Opening of the blood-brain barrier with an unfocused ultrasound device in rabbits

Laboratory investigation

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Object. The blood-brain barrier (BBB) is a major impediment to the intracerebral diffusion of drugs used in the treatment of gliomas. Previous studies have demonstrated that pulsed focused ultrasound (US) in conjunction with a microbubble contrast agent can be used to open the BBB. To apply the US-induced opening of the BBB in clinical practice, the authors designed an innovative unfocused US device that can be implanted in the skull and used to transiently and repeatedly open the BBB during a standard chemotherapy protocol. The goal of this preliminary work was to study the opening of the BBB induced by the authors’ small unfocused US transducer and to evaluate the effects of the sonications on brain parenchyma.

Methods. Craniectomy was performed in 16 healthy New Zealand White rabbits; epidural application of a single-element planar ultrasonic transducer operating at 1 MHz was then used with a pulse-repetition frequency of 1 Hz, pulse lengths of 10–35 msec, in situ acoustic pressure levels of 0.3–0.8 MPa, and sonication for 60–120 seconds. Sonovue was intravenously injected during the US applications, and opening of the BBB was determined by detecting extravasation of Evans blue dye (EBD) in brain tissues, quantitative measurement of EBD with UV-visible spectrophotometry, and contrast enhancement after Gd injection in 4.7-T MRI. A histological study was performed to determine adverse effects.

Results. An opening of the BBB was observed over a large extent of the US beam in the brain corresponding to in situ pressures of greater than 0.2 MPa. The BBB opening observed was highly significant for both EBD (p < 0.01) and MRI Gd enhancement (p < 0.0001). The BBB opening was associated with minor adverse effects that included perivascular red blood cell extravasations that were less than 150 μm in size and not visible on MR images. Moderate edema was visible on FLAIR sequences and limited to the extent of the sonication field.

Conclusions. The results demonstrate that the BBB can be opened in large areas of the brain in rabbits with low-power, pulsed, and unfocused US with limited damage to healthy tissue.

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Key Words • blood-brain barrier • unfocused ultrasound • brain tumor • Evans blue dye • MRI • chemotherapy • oncology
of a monolayer of endothelial cells that line the cerebral microvessels and connect to each other via tight junctions. The BBB represents active and passive transport mechanisms that limit the passage of potentially toxic molecules from the blood to the brain.\textsuperscript{1,2} As a consequence, approximately 98\% of small-molecule drugs (< 0.5 kD) and 100\% of large-molecule drugs do not cross the intact BBB.\textsuperscript{26}

Several methods have been investigated for their potential to increase the permeability of the BBB to therapeutic molecules. These methods include chemical modifications of drugs to make them more lipophilic or linkage to carriers that can cross the BBB.\textsuperscript{27,28} However, the development for therapeutic applications of such molecules is still limited and these chemical approaches result in a diffuse and nontargeted passage through the BBB. Balis et al. in 1985 proposed to use high-dose intravenous methotrexate to reach higher doses in the central nervous system to treat children with acute lymphoblastic leukemia and meningeal disease.\textsuperscript{2} The study reported good results with this method, but this technique is limited by the systemic adverse effects induced by high systemic drug concentrations. In addition, several clinical studies of patients with cerebral lymphoma or brain tumors have used arterial injection of hypertonic solutions to temporarily increase the BBB permeability.\textsuperscript{5,24,38} Although encouraging results have been obtained with this technique, it is invasive, requires general anesthesia, and is associated with significant complications, including vascular lesions due to arterial catheterization, transient neurological deficits, or seizures.

Seventeen years ago, Vykhodtseva et al. noted that pulsed US could induce local disruption of the blood-brain barrier in rabbit brain.\textsuperscript{35} A few years later, Hynynen et al.\textsuperscript{13} studied the effects of 1.63-MHz sonication in the presence of an intravenous microbubble contrast agent under MRI monitoring of rabbit brain. The authors used varying acoustic power levels (0.2–11.5 W) and burst lengths (10–100 msec) and confirmed that focused US combined with the application of microbubble contrast agent could be potentially used for noninvasive image-guided focal opening of the BBB.\textsuperscript{13} With low-frequency bursts (0.26 MHz), a focused US-induced opening of the BBB can be performed with in situ acoustic pressures of less than 0.2 MPa.\textsuperscript{14} Opening of the BBB is dependent on acoustic parameters such as burst length,\textsuperscript{20} pressure amplitude,\textsuperscript{12,13} and more generally the mechanical index;\textsuperscript{19} the concentration\textsuperscript{39} and type\textsuperscript{21} of the contrast agent used during the sonications also plays a role in the magnitude of the BBB opening. Depending on the in situ acoustic pressure applied, the sonications used for opening of the BBB may result in red blood cell extravasation, petechial bleeding, and large areas of hemorrhage or necrosis and apoptosis.\textsuperscript{12} However, by using low acoustic pressures (mechanical index < 1), opening of the BBB can be achieved with minimal or no tissue damage.\textsuperscript{10,16,25,34} All of these results, initially obtained in small animal models, have been recently confirmed in nonhuman primate studies, opening the way for clinical translation.\textsuperscript{17,18}

The human skull bone represents the principal barrier to the use of US for intracranial pathologies since it attenuates and distorts the propagation of US.\textsuperscript{32} While transcranial delivery of therapeutic US into the brain is feasible with the use of phased array transducers,\textsuperscript{31} this technique requires complicated electronics and corrections for phase distortion and attenuation by the skull.

