Permeability Dependence Study of the Focused Ultrasound-Induced Blood–Brain Barrier Opening at Distinct Pressures and Microbubble Diameters Using DCE-MRI

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Blood–brain barrier opening using focused ultrasound and microbubbles has been experimentally established as a non-invasive and localized brain drug delivery technique. In this study, the permeability of the opening is assessed in the murine hippocampus after the application of focused ultrasound at three different acoustic pressures and microbubble sizes. Using dynamic contrast-enhanced MRI, the transfer rates were estimated, yielding permeability maps and quantitative $K_{\text{trans}}$ values for a predefined region of interest. The volume of blood–brain barrier opening according to the $K_{\text{trans}}$ maps was proportional to both the pressure and the microbubble diameter. A $K_{\text{trans}}$ plateau of $-0.05 \, \text{min}^{-1}$ was reached at higher pressures (0.45 and 0.60 MPa) for the larger sized bubbles (4–5 and 6–8 μm), which was on the same order as the $K_{\text{trans}}$ of the epicranial muscle (no barrier). Smaller bubbles (1–2 μm) yielded significantly lower permeability values. A small percentage (7.5%) of mice showed signs of damage under histological examination, but no correlation with permeability was established. The assessment of the blood–brain barrier permeability properties and their dependence on both the pressure and the microbubble diameter suggests that $K_{\text{trans}}$ maps may constitute an in vivo tool for the quantification of the efficacy of the focused ultrasound-induced blood–brain barrier opening. Magn Reson Med 66:821–830, 2011. © 2011 Wiley-Liss, Inc.

Key words: permeability; DCE-MRI; focused ultrasound; blood–brain barrier

The key to the treatment of various neurological diseases resides in the safe opening of the blood–brain barrier (BBB), a specialized structure that impedes the delivery of therapeutic agents to the parenchyma (1,2). Under normal physiological conditions, the BBB blocks molecules that are larger than 400 Da (3) from penetrating the brain parenchyma. Several techniques have been proposed over the years for the opening of the BBB. Hyperosmolar solutions, such as lactamide or mannitol, have been used clinically and experimentally (4,5) and have been found to increase the permeability of the interendothelial tight junctions. Intracranial operations with a needle have also been performed in patients allowing for a more localized BBB opening (6). More recently, focused ultrasound (FUS) in the presence of circulating microbubbles has been suggested as the only non-invasive, localized, and transient method for BBB opening (7,8).

A series of studies have assessed the extent of the FUS-induced BBB opening and its dependence on various acoustic parameters. With the use of MR (7,8) and fluorescence imaging (9,10), the opening has been found to have a significant dependence on the acoustic pressure, the microbubble size distribution (9) and the molecular size of the administered pharmacological compound (10). More specifically, the BBB opening threshold has been found to lie between the peak rarefactional pressures of 0.30 and 0.45 MPa for both the commercial and the custom-made microbubbles that were tested, whereas a maximum compound size of 2 kDa was found to sufficiently penetrate the brain parenchyma after the FUS procedure. Other studies have focused on the mechanism of the BBB opening by providing detailed descriptions of the different transcapillary molecular pathways that increase the BBB permeability using electron and immunoelectron microscopy (11,12) or by quantifying the passive acoustic emissions of the FUS procedure (13,14).

Tung et al. (13), in particular, have measured the broadband spectral response acquired with a passive cavitation detector and the findings indicated that inertial cavitation occurs at and beyond the peak rarefactional pressure of ~0.45 MPa. Finally, the safety of the technique has been thoroughly investigated using both ex vivo (15,16) and in vivo (17) methods. For example, Baseri et al. (15) have indicated that the safest acoustic pressure is within the range of 0.3–0.46 MPa based on hematoxylin and eosin (H&E) findings.

