Expert Opinion

Focused ultrasound for blood–brain disruption and delivery of therapeutic molecules into the brain

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Noninvasive, transient and local image-guided blood–brain barrier disruption can be accomplished using focused ultrasound exposure with intravascular injection of preformed microbubbles. MRI-guided blood–brain barrier disruption has been demonstrated and has been shown to heal in within a few hours after exposure. The delivery of several marker molecules has been demonstrated in different animal models with minimal or no damage to the brain tissue. Most notably, the delivery of antibodies and liposomal doxorubicin has been shown. The method may potentially open a new era in CNS drug delivery and perhaps also aid in molecular imaging and targeting. However, effective clinical devices and methods need to be developed further and the clinical feasibility demonstrated.

Keywords: blood–brain barrier, focused ultrasound, HIFU, image-guided therapy, targeted drug delivery, ultrasound

Expert Opin. Drug Deliv. (2007) 4(1):27-35

1. Introduction

The blood–brain barrier (BBB) limits the passage of many molecules from the circulation into the brain parenchyma, thus precluding their use in drug treatments of the brain [1-4]. The BBB is formed by the endothelial cells of the cerebral microvessels that connect to each other by intracellular attachments known as tight junctions [1,2]. In addition, there is a physiological barrier at the level of basal lamina [5] that actively removes undesirable molecules from the brain. The factors that determine penetration of substances from the blood to the CNS are lipid solubility, molecular size and charge. The BBB prevents penetration of ionized water-soluble materials with molecular weight > 180 [2]. There are two ways to enhance propagation through the barrier: chemical modification of the drugs to make them lipophilic, or the use of other carriers, such as amino acid and peptide carriers. The tight junctions can also be opened temporarily by an intra-arterial injection of certain chemicals, such as mannitol, or other hyperosmotic solutions. These cause the endothelial cells to shrink, resulting in an opening of the tight junctions that lasts for a few hours [6]. Both the osmotic and chemical methods produce diffuse BBB openings within the entire tissue volume supplied by the injected artery branch [1,2] without the ability to selectively target a brain region. With the development of non-invasive imaging methods that can visualize not only the anatomy, but also the physiology, function and location of specific molecules of the brain, it would be desirable to use this information for the delivery of the therapeutic agents only in specific locations targeted for treatment or diagnosis. This requires disruption of the BBB only in locations specified by imaging, while protecting the surrounding regions of the brain from potential side effects. This type of localized drug delivery can currently be accomplished by direct injection of agents through a needle or catheter into the targeted region of the brain [2].

Focal, non-invasive, image-guided disruption of the BBB will require some sort of energy delivery deep into the brain. The only non-invasive method that can focus energy deep into the body is ultrasound. Although trans-skull focal ultrasound exposure of human brain tissue was considered very difficult for a long time [7], recent technological development has made it possible to deliver ultrasound through the intact skull to precise volumes of tissue under MRI guidance and monitoring [8]. It has been known for several decades that deep brain tissue sonications can be associated with the disruption of the BBB [9-11]. In the first report that specifically explored the disruption of the BBB, Patrick et al. studied blood–brain barrier disruption (BBBD) around ultrasound-induced thermal lesions in animals and proposed it for delivery of chemotherapy for brain tumour treatments [12]. However, in these experiments, the disruption of the BBB was always accompanied by tissue damage. The disruption of the BBB around ultrasound-induced tissue damage resulting from elevated temperature has been later verified by contrast-enhanced MRI [13]. Recently, long-duration temperature elevations have been shown to be associated with BBBD in an *in vitro* cell culture model [14].

High-pressure amplitude ultrasound exposures can generate oscillating and collapsing gas bubbles in tissue. This phenomenon, called cavitation, is associated with high shear stress, temperature and pressure in the proximity of the micrometer-sized bubbles. Vykhodseva *et al.* demonstrated that short (ms) ultrasound exposures can also induce BBB disruption and that sometimes there was no damage to the neurons in the locations with the compromised BBB [15]. However, these results were not consistent, and so far there have been no reports describing exposure parameters that could consistently produce BBBD without associated tissue damage. Similar results were observed at higher frequency short-pulse sonications by others [16]; however, it is not known if the BBBD was caused by cavitation in these studies.