Surgery and removal of a small portion of the skull is a common practice for the treatment of brain tumors either to resect the tumor or to remove a tissue sample for histological analysis.\textsuperscript{30,31} Moreover, after tumor resection, drug delivery has to be targeted to a large area around the tumor cavity to create a wide field of diffusion for efficient action of the drug on infiltrated tumor cells that have the potential for generating tumor recurrence. In view of these 2 observations, our group is developing a small planar US transducer that can be implanted into the skull bone for opening of the BBB in the vicinity of tumor cavities. By emission of unfocused US, such an MRI-compatible device can increase the permeability of the BBB in a large volume of brain tissue. Moreover, by implanting the US transducer in a skull burr hole, attenuation and distortion of the US energy by the skull bone can be avoided, and sonications can be repeated regularly without anesthesia and without the requirement of MRI control being synchronized with a standard chemotherapy regimen.

The purpose of this study was to characterize a prototype US device, to test its capacity to open the BBB in rabbit brain, and to evaluate potential adverse effects of sonications. The experiments were performed on healthy rabbits, with a 1-MHz unfocused transducer operating at pressures ranging from 0.3 to 0.8 MPa, in combination with a microbubble contrast agent. The opening of the BBB was detected and quantified through EBD diffusion in brain parenchyma and MRI contrast enhancement after Gd intravenous injection. A histological study was performed to evaluate the safety of this approach.

Methods

Preparation of Animals

All of the experiments described were approved by the faculty ethics committee (Comité d’Ethique en matière d’Expérimentation Animale, Paris-Descartes, France). Experiments were performed by researchers authorized and certified to perform animal experiments. This study used 16 healthy male New Zealand White rabbits, weighing 2.5–3.5 kg (CEGAV SSC). Animals were anesthetized with an intramuscular injection of a mixture of xylazine (9 mg/kg; Rompun, 2\% solution, Bayer) and ketamine (50 mg/kg; Imalgène, solution of 1000 mg/10 ml, Merial); additional injections were performed if necessary during the experiments. A femoral vein was catheterized for perfusion of isotonic saline during the experiment and for injection of drugs, US-contrast agent, and EBD. Analgesia was ensured with a 2-mg intravenous bolus of morphine (Morphine Lavoisier, 10 mg/ml); the scalp was infiltrated with Xylocaine (at 1\%) before the skin incision. A unilateral craniectomy was performed from the coronal suture to 2 cm posteriorly. Killing of the animals was performed by intravenous injection of pentobarbital sodium salt (54.7 mg/100 ml). Brains were extracted at the end of each experiment.

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Ultrasound Experimental Setup

A custom-built air-backed 1-MHz single-element transducer was constructed with a 10-mm flat piezoceramic disk (PZ 26, Ferroperm Piezoceramics). The transducer was driven with a function generator (HP 33120A, Hewlett Packard) and a radiofrequency amplifier (25 W, Kalmus 125C CE, Kalmus). The electrical power was monitored with a power meter (Rhode and Schwarz). To characterize the acoustic output of the transducer, a calibrated hydrophone (HGL-0200, Onda) was used to perform field scans of the pressure distribution at low amplitude. Figure 1 shows the results of field scans in the plane along the acoustic axis. The pressure contours in the figure were normalized to the value measured at a distance of 12 mm from the transducer. Here, the pressure contours corresponding to 0.25, 0.5, 0.75, and greater than 1.25 of the normalized pressure are shown. Calibrated hydrophone measurements were performed at a distance of 12 mm on the acoustic axis at high power in water under the same conditions as used in the in vivo experiments and were derated by a factor of 0.5 dB/cm to account for attenuation in the rabbit brain. This pressure amplitude, hereafter referred to as the “in situ pressure,” is used throughout this paper to describe the exposure conditions used in each animal and is shown marked by an “x” in Fig. 1.

The sonications were performed with the generator and the amplifier described above. After craniectomy 1 cm behind the coronal suture, the transducer was placed on and coupled to the dura mater with a thin layer (1 mm) of US-coupling gel (Sonogel, Sonogel Vertriebs GmbH). The transducer was operated at a center frequency of 1 MHz and a pulse repetition frequency of 1 Hz, with pulse lengths ranging from 10 to 35 msec and in situ acoustic pressure levels ranging from 0.3 to 0.8 MPa. At the beginning of the sonication, a bolus of 0.03 ml of US contrast agent (Sonovue, Bracco Imaging) was injected into the femoral vein, followed by a flush of 2 ml of saline. One sonication was delivered into 1 of the hemispheres of each animal; the contralateral hemisphere served as a control. Thirteen rabbits were sonicated for 120 seconds to evaluate the effects of varying US parameters; pulse lengths and in situ acoustic pressures for each exposure condition are given in Table 1. One animal received a shorter sonication treatment of 60 seconds. Two additional animals served as controls: 1 had a unilateral craniectomy, without sonication or injection of US contrast agent, and another received sonication without injection of US-contrast agent.

