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Evaluating the safety profile of focused ultrasound and microbubble-mediated treatments to increase blood-brain barrier permeability

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Abstract

Introduction: Treatment of several diseases of the brain are complicated by the presence of the skull and the blood-brain barrier (BBB). Focused ultrasound (FUS) and microbubble (MB)-mediated BBB treatment is a minimally invasive method to transiently increase the permeability of blood vessels in targeted brain areas. It can be used as a general delivery system to increase the concentration of therapeutic agents in the brain parenchyma.

Areas covered: Over the past two decades, the safety of using FUS+MBs to deliver agents across the BBB has been interrogated through various methods of imaging, histology, biochemical assays, and behaviour analyses. Here we provide an overview of the factors that affect the safety profile these treatments, describe methods by which FUS+MB treatments are controlled, and discuss data that have informed the assessment of treatment risks.

Expert opinion: There remains a need to assess the risks associated with clinically relevant treatment strategies, specifically repeated FUS+MB treatments, with and without therapeutic agent delivery. Additionally, efforts to develop metrics by which FUS+MB treatments can be easily compared across studies would facilitate a more rapid consensus on the risks associated with this intervention.

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Declaration of interest

K Hynynen is an inventor on issued patents and patent applications related to transcranial focused ultrasound technology. K Hynynen owns stock in FUS Instruments, which has licensed IP related to the methods described in this review for pre-clinical use. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

A reviewer on this manuscript has disclosed that they are a founder of CarThera, a company developing an approach to disrupt the blood-brain barrier using an implantable ultrasound approach. Another reviewer on this manuscript has disclosed that they have a number of filed and issued patents related to both intranasal delivery of drugs to the brain and to focused ultrasound delivery of drugs to the brain. Peer reviewers on this manuscript have no other relevant financial relationships or otherwise to disclose.

Keywords

blood-brain barrier; focused ultrasound; microbubbles; targeted drug delivery; low intensity pulsed ultrasound; image-guided therapy

1. Introduction

Drug delivery to the brain is limited by the BBB. Gaseous or small hydrophobic molecules (< 400 Da) can diffuse across the BBB, however more than 98% of small-molecule drugs are excluded from entry into the brain parenchyma [1]. A major constituent of the BBB that limits entry of substances into the brain is the presence of tight junction proteins between endothelial cells, contributing to the physical barrier between the systemic circulation and brain [2]. Astrocytic endfeet processes, which enwrap almost all brain capillaries [3], as well as pericytes [4] and the anionic extracellular matrix also act to limit drug penetration and diffusion into the CNS [5,6]. Carrier proteins allow the transport of specific molecules into and out of the brain parenchyma, such as glucose and neutral amino acids [7]. The BBB limits exposure of the brain parenchyma to pathogens and aids in the maintenance of homeostasis, ensuring optimal conditions for neural function (Figure 1).

Currently, strategies to circumvent the BBB for the delivery of therapeutics rely on altering paracellular transport (eg. hyperosmotic solutions [8]), transcellular transport (eg. carrier protein mediated transport [9]), or on utilizing delivery routes outside of the circulatory system (e.g. intracranial injections [10], intranasal delivery [11], hydrogels [12]). Although hyperosmotic solutions may be helpful for neurological diseases that require treating large volumes of brain tissue, the use of such reagents can lead to structural alterations to neurons, lesions, macrophage accumulation, and glial activation [15]. Other strategies for bypassing the BBB suffer from their invasive nature, non-targeted delivery, or non-therapeutically relevant concentrations of drug delivery.

For many brain diseases, such as Parkinson’s disease and Alzheimer’s disease, passage of therapeutics across the BBB should be targeted to specific brain regions, and induced changes to BBB permeability must be reversible. With these considerations in mind, FUS combined with intravenous MBs has shown great potential. With appropriate FUS+MB parameters, controlled levels of increased BBB permeability can be achieved transcranially in targeted brain areas [16]; baseline BBB permeability levels are restored within six to 24 h [17]. Importantly, FUS+MBs can be used in conjunction with magnetic resonance imaging (MRI), enabling precise localization and targeting of pathological tissue. The size of treated tissue resulting from FUS+MB treatments is determined largely by transducer geometry and driving frequency and can be on the order of cubic millimeters [18]. Larger volumes of brain vasculature can be treated by sonicating multiple spots [17,18]. Thus, the minimally invasive nature of this technique and its transient effect on BBB permeability renders FUS+MBs a promising tool for the treatment of a variety of neuropathologies. In recent years, FUS+MB-mediated BBB treatments have progressed to clinical trials, bringing renewed attention to safety studies investigating the impact of FUS+MB treatments on brain tissue and neural function.

