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Quantitative evaluation of the use of microbubbles with transcranial focused ultrasound on blood–brain-barrier disruption

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Abstract

It has been shown that focused ultrasound (FUS) can disrupt the blood–brain barrier (BBB) noninvasively and reversibly at target locations when applied in the presence of ultrasound contrast agent (UCA). In this study, the dose-dependent effects of UCA on BBB disruption were investigated in the brains of 16 male Wistar rats sonicated by 1.0-MHz transcranial FUS, with the UCA present at four doses. The BBB disruption was evaluated quantitatively based on the extravasation of Evans blue (EB). The amount of EB extravasation in the brain increased with the quantity of UCA injected into the femoral vein prior to sonication. Moreover, the use of a suitable dose of UCA resulted in the BBB disruption being concentrated in the focal region instead of the entire brain. Our results indicate that injecting an appropriate quantity of UCA effectively increases and localizes the BBB disruption induced by transcranial FUS sonications.

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Keywords: Focused ultrasound; Ultrasound contrast agent; Transcranial; Blood–brain-barrier disruption

1. Introduction

It is difficult to deliver many potent therapeutic agents to the brain due to the presence of the blood–brain barrier (BBB), which is a specialized system of capillary endothelial cells that protects the brain from harmful substances [1]. The BBB exhibits a low permeability to ionized water-soluble molecules that have molecular weights greater than 180 Da [2], and hence it represents a significant obstacle to the delivery of both small molecules and macromolecular agents to the brain.

The direct delivery of drugs to the central nervous system would make the resulting interactions highly target-specific and thereby dramatically improve the therapeutic effects and reduce possible side effects. Although many methods have been developed to overcome BBB impermeability when delivering drugs, such as increasing their lipid solubility, making them water-soluble with high affinities for carriers at the BBB, and by the using vectors such as amino acids and peptide carriers [3–5], none has been applied clinically. The major defect of these techniques is that they promote the permeability of drugs throughout

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the brain. Drug delivery can be localized by using a catheter or needle [6], but this is invasive and is associated with the risk of neurologic damage, bleeding, and infection.

Recent studies suggest that focused ultrasound (FUS) in the presence of microbubbles can be used to transiently disrupt the BBB and thereby aid the noninvasive delivery of treatment agents to specific regions in the brain [7,8]. The administration of microbubbles can effectively enhance cavitation activity in noninvasive ultrasound surgery [9]. The application of FUS at an appropriate acoustic pressure in the presence of an ultrasound contrast agent (UCA) can locally disrupt the BBB without visible damage to the brain tissue [10]. Recent experiments have shown that the normalized signal intensity of magnetic resonance imaging (MRI) in the focal volume is proportional to the applied pressure amplitude when MRI contrast agent is present [8,10]. Furthermore, this relationship is much weaker in sonications without the simultaneous injection of UCA. Hence, introducing microbubbles into the bloodstream can both restrict the ultrasound effects to the vasculature and reduce the pressure required to disrupt the BBB. The cellular mechanisms by which FUS causes BBB disruption are still unclear, but are probably closely related to direct interactions between microbubbles and the ultrasound [11]. Therefore, UCA is potentially the most important factor influencing the induction of BBB disruption in the presence of ultrasound through the intact skull.

The purpose of this study was to quantitatively evaluate the dose-dependent effects of UCA on BBB disruption in trans-skull FUS sonications, and to investigate the impact of the skull bone on this noninvasive surgery technique.

2. Materials and methods

2.1. Experimental animals

A total of 16 male Wistar rats weighing from 280 to 350 g were used in two sets of experiments. Each rat was anesthetized intraperitoneally with chloral hydrate (400 mg/kg), and the body temperature was maintained at 36.5–37.5 °C using a thermostatically controlled heating blanket. The scalp overlying the skull was removed to facilitate using the bregma of the rat skull as an anatomic landmark for targeting purposes. In the first set of experiments, FUS was applied to 12 rats in the presence of UCA at four doses. In the second set of experiments, the temperatures at the outer and inner skull surfaces were measured in four rats. Our institutional animal committee approved all of the experiments.