Evans Blue Dye Study

The opening of the BBB was quantified by measuring the diffusion of EBD in the brain parenchyma. The EBD binds to albumin and does not freely cross the intact BBB. The dye (Evans blue, pure grade, Réactifs RAL) was injected intravenously 30 minutes after sonication, at a dose of 100 mg/kg in 6.5 ml of saline. The animals were killed 240 minutes after the dye injection, and brains were extracted immediately. Two different analysis methods were performed on the extracted brains. First, a qualitative analysis examined the blue coloration in the brain to characterize the BBB opening (situation, volume, and intensity); examination for potential hemorrhagic lesions was also performed during this first macroscopic evaluation. Second, a quantitative analysis measured the EBD concentration in different sites of the brain parenchyma: after the macroscopic analysis, a coronal slice of the brain centered on the sonication site was prepared. Six samples were then taken from this slice: 3 samples in each hemisphere, at 3 different levels of depth (cortical, subcortical, and deep; Fig. 2). The samples were weighed. Calibration standards, quality controls, and samples were mixed with 100 ml of water and 750 ml of acetonitrile. The mixture was then homogenized and centrifuged; absorbance in the supernatant due to EBD was measured using UV-visible spectrophotometry at a wavelength of 610 nm. Brain calibration standards and quality controls were prepared by spiking blank brain samples with 100 µl of appropriate working solutions of EBD (1–50 µg/ml). This quantification method was linear between 0 and 50 µg/g. The minimum sensitiv-

![Fig. 1. Acoustic pressure measured in water generated by the 1-MHz flat 10-mm piston transducer used in this study for opening of the BBB. The pressure field was mapped in a plane perpendicular to the transducer. The pressure field shown was normalized to the acoustic pressure at a distance of 12 mm, indicated by the small "x" in the graph.](image-url)
ity was 1 μg/g. To evaluate the EBD diffusion in 2 different tissues, 2 more samples from 1 animal were extracted: 1 sample from the gray matter and the other from the white matter.

**Histological Study**

The animals were killed, and brains were immediately removed and cut into coronal slices. Some slices were used for the EBD study described above; others (2–3 per animal) were fixed by immersion into 10% formalin for histological analysis. After fixation of the tissues, the slices were embedded in paraffin, sectioned into slices of 3 to 4 μm thickness, and stained with hematoxylin and eosin. A light-microscopy study was performed, and the hemorrhagic lesions observed were graded following the system proposed by Hynynen et al.12 Lesions were graded on a scale ranging from 0 to 3 where “0” corresponded to no damage of the tissue, “1” corresponded to 1 or a few red blood cell extravasations, “2” corresponded to petechial hemorrhages or mild damage to the brain parenchyma, and “3” corresponded to hemorrhagic or nonhemorrhagic local lesions. The neuropathologist who performed the histological examination was blinded to the US parameters used but had general knowledge of the sites targeted by the US because of EBD coloration.

**Magnetic Resonance Imaging Study**

An MRI study was performed on 5 rabbits (Table 1) not included in the histological study and EBD experiments. After craniectomy, 1 hemisphere was sonicated in combination with intravenous contrast agent. The animals were under general anesthesia during the procedure and immobilized in a customized support system. The MR imager was a Bruker Biospec 47/40, 4.7 T. Just after the sonication, several MRI sequences including T1, T2*, T2, diffusion, and FLAIR were performed. A first dose of MRI contrast agent (Gd oxide, Dotarem, 0.5 mmol/ml) was injected just before the first FLAIR sequence, followed by a T1-weighted sequence. Then, 2 to 3 more sequences were acquired at different times: a T1-weighted sequence was acquired before any additional Gd injection to determine the residual Gd concentration. After Gd injection, a FLAIR and 1 or several T1-weighted sequences were acquired at various time intervals of up to 473 minutes after sonication. The MRI parameters used were the following: T1: TR 220 msec, TE 6 msec, flip angle 40°.

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**TABLE 1: Summary of the experiments in this study**

<table>
<thead>
<tr>
<th>Experimental Protocol*</th>
<th>Craniectomy</th>
<th>Sonication Length (sec)</th>
<th>Sonovue Dose (ml)</th>
<th>No. of Rabbits</th>
<th>In Situ Acoustic Pressure (MPa)</th>
<th>Pulse Length (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control: no ultrasound, no microbubble contrast agent</td>
<td>unilat</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control: w/ ultrasound, no microbubble contrast agent</td>
<td>bilat</td>
<td>120</td>
<td>0</td>
<td>1</td>
<td>0.5</td>
<td>35</td>
</tr>
<tr>
<td>treatment: short sonication</td>
<td>unilat</td>
<td>60</td>
<td>0.3</td>
<td>1</td>
<td>0.5</td>
<td>35</td>
</tr>
<tr>
<td>treatment: long sonication</td>
<td>unilat</td>
<td>120</td>
<td>0.3</td>
<td>2</td>
<td>0.3</td>
<td>25, 35</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment: long sonication</td>
<td>unilat</td>
<td>120</td>
<td>0.3</td>
<td>1</td>
<td>0.4</td>
<td>25</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