In this review, we will discuss studies that have sought to evaluate and improve on the safety of FUS+MB-mediated increases in BBB permeability through post-treatment analysis and novel acoustic feedback techniques. In this first section, we review current methods to circumvent the BBB. In the second section, we provide an overview of the *Safety Considerations* associated with FUS+MB-mediated BBB treatments. In the third section, *Assessments of Safety*, we examine the post-treatment methods by which safety has been analyzed in previous studies, including imaging, histology, behaviour, and inflammatory response analyses. In the fourth section, *Clinical Trials*, we review the published clinical trials on FUS+MB treatments. Finally, we provide our *Expert Opinion* on the topic of the safety profile in FUS+MB-mediated BBB treatments.

1.1 FUS-mediated increase in BBB permeability

In the context of neuroscience research, FUS was first investigated as a noninvasive tool to induce thermal lesions in the brain [19]. Changes in BBB permeability were found in the periphery of lesions exposed to high-intensity focused ultrasound (HIFU) [20]. Such changes in BBB permeability were only observed within 72 h of lesion formation; hemorrhages were not observed [21,22]. In 2001, Hynynen *et al.* introduced the use of ultrasound contrast agents (MBs), typically used in diagnostic ultrasound [23], to FUS brain treatments (Figure 1). These ultrasound contrast agents consist of MBs that act as cavitation sites, thereby enabling increases in BBB permeability to be achieved using temporal average powers more than three orders of magnitude below the level that causes thermal damage in brain tissue. Thermally induced increases in BBB permeability were shown to be achievable using HIFU, but results were inconsistent and at times accompanied by tissue damage [22]. In contrast, FUS treatment with MBs reliably caused increases in BBB permeability without causing permanent tissue damage and avoiding neuronal damage [16]. Since then, techniques have been developed to better control FUS+MB parameters to minimize the risk of tissue damage [24].

Since this initial study [16], FUS+MB treatments have been used to successfully deliver a variety of agents across the BBB, resulting in functional benefits. Chemotherapeutics delivered to the brain using FUS+MBs resulted in increased concentrations of drug within brain tumours, reduced tumour growth, and improved median survival in rodents [25–29]. Antibodies have been delivered to the brain parenchyma using this technique [30,31], leading to functional effects such as β-amyloid plaque reduction [32]. FUS+MB treatments have also been used to deliver cells [33,34], neurotrophic factors [35], nucleic acids [36], viral vectors [37–39], non-viral genes encapsulated in liposomes [40], and brain-penetrating nanoparticles [41,42]. FUS+MBs alone, without agent delivery, has been shown to have effects that may be beneficial in specific contexts. In wild-type animals, FUS+MB-mediated BBB treatments have been reported to result in neurogenesis [43,44]. In transgenic mouse models of Alzheimer's disease, FUS+MBs alone has resulted in reductions in β-amyloid plaque load [45–48] and phosphorylations of tau [49], and improvements in behaviour [45,46,49]. Surface cortical structures [32,46,49,50] and deep brain structures, such as the hippocampus [45] and basal ganglia [37], have been successfully targeted in animal models. By sonicating multiple overlapping spots, larger volumes of the BBB (e.g. half of an adult canine brain [51]) can also be treated [51].

One substantial obstacle and source of heterogeneity for FUS+MB treatments is the presence of the skull. The thickness and heterogeneity of skulls in larger animal models and humans poses difficulties due to the attenuating and aberrating effects of bone on ultrasound propagation [52]. Transcranial ultrasound propagation suffers from less distortion in small animal models with thinner skulls. Skull-based distortions can also be avoided by using implantable transducers [53,54]. Phased array transducers and computed tomography based density measurements have been used to calculate skull corrections in order to reduce focus distortion in larger animal studies [55,56], some of which utilize *ex vivo* human skulls in the path of ultrasound [57–59]. The success of large animal studies investigating the efficiency and safety of FUS+MB treatments [18,59,60] have facilitated the field's progression to clinical trials [53,61,62].