Although all the aforementioned studies have examined the FUS-induced BBB opening from different points of view, to our knowledge, no study has emphasized on the in vivo drug kinetics in the sonicated region. Recently, we compared two standardized MR-based pharmacokinetic models, estimating for the first time a numerical permeability value using dynamic contrast-enhanced MRI (DCE-MRI) (18). According to the study, the generalized Tofts and Kermode kinetic model (19) yielded reliable transfer rate constants that demonstrated...
the significant permeability increase in the sonicated hippocampal region compared with the contra-lateral (control) side in mice. Therefore, DCE-MRI could be established as a standardized in vivo evaluation tool for the efficacy of the FUS procedure.

The objective of this article is to assess the BBB permeability changes when two of the most influential FUS parameters, i.e., the peak rarefactive pressure and the microbubble size distribution, are varied. The general kinetic model (GKM) (19) was used to generate permeability maps and measure the transfer rates in a specific region within the sonicated murine hippocampus at each acoustic pressure and microbubble size. The permeability of the epicranial muscle (no barrier) was also measured and compared with the values derived from the BBB-opened region. $T_2$ imaging and H&E staining were also used to assess the impact of FUS on the neuronal and vascular cells.

MATERIALS AND METHODS
Focused Ultrasound Setup

A single-element, spherical-segment FUS transducer (center frequency: 1.5 MHz; focal depth: 60 mm; outer radius: 30 mm; model: cdc7411–3, Imasonic, Besançon, France) was used with a central void (radius: 11.2 mm) that held a pulse-echo diagnostic ultrasound transducer (center frequency: 10 MHz, focal length: 60 mm, Olympus NDT, Waltham, MA, USA). The transducer assembly was positioned so that the two foci overlapped. A cone filled with degassed and distilled water was mounted onto the transducer system and a fitted polyurethane membrane (Trojan; Church & Dwight Co., Inc., Princeton, NJ) supported the water in the cone. The system was attached to a computer-controlled, three-dimensional system (Velmx Inc., Lachine, QC, Canada). The FUS transducer was connected to a matching circuit and was driven by a computer-controlled function generator (Agilent, Palo Alto, CA) and a 50-dB power RF-amplifier (ENI Inc., Rochester, NY). The pulse-echo transducer was driven by a pulser-receiver system (Panametrics, Waltham, MA) connected to a digitizer (Gage Applied Technologies, Inc., Lachine, QC, Canada) (Fig. 1).

A needle hydrophone (Precision Acoustics Ltd., Dorchester, Dorset, UK, needle diameter: 0.2 mm) was used to measure the three-dimensional pressure field in a degassed water-tank before the in vivo experiments. The calculated peak-negative and peak-positive pressure values were attenuated by 18% to correct for the murine skull attenuation (8), whereas the lateral and axial full-width-at-half-maximum intensities of the beam were 1 and 7.5 mm, respectively.

Sonication Protocol

All procedures performed were approved by the Columbia University Institutional Animal Care and Use Committee. A total of 40 seven-week-old male mice (C57BL/6) of mass 23.87 ± 1.82 g were used for this study. Before the experiment, each mouse was anesthetized using 1–3% isoflurane gas (SurgiVet, Smiths Medical PM, Inc., Wisconsin, USA). Subsequently, the mouse head was shaved and positioned on the stereotactic apparatus (David Kopf Instruments, Tujunga, CA) under the transducer assembly during the entire experiment. Coupling gel and degassed water were placed between the skin of the mouse head and the transducer, enabling the focus of the transducer to overlap with the hippocampus and the posterior cerebral artery. A plastic container and an acoustically and optically transparent surface (Saran, SC Johnson, Racine, WI) maintained the water over the mouse head. The lateral positioning of the transducer was assessed with a grid-positioning method that used the pulse-echo diagnostic transducer as described in previous studies (8).