* In total 16 animals were used in this study, including 2 controls. The 10 treated rabbits in the EBD study were exposed to a range of ultrasound conditions in which the pulse lengths ranged from 10 to 35 msec and the acoustic pressure levels from 0.3 to 0.8 MPa. The 5 rabbits in the MRI study were exposed to a range of ultrasound conditions in which the pulse length was 25 msec and the acoustic pressure levels ranged from 0.4 to 0.7 MPa. All exposures were performed using a 1-MHz transducer.
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and number of excitations 5; T2: TR 4000 msec, TE 50 msec, flip angle 170°, number of excitations 3, and echo train length 8; T2*: TR 340 seconds, TE 17 msec, flip angle 40°, number of excitations 5, and echo train length 1; FLAIR: TR 10,000 msec, TE 39 msec, inversion time 1800, flip angle 170°, number of excitations 2, and echo train length 8; diffusion: TR 2250 msec, TE 34 msec, flip angle 90°, number of excitations 1, echo train length 21; and T1 and Gd: TR 220 msec, TE 6 msec, flip angle 40°, number of excitations 5, and echo train length 1. The matrix size was 256 × 256 and the field of view was 90 × 90 mm with a slice thickness of 2 mm for all sequences (voxel size 0.35 × 0.35 × 2 mm) except for the diffusion sequence, where the matrix size was 128 × 128 (voxel size 0.7 × 0.7 × 2 mm). Although our transducer was MRI compatible, sonications were not performed inside the coil but just outside the MR bore. Results of the MRI were analyzed using OsiriX (Pixmeo) and ImageJ (Rasband, W. S., ImageJ, U. S. National Institutes of Health: http://imagej.nih.gov/ij/, 1997–2012). To study the contrast enhancement after Gd injection, the most representative slide was chosen. Next, 2 ROIs were selected in each cerebral hemisphere: one in the cortex and the other in the deep-brain structures. The average signal intensity and its standard deviation for all the pixels in each ROI were calculated. Statistical analysis was performed to compare the signal intensity in the ROI in the sonicated and nonsonicated hemispheres.

Statistical Analysis

Statistical analysis was performed by an independent, blinded statistician using a statistical software package (JMP software, SAS Institute Inc.). Statistical significance was tested using a paired t-test. Probability values of <0.05 were considered statistically significant.

Results

Evans Blue Dye Study

Except for the 2 control rabbits, an opening of the BBB was achieved with all of the US exposure parameters used in this study. A typical EBD-stained brain sample after sonication is shown in Fig. 3. The increased permeability of the BBB was observed as a blue coloration of the brain parenchyma. The coloration was unilateral (limited to the sonicated hemisphere) and extended roughly within the spatial extent of the sonication field (a cylinder 10 mm in diameter extending from the dura mater to the skull base, corresponding to a volume of approximately 1.2 cm³). This sonication field roughly corresponded to the region limited to the 0.25 pressure level contour shown in Fig. 1. The EBD staining was not homogeneous within the sonicated region, and the staining also varied depending on the acoustic parameters used. In general, when acoustic parameters (in situ acoustic pressure and pulse lengths) were increased, diffusion of the EBD in the brain parenchyma resulted in more intense staining, and the area of the BBB disruption extended from the surface to deeper regions in the brain. In addition, the EBD staining decreased with the depth of the pressure field and was heterogeneous among different regions of the brain. In particular, more intense coloration was observed in the gray matter than in the white matter (Fig. 4). A sample of each type of brain tissue was extracted from the sonicated hemisphere in 1 of the rabbits to measure EBD concentration. Concentrations of EBD were 64.2 µg/g in the gray matter sample and 8.7 µg/g in the white matter sample, indicating that the EBD concentration was 7 times greater in the gray matter than in the white matter.

The concentration of EBD in the brain parenchyma was measured in 66 samples. The data of the 8 rabbits (Rabbits 1–8; see Table 3) that had received 120-second-long sonications in the presence of US contrast agent were statistically analyzed by using a unilateral paired t-test. The analysis compared the EBD concentration in the sonicated hemispheres and in the nonsonicated hemispheres (contralateral hemispheres) at 3 different depth levels (cortical, subcortical, and deep). A statistically significant difference was observed between the EBD concentrations in the sonicated cortex and the concentrations in the nonsonicated contralateral cortex (p < 0.01). A sim-

![Fig. 3. Representative example of EBD extravasation observed in rabbit brain after sonication. In situ acoustic pressures and pulse lengths of 0.8 MPa and 10 msec (left) and 0.5 MPa and 25 msec (right), respectively, were used with a pulse repetition frequency of 1 Hz for 120 seconds. The sonicated cortex is colored by the blue dye (left, black circle). The coloration is visible at depth in coronal slices of brain: it is unilateral and spatially limited to the extent of the ultrasound field (right, dashed lines). Some very small areas of petechial bleeding are visible in the proximal part of the pressure field (right, arrows).](image)

![Fig. 4. Brain tissue sonicated with a 0.5-MPa acoustic pressure and 35-msec pulse length using a pulse repetition frequency of 1 Hz and 120 seconds of sonication. Note that coloration with EBD is more intense in the gray matter (asterisks) than in the white matter (daggers). Very small areas of petechial hemorrhages are predominantly situated in the cortical gray matter (arrows).](image)
ilar statistically significant difference was also observed for the subcortical samples (p = 0.04) but not for the deep-brain samples (p = 0.37) (Table 2).

Microscopic petechial hemorrhages (<150 μm in diameter) were observed in all of the sonicated brain tissues (Fig. 3). These hemorrhages were limited to the blue-stained area and predominated in the proximal part of the pressure field, particularly in the cortex. Their number increased with the acoustic parameters (pulse length and in situ acoustic pressure). Subarachnoid hemorrhages were observed in 3 animals with higher sonication parameters of 25 msec pulse length and 0.5 MPa (Rabbit 5; see Table 3), 15 msec pulse length and 0.8 MPa (Rabbit 2), and 25 msec pulse length and 0.8 MPa (Rabbit 1). In the latter 2 animals, which were sonicated with the highest parameters, brain tissues presented larger hemorrhagic areas with an aspect of subcortical hematoma. However, it was difficult to macroscopically distinguish a hematoma from an intense blue staining.