The BBB is a physiological barrier that is vital in maintaining homeostasis in the brain. Circumventing the BBB, albeit in a minimally invasive manner, raises concerns, including entry of pathogens into the brain and the induction of inflammatory processes [63–65]. Mechanisms by which FUS+MBs increase BBB permeability, as well as factors that contribute to the return to baseline permeability, remain important questions to address in fully characterizing risk factors. In this review, we will highlight studies that have sought to evaluate and improve on the safety of FUS+MB-mediated increases in BBB permeability, as well as discuss considerations for interpreting results from preclinical research.

2. Safety considerations

2.1 FUS parameters

With the use of MBs, FUS is capable of inducing increases in BBB permeability at low powers. When MBs are driven by the cycles of compression and rarefaction of ultrasound, stresses are exerted on blood vessel walls. However, there is a narrow window of MB oscillation characteristics that have been shown to induce the desired effects on vascular permeability while minimizing the risk of permanent detrimental effects to brain tissue. This section will briefly discuss the ultrasound parameters that have been shown to alter biological responses following FUS+MB treatments.

While acoustic frequencies ranging between 28 kHz [66] and 8 MHz [67] have been employed to increase BBB permeability, it is important to consider that as ultrasound frequency increases, so too does the degree of tissue attenuation. This can lead to skull heating and distortion of the ultrasound focus [68–70]. Conversely, the use of lower frequencies is accompanied by larger focal sizes, which may be undesirable for small targets that require precise targeting, but may be applicable for large volume treatments. For clinical, transcranial applications, the range of effective frequencies has been proposed to lie between 200 kHz and 1.5 MHz [71]. Additionally, as driving frequency increases, the peak negative pressure (PNP) required to induce increased BBB permeability also increases (provided all other parameters remain constant). This relationship is captured in the equation for mechanical index ($MI = PNP/\sqrt{f}$, where f is the center frequency of the ultrasound wave). In one study, sonications with an MI of approximately 0.42–0.50 resulted in increased BBB permeability for a range of frequencies using a routine clinical ultrasound imaging dose of Optison MBs [72]. It is important to note that the range of MIs reported in

this study to produce increased BBB permeability should not be considered universally applicable; factors such as pulsing scheme (discussed below) and MB dose will have substantial impact on the biological outcomes of sonications at any MI. If parameters like PNP are inappropriately chosen, hemorrhage, edema, extensive inflammation, prolonged impact on BBB permeability, ischemia, apoptosis, and necrosis will result [63,64,73–75].

The magnitude of stress exerted on vasculature by oscillating MBs has a large impact on the resulting biological responses and is influenced by a host of parameters, including PNP, MB size, MB composition, driving frequency, and blood vessel size. Studies investigating the effects of factors that influence the temporal distribution of these stresses, such as pulse length, pulse repetition frequency, and sonication duration, have shown that these parameters also influence the outcomes of FUS+MB treatments. McDannold *et al.* demonstrated that as pulse length is increased from 0.1 to 10 ms, the degree of BBB permeability enhancement also increases [76]; earlier work showed no further increases in permeability between 10 and 100 ms [16]. It has been postulated that pulse lengths beyond 10 ms do not result in additional increases in BBB permeability due to a complete destruction of MBs in the focal region during this time [76]. Conversely, increased BBB permeability has also been shown with short pulse lengths, down to single cycle sonications, provided PNP is sufficiently high [77]. Pulse repetition frequency [76,78] and sonication duration [79] have also been shown to influence the degree of increased BBB permeability following FUS+MB treatment.

When discussing parameters that influence the safety profile of FUS+MB treatments, it is important to consider that as the degree of BBB permeability is increased, the risk of tissue damage also rises. Developing sonication protocols for increasing therapeutic agent delivery to the brain involves adjusting parameters to achieve both adequate BBB permeability enhancement and an acceptable impact on tissue health. While drawing on published methods is a good starting point, it should not be a substitute for internal, in-depth safety evaluation pilots studies when establishing new FUS+MB protocols or using emissions-based feedback control schemes.