The mice were divided in nine cohorts (Table 1). Each cohort was sonicated using a different combination of microbubble size and acoustic pressure. A minimum of three mice per cohort were used. However, additional mice per cohort were sonicated in most cases, to further strengthen the statistical analysis in those cases. The lipid-shelled microbubbles were manufactured in-house by differential centrifugation (20) at mean diameter sizes of 1–2, 4–5, and 6–8 μm. The microbubbles were injected intravenously (IV) through the tail vein at a concentration of $\sim 10^7$ numbers/mL, using a 30-G needle. The right hippocampal region of the brain was sonicated immediately after the microbubble injection, using pulsed FUS (burst rate: 10 Hz; burst cycles: 100; duty cycle: 0.067%; frequency: 1.5 MHz) for a duration of 60 s. For each microbubble size, three cohorts of mice were sonicated at three different acoustic pressures (0.30, 0.45, and 0.60 MPa peak rarefactive). Previous studies (9) have shown that the safe acoustic pressure for microbubble-mediated BBB opening

<table>
<thead>
<tr>
<th>Number of Sonicated Mice ($N = 40$)</th>
<th>Acoustic pressure (MPa)</th>
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<tbody>
<tr>
<td>Number of mice</td>
<td>0.30</td>
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<td>Microbubble size (μm)</td>
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lies between 0.30 and 0.45 MPa. However, different sonication parameters (i.e., pulse length and pulse repetition frequency) were used in this study, which can affect the BBB opening properties significantly (21). Thus, a higher acoustic pressure of 0.60 MPa was also included as an input parameter to ensure BBB opening in every case.

### MRI Protocol

All the mice were imaged in a vertical 9.4 T MRI system (DRX400, Bruker Biospin, Billerica, MA). Each mouse was scanned 30–40 min after sonication, using a 30-mm-diameter birdcage coil. Isoflurane gas (1–2%) was used to anesthetize the mouse at 50–70 breaths/min during the entire MRI procedure. DCE-MRI was performed using a 2D FLASH $T_1$-weighted sequence (temporal resolution: $130 \times 130 \, \mu m^2$; slice thickness $600 \, \mu m$ with no inter-slice gap; flip angle: $70^\circ$; pulse repetition time/echo time $= 230/2.9 \, ms$; Number of Excitations = 4; scan time: $88 \, s$). Forty dynamic acquisitions were made over a total period of 60 min. Each acquisition produced 20 horizontal slices that covered the entire mouse head. Upon completion of the second dynamic acquisition, a $0.30 \, mL$ nondiluted bolus of gadolinium diethylenetriamine penta-acetic acid (Gd-DTPA) was injected intraperitoneally (IP) through a catheter at a rate of $\sim 10 \, \mu L/s$. The relatively large dosage of contrast agent (CA) was preferred to secure the presence of a bolus peak in the vascular system, which is essential for the arterial input function (AIF) determination, but also to have a clearer depiction of the extent of BBB opening (22). IP CA administration was preferred over IV, because it allowed dynamic acquisitions with lower temporal and higher spatial resolution. In addition, several attempts to perform IV cathe terization in the tail or femoral vein resulted in clots or scars, which impeded contrast administration (18). Gd-DTPA has been shown to reduce the longitudinal relaxation time when excreted in the extravascular extracellular space (EES), thus enhancing the $T_1$ signal intensity, where the BBB opening has occurred. Upon completion of DCE-MRI, a 2D FLASH $T_2$-weighted sequence (pulse repetition time/echo time $= 230/3.3 \, ms$; flip angle: $70^\circ$; NEX $= 18$; scan time: $9 \, min \, 56 \, s$) with higher spatial resolution (matrix size: $256 \times 192$; spatial resolution: $86 \times 86 \, \mu m^2$; slice thickness: $500 \, \mu m$ with no interslice gap) and a 2D RARE $T_2$-weighted sequence (pulse repetition time/echo time $= 3300/43.8 \, ms$; echo train: 8; NEX $= 10$; scan time: $9 \, min \, 54 \, s$; matrix size: $256 \times 192$; spatial resolution: $86 \times 86 \, \mu m^2$; slice thickness: $500 \, \mu m$ with no interslice gap) were acquired.