A few years ago, the current author's group demonstrated non-invasive and reversible disruption of the BBB at targeted locations using focused ultrasound bursts in conjunction with an ultrasound contrast agent [17]. These contrast agents, activated by ultrasound, are now extensively explored for many therapeutic applications (see [18]). Subsequent studies have expanded on that initial work, investigating the disruption with electron microscopy [19,20], and showing that the ultrasound exposures reliably produce negligible damage to the brain parenchyma, with no delayed effects [21]. Other groups have also followed these initial reports and verified the effectiveness of the proposed method [22]. This method will be the main subject of this review.

2. Microbubble-aided focal ultrasound disruption of the blood–brain barrier

The method for BBBD with ultrasound utilizes preformed microbubbles injected into the bloodstream before the ultrasound exposures. These bubbles, developed for ultrasound imaging, contain gas (air or perfuorocarbon) encased in a shell – most often made of albumin or lipids – and have diameters of ~ 1 and 5 μm, such that they pass through the capillary network. The bubbles are injected intravenously, and the sonications are delivered during their first pass through the capillary network. When the bubbles pass through the tissue volume exposed to ultrasound, they expand and contract at the frequency of the propagating acoustic wave due to the cyclic pressure reductions and increases associated with the exposure. The bubble oscillations also cause the surrounding fluid to move at the frequency of the ultrasound, thus creating large shear forces around the bubbles. In addition, the bubbles are pushed by a radiation force [23] in the direction of wave propagation. Above a threshold, the bubble oscillations become so large that the inertia of the surrounding fluid cause the bubble to collapse, inducing high temperatures and pressures and causing a shock wave to propagate at supersonic speed radially from the collapse site. If the bubbles collapse close to a wall, they can create fluid jets that can damage the wall [24-26]. The exact physical mechanism of the disruption of the BBB is not unknown yet, but it has been shown that the bubble collapse is often associated with BBBD, although it is possible to induce BBBD with only bubble oscillations and no detectable collapse [27]. The bubble oscillations may trigger receptors in the vessel wall to cause the BBBD. One possible explanation is that the bubbles reduce the oxygen transfer temporarily, but for long enough to trigger ischemia-related receptors. It is known that ischemia can compromise the BBB [28]. Regardless of the actual biological mechanism, the bubbles absorb and concentrate energy from the ultrasound wave- reducing the power levels by at least two orders of magnitude from that required to induce bio-effects without the bubbles [17]. As the microbubbles are contained in the blood, the induced biological effects are mostly confined to the blood vessel walls.

The microbubbles used so far are standard, clinically proven, diagnostic ultrasound contrast agents. The most experience has been collected with Optison™ (GE Healthcare), containing bubbles of perfluorocarbon gas (perflutren) with a human albumin shell. According to the manufacturer, the bubbles have a mean diameter of 2.0 – 4.5 μm, with the maximum diameter of 32 μm. The bubble concentration in the agent is $5 - 8 \times 10^8$ bubbles/ml. Most of the animal experiments have been performed at the clinically recommended diagnostic dose of 0.2 ml of Optison/kg. The prefluorocarbon gas is not metabolized and is removed from the body via lungs. The albumin shell is assumed to be handled by normal metabolic routes for human albumin. The clearance of Optison is fast, with a $t_{1/2}$ of 1.3 min in healthy individuals. Optison can most likely be replaced by other ultrasound contrast agents. This was demonstrated in animals when similar BBBD was achieved by using Definity™ (Bristol-Myers Squibb), which is another diagnostic agent