2.2. Ultrasound equipment

Ultrasound waves were generated by a single-element FUS transducer (A392S, Panametrics, Waltham, MA) with a diameter of 38 mm, a radius of curvature of 63.5 mm, and a center frequency of 1 MHz. The transducer was mounted on a removable cone filled with deionized and degassed water whose tip was capped by a polyurethane membrane. The center of the focal spot is at approximately 5.3 mm below the cone tip. The FUS was precisely targeted using a stereotaxic apparatus (Stoelting, Wood Dale, IL) that utilized the bregma of the skull as the anatomical landmark. The transducer was positioned using the stereotaxic apparatus with the removable cone directing the acoustic beam to the brain. A function generator (33220A, Agilent, Palo Alto, CA) connected to a power amplifier (500-012, Advanced Surgical Systems, Tucson, AZ) drove the FUS transducer (Fig. 1). The rat was laid prone beneath the cone tip, and ultrasound transmission gel (Pharmaceutical Innovations, Newark, NJ) was used to maximize the transmission of ultrasound between the transducer and the brain. The axial and lateral acoustic fields induced by the 1 MHz transducer and cone were measured in distilled and degassed water using a needle hydrophone. The half-maximum of the pressure amplitude of the focal zone had a diameter and length of 3 and 26 mm, respectively. Using extrapolation, the focal pressure in water was estimated to be 1.2 MPa (peak negative pressure) for an acoustic power of 24.5 W.

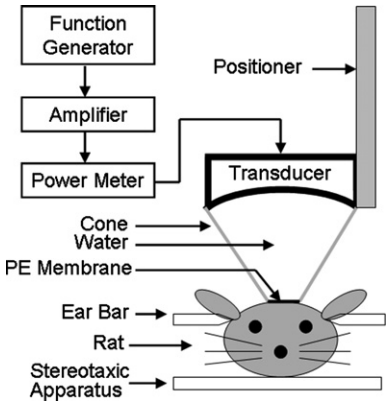

Fig. 1. Schematic diagram of the FUS sonication setup. PE, polyurethane.

2.3. Sonications

The rat's head was mounted on the stereotaxic apparatus with the nose bar positioned 3.3 mm below the interaural line. Pulsed sonications were applied with a burst length of 50 ms, a duty cycle of 5%, and a repetition frequency of 1 Hz. Each sonication protocol lasted 60 s. The FUS was delivered to one location in the right hemisphere brain (3.5 mm posterior and 2.5 mm lateral to the bregma, and 5.3 mm below the skull surface). The UCA (SonoVue, Bracco International, Amsterdam, The Netherlands), which contains phospholipid-coated microbubbles (mean diameter = 2.5 μm, concentration = $1–5 \times 10^8$ bubbles/mL), was injected into the femoral vein of the rats about 20 s before each sonication. In in vivo experiments, the rats were sonicated with UCA at four doses (0, 150, 300, and 450 μL/kg) at an acoustic power of 24.5 W.

2.4. Assessment of blood–brain-barrier integrity

BBB permeability was evaluated based on the extravasation of Evans blue (EB), which binds with albumin for the measurement of vascular permeability [12]. EB (Sigma, St. Louis, MO) (100 mg/kg) was injected intravenously 5 min after FUS sonication. The animals were sacrificed approximately 4 h after the injection of EB. After perfusion and brain removal, the brain was sectioned into six slices (6 mm anterior and 6 mm posterior to the bregma) and mounted on glass slides. Samples were weighed and then soaked in 50% trichloroacetic acid solution. After homogenization and centrifugation, the extracted dye was diluted with ethanol (1:3), and its fluorescence was measured using a multiwell-plate-reading fluorometer (Cytofluor 2300, Millipore, Bedford, MA; 620-nm excitation and 680-nm emission). The EB extravasation in the brain was quantified by a linear regression standard curve derived from seven concentrations of the dye, and was expressed per gram of tissue. All results are presented as mean and standard error of the mean (SEM) values.

2.5. Temperature measurements

A 127-μm-diameter T-type copper–constantan thermocouple (5TC-TT-T-36-36, Omega Engineering, Stamford, CT) used to measure the temperatures at the outer and inner skull surfaces was connected to a data-acquisition system (TC-2190, National Instruments, Austin, TX). One thermocouple was inserted under the forward edge of the cone on the outer surface of the skull bone, and another was embedded in the bone–dura interface through a cranial cleft fashioned with a high-speed drill. Ultrasound gel was used as the coupling medium between the transducer and the skull. The exposure method used in this study involved the ultrasound beam passing through the gel and the skull bone before reaching the focus. For the intact-skull experiments, the exposure and temperature recordings were repeated for each rat with different UCA doses to assess the effects of UCA.