### Image Processing

The GKM was used to measure the BBB permeability in the targeted region. Previous studies (18) have validated the reliability of GKM in the FUS-induced BBB opening. GKM classifies tissues in two compartments, the blood plasma and the EES (23):

$$\frac{dC_i}{dt} = K_\text{trans}C_p - K_{ep}C_i$$

where $K_\text{trans}$ and $K_{ep}$ are the transfer rate constants from the blood plasma to the EES and from the EES to the blood plasma, respectively, and $C_p$ and $C_i$ are the concentrations of Gd-DTPA in the blood plasma and the EES, respectively. $C_p$ provides the AIF, which is fitted to a biexponential equation:

$$C_p(t) = A_1e^{-mt} + A_2e^{-nt}$$

where $A_i$, $m_i$ ($i = 1, 2$) are the amplitude and decay rates of $C_p$, respectively, and $t$ is time. The plasma concentration $C_p$ is calculated as a fraction of the blood concentration $C_{bi}$, $C_p = (1 - H_{bi})C_{bi}$, where $H_{bi} = 0.45$ is the hematocrit level for wild-type mice. The difficulty in obtaining an accurate AIF from a detectable vessel in the dynamic images has been reported and assessed with various estimating models (24). However, recent studies (24) have demonstrated that selecting a population average from a large group of the same strain of animals to determine the AIF can be both accurate and robust. In this study, the entire cohort of mice was used to determine the AIF, by averaging the Gd-DTPA concentration changes in the internal carotid artery, as shown in previous studies (18).

Signal intensity in $T_1$ images is translated to tracer concentration, using the Solomon-Bloembergen equation (25). The equation assumes a linear relationship between the concentration of the CA ([Gd]) and the relaxation rate difference ($\Delta R_t$). Relating the relaxation rate to signal intensity ($S$) in the gradient echo MR images yields a linear relationship between $S$ and [Gd]:

$$[\text{Gd}] = \frac{S_{\text{post}} - S_{\text{pre}}}{T_{\text{1,pre}} \times r_1 \times S_{\text{pre}}}$$

where $T_{\text{1,pre}}$ is the longitudinal relaxation time of the corresponding tissue before the CA administration, $r_1$ is the longitudinal relaxivity of the CA and $S_{\text{pre}}$ and $S_{\text{post}}$ are the signal intensities before and after Gd-DTPA injection, respectively. Phantom experiments in the 9.4 T MRI system have shown that the $r_1$ relaxivity of gadodiamide (Omniscan®, molecular weight of 530 Da) is $\sim 2.6 \, \text{mM}^{-1} \, \text{s}^{-1}$, whereas the longitudinal relaxation times of brain tissue (0.9 s) and arterial blood (1.5 s) in mice have been reported in previous studies, using arterial spin labeling techniques (26).

Before the quantitative $K_\text{trans}$ measurements, all the images were smoothed using a 3-by-3 averaging linear filter from the Image Processing Toolbox® of Matlab (MathWorks, Inc., Natick, MA). In addition, because the actual Gd-DTPA injection time occurred $\sim 3 \, min$ after the beginning of DCE-MRI, exclusion criteria were set for the estimated Gd-DTPA injection time ($t < 0$ or $t > 30 \, \text{min}$) to avoid any fitting divergences. The 30-min threshold was selected in accordance with the AIF curve, which showed that Gd-DTPA uptake in the intravascular space reached a “steady-state” $\sim 30 \, \text{min}$ after the beginning of the dynamic acquisition (Fig. 2). Thus, it was assumed that any CA uptake by the tissue after 30 min is the result of diffusion. The spatial permeability distribution was estimated by counting the voxels that exhibited a $K_\text{trans}$ value over a predefined threshold (0.005 min$^{-1}$). The threshold was selected in the mouse that showed the smallest BBB opening, using the quantification method described below. The estimations represented the volume area in the sonicated region where there was...
a clear permeability increase. The $K_{trans}$ values were measured both pixel-by-pixel, generating transverse and reconstructed coronal permeability maps of the mouse brain, and for a circular region of interest (ROI) of 1 mm in diameter in the targeted hippocampal area and the control side. The ROI was applied on the slice with the highest $T_1$ signal enhancement due to BBB opening and the ROI size was selected so it matched the axial full-width-at-half-maximum intensity area dimension of the beam. If the temporal Gd-DTPA concentration profile of a pixel fitted the AIF curve (Fig. 2), then that pixel was excluded and the remaining pixels within the ROI were averaged to extract the $K_{trans}$ value. The permeability was also estimated in the epicranial muscle (Fig. 3g), where the vessels lack a protective barrier, to compare the $K_{trans}$ value of muscle tissue (no barrier) with the one in the BBB-opened region. More specifically, a circular ROI with similar dimensions to the one described above was applied on the anterior left epicranial muscle on the transverse MR images, away from the sonicated region and $K_{trans}$ was averaged over the entire mouse cohort ($N = 40$).