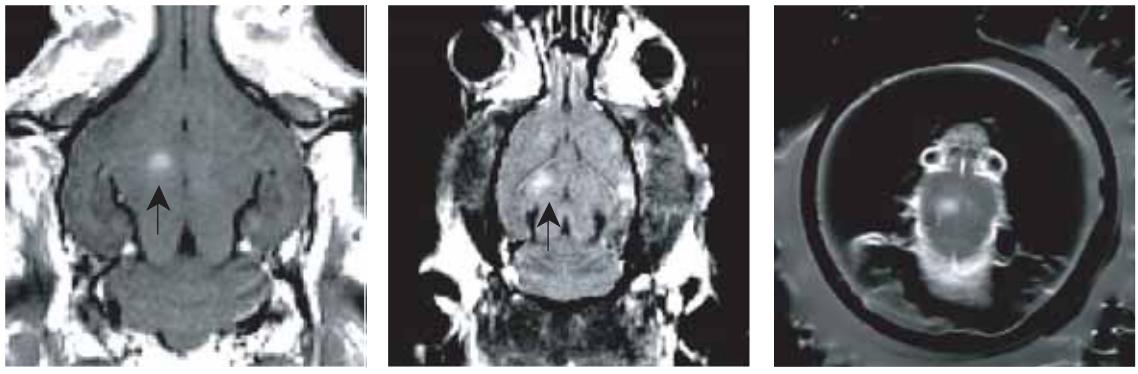

Figure 1. Examples of T1-weighted MR images obtained after sonication of a rabbit, rat and mouse brain *in vivo* and an injection of a bolus of gadolinium contrast agent. The images are acquired across the focus of the ultrasound beam and show local contrast enhancement at the sonicated locations.

with microbubbles containing the same perfluorocarbon gas, but surrounded by a lipid shell. The mean diameter of the bubbles is 1.1 – 3.3 µm, with a maximum diameter of 20 µm. The bubble concentration is 1.2×10.0^{10} bubbles/ml. The mean $t_{½}$ of the contrast agent in blood in healthy subjects is 1.3 min.

Both of these agents contain some bubbles that are too large to pass through the capillary network. As the contrast agent is intravenously injected, the large bubbles are filtered away by the lungs. However, if intra-arterial injection is needed, then the large bubble should be removed to avoid development of microembolizations. These large bubbles were the most likely cause for the BBB disruptions seen in experiments with intra-arterial injection of Optison without sonications in rats [29] as similar BBBD has not been seen with intravenous injections.

3. Animal studies using MRI contrast agents

The disruption of the BBB with focused ultrasound and microbubbles has been investigated extensively using standard commercial MRI agents, such as gadopentetate dimeglumine (Magnevist®, Berlex Laboratories; molecular weight of 928), which does not penetrate through the BBB. These studies showed that focal disruption of the BBB is feasible at pressure amplitudes that do not induce necrosis, ischemia or apoptosis of the brain tissue (0.4 – 0.5 MPa at 0.69 MHz) [20]. Figure 1 shows the focal enhancement induced in rabbit, rat and mice brains. The amount of contrast enhancement was found to be dependent on the pressure amplitude; however, at higher pressures, the exposures induced tissue damage (Figure 2A). A frequency of 1.7 MHz was associated with a few locations of extravasated red blood cells (RBC) that seemed not to have any long-term effect on the brain tissue [30]. At the lower frequencies, the pressure threshold of the BBB disruption decreased and the number of extravasated RBC decreased such that, at the frequency of 250 kHz, the BBB disruption was possible without any extravaced RBC [31]. The disruption of the BBB seemed to be self-healing, such that 6 h after the sonications, only a small signal enhancement was observed with contrast-enhanced MRI (Figure 2B). The imaging studies performed at 2 – 5 days, and 4 weeks after the sonications revealed that the BBB was completely healed [20,30]. Similarly, a light microscopy study demonstrated intact brain tissue and vasculature at these follow-up time points.

A larger intravascular MRI contrast agent, monocrystalline iron oxide nanoparticles ([MION], 20 nm nanoparticulate contrast agent, MION-47, Center for Molecular Imaging Research) was also used in BBB disruption tests. These tests demonstrated that MION leaks into the brain in the sonicated locations. However, the leakage was much stronger when the agent was present in the blood during the sonications than when injected after the exposure. After the exposure, only a slight enhancement was observed [31]. These experiments demonstrated that large molecules and particles can be delivered through the BBB and that the maximum delivery is achieved when the agent is in the blood during the sonication. More work needs to be done to characterize the duration of the BBBD for each molecule of interest and to establish the feasibility of using longer or repeated exposures to increase the amount of molecules delivered.