2.6. Cavitation detection

The cavitation was quantified as the inertial cavitation dose (ICD), corresponding to the spectral broadband signal enhancement during microbubble destruction [13]. The in vitro experiment quantitatively investigated the effects of cavitation induced by the 1 MHz pulsed ultrasound transducer (A392S, Panametrics, Waltham, MA) and the doses of UCA. The ultrasound parameters are the same as the in vivo study. A test tube 5 mL in volume was made in plastic as a container for the microbubble solution. An unfocused 10 MHz transducer (V315, Panametrics) was used to receive signals from the microbubbles during sonication. The two transducers were positioned perpendicular to each other and both were focused at the same region. The distances between the focal zone and the 1 MHz and 10 MHz transducer were 63.5 mm and 30 mm, respectively. The ICD was quantified as the root-mean-square (RMS) value of the spectrum between 9.5 and 10.5 MHz. Finally, the background time–amplitude curve (the measured RMS amplitudes from water only) was subtracted and the resulting amplitude is referred to as the differential ICD that was used for the subsequent signal analysis.

2.7. Light microscopy

Four rats were sacrificed approximately 24 h after the sonication for histologic evaluation. For our preliminary studies, the histologic effects of each FUS sonication at an acoustic power of 24.5 W with UCA at doses of 300 and 450 μL/kg were determined by taking a series of 10-μm-thick frozen sections starting at 2 mm anterior to the blue-stained focus region and extending through this region to 2 mm posterior to the blue region. After having identified the bluest sections, we applied TUNEL staining to the neighboring sections (DeadEnd Colorimetric TUNEL system, G7130, Promega, Madison, WI, USA). Some sections subjected to DNase treatment were stained as a positive control. The TUNEL system measures nuclear DNA fragmentation, which is an important biochemical indicator of apoptosis. The histology evaluation was performed using light microscopy (Axiophot 2, Zeiss, Göttingen, Germany).

3. Results

The BBB disruption was observed in the focal zone of the ultrasound beam with EB extravasation. Fig. 2a shows the relationship between the injected dose of UCA and the degree of EB staining in the right and left hemispheres with and without sonication at an acoustic power of 24.5 W. Both the size and color intensity of EB staining in the right brain increased with the injected dose of UCA. In addition, there was no visible EB staining in the nonsonicated left hemispheres for the four doses of UCA used. Fig. 2b indicates that EB extravasation occurred at the brain surface in response to the injection of 300 or 450 μL/kg UCA, with it clearly being greater for the latter dose.

Fig. 3a shows the average extravasation of EB (in micrograms per gram of tissue) in the six coronal slices 4 h after sonication at an acoustic power of 24.5 W in the presence of UCA at four doses. The EB extravasation at the focal zone was correlated with the dose of UCA. The BBB disruption upon sonication was significantly greater with the injection of 300 or 450 μL/kg UCA than with either 0 or 150 μL/kg UCA, and there was no detectable EB extravasation when no sonication was applied. The measurements of EB fluorescence are shown in Fig. 3b, which confirms that EB extravasation was significantly greater in the focal region (slices 4, 5, and 6) than in the neighboring region