2.2 Microbubble dose

While there are a host of parameters that can influence the outcome of sonications intended to increase BBB permeability, one of the more difficult to compare between studies is MB dose. Complexities arise from the number of factors that can influence how MBs respond to ultrasound and interact with biological systems, as well as the lack of accepted practices for MB preparation, handling, and administration. This may manifest as significant disparity in results between studies that report using similar exposure settings and MB doses. Even with identical MB administration protocols, there will be variability in MB concentration at target locations as a function of time due to intersubject differences in cardiac function and vascular density.

MB dose has been shown to influence the degree to which the permeability of the BBB is increased, as well as the transcription of acute inflammatory markers 6 hrs post-FUS+MBs [64]. While MB number is important to consider when comparing doses, this measure alone does not account for variance in MB responses. Factors such as mean size, size distribution, shell composition, and half-life in circulation of different MBs will have a substantial

influence on biological outcomes [75,80–85]. For example, all sonication parameters being equal, a population of MBs with a mean diameter of 1 μm will not have the same impact as a population with a mean diameter of 3 μm. One of these populations will have a larger proportion of MBs that are closer to resonance size and thus display a stronger response to the incident ultrasound field.

There have been efforts to develop strategies for determining equivalent MB doses. Song *et al.* have proposed gas volume to be a unifying parameter, demonstrating a strong correlation between gas volume and Evans blue extravasation following sonication when comparing MBs with mean diameters of 2 and 6 μm [80]. It is unclear if this relationship holds true between MBs with different shell compositions or for MBs with wide size distributions. Another approach has been to use the clinical imaging dose of MBs as a normalizing factor. McDannold *et al.* found that the probability of increased BBB permeability as a function of pressure amplitude was similar between the clinical imaging doses of Definity and Optison [86]. While these methods may have value in approximating equivalent doses, the development of more robust dose-equivalence strategies remains an unresolved issue, though continued advancement of acoustic control schemes, may alleviate some of this need.

It is also important to consider factors that can alter MBs before they enter vascular beds in the targeted tissue. Three relevant factors in this regard are handling procedure, method of administration, and blood oxygen levels. For Definity, size distribution and ultrasound attenuation has been shown to be greatly influenced by the length of time that passes between decanting and use [84]. Similarly, the size distribution of Definity MBs was found to be strongly dependent on preactivation vial temperature [87]. It is conceivable that subtle differences in MB handling and preparation procedures could lead to different concentrations and size distributions, and ultimately in substantially different downstream biological responses, even in studies that report using the same MB dose.

The method of administration (bolus versus infusion) will affect the number of cavitation nuclei available to act at any given time over the course of sonication. While many studies have employed bolus MB delivery, slow infusion is likely to produce a more stable rate of MB-vascular wall interactions over the duration of FUS exposures [77]. This is due to the short half-life in circulation of most commercially available MBs [88,89], leading to a large change in the systemic concentration of MBs over a relatively short amount of time when delivered as a bolus. Also relevant to the persistence of MBs in circulation is oxygenation status. The choice of medical air or oxygen as a carrier gas for anesthesia has been shown to affect the degree of increased BBB permeability following FUS+MBs, with medical air resulting in a greater increase in signal intensity on contrast enhanced T1-weighted (CE-T1w) images [85]. These results may be explained in part by the effect of dissolved gases in circulation on MB stability, as others have shown that MB circulation time is reduced in animals breathing oxygen compared to medical air [88,89].

2.3 Acoustic feedback control

Local differences in vascularity and inhomogeneities in MB dispersion can lead to inconsistent effects of FUS+MBs on BBB permeability throughout the brain [18,90,91]. In addition, defocusing of the ultrasound beam(s) by the skull (due to skull thickness, non-

normal ultrasound propagation, and standing waves) can lead to inaccuracies in predicting *in situ* ultrasound pressures [69,70]. Given the relatively narrow safety window [18] between a clinically relevant increase in BBB permeability and widespread distribution of microhemorrhages, methods of monitoring and controlling FUS+MB treatments in real-time are essential for minimizing the chance of causing substantial tissue damage.