4. Multi-photon *in vivo* microscopy observations

In order to determine the time course between the sonications and the disruption of the BBB, a series of mouse experiments were performed using *in vivo* multi-photon microscopy [32]. In these experiments, two

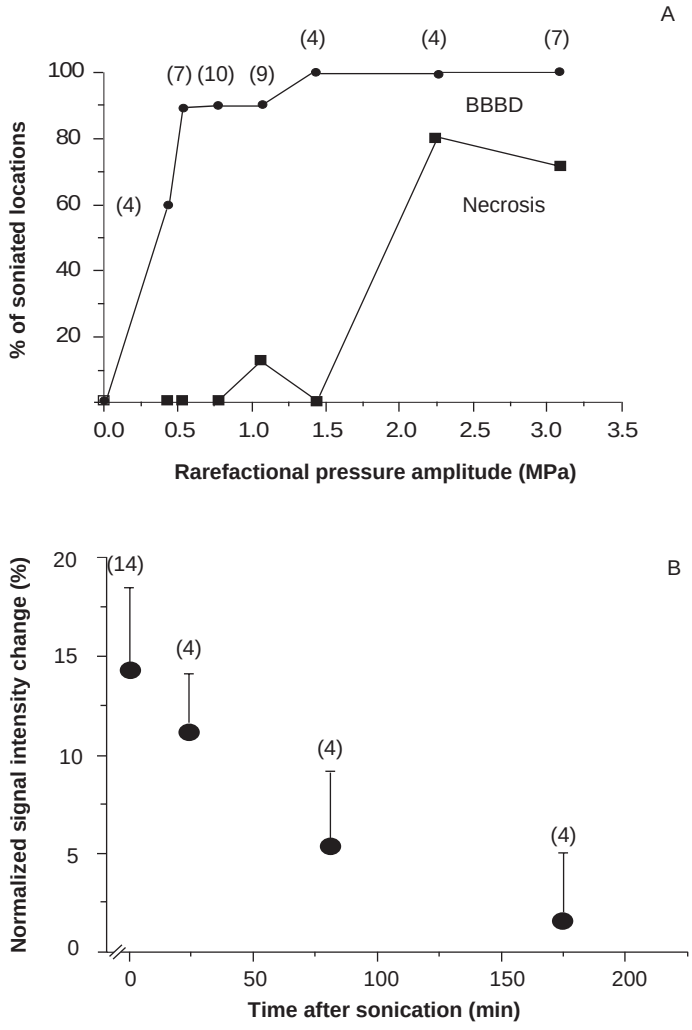

Figure 2. A. The percentage of sonicated locations that showed contrast enhancement in T1-weighted MR images after sonication as a function of the pressure amplitude during the 10 ms ultrasound bursts. The percentage of the locations that showed light microscopy evidence of neural damage is also plotted in the graph. **B. The magnitude of the contrast enhancement as a function of time after the sonications.** Each point was a separate injection of contrast agent. These sonications were performed at the frequency of 0.69 MHz and with 10 ms bursts repeated at the frequency of 1 Hz for 20 s. The number of locations (animals) are presented in parenthesis. The figures are replotted from the data presented in [20].

dyes of different molecular weights were injected intravenously into a mouse positioned in the microscopy system that allowed simultaneous ultrasound exposures. The results showed that the dye leakage occurred without extravasation of RBC (which was also seen on one occasion) via two observed routes. First, microdisruptions in which the dye rapidly leaked from a point on the vessel wall were observed. This could potentially be caused by bubble collapse with associated jet formation that punctures the vessel wall. These microdisruptions happened more in arteries than veins and were associated with points of bifurcation. Second, the dye leaked slowly through apparently intact endothelium. The results demonstrated that the sonications are associated with an almost instantaneous constriction of at least some of the arteries and arterioles followed by slow leakage of the dye molecules through the vessels' walls. The smaller (10 kDa) molecule leaked at higher rate than the larger (70 kDa) molecule, which showed a much lower intensity. The vessel diameter relaxed slowly after reaching the minimum during and after the sonication. It is not known whether or not the constriction was the cause of the BBBD or if it was just an unrelated by-product of the sonications. The most likely reason for the vessel constriction is mechanical stimulation induced by the radiation force caused by the sonication.