In the 2 controls (1 without sonication [Rabbit 11] and 1 with sonication and without contrast agent injection [Rabbit 10]), no significant blue coloration of the brain parenchyma was observed (Fig. 5). No macroscopic petechial hemorrhages were observed in either rabbit. In the animal that did not receive US, EBD concentrations were similar in both sides of the brain regardless of the site of the craniectomy, indicating that the opening of the BBB was not due to the craniectomy. In the control animal sonicated without contrast agent, EBD concentrations were comparable to concentrations observed in the nonsonicated hemispheres of the animals receiving sonication in the presence of the contrast agent. The animal that had received a short sonication of 60 seconds (Table 1) showed a blue staining of the sonicated hemisphere comparable to the staining observed with a long sonication with the same parameters (35 msec and 0.5 MPa). The concentration of EBD was similar in the brains of the 2 control rabbits.

Histological Study

For the histological study, 29 brain slices were analyzed, and grading of the lesions was performed as proposed by Hynynen et al. For each rabbit, the highest grade observed among all the slices analyzed was determined (Table 3). Figure 6 shows histological sections of brain parenchyma illustrating the 4 levels of grading used.

Grade 0 (no detected damage, Fig. 6A) was the only grade observed for the control animal (no US, no microbubble contrast agent), and the higher grade observed for the animal treated with sonication without US contrast agent. This grade was also observed in other animals, in tissue slices taken from the periphery of the sonication field; in those cases, Grade 0 lesions were associated with higher-grade lesions in the center of the sonication field.

Grade 1 (1 to a few tiny red blood cell extravasations, Fig. 6B) was the highest grade observed in 1 animal (Rabbit 8 [Table 3]) treated with acoustic parameters of 25 msec and 0.3 MPa. In the 4 other animals, Grade 1 lesions were observed in the periphery of the sonication field; higher grades were noted in slices taken from the center of the sonication field. These lesions were generally situated in the cortical and the subcortical tissue; sometimes, they were also observed in the subependymal and paraventricular regions.

Grade 2 (petechial hemorrhages; mild damage to the brain parenchyma, Fig. 6C) was the histological grade that was the most frequently observed in these experiments. For 7 of 11 rabbits, Grade 2 was the highest grade observed among all the brain slices studied. It corresponded to petechial hemorrhages from red blood cell extravasations, generally centered on a blood vessel. Sometimes, they were surrounded by a circular zone of vacuolization due to the vascular leakage induced by the sonication. The maximum lesion size observed was approximately 150 μm in diameter. The petechial hemorrhages, when observed, were scattered within the sonication field.

Grade 3 (hemorrhagic or nonhemorrhagic local lesions, Fig. 6D) was the highest grade observed in 1 rabbit (Rabbit 1 [see Table 3], which had received the highest intensity of the acoustic parameters: 25 msec and 0.8 MPa): hemorrhages were diffuse on 2 different slices in the sonication field. In 3 other animals, the lesions corresponded to a single frontopolar lesion distant from the US beam, and ipsilateral or contralateral to the sonication, and these lesions were probably due to traumatic extraction of the brain.

All of the microscopic petechial hemorrhages associated with the sonication were situated and limited to the extent of the sonication field (Grade 0: no detected dam-

![Fig. 5. Brain slices from control animals. Left: Rabbit with right craniectomy (C) without sonication. Right: Rabbit with right sonication (US) and without microbubble contrast agent. No significant blue dye coloration of the parenchyma is present in either sample: craniectomy alone and US without microbubbles do not cause significant opening of the BBB. Both controls do not show any signs of macroscopic petechial hemorrhages.](image-url)

**TABLE 2: Mean EBD concentrations in sonicated and nonsonicated hemispheres**

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Sonicated Hemisphere</th>
<th>Nonsonicated Hemisphere</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortex</td>
<td>26.9</td>
<td>6.5</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>subcortical region</td>
<td>10.0</td>
<td>5.2</td>
<td>0.04†</td>
</tr>
<tr>
<td>deep region</td>
<td>5.4</td>
<td>4.9</td>
<td>0.37†</td>
</tr>
</tbody>
</table>

* Evans blue dye concentrations in the 8 rabbits that received 120-second-long sonications with ultrasound contrast agent.
† Concentrations were significantly different between the sonicated and nonsonicated hemispheres of the cortex and subcortical brain samples; concentrations were not significantly different between the sonicated and nonsonicated hemispheres of the deep-brain samples.
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TABLE 3: Summary of the data in this study