The general kinetic algorithm determined both the $K_{trans}$ and $K_{ep}$ values, but this study emphasized only on $K_{trans}$, which represents the Gd-DTPA leakage from the systemic circulation and is mostly influenced by the concentration changes immediately after the Gd-DTPA injection. $K_{ep}$ values are mostly influenced by the “steady-state” time points of the concentration curves (Fig. 2), when equilibrium is reached between the EES and the blood plasma concentrations (27, 28).

**Physiological Assessment**

The mice were sacrificed 7 days after sonication and prepared for histology to assess the long-term bioeffects of FUS and separate reversible from irreversible damage. In addition, a parallel study was conducted by our group, which assessed these effects 3 h after sonication, which is currently under review elsewhere. A mixture of oxygen (0.8 L/min at 1.0 Bar, 21°C) and 1.5–2.0% vaporized isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL) was used to anesthetize the mice and their subsequent transcardial perfusion with phosphate-buffered saline (5 min) and 4% paraformaldehyde (8 min) at a flow rate of 6.8 mL/min. After soaking the head in paraformaldehyde for 24 h, the skull was removed and the brain was fixed again in 4% paraformaldehyde for 144 h. The brain was embedded in paraffin, serially sectioned into 6-μm-thick transverse sections, and then stained with H&E. The evaluator searched for dead neurons and erythrocyte extravasations around the sonicated region using microscopy and was blinded with respect to the pressure amplitude and microbubble size. The contrast-enhanced MR images were used as a guide for the determination of the sonicated area. The microscopic examination was also performed on the left hippocampal area, which was used as a control.

The postcontrast $T_2$ images were used as complementary information for the assessment of any physiological changes in the targeted region ~2 h after sonication. No quantification was performed in the $T_2$ images, because the field inhomogeneities that the susceptibility effects generate render such quantifications nonreproducible (29).

**RESULTS**

The population-averaged AIF of the IP-injected CA in the blood stream showed a significantly slower Gd-DTPA uptake compared with previously reported IV-administered AIFs (30), where a bolus peak is detected only seconds after the injection. Figure 2 shows that the bolus peak occurred 5–6 min after the IP injection. The Gd-DTPA concentration decayed exponentially for ~25 min after the bolus peak and reached a steady-state concentration onward. The slow Gd-DTPA uptake, associated with the IP administration, allowed the acquisition of lower temporal resolution dynamic images with relatively increased spatial resolution. The biexponential fitting of the AIF yielded the amplitude and decay rates ($A_1$, $m_1$, $A_2$, $m_2$) = [0.02, -0.06, 1.24, 0.17], which were subsequently used for the EES concentration fits.

The permeability maps (Fig. 3) assessed both the efficacy of the targeting and the spatial extent of the opening. The 1–2 μm bubbles exhibited no uptake of Gd-DTPA at 0.30 MPa and a small uptake at 0.45 MPa, which covered a volume of 2.3 ± 1 mm$^3$. At 0.60 MPa, a larger area of 12.5 ± 2.8 mm$^3$ exhibited a $K_{trans}$ higher than 0.005 min$^{-1}$. Similar $K_{trans}$ distributions were found between the 4–5 μm/0.30 MPa and 6–8 μm/0.30 MPa cases (7.5 ± 4.2 and 9.7 ± 2.7 mm$^3$, respectively), resulting in mildly permeable BBB openings (Fig. 4a). Higher BBB opening volumes were measured in the cases of 4–5 μm/0.45 MPa, 6–8 μm/0.45 MPa, 4–5 μm/0.60 MPa, and 6–8 μm/0.60 MPa (15.4 ± 4.4, 28.8 ± 3.3, 26.1 ± 5.7, and 34.8 ± 3.1 mm$^3$, respectively) (Table 2). The statistically significant P-values for each acoustic pressure and microbubble size using a two-tailed Student’s t-test with unequal variances are depicted on the corresponding graphs (Fig. 4a,b, respectively). A linear increase of the BBB opening volume with pressure ($R^2 = 0.987$) and bubble diameter ($R^2 = 0.995$) was found. The linearity was mostly evident for...
the higher pressures (0.45 and 0.60 MPa) and larger bubble diameters (4–5 and 6–8 μm) (Fig. 4a,b).