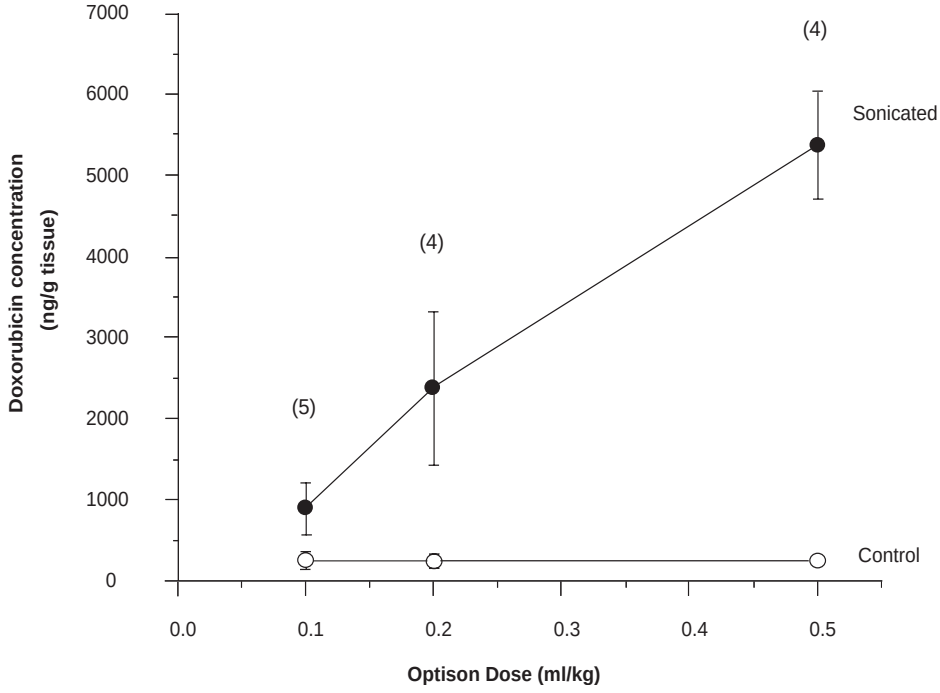

Figure 3. The concentration of doxorubicin in the sonciated rat brains and in the contra-lateral control volume as a function of the ultrasound contrast agent dose injected in the animal. The injected doxorubicin concentration was 5.7 mg/kg administered intravenously. The number of animals is indicated in the figure.
The graph was drawn based on the data from [34].

5. Post mortem light microscopy observations

In order to determine the potential impact of the BBBD on the brain, most of the brain tissue exposed to ultrasound in the previous BBBD studies were examined with light microscopy and several histology stains. As stated earlier, the only tissue effect that was seen at the pressure amplitude levels close to the threshold of BBBD were occasional extravasations of RBCs. The number of extravasations decreased with decreasing ultrasound frequency and increased with increasing pressure amplitude. For example, at 1.7 MHz, ~ 5% of the detected vessels had extravasated RBCs. These extravasations seemed to be absorbed by the tissue over the course of 4 weeks, with no detectable adverse reaction. No apoptotic or ischemic tissue regions were observed at these exposure levels. However, when the pressure amplitude was increased, both ischemic and apoptotic cells were detected, with the number increasing with the pressure amplitude. A further elevation in the pressure amplitude resulted in tissue necrosis and hemorrhage [30].

6. Electron microscopy observations

There have also been two electron microscopy studies evaluating the ultra structural changes that may be responsible for the BBBD [19,20]. The marker molecules used were immunoglobulin and horseradish peroxidase. These studies identified three main mechanisms: first, rupture of the blood vessel was associated with extravasations of the RBCs. Second, there was an occasional widening of the tight junctions. This was similar to that shown by Massiawala [33], with high-frequency ultrasound exposures without microbubbles. Third, it was observed that vacuoles were transporting marker molecules through the endothelial cells.