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Parameters (MPa, msec)*</th>
<th>Histological Lesions (higher Hynynen grade)†</th>
<th>Contrast Enhancement on MRI‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8, 25</td>
<td>hemorrhagic local lesions (3)</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>0.8, 15</td>
<td>&lt;150-mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>0.8, 10</td>
<td>&lt;150-mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>0.5, 35</td>
<td>&lt;150-mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>0.5, 25</td>
<td>&lt;150-mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>0.5, 15</td>
<td>&lt;150-mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>0.3, 35</td>
<td>&lt;150 mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>0.3, 25</td>
<td>few red blood cell extravasations (1)</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>0.5, 35; short sonication (60 sec)</td>
<td>&lt;150-mm petechial hemorrhages (2)</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>0.5, 35; no microbubble contrast agent</td>
<td>no damage detected (0)</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>no US, no microbubble contrast agent</td>
<td>no damage detected (0)</td>
<td>NA</td>
</tr>
<tr>
<td>12§</td>
<td>0.4, 25</td>
<td>NA</td>
<td>slightly visible</td>
</tr>
<tr>
<td>13§</td>
<td>0.7, 25</td>
<td>NA</td>
<td>clearly visible</td>
</tr>
<tr>
<td>14¶</td>
<td>0.6, 25</td>
<td>NA</td>
<td>clearly visible</td>
</tr>
<tr>
<td>15¶</td>
<td>0.6, 25</td>
<td>NA</td>
<td>clearly visible</td>
</tr>
<tr>
<td>16¶</td>
<td>0.6, 25</td>
<td>NA</td>
<td>clearly visible</td>
</tr>
</tbody>
</table>

* Ultrasound parameters were in situ acoustic pressure (in MPa) and pulse length (in msec).
† Histological lesions and corresponding highest histological grade (in parentheses) were assessed as described by Hynynen.10
‡ Observation of MRI signal enhancement after Gd injection. NA = not assessed.
§ Statistical analysis was not performed because these 2 animals had US parameters that were different from those used for the other animals. NA = not assessed.
¶ A statistically significant contrast enhancement of the voxel intensity was observed in the sonicated cortex (t-test, p < 0.0001) compared with the nonsonicated contralateral cortex. NA = not assessed.

age outside of the sonication field); these hemorrhages were essentially concentrated in the first 0.6 mm from the surface of the cortex. In 5 of the 11 animals, the hemorrhage grade in brain slices progressively decreased with increasing distance from the center toward the periphery of the sonication field. Neither focal ischemic lesions directly associated with the US beam nor interstitial edema was observed in the histological study.

Paraventricular Grade 2 hemorrhages (petechial hemorrhages) and areas of subarachnoid hemorrhage on the side of the craniectomy were observed in the slices taken from the animal treated by sonication without the US contrast agent. For 3 sonicated rabbits, Grade 3 lesions (hemorrhagic local lesions) were observed in the frontal pole, ipsilateral or contralateral to the sonication and were distant from the US beam. All of these lesions were most likely due to a traumatic extraction of the brain and, therefore, are not reported in Table 3.

Magnetic Resonance Imaging Study

The MRI study was performed on 5 rabbits (Tables 1 and 3). Rabbit 12 was exposed to a 0.4-MPa and 25-msec sonication. Contrast enhancement was observed after a total injection of 3 ml of Gd beginning 108 min after the sonication and was limited to the sonication field, from the cortex to the depth of the brain. Rabbit 13 was exposed to a 0.7-MPa and 25-msec sonication and received 3 ml of Gd. A contrast enhancement was observed 49 to 473 minutes after the sonication, with a stable intensity and the same radiological characteristics as described above. A 3 × 4–mm image was observed in the deep-brain structures on the T2* (hyposignal), T1 (hyposignal), and FLAIR (hypersignal) sequences but not on the T2 diffusion and Gd T1 sequences. Because no MRI exam was performed before the sonication, we do not know whether this image had appeared before or after the sonication. This region was not found to be hemorrhagic on macroscopic observation of brain slices. Rabbits 14–16 were each exposed to a 0.6-MPa and 25-msec sonication with the same Gd dose as Rabbit 2. The same contrast enhancement as described above was observed in the sonication field, from 49 to 473 minutes after the sonication. No adverse event was observed on any sequences (Fig. 7A–H).

A statistical analysis was performed to compare the sonication results of Rabbits 14–16. A statistically significant contrast enhancement of the voxel intensity was observed in the sonicated cortex (t-test, p < 0.0001) compared with the nonsonicated contralateral cortex. NA = not assessed.
trast enhancement was significantly higher in the sonicated hemisphere than in the control hemisphere (t-test, p < 0.0001).

The last Gd injection with MRI acquisition was performed about 8 hours after sonication and was followed by the same procedure for contrast enhancement as described above. We noted that in the T1-weighted images, the signal intensity in the sonicated cortex before Gd injection was lower than in the contralateral cortex; thus, the sonicated cortex appears to yield an intrinsically lower image signal compared with the nonsonicated cortex.

Finally, with acoustic pressures ranging from 0.4 to 0.6 MPa, no hemorrhagic or ischemic lesions were observed (Fig. 7F). Edema was observed in FLAIR sequences after the sonication on all of the animals exposed to 0.6 MPa (Rabbits 14–16; Fig. 7G). It was limited to the extent of the sonication field and was not associated with any mass effect. No steroids were administered that would have limited the edemas.

**Discussion**

It is important to first discuss the motivation for performing the studies described herein. In this study, we have used a single-element unfocused transducer to induce a broad opening of the BBB. This approach is different from those described in most other studies, which have used focused transducers to induce BBB openings that are only very localized.10–13,15–21 In contrast, our goal is to develop a device for increasing the permeability of the BBB that can be used for broad delivery of chemotherapy drugs for diffuse glioma pathologies, can be easily applied in clinical practice during multiple chemotherapy sessions, and does not require head immobilization and anesthesiology. Because US does not easily pass through the skull, we propose that implantation of a US transducer for efficient transmission could take advantage of existing bur holes from conventional open-brain surgery. In addition, since it is necessary to perform repeated follow-up MRI during glioma treatments, the US device has to be entirely compatible with MRI by not inducing artifacts and not requiring an integrated power supply. To achieve this, we plan to develop an electric power supply that is easily connected to the device during sonications by using a transdermal bipolar needle that plugs into the device (Fig. 9).