Assessing the spectral frequency content of acoustic emissions from MBs during sonication can give insight into the behaviour of MBs *in vivo* [92,93] (Figure 2). When driven at sufficient pressures, MBs oscillate linearly and nonlinearly in size around their equilibrium state. This regime of activity, referred to as stable cavitation, results in an increase in magnitude of acoustic emissions at harmonics of the driving frequency [94]. If the pressure amplitude is increased above a threshold value, sub- and ultraharmonics will be emitted [95]. Stable cavitation can induce hoop stress in vascular walls as MBs expand in close proximity to the endothelial lining, as well as cause microstreaming of plasma surrounding MBs, contributing to shear stress on endothelial cells [96,97]. These physical forces are believed to contribute to increased BBB permeability [98,99]. While stable cavitation can lead to increased BBB permeability with minimal negative effects on tissue health [18,100], it would be overly simplistic to state that stable cavitation is “safe”. If the magnitude of stress generated by stably oscillating MBs is sufficient, blood vessel rupture can occur [97]; thus, the component of magnitude needs to be considered.

As the applied PNP is further increased, MBs will begin to collapse in the compression phase of the ultrasound wave. This behaviour, termed inertial cavitation, can generate shockwaves, jets streams, free radicals, and extreme heat, and is characterized by a sharp increase in the production of broadband emissions [94]. This violent collapse can result in ischemia, apoptosis, necrosis, edema, and hemorrhage [74]; thus, efforts to reduce inertial cavitation are essential in the context of increasing BBB permeability with FUS+MBs. Studies have demonstrated that increased BBB can be achieved without wideband emissions indicative of inertial cavitation [100,101].

A number of strategies have been developed to control applied pressure in real-time based on acoustic emissions. In one method, PNP is adjusted to produce an empirically determined magnitude of harmonic emissions [102]. Recently, Sun *et al.* demonstrated that a closed-loop algorithm based on the amplitude of harmonic emissions can be used to consistently increase BBB permeability and may be effective in modulating the degree of permeability [103]. One potential drawback of this approach is the necessity to determine the target harmonic setpoint based on the animal model, MB type, acoustic field, and hydrophone sensitivity. Furthermore, the method relies on the amplitude of the signal emitted by the MB cloud at the focus, thus rendering the signal dependent on the MB distribution and the vascular network; however, the ability to control the degree of BBB permeability enhancement represents a substantial advancement in this field.

Another approach for controlling FUS+MB treatments is to incrementally increase the applied pressure until detecting a threshold event, such as ultraharmonic [24] or subharmonic emissions [45], then reducing the applied pressure to a fraction (aka scaling factor) of the triggering pressure. O’Reilly and Hynynen first demonstrated the effectiveness

of this approach in consistently producing increases in BBB permeability. They also showed a linear relationship between the scaling factor after a threshold event and mean intensity on CE-T1w MR images. A variation of this approach has been used in clinical trials that employ transcranial ultrasound propagation [62]. Potential drawbacks of this approach include the necessity to adjust the scaling factor for MB type and hydrophone sensitivity.

Whether calibrating applied pressure based on the amplitude of harmonic emissions or a threshold event, it is important to note that MB size in most commercially available formulations is polydispersed. Given that the resonance frequency of a MB is largely influenced by its size, this can result in a growing fraction of the MB population cavitating as applied pressure is increased. The point at which a sufficient number of MBs are oscillating and producing acoustic emissions that are detectable above baseline noise will influence the function of any acoustic control algorithm. Thus, when assessing the efficacy or modifying the parameters of an acoustic control algorithm, the sensitivity of the detector(s) implemented, which is influenced by size, shape, and material, should be taken into account.

Acoustic feedback control strategies have been essential in improving consistency and reducing the risks associated FUS+MB treatments, thereby facilitating progression to clinical testing. There continues to be efforts directed at improving the accuracy of predicting biological outcomes based on acoustic emissions and refining strategies for large volume treatments. This work will add flexibility in the clinical application of FUS+MBs for increasing BBB permeability.

2.4 Risks/benefit assessment

As with any medical intervention, the use of FUS+MBs to facilitate drug delivery to the brain is not benign. There are risks associated with transiently increasing the permeability of the BBB. Current clinical trials have been cautious in this regard; patients with active infections have been excluded due to unknown hazards [62]. However, if used in the appropriate contexts and with fully characterized FUS+MB parameters, the benefits of treatment should, by design, outweigh potential detrimental effects. Given the range of pathologies that FUS+MBs has the potential to aid in treatment, as well as the range of ultrasound parameters to optimize for each condition, there are many considerations in assessing the suitability of this developing treatment option.

