prior to reaching the endothelium and not interact with the vessel wall (28). Therefore, it could be concluded that the smaller microbubbles were not capable of inducing opening in sites away from the bigger vessels, because they may have undergone fragmentation at the beginning of the FUS pulse before perfusing the microvasculature. Thereby, the therapeutic efficacy of 1–2 μm bubbles in the capillaries might be decreased, an effect noted in previous studies (36,37).

In this study, the timeline required for closing is longer than what has been reported by previous studies, i.e., 3–24 h (6,10,11,13–17) as previously explained. The discrepancy with the findings presented here could be due to multiple factors. First of all, the microbubble formulation, manufactured in-house, used in this study is different than the commercially available contrast agents used in the other BBB restoration studies, in terms of the combination of shell properties, gas core and diameter size; therefore, this microbubble formulation used here is a parameter that could also induce variations. However, the size range of the commercially available microbubbles used in those studies is closer to the case of the 1–2-μm bubbles used in our study, which are also shown here to induce BBB opening that can close within 24 h.
At lower PRPs, we showed that the BBB closes faster, and in most of these studies, the PRPs used were below 0.50 MPa. Moreover, the FUS frequency (1.5 MHz) used in this study might also introduce differences compared to other studies. The resonance frequency decreases with the microbubble radius, and also decreases with decreasing microvessel radius (30). Therefore, in this study, with the use of a lower frequency for example, which would be closer to the resonance frequency of the microbubbles within the microvessels and capillaries, and the aforementioned effects could be further enhanced. Finally, in other timeline studies, the agents used to cross the BBB for the detection of opening, had larger MWs (Magnevist®: 938 Da, HRP: 40 kDa, Evans Blue: 961 Da) while it has been shown that the BBB becomes less permeable at higher MWs (9,38). Since we administered Gd-DTPA-BMA, which is a relatively smaller (574 Da) agent and only above the size threshold to cross the BBB (400 Da), our method may have an increased sensitivity regarding the detection of BBB opening, which could contribute to the longer times shown here.

In the five cases where cell loss was detected in the H&E stained brain sections on the sonicated region, hyperintensity was also detected on the precontrast MRI images at the corresponding regions. The signal enhancement detected in these areas in the precontrast $T_1$-w images could be therefore due to permanent damage, blood present in the brain or arrested Gd-DTPA-BMA in possibly damaged vasculature, and BBB could not be completely restored.

Another interesting finding in this study is that the BBB remained opened over several days in some cases. It was thereafter restored and 87% of the cases showed no detectable damage, while the remaining 13% showed minimal cell loss. In the past, more significant damage has been reported (39) including microvacuolated sites, dark neurons and sites with extravasated erythrocytes, when the BBB opening was induced in mice at similar PRPs, with Definity microbubbles (mean diameter: 1.1–3.3 μm) and the same PRF (10 Hz) but longer burst lengths (20 ms) than the one used here. Also, in that study (39), survival times periods were 30 min and 5 h, whereas in this study it was 7 days. It could therefore be concluded that, first, at shorter burst lengths openings is induced (40) with less damage, and second, that the self-repairing mechanism of the BBB could restore certain types of injury induced to the brain after FUS provided sufficient time is allowed.

There are certain limitations to this study. Firstly, BBB may have been disrupted longer than what could be detected by the sensitivity the MRI system used and the resolution of the images acquired. Another limitation is that the first acquisition or postcontrast $T_1$-w images was 1 h after FUS, and within this time it is possible that some cases with very small BBB opening might have not been detected. Finally, it was assumed that the circulation times and persistence of all the different sizes of microbubbles were similar for the 10–20 s interval between injection and FUS, however, it must be taken into consideration that any differences could have had an impact on our results.

In this longitudinal study we showed dependence of the volume of BBB opening and the time required for the BBB to be reinstated on the microbubble size and the acoustic pressure. In addition, the time required for closing was found to be proportional to the volume of opening induced by FUS, and BBB was shown to recover its functionality between 24 h and 5 days after. The BBB opening volume was shown to decrease radially towards the center of the focal spot over time. At lower acoustic pressures, the microbubble size becomes more important, with smaller microbubble diameters inducing a lower volume of BBB opening and the closing timeline being significantly different than with larger microbubbles. As the PRP increases, the differences in BBB opening and closing between the different microbubble sizes become less significant. Overall, the BBB closes faster when small microbubbles are used, while for the 4–5 and 6–8 μm the same duration for closing was required. Finally, hyperintensity in the area of BBB opening was detected in the precontrast MR images only in the cases where damage was concluded in histology. In conclusion, this study may offer some further insight in the understanding of the FUS-opened BBB self-repairing characteristics, spatially and temporally, and the methodology may be adjusted to fit the pharmacokinetic needs of the administered CNS drugs.

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