The aim of this preliminary study was to determine if broad opening of the BBB could be achieved with a 1-MHz unfocused US transducer. As reported in the results, 2 methods were used to evaluate the opening of the BBB: EBD diffusion in the brain parenchyma and contrast enhancement after Gd injection on MRI acquisitions.13 In our study, a significantly increased permeability of the BBB after brain sonication was detected with both methods. The concentrations of EBD were significantly higher in the sonicated hemisphere, particularly in the cortex and the subcortical regions. Measurements of MRI contrast enhancement indicated significantly higher contrast in the sonicated hemisphere, particularly in the cortex. Although this is not the first time BBB disruption has been achieved with an unfocused US,15,23 to our knowledge, our study is the first that has tested a small unfocused US transducer that can be adapted for use as an implantable US device in a bur hole.

Disruption of the BBB was achieved with in situ acoustic pressures of 0.3 to 0.8 MPa and pulse lengths of 15 to 35 msec. In all cases, the transducer was operated at a center frequency of 1 MHz and a pulse repetition frequen-
Ultrasound-induced blood-brain barrier opening in rabbits

The BBB opening observed in this study displayed heterogeneity as a function of tissue depth and tissue characteristics. Intracerebral diffusion of the blue dye followed a decreasing intensity from the surface to the depth of the sonicated brain. This observation, made from the EBD qualitative analysis of the brain slices, was confirmed by the EBD quantitative analysis and the MRI study. The difference in blue dye concentration between the sonicated and the nonsonicated hemispheres was statistically significant in the cortex and in the subcortical region but not in the deep-brain regions; in the MRI analysis, the contrast enhancement after Gd injection was more heterogeneous in the deep-brain structures than in the cortex. Although attenuation of the US by the brain tissue is one plausible explanation for this effect, attenuation does not adequately explain these observations. The depth of the rabbit brain is only approximately 2 cm, so the maximum attenuation is limited to only 1 dB in the deep brain of the rabbit or 90% of the free-field pressure. The BBB opening, as detected with EBD, extends to the edges of the acoustic field (corresponding to ±5 mm in the x-direction in Fig. 1), where the pressure is less than 25% of the pressure in the intermediate brain along the acoustic axis. This difference in pressure does not adequately explain the observation that there was significantly more blue dye in the cortex than in the deep brain. Another explanation for these observations may be that cavitation activity in the near-field of the transducer at the gel-tissue interface or microbubble contrast agent in vessels in the cortex created additional US attenuation and limited the pressure in the far field.

Macroscopic observation of the sonicated brain slices indicated that the pattern of EBD diffusion differed among the tissues of the brain. In particular, EBD diffusion and BBB disruption was more intense in the gray matter than in the white matter. Comparison of the EBD concentration in 2 samples of gray and white matter sonicated with the same acoustic parameters further showed...
that the EBD concentration was 7 times higher in gray matter than in white matter. McDannold and colleagues are the only other authors who have also reported such a difference. Using rhesus macaques, the authors observed opening of the BBB in gray matter only in the trypan blue–staining experiments. In contrast, the same authors observed opening of the BBB in white matter only in the trypan blue experiment, and this blue staining was only slight in the white matter compared with the gray matter. McDannold et al. suggested that opening of the BBB was less intense in the white matter compared with the gray matter. McDannold and colleagues also determined that in situ acoustic pressures decreased from the center to the periphery of the sonication field, and we also noted that the grade of the microhemorrhages decreased from 4.7-T MRI used here. As McDannold et al. suggest, we hypothesize that the difference between gray and white matter is directly linked to variable density of microvessels in the brain. Indeed, it is known that in human brain, although there is no difference in the quality of the microvessels, the microvascular network is more dense in gray matter than in white matter. Some authors believe that the high density of microvessels in the gray matter is the result of the synaptic activity in this tissue. Thus, the microvascular interface that is likely to be reached by US during sonication is higher in the gray matter than in the white matter; accordingly, the surface of exchange between blood circulation and brain parenchyma is higher in the gray matter than in the white matter. This point will need to be considered for a future clinical application of our technique because US-assisted drug exposure will be different depending on the location of the drug target in the brain parenchyma.

We investigated the possibility of adverse effects of sonications and BBB opening primarily by using histological observations. The main result of our histological study was the rare occurrence of diffuse hemorrhagic lesions observed in only 1 rabbit exposed to the highest acoustic parameters (25 msec and 0.8 MPa). For in situ acoustic pressures of less than 0.8 MPa, Grade 2 hemorrhagic lesions (microscopic petechial hemorrhages < 150 μm) were observed in all of the brains analyzed, including those exposed to lower in situ acoustic pressure, and these lesions were limited to the extent of the US beam. This observation is consistent with the results reported by Hynynen et al. indicating that Grade 2 damage was the dominant damage resulting from exposures to pressures between 0.5 and 1.4 MPa. The grading used in this study is more qualitative than quantitative, and we only studied the effects of 3 different in situ acoustic pressures. Thus, we were unable to establish a direct and statistically significant correlation between the likelihood of hemorrhage and in situ acoustic pressures in the range of 0.3–0.8 MPa. However, in 5 of 11 animals we noted that the grade of the microhemorrhages decreased from the center to the periphery of the sonication field, and we also determined that in situ acoustic pressures decreased by up to 75% of the maximum acoustic pressure applied in the pressure field of the transducer. Thus, as also noted by other authors, we hypothesize that vascular modifications induced by our transducer are correlated to the in situ acoustic pressure applied.