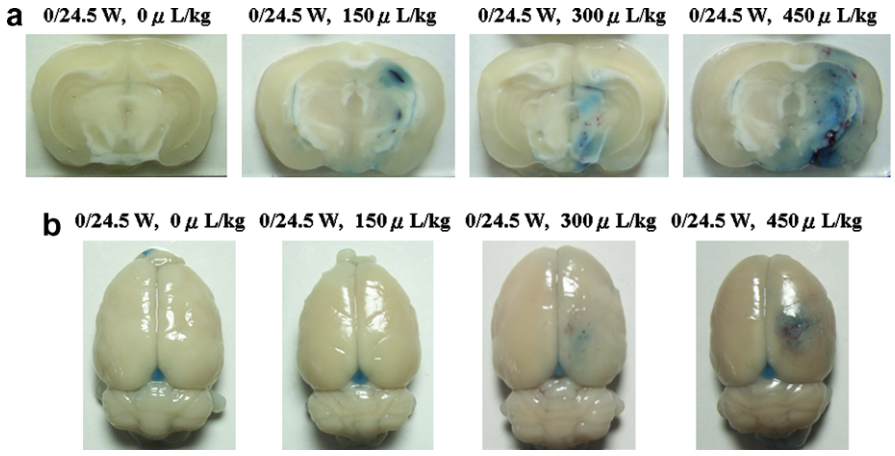

Fig. 2. (a) Distribution of BBB disruption for four doses of UCA as evaluated by the extravasation of EB into the brain in the focal region. Right brain: 24.5 W acoustic power sonication. Left brain: no sonication. (b) Impact of the sonication at the brain surface.

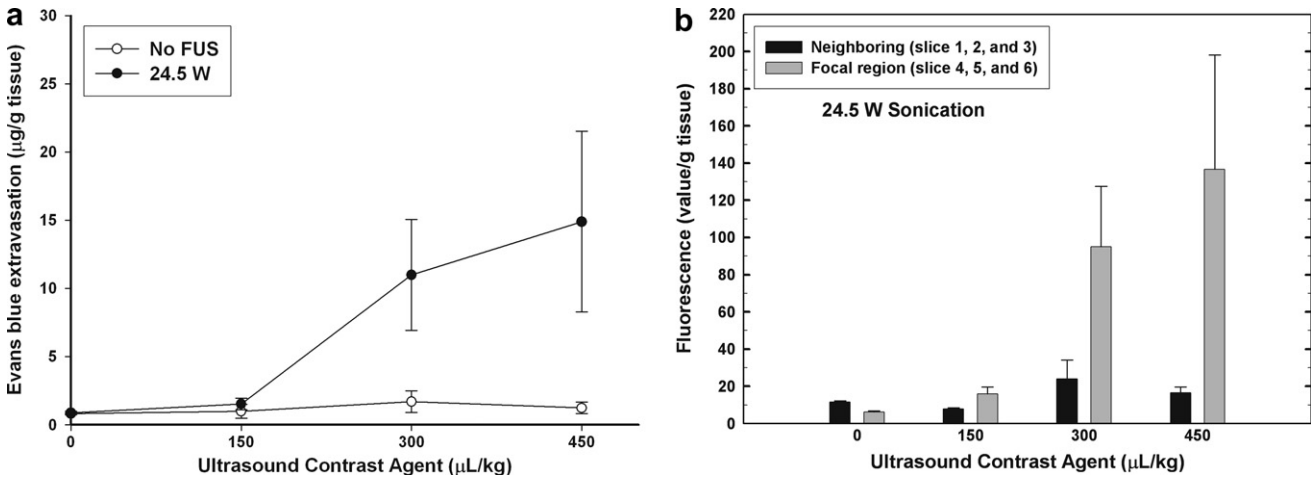

Fig. 3. (a) Relationship between EB extravasation (mean ± SEM values) and the injected dose of UCA in each brain hemisphere with and without sonication at an acoustic power of 24.5 W. The values plotted are the between-rat means of data averaged over all six slices within a rat. The EB extravasation as a function of the UCA dose in the presence of sonication. (b) EB in the focal and neighboring regions in response to different doses of UCA for sonication at an acoustic power of 24.5 W. Data are mean ± SEM values.

(slices 1, 2, and 3) for different doses of UCA, especially for 300 and 450 μL/kg.

The temperatures recorded by the thermocouples placed at the inner and outer skull surfaces during 60 s of sonication at an acoustic power of 24.5 W in the presence of UCA at the four doses are plotted in Fig. 4. The temperatures reached a steady state approximately 20 s after sonication, and then decayed exponentially after sonication stopped. The peak temperature increases at the inner and outer skull surfaces during the sonication did not differ significantly with the dose of UCA (approximately 2.9 °C and 6.9 °C, respectively). However, the temperature at the outer skull surface varied in each rat during the pulsed sonication.

Fig. 5a shows that the inertial cavitation of different doses of UCA was quantified by measuring ICD and Fig. 5b shows that the differential ICD increased as the UCA dose increased from 150 to 450 μL/kg. Fig. 6 shows representative TUNEL-stained sections for UCA at 300 and 450 μL/kg at an acoustic power of 24.5 W. The location of BBB disruption was confirmed by the region stained with EB. Fig. 6B indicates that there was a few cells damage at the UCA dose of 300 μL/kg. Fig. 6C shows that many of the cells appeared apoptotic after the application of 450 μL/kg UCA and 24.5 W sonication.