One important consideration is the desired treatment outcome. Consider the delivery of chemotherapeutic agents to glioblastoma. Since the goal in this context is to aid in killing tumour cells and to slow cancer progression, concerns of inducing an acute inflammatory response in the core of the tumour or its sonicated margins may be irrelevant in comparison. Conversely, if the goal of treatment is to restore or preserve tissue function, more attention must be paid to the consequences of transiently causing glial cell activation. This notion may be especially relevant in the treatment of pathologies that are associated with chronic inflammation, such as amyotrophic lateral sclerosis and Alzheimer’s disease.

Another important consideration when evaluating the risk of intervention or comparing results of preclinical FUS+MB studies is the volume of sonicated tissue. While O’Reilly *et*

al. demonstrated that the time required for BBB permeability to return to baseline following FUS+MBs is independent of affected tissue volume [17], other aspects of brain health have yet to be thoroughly evaluated through this lens. Behavioural studies in wild-type mice following four weekly treatments targeted to the bilateral hippocampi show no impairment in performance in hippocampal-dependent tasks compared to untreated controls [45]. Given the volume of brain tissue treated in this study, relative to the size of the mouse brain, this should be considered a 'large volume' sonication. This study is one piece of evidence suggesting that large volume sonications can be performed without overt detrimental behavioral effects, however, more comprehensive investigations in wild-type animals are required to determine the added degree of risk associated with treatment volume. Recent technological advances in the field have enabled large volume sonications to be controlled effectively using acoustic emissions in larger animal models [104]. This capability should be utilized to investigate the question in animals that more closely model humans.

Treatment schedule is a consideration that will have great relevance as FUS+MB-mediated drug delivery strategies develop. A single sonication with the goal of delivering a virus carrying a CRISPR-Cas9 system will have a different safety profile than 8 biweekly sonications intended to increase the delivery of anti-β-amyloid antibodies [47,48,50]. The risks of cumulative detriments to tissue and vasculature will rise with increasing treatment frequency and number. This is an area of preclinical research that would benefit from further investigation, including the examination of a variety of treatment repetition frequencies with and without therapeutic agent delivery.

Finally, the toxicity of therapeutic agents need to be evaluated in the context of brain delivery. With FUS+MB treatment, systemically administered drugs will be permitted to accumulate in the brain parenchyma and reach concentrations higher than that achieved in clinical trials executed without sonication. Thus, the sensitivity of brain tissue to the agents that are to be used in conjunction with FUS+MBs need to be carefully evaluated. Additionally, undesired side-effects on non-targeted organs also warrants thorough attention, since therapeutics are administered systemically in FUS+MB treatments. For some agents, immunoliposome encapsulation may be helpful in reducing systemic toxicity by biologically targeting liposomes through ligand-antibody interactions [14,105,106]. Immunoliposomes that have accumulated in regions of the brain where BBB permeability has been increased can then be stimulated to release their payload.

3. Assessments of safety

Promising results from clinical trials testing the feasibility of using FUS+MBs to increase BBB permeability in human patients have been demonstrated in the context of malignant brain tumours [53,61,107] and Alzheimer's disease [62]. These studies have been informed by data generated from preclinical work. The safety of FUS+MB treatments has been assessed by evaluating the extent of BBB permeability, duration of increased permeability, effects on surrounding cells and vasculature, and changes in behaviour.

3.1 Magnetic resonance imaging

MRI is useful in FUS+MB treatments as a way to target specific brain regions and to evaluate treatment effects. T2-weighted (T2w) imaging can provide anatomical details (e.g. ventricles and sulci) to inform targeting. After sonication, a variety of MRI sequences can be used to evaluate tissue health. Commonly, hyperintensities in T2w images and hypointensities in T2*w are used to assess the presence of edema [108] and microhemorrhages [109], respectively. In addition, CE-T1w images can be used to assess BBB permeability following FUS+MB treatments: elevated signal intensity in targeted areas indicate increased BBB permeability.

Results from CE-T1w imaging have shown FUS+MBs to be a reliably precise method of causing increased BBB permeability in targeted brain regions. Given appropriate exposure settings, areas exhibiting gadolinium enhancement are limited to the targeted volume [18].