7. Chemotherapy and antibody delivery

The first potential clinical use of targeted BBBD could be the delivery of therapeutic agents in brain tumours [34]. The feasibility of delivering clinically effective amounts of liposome encapsulated doxorubicin was demonstrated by focusing an ultrasound beam on one side of a rat brain, and simultaneously injecting the agent and microbubbles in the tail vein. After a few hours of survival, the animals were sacrificed and brain samples harvested and the drug concentration measured in the sonicated and unexposed control samples. The sonicated locations showed a significantly higher concentration of doxorubicin than the contra-lateral side. The concentration of the drug in the brain tissue increased with increasing microbubble concentration, with ~ 1000 ng/g of tissue at the microbubble dose of 0.1 ml/kg, to 4500 ng/g at 0.5 ml/kg (Figure 3). This

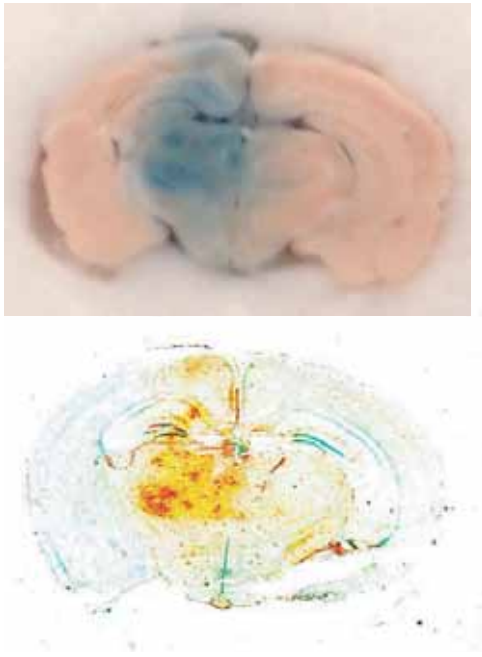

Figure 4. Top: Post mortem picture of a mice brain after sonication and injection of Trypan blue. Bottom: the same location showing the antibody distribution in the brain [36].

measured drug concentration is higher than the concentration that has been found to produce a clinically significant response [35]. The targeting of the ultrasound exposure was done under MRI guidance and monitoring. BBBD was verified by signal enhancement on contrast-enhanced MRI, which correlated with the drug concentration in the brain. More studies of the long-term impact of the chemotherapy agent and the ultrasound delivery on the brain tissue and tumours needs to be performed prior to clinical use.

The current authors have also tested antibody delivery through the BBB with ultrasound. In their first study, they used a dopamine D_4 receptor-targeting antibody to test the ability of ultrasound to disrupt the BBB for the diffusion of the antibody and to test the functionality of the antibody after the exposure [36]. In this study, the antibody was injected through the tail vein of a mouse simultaneously with the microbubbles, while exposing a selected location of the brain to ultrasound. MRI was used to aim the ultrasound beam and to verify BBBD with contrast-enhanced scans. Post mortem evaluation showed Trypan blue staining of the exposed tissue verifying the BBBD. Antibody staining of the brain sections of the exposed volume showed positive signals in the brain (Figure 4). When the sections were evaluated under the microscope, the signals were detected in the hippocampus and, small cells in the basal ganglia within the sonicated tissue volume. These sites are characteristic for location of the dopamine D_4 receptor. There was no obvious staining in the contralateral site, suggesting that the antidopamine D_4 receptor antibody (which recognizes the third extracellular domain of the human dopamine D_4 receptor) was delivered only at sites where BBB disruption had been produced by the sonication. When the staining intensity was compared with the MRI contrast enhanced signal, a good correlation was found. This indicated that MRI may be used not only to target, but also to quantify the delivery of antibody through the BBB.