4. Discussion

This study demonstrates that UCA can both enhance and concentrate the BBB disruption in the focal region elicited by an FUS beam passing through the intact skull (Fig. 2a). A higher dose of UCA means that there are more microbubbles in the vasculature to serve as nuclei for cavitation, which results in more cavitation, more extravasation, and darker staining. Fig. 2a shows that both the

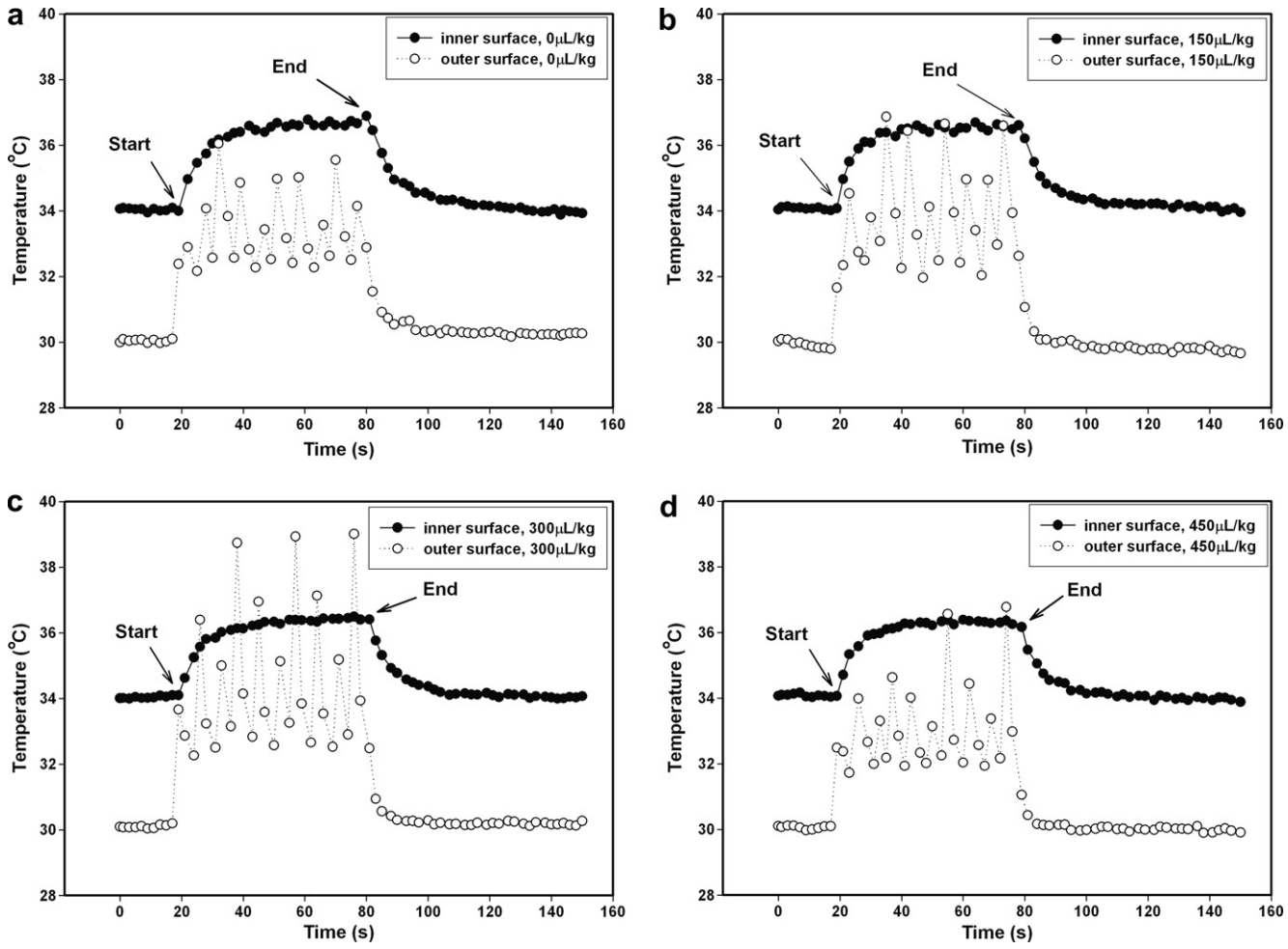

Fig. 4. Measured temperature elevations at the inner and outer skull surfaces for UCA doses of 0 μL/kg (a), 150 μL/kg (b), 300 μL/kg (c), and 450 μL/kg (d).