For in situ acoustic pressures ranging from 0.4 to 0.6 MPa, no hemorrhagic lesions or petechial hemorrhages were observed in MRI; in histological studies, Grade 2 lesions that corresponded to red blood cell extravasations were the only lesions observed at these acoustic pressures. If only isolated and localized, we suppose that such blood cell extravasations would likely not have any affect on a patient’s clinical status. We also note that no focal ischemic lesions directly linked to the US beam were observed in the histological study and no hypersignal indicating ischemic injury of the brain parenchyma was observed in MRI diffusion sequences. A moderate edema limited to the extent of the US field was observed in FLAIR sequences in Rabbits 14–16, but no midline deviation nor brain herniation was associated with this edema. Thus, such edemas may be acceptable in clinical practice.

In view of our results, we conclude that for in situ acoustic pressures...
acoustic pressures of up to 0.5 MPa in rabbits, a single-element contact US can be safely used to increase permeability of the BBB. We could have performed experiments with a higher number of animals to enable a more exhaustive analysis; however, the sensitivity of the BBB to US likely differs between rabbits and humans. Therefore, similar studies will have to be conducted in animal models with brain sizes and characteristics that are more similar to those of the human brain, and such experiments are the focus of other research groups using primates.\(^{17,18}\) Moreover, complementary experiments are being performed to study the consequences of repeated opening of the BBB on brain histology and function.

Several criteria are of importance to apply our technique to patients with brain tumors. First, tumor infiltration is responsible for up to 80% of the recurrences of gliomas in the tissue surrounding a tumor where the intact BBB limits the diffusion of drugs. By using unfocused US, we have shown here that a broad but targeted volume of BBB could be opened; thus, a large volume of brain parenchyma around a tumor cavity could be targeted with our device to kill quiescent neoplastic cells after surgery. Second, considering that chemotherapy protocols are based on repeated courses, our device has been designed to be easily implanted in a skull bur hole and activated on demand during chemotherapy sessions. Its size (12 mm in diameter) corresponds to the size of a current bur hole commonly cut during craniotomies; a transcutaneous connection is being developed to connect the plug when necessary and open the BBB synchronously with drug injections. Moreover, such an implantable US device must only minimally interfere with MRI in patients who require repeated MRI to monitor their treatment. Other authors have shown that implantable US devices could be safely used with MRI,\(^4\) and in our case, preliminary tests have shown that our device is also MRI compatible.

Finally, to be used in clinical practice, our device has to be safe for patients. In this study, a preliminary evaluation of the histological effects of our method has shown that few hemorrhagic effects and moderate edema were induced by the sonications, especially as very low in situ acoustic pressures had to be applied to open the BBB. No ischemic lesions were observed in our histological and MRI studies. Additional studies are being performed by our group to evaluate the effects of sonications on the neuronal activity and on the histological and functional consequences of repeated sonications in brain parenchyma of primates.

Conclusions

A 1-MHz unfocused US transducer was used to successfully open the BBB in rabbits using in situ acoustic pressures ranging from 0.3 to 0.8 MPa. For in situ acoustic pressures of 0.3 and 0.5 MPa, opening of the BBB was associated with rare extravasations of red blood cells, indicating that safe opening of the BBB can be achieved with in situ acoustic pressure of up to 0.5 MPa. Our device is designed to be implanted into skull bur holes to avoid bone attenuation. The BBB opening can be performed under MRI control. Before application of our technique to human subjects, further studies are required to investigate the potential toxicity of repeated BBB openings, potential electrophysiological consequences, and potential metabolic disturbances. These additional studies should also investigate whether the acoustic pressure parameters determined from small animals studies can be safely applied to clinical use in humans.

Disclosure

This work was supported by CarThera SAS and Centre Francais de l’Innovation. Olivier Clément, Gwennhadel Autret, Julie Piquet, Lauriane Goldwirt, Christine Fernandez, and Clovis Adam had no potential conflicts of interest. Alexandre Carpentier, Michael Canney, Cyril Lafon, and Jean-Yves Chapelon had ownership interest in CarThera SAS. Kevin Beccaria and Michael Canney were employees of CarThera SAS. Alexandre Carpentier, Kevin Beccaria, Michael Canney, Cyril Lafon, and Jean-Yves Chapelon have submitted a patent application related to this work.

Author contributions to the study and manuscript preparation include the following. Conception and design: Carpentier, Beccaria, Canney, Fernandez, Clément, Lafon, Chapelon. Acquisition of data: Carpentier, Beccaria, Canney. Goldwirt, Adam, Autret, Clément. Analysis and interpretation of data: Carpentier, Beccaria, Canney, Goldwirt, Fernandez, Adam, Autret, Clément, Lafon, Chapelon. Drafting the article: Carpentier, Beccaria, Canney, Lafon, Chapelon. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Carpentier. Administrative/technical/material support: Piquet, Autret. Study supervision: Carpentier.

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