The quantitative measurements provided numerical permeability values of the BBB opening (Fig. 4b). The opening threshold for the 1–2 μm bubbles was 0.45 MPa, yielding a $K_{\text{trans}}$ value of $0.011 \pm 0.004 \text{ min}^{-1}$, whereas at 0.60 MPa $K_{\text{trans}}$ reached a value of $0.039 \pm 0.008 \text{ min}^{-1}$. The 4–5 μm bubbles exhibited higher $K_{\text{trans}}$ values, i.e., $0.028 \pm 0.013 \text{ min}^{-1}$, $0.044 \pm 0.011 \text{ min}^{-1}$, and $0.052 \pm 0.007 \text{ min}^{-1}$, for the pressures of 0.30, 0.45, and 0.60 MPa, respectively. Similar results with the 4–5 μm bubbles were found for the 6–8 μm, where the estimated $K_{\text{trans}}$ was $0.033 \pm 0.007 \text{ min}^{-1}$, $0.049 \pm 0.001 \text{ min}^{-1}$, and $0.049 \pm 0.006 \text{ min}^{-1}$ for the pressures of 0.30, 0.45, and 0.60 MPa, respectively (Table 2). The statistically significant $P$-values for each acoustic pressure and microbubble size using a two-tailed Student’s $t$-test with unequal variances are depicted on the corresponding graphs (Fig. 4c,d, respectively). The permeability of the epicranial muscle of the 0.60 MPa/1–2 μm mouse case is presented for comparison.

The histological findings showed that only three out of all mice used in this study revealed some structural neuronal damage and cell loss in the sonicated hippocampal area. In two of these mice, which were sonicated at 0.45 and 0.60 MPa, using 4–5 μm microbubbles (Fig. 5c–h,k–p, respectively), cell loss was detected in the granule cell layer of the right dentate gyrus (GrDG). Moreover, the presence of dark neurons in the layer could be an indication of apoptosis. More serious damage was found in the 6–8 μm/0.60 MPa case (Fig. 5s–x), which exhibited major damage in the CA3 region of the right hippocampus, accompanied by cell loss and multiple dark neurons.
However, the H&E slices of all the mice showed no red blood cell extravasations that could indicate hemorrhage. The $T_1$ images (Fig. 5a,i,q) and the permeability maps (Fig. 5b,j,r) of the corresponding mice, acquired $\sim 1.5$ h after sonication are presented along with the histological findings, but no direct correlation could be found between the two imaging techniques.

$T_2$ imaging was used as a complementary tool for the assessment of any physiological changes in the sonicated region. Dark regions were detected in the sonicated region in most of the cases of higher pressures and larger microbubbles (Fig. 6), but a distinct correlation with the histological findings could not be established, because H&E staining showed no signs of erythrocyte extravasations. This led to the conclusion that the dark regions in $T_2$ imaging were the result of susceptibility artifacts from the excessive Gd-DTPA excreted in the EES, rather caused by hemorrhage.