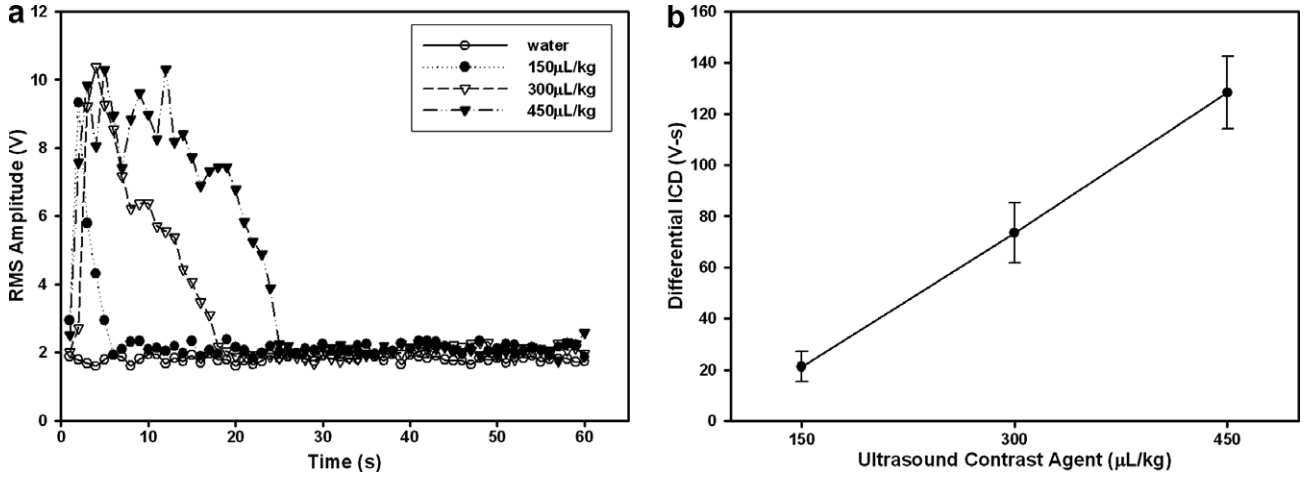

Fig. 5. (a) RMS amplitude as a function of time with various doses of UCA. (b) Differential ICD as a function of UCA dose (acoustic power: 24.5 W). Data are presented as the mean ± SEM for three independent trials.

size and degree of EB staining were dependent on the UCA dose at an acoustic power of 24.5 W.

Fig. 3a shows that the EB extravasation did not increase with the dose of UCA in the absence of sonication. The small amount of EB extravasation in the absence of sonication may be attributable to the absence of the BBB at some parts of the brain. A recent study found that UCA circulating in the vasculature concentrates the effects of ultrasound