If this delivery of antibodies can be done in a clinical setting, it will open the potential of using many therapeutic antibodies against CNS diseases. For example, the anti-HER2 monoclonal antibody, trastuzumab, could be used for some brain metastases of breast cancer, and the anti-$CD20^+$ monoclonal antibody, rituximab, for malignant lymphoma. There is also evidence suggesting that antibodies against the Abeta may reverse cognitive deficits in early Alzheimer's disease [37]. There are major problems in using these promising agents *in vivo* in the CNS because the antibodies have a large molecular size, and thus, they are blocked by the BBB if administered into the circulation. The first delivery of a therapeutic antibody agent with ultrasound induced BBBD was done by Kinoshita *et al.*, demonstrating the potential of this method for the antibody delivery [38].

8. Ultrasound exposure through the skull

The first demonstration of disrupting the BBB with focused ultrasound was done in rabbits by removing the skull [17]; however, this has now been able to be performed through intact skull in rabbits [20], rats [34] and in mice [39]. The situation is more complicated when ultrasound delivery through the human skull is attempted. The skull has several factors that make it a barrier for the use of both diagnostic and therapeutic ultrasound in the brain, and for several decades it was believed that the bone had to be removed to perform ultrasound treatments in the brain [40-42]. First, it had a density and speed of sound that are larger than in the surrounding soft tissues. This results in an acoustic impedance mis-match at the outer and inner bone surface, resulting in a significant reflection of sound at each interface. With continuous wave sonication this can result in the formation of a standing wave between the transducer and the skull, which can change the characteristics of the emitted wave [43]. The magnitude of the reflected wave is strongly angle dependent, resulting in a complete reflection of the longitudinal waves at a $> 30°$ entrance angle. Although energy is still transmitted through the bone via shear waves at larger entrance angles, the attenuation losses are large [44]. The high speed of sound in the bone, coupled with its dependence on the density of the bone, further complicates ultrasound transmission through the skull by distorting the propagating wave front. This is due to the variable thickness of the bone as a function of location, resulting in a situation in which parts of the beam is propagating through a thinner, and parts through a thicker, layer of bone. As the speed of sound in skull bone is almost twice the speed of sound in soft tissues, the wave propagating through a thicker piece will advance further than

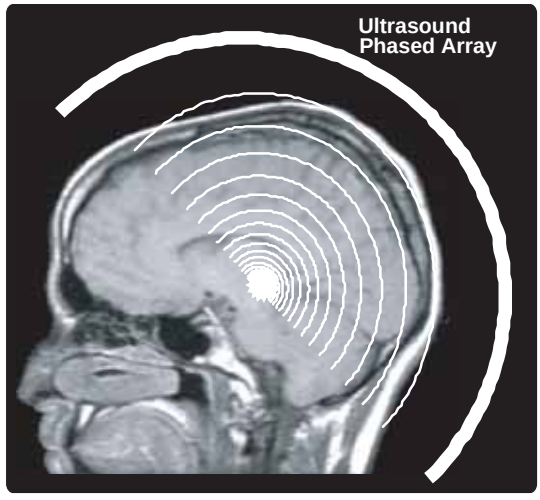

Figure 5. A diagram illustrating the potential clinical BBBD by focused ultrasound guided by an online MRI.
BBBD: Blood–brain barrier disruption.

the part propagating through a thinner piece when the wavefront has propagated through the skull. This makes focusing through the skull difficult with traditional single-focus transducers [45]. Phased arrays with CT-derived phase and amplitude corrections have been proposed to overcome this problem [46,47]. Such a system has been able to focus through human skulls and create thermal lesions in rabbit brains *in vivo* [8], and is currently in a clinical Phase I trial for thermal ablation of malignant brain tumors. As the time-averaged ultrasound energy is at least two orders of magnitude larger for thermal ablation than for the BBB disruption it is clear that such systems can be used in humans for BBB disruption (Figure 5). It has also been proposed that the skull induced distortions could be limited by reducing the frequency of the sound beam, thus increasing the wavelength. Both experiments [45] and simulations [48] have shown that the use of a low frequency (~ 250 – 300 kHz) will allow focusing through human skull without skull-specific distortion corrections. There is also another route for using higher frequencies that allow more precise focusing without the need for CT derived corrections. This method uses the transmission of shear waves that distort less than longitudinal waves [44].