**DISCUSSION**

In this study, the permeability properties of the FUS-induced BBB opening at different microbubble sizes and acoustic pressures were mapped and quantified. We
used GKM to measure the $K_{\text{trans}}$ values in 40 mice, which were sonicated at three peak rarefractional acoustic pressures (0.30, 0.45, and 0.60 MPa), using three different microbubble sizes of 1–2, 4–5, and 6–8 μm in diameter. Transverse and coronal permeability maps were generated, which exhibited the extent of the Gd-DTPA uptake in the right hippocampal area and its proximity. Quantitative measurements of $K_{\text{trans}}$ were also carried out, providing a numerical evaluation of the efficacy of the FUS-induced BBB opening. H&E staining was performed in all mice 7 days after sonication, to assess any structural macroscopic damage. Our findings showed that the volume of the opening is directly proportional to both the acoustic pressure and the microbubble size for the larger bubbles. The volume spanned from 4.1 to 31.1 mm$^3$ for the 4–5 μm bubbles and from 6.8 to 37.7 mm$^3$ for the 6–8 μm bubbles (Fig. 4a). The 1–2 μm bubbles yielded a mean volume of 8.4 ± 3.7 mm$^3$ (0.45 and 0.60 MPa together) (Fig. 4b). The quantitative permeability measurements demonstrated that the pressure of 0.30 MPa with 4–5 and 6–8 μm bubbles yielded a subtle BBB opening with a mean $K_{\text{trans}}$ of 0.03 ± 0.011 min$^{-1}$ (Fig. 4d). For the same bubble sizes, a $K_{\text{trans}}$ plateau (0.048 ± 0.008 min$^{-1}$) was reached at higher acoustic pressures (Fig. 4d). The plateau was in excellent agreement with the $K_{\text{trans}}$ that was found in the epicranial muscle (0.047 ± 0.007 min$^{-1}$) (Fig. 3g), implying that at higher pressures and for larger bubbles the permeability properties of the sonicated hippocampal vessels are similar to the properties of the vessels that do not possess a barrier. On the other hand, relatively smaller BBB openings were detected with the 4–5 and 6–8 μm microbubbles (Fig. 4c). At 0.45 MPa, a clear opening was observed with a mean $K_{\text{trans}}$ of 0.039 ± 0.008 min$^{-1}$.

The 4–5 and 6–8 μm microbubbles appeared to have similar permeability values for each acoustic pressure that was used, although the BBB opening volume measurements exhibited a linear increase. This could be explained by the oscillatory behavior of the bubbles within the sonicated vessel. The wall boundaries of the capillaries, which span from 6 to 10 μm in diameter in the hippocampal region of the mouse brain, could be an important limiting factor in the oscillatory expansion of the bubbles during sonication. Hence, both the 4–5 and 6–8 μm bubbles are expected to expand up to the same threshold within a compliant vessel, leading to similar transfer constants. Relatively lower $K_{\text{trans}}$ values were reported in the case of 1–2 μm bubbles. These bubbles have a smaller contact area with the vessel wall and thus, may induce smaller regions with BBB opening. Also, although the number distribution for the 1–2 μm bubbles was consistent before and after the experiments, the bubble volume distribution was shifted to higher microbubble diameter sizes, sometimes within less than

**FIG. 5.** Permeability and histological findings of the only three mice that exhibited neuronal damage and cell loss. a: $T_1$ image of the first mouse, sonicated at 0.45 MPa using 4–5 μm bubbles. b: Corresponding permeability map of the first mouse. c–h: H&E sections of the first mouse. i: $T_1$ image of the second mouse, sonicated at 0.60 MPa using 4–5 μm bubbles. j: Corresponding permeability map of the second mouse. k–p: H&E sections of the second mouse. q: $T_1$ image of the third mouse, sonicated at 0.60 MPa using 6–8 μm bubbles. r: Corresponding permeability map of the third mouse. s–x: H&E sections of the third mouse. The black boxes in (c), (f), (k), (o), (s), and (v) refer to the regions of interest depicted in (d), (g), (l), (i), (t), and (x), respectively. The black boxes in (d), (g), (l), (i), (t), and (x) refer to the regions of interest depicted in (e), (h), (m), (p), (u), and (x), respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
the time-dependence of reference region model (31). The IP CA administration, the Gd-DTPA concentration fits for both GKM and the DCE-MR acquisitions, to be able to extract solid constriction timeline needs to be followed during the FUS and decrease over time, as BBB properties will change. A specific and kinetic model can significantly increase the accuracy of the permeability measurements, especially in BBB disruptive models (34). Future studies will include periodic $K_{\text{trans}}$ measurements while the BBB remains open, which will allow us to better understand the closing mechanism of the barrier. Finally, additional parameters that permit to evaluate the efficacy of the BBB opening, such as the maximum uptake and washout slope, the Gd-DTPA diffusion in the EES and the EES volume fraction ($v_e$) will be added in our future model estimations, which will provide additional information on the pharmacokinetics.