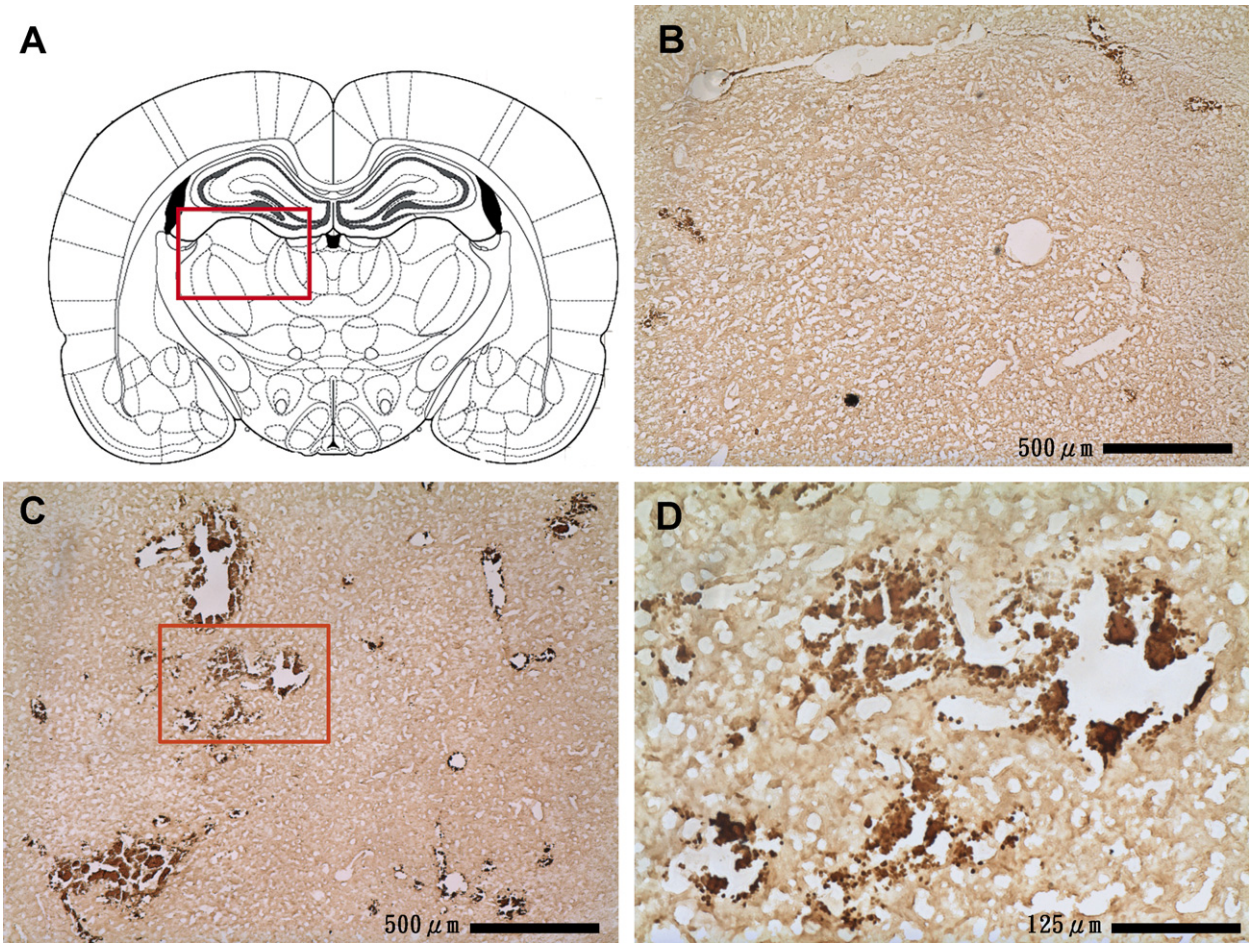

Fig. 6. Examples of apoptotic cells after sonications with 300 μL/kg (B) and 450 μL/kg (C). (A) Illustration of the position of ultrasound sonication. (B) A few cells were seen to be undergoing apoptosis (50×). (C) Many of the cells within the focal zone appeared apoptotic (50×). (D) Apoptotic cells in (C) at a higher magnification (200×).

in the microvasculature, with few effects in the surrounding tissue [14]. Our results indicated that a higher dose of UCA is effective at enhancing the BBB disruption and concentrating the effects of transcranial sonication in the focal region, especially at higher UCA doses (300 and 450 μL/kg) (Fig. 3b). The UCA doses, duty cycle, and sonication time were all greater in this transcranial study than in our previous craniotomy study [15] (with the same acoustic power of 24.5 W) so as to compensate for the attenuation of acoustic propagating waves at the skull due to scattering and absorption.

There was no EB extravasation at the brain surface in the low-dose UCA groups (0 and 150 μL/kg). However, EB extravasation with a few petechial hemorrhages appeared at the brain surface directly in the path of the ultrasound beam for the high-dose UCA groups (300 and 450 μL/kg), especially the latter (Fig. 2b). Fig. 7 shows that the blood vessels at the brain surface of the sonicated area were unevenly distributed and of variable sizes. The region of sonication is circled by the white line. The higher density of microbubbles resulted in a lower pressure-amplitude threshold for inducing BBB disruption in this area. According to our pressure measurements in water, the pressure amplitude at the brain surface was approximately 70% of the peak pressure at the focus. Moreover, the acoustic impedance mismatch at the bone–dura interface will have resulted in part of the remaining waves being reflected back to the skull. The results showed that BBB disruption at the brain surface was possible at a lower pressure amplitude but higher density of UCA due to a larger volume of vessels. Therefore, pretreatment imaging planning should aim to direct the ultrasound beam so as to avoid large blood vessels and BBB disruption at unwanted regions.