CE-T1w imaging has also been used to investigate the duration of time for which the BBB exhibits increased permeability after sonication. Such studies have reported various BBB closure times, ranging from one to 24 hrs [16,110–112]. Disparities may be due to both differences in the initial magnitude of BBB permeability enhancement as well as the methods used to evaluate permeability. Marty *et al.* demonstrated that the half closure time ($t_{1/2}$) of the BBB following FUS+MB treatment is dependent on the size of MRI contrast agent used, ranging from ~ 0.5 hrs for 7 nm particles, to 5.5 hrs for 1 nm particles in rats [113]. Closure time has been shown to be dependent on MB size [75], but not on volume of treated tissue [51]. Using controlled acoustic pressures, single- and multi-point FUS+MB exposures have been shown to cause transient increases in BBB permeability for MRI contrast agents (molecular weight < 1 kDa) that returns to baseline permeability levels within 6 hrs, and are devoid of edema or hemorrhage in canines [51]. Similarly, CE-T1w imaging revealed that FUS+MB-mediated increases in BBB permeability resolved within 24 hrs of sonication in human patients with Alzheimer's disease [62]. These studies emphasize the point that BBB permeability is not a binary classification (open or closed), and that the extent of FUS+MB-mediated increases in BBB permeability is dependent on a wide range of factors, relating to both sonication parameters and the compounds crossing the BBB. Of note, the signal-to-noise ratio of an imaging sequence is likely to have a significant bearing on the ability to detect contrast enhancement.

One consideration when using CE-T1w imaging to assess BBB permeability is the variability in vascular density between brain regions. White matter, which is less vascularized than grey matter, has been shown to exhibit a lower extent of contrast enhancement at the same acoustic pressures [18]. In preclinical studies, post-mortem analyses of dye extravasation may be more sensitive than CE-T1w imaging in detecting changes in permeability, in large part due to experimental designs which allow dyes to circulate for extended periods of time [18].

MRI can also be used to infer the effects of FUS+MB treatments on vascular and tissue health. Hypointensities on T2*w MRI are indicative of hemosiderin or extravasation of red blood cells [114,115], and suggest damage to vasculature. In a comprehensive non-human primate study that tested the effects of repeated focal and volumetric (~ 1 cm^3) sonications

in seven rhesus macaques over several weeks, McDannold *et al.* observed that hypointensities were present in 5–15% of the 185 targets (ExAblate 400, InSightec, 220 kHz) [18]. In another study, macaques exposed to multiple unilateral FUS+MB-mediated BBB treatments (500 kHz, 200 – 400 kPa) over a maximum of 20 months did not show any indications of hemorrhage on susceptibility-weighted imaging (SWI), although this was not confirmed by histology [116]. Hypointense regions were often seen in sonications that resulted in wideband emissions (indicating inertial cavitation), whereas increased emissions at the second and third harmonics (without wideband emissions) were accompanied by increases in contrast enhancement on T1w images without hypointensities on T2*w images [18].

Hyperintensities on T2w images, which can be indicative of edema, have been observed following FUS+MB treatments. When interpreting these results, it is important to consider sonication parameters as well as treatment repetition frequency. Downs *et al.* noted that approximately 6% of BBB treatments targeted to the putamen and caudate nucleus of the basal ganglia in macaques resulted in hyperintensities on T2w images 30 mins to 30 hrs after FUS+MB treatment. Animals in this study underwent multiple unilateral FUS+MB-mediated BBB treatments (500 kHz, 200 – 400 kPa) over a maximum of 20 months with an average affected volume of 203 mm^3. All hyperintensities were only observed following the last one to three FUS+MB treatments, and were resolved within one week of sonication. Similar results were obtained by the same group using acoustic pressures of 300 kPa, in an unanesthetized, alert macaque [117]. Conversely, others have demonstrated that increased BBB permeability can be achieved without hyperintensities on T2w images [17,51].