The histological findings of this study revealed that no significant damage was induced in most of the sonicated mice, demonstrating that the permeability can increase, but not at the expense of safety. Three out of 40 mice (7.5%) showed signs of neuronal damage or cell loss in the sonicated hippocampus 7 days after sonication at pressures higher than 0.45 MPa and microbubble sizes larger than 4–5 μm (Fig. 5). The damage was represented by the presence of necrotic or apoptotic cells. In one particular case, a significant deformation of the right hippocampal anatomical structure was observed. Overall, the acoustic pressure of 0.30 MPa for larger bubbles and the pressures of 0.45 and 0.60 MPa for the 1–2 μm bubbles appeared to always induce safe BBB opening, according to the 7-day H&E findings. These findings indicate that larger bubbles and the 1–2 μm bubbles may induce larger and smaller BBB openings, respectively, than the commercial microbubbles that have been used in other studies (15).

The presence of dark regions in $T_2$ imaging (Fig. 6) at higher FUS pressures using larger sized bubbles was found not to correlate with hemorrhage, because no red blood cell extravasations were found at H&E examination of any of the mice used in this study. Thus, the dark regions were assumed to be directly related to field inhomogeneities, because of the excessive Gd-DTPA presence in the EES. Liu et al. (17) have suggested that susceptibility-weighted imaging can detect massive hemorrhagic regions after FUS at the acoustic pressure of 3.47 MPa, but more subtle effects on the brain, as was the case at the low acoustic pressures studied in this article, appear to be a challenge on the sensitivity and spatial resolution of the MRI used. Future studies will entail histological assessment of the mouse brains on the day of sonication with additional histological examination, which will allow us to accurately detect and characterize other types of tissue effects induced and subsequently relate them to the measured permeability increase.

CONCLUSIONS

This study investigated the relationship between permeability, the diameter of the administered microbubbles, and the peak rarefractional pressure of the FUS-induced BBB opening. Nine different groups of mice were
sonicated using three microbubble sizes (1–2, 4–5, and 6–8 μm) and three acoustic pressures (0.30, 0.45, and 0.60 MPa). The volumetric and quantitative permeability measurements showed that $K_{\text{trans}}$ in the sonicated region increases with the bubble size and the acoustic pressure. The volumetric area of the BBB opening spanned from 1.5 to 37.7 mm², showing a linear increase with the bubble size for the higher acoustic pressures ($R^2 \geq 0.98$). With the exception of the 1–2 μm bubbles, the acoustic pressure of 0.30 MPa yielded a mean $K_{\text{trans}}$ of 0.03 min⁻¹, whereas a plateau was reached above 0.45 MPa at ~0.05 min⁻¹. An excellent agreement was found between the $K_{\text{trans}}$ plateau and the permeability value of the epithelial muscle, i.e., at the absence of a barrier. The 1–2 μm bubbles did not open the BBB at 0.30 MPa, whereas at 0.45 and 0.60 MPa they yielded mean $K_{\text{trans}}$ values of ~0.01 and 0.04 min⁻¹, respectively. Histological examination of H&E sections revealed some structural damage in 7.5% of the mice and only at higher pressures and larger bubbles, but unlike in previous reports no hemorrhagic regions were detected, including in the cases of the highest acoustic pressure studied (0.60 MPa) and the largest bubbles (6–8 μm). This points to the effect of the shorter pulse length used here. The results indicate that DCE-MRI could be used as a diagnostic method for the assessment of the permeability of the barrier and thereby efficacy of drug delivery through the FUS-induced BBB opening.

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