The effects of different UCA doses on the temperature rise in the skull are shown in Fig. 4. The body temperature of the animals was found to decrease by several degrees when anesthesia was induced [16], and the temperature at the outer skull surface decreased by about 4 °C due to the resulting lower temperature of the US transmission gel. This phenomenon is equivalent to reducing the temperature at the skin–bone interface in clinical applications, which is desirable. Comparing the effects of UCA at the various doses reveals no obvious differences in the

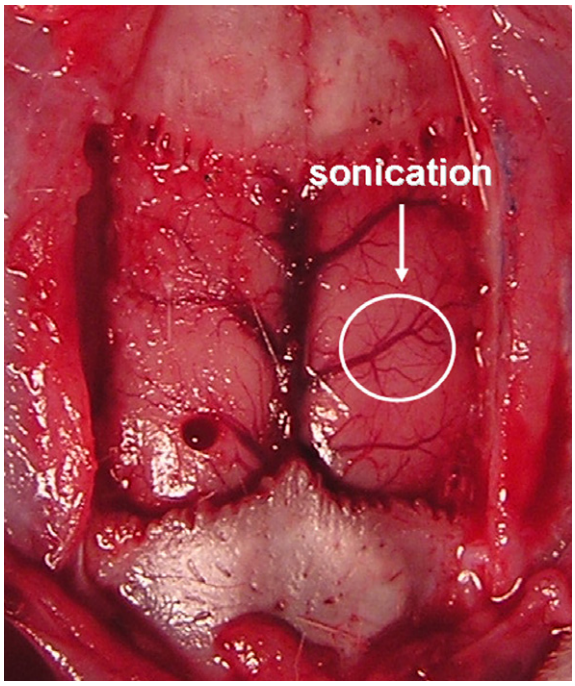

Fig. 7. There were some larger blood vessels present in the path of the ultrasound beam at the brain surface.

temperature rise at the inner skull surface. The highest UCA dose used in the in vivo experiment produced a temperature rise of approximately 2.9 °C at the inner skull surface. This temperature rise is acceptable given that the actual temperature of the skull is below 37 °C and it has previously been shown that hyperthermia can induce BBB disruption only once the temperature of the brain exceeds 40.5 °C [17–19]. Hence, the EB extravasation in our study (Fig. 2b) most likely resulted from the interaction of FUS with microbubbles in the larger vessels at the brain surface, and not from the temperature elevation. Further research is needed to determine the ultrasound pressures that would be sufficient when using lower UCA doses to induce BBB disruption without damage to normal tissue outside the desired region.

The mechanisms of transient barrier disruption by FUS are currently unknown. Some previous studies have revealed that the effects of inertial cavitation on the vasculature, which could include high temperatures, shockwaves and free radicals, are responsible for BBB disruption. However, inertial cavitation could cause damage to the brain tissue. The results of Figs. 5 and 6 indicate that both inertial cavitation and apoptotic cells were highly correlated with UCA doses at the same acoustic power. Thus additional experiments must demonstrate that BBB can be disrupted with minimal brain tissue damage by an adequate dose of UCA and optimal ultrasound parameters.

Besides, the duration of the BBB disruption after sonication with ultrasound and microbubbles has been variable. One group reported that magnetic resonance imaging (MRI) can be used to monitor BBB disruption during sonication. The signal intensity change was greatest immediately after the sonication and declined after 6 h. However, no contrast enhancement was evident after 24 h [8]. In another study, BBB disruption appears to persist at least 72 h after initial treatment with FUS [20]. Therefore, more work is needed to investigate the effects of ultrasound and microbubbles upon the various duration of BBB disruption.

In conclusion, our quantitative experiments revealed that the region of BBB disruption induced by transcranial ultrasound increased with the injected dose of UCA. The use of UCA at an appropriate dose results in BBB disruption being localized to the focal region. Future studies should investigate the use of phased arrays to eliminate the phase distortion caused by the intact skull so as to both enhance the focusing at the target region and prevent undesired damage at the brain surface.

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