When BBBD is performed in the brain with a closed skull, it is important to be sure that the blood vessels stay intact and that no bleeding is caused. The vasculature of elderly patients or those with pretreatment with radiation or chemotherapy may have compromised integrity of the blood vessel walls. Ultrasound (without microbubbles) may have caused bleeding in some patients exposed to low-frequency, unfocused ultrasound for thrombolysis [49]. Therefore, the field should progress cautiously when testing ultrasound-induced BBBD in these patient populations.

Overall, the progress made in the delivery of ultrasound through human skulls has reached a point where, from a physics point of view, it is almost certain that BBB disruption can be performed in man through an intact skull. However, it still remains to be seen how well the exposures can be localized without unintended exposures of surrounding tissues.

9. Conclusions

A number of animal studies have demonstrated that local BBBD is possible with focused ultrasound and microbubbles. The required acoustic power values are > 100-times smaller than those required to produce thermal damage in tissue, and are thus safe to deliver through intact skull. Two major mechanisms have been identified for the disruption, including opening of the tight junctions and active transport by vacuoles. BBBD is associated with minimal or no damage to the vasculature or the surrounding brain tissue. The survival studies have verified the lack of adverse event in the test animals. Focal delivery of antibodies and chemotherapy agents has been shown. However, the effectiveness of the delivery for a specific intervention or diagnosis has not been demonstrated.

10. Expert opinion

The BBB is a major barrier for the delivery of diagnostic or therapeutic agents into the brain. However, it can be used to localize the delivery of such agents if it is focally and transiently disrupted, thus allowing the molecules to leak only in the targeted site. This approach will minimize the impact of the agents to the CNS and allow a wide variety and more potent agents to be used. The only method that currently holds promise to non-invasively facilitate BBBD is ultrasound. Animal experiments have shown that local image-guided focused ultrasound disruption of the BBB can be induced with the aid of microbubbles in such a manner that minimal or no damage is induced to the surrounding brain tissue. This disruption is transient and will last ~ 6 h for a small molecular MRI contrast agent. The disruption has been demonstrated for several agents in multiple animal models. However, the clinical feasibility has yet to be demonstrated. The ability to perform image-guidance will allow the precise delivery of agents for therapeutic and/or diagnostic purposes into anatomical, physiological, functional or molecular targets in the CNS. For example, the BBBD based on image information will eventually be used to treat patients with brain metastasis and other sensitive/non-resistant brain tumours by targeting the tumour periphery where the BBB is still relatively intact. The exposure will allow a chemotherapy agent, which does not penetrate the BBB, but is effective against the tumour elsewhere in the body, to be used in the brain. Antibody-based treatments such as trastuzumab are especially promising in this regard.

Although the basic feasibility has been demonstrated in animals, much work is still required prior to fully utilize this methodology in clinical medicine. First, more *in vivo* studies need to be performed to quantify and optimize the delivery of each molecule of interest. Second, the effectiveness of a specific therapy or diagnosis needs to be demonstrated. For example, chemotherapy tests in an animal tumor model needs to be done. Third, clinical devices, with controlling and monitoring methods need to be developed. The basic feasibility of delivering ultrasound through intact animal or human skull has been demonstrated for thermal ablation purposes, thus, it is highly likely that a clinical delivery system for BBBD can be constructed. This step requires a contribution from the medical device manufacturers and is critical for the clinical utilization of the method. Finally, after the animal feasibility studies and clinical device development, the clinical trials need to be performed to test the effectiveness of any of the potential uses of the method. In the author's opinion, image-guided focused ultrasound BBB disruption is a method that has the potential to change the diagnosis and treatment of many CNS diseases and it will most likely have a major role in future patient care.

7. Acknowledgements

This research was supported by NIH grants (R01EB003268 and R33EB000705) and CRC program. The author wishes to thank Nathan McDannold and Manabu Kinoshita for providing some of the figures.

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