3.2 Histology

Histological methods have been used extensively to characterize the impact of FUS+MB treatments on both BBB permeability and tissue health (Figure 3). Extravasation of dyes, such as trypan blue and Evans blue [30,46], are sensitive tools for confirming changes in BBB permeability in preclinical studies. Immunohistochemistry and immunoblot analyses of extravasated exogenous [30,118,119] and endogenous antibodies [32,118,120] have also been used for this purpose. Significant increases in the levels of IgG and IgM in the brain parenchyma have been observed from one hour to four days after FUS+MB treatment in mice and rabbit brains [32,118,120]. Of note, the extent of increased BBB permeability caused by FUS+MBs, analyzed as the rate of enhancement and maximum enhancement on CE-T1w images, has been shown to correlate with increased levels of IgM, but not IgG, detected in the brain four days after treatment [32].

To assess the impact of FUS+MBs on tissue health, basic histological stains, such as H&E and Nissl, have been used. Damaged vasculature can be identified in H&E stained sections as regions containing extravasated erythrocytes. Such sites have been observed as early as 30 mins post-FUS+MB treatment in rodents and non-human primates [17,18,51,54,121] and may present as hemosiderin deposits (Prussian blue stain) months later [18]. In sonicated brain regions exhibiting low levels of erythrocyte extravasation in H&E stained sections, dark, potentially ischemic neurons (H&E), have been observed, but without the presence of apoptotic bodies (TUNEL staining) [18].

Studies evaluating the effects of a range of PNPs have shown substantial damage to brain vasculature and parenchyma at high pressures. For example, H&E stained brain sections revealed that FUS+MB exposures at a PNP of 0.6 MPa (driving frequency 0.69 MHz) resulted in a few extravasated erythrocytes visible four hours post-FUS+MBs, but a significant increase in the number and size of erythrocyte extravasations at 0.8 MPa [86]. TUNEL-positive sites, indicating apoptotic cells and DNA fragmentation, were coincident with areas of severe extravasation, but major ischemic sites were not observed (vanadium acid fuchsin staining) [119]. Erythrocyte extravasations observed in H&E stained sections have been shown to coincide with the presence of wideband emissions [30]. Additionally, high exposure levels (444 – 700 kPa, 220 kHz) have also been associated with hemosiderin-filled macrophages in the meninges (H&E) and persistent hypointense regions on T2*w images [18].

Other studies have demonstrated FUS+MB-induced increased BBB permeability without tissue abnormalities, extravasated erythrocytes, hemosiderin deposits, or altered neuron health (Bielschowsky's silver stain)[18,101], in sonicated brain regions. When considering the histological observations of any study, it is necessary to consider both the FUS+MB parameters employed and the amount of time that has passed between sonication and euthanasia. The former affects the magnitude of impact on tissue health and the latter influences the opportunity for lesion formation or tissue repair.

3.3 Behaviour

Effects of FUS+MB-mediated BBB treatments on behaviour have been thoroughly assessed in a small number of studies utilizing non-human primates. McDannold *et al.* conducted a comprehensive study in rhesus macaques using a clinical-prototype MRgFUS brain system (ExAblate 4000, InSightec) to investigate the effects of a range of acoustic power levels, and MB injection/infusion parameters. Behavioral responses were evaluated by observing activities of daily living and visual function and acuity after repeated FUS+MB treatments to the lateral geniculate nucleus (relay system for the visual pathway) and primary visual cortex. After five successive volumetric (~ 1 cm^3) treatments targeting the primary and secondary visual cortices bilaterally over the course of five to nine weeks, visual performance, visual acuity, motor skills, and species-specific behaviours were unaffected, although a few hypointense regions in T2*-weighted images were observed [18].

Similarly, Downs *et al.* assessed the effects of repeated FUS+MB treatments on decision-making and motor control. FUS was targeted to the putamen and caudate nucleus of the basal ganglia in one hemisphere over a maximum of 20 months in macaques. Of the 61 spots treated, four exhibited possible edema (hyperintensities in T2w MRI), all of which resolved within one week. Animal physiology (locomotion, eating, drinking, social behaviors) was unaffected by FUS+MB treatments. Visual perception, decision making, motivation, and motor function were evaluated using the reward magnitude bias and random dot motion tasks. All three animals exhibited variability in behavioral tasks. Authors noted that responses differed between high and low rewards on non-sonication days, suggesting that FUS+MB treatments may impact motivation. In addition, behavioral data on days when T2w images showed hyperintense voxels were not significantly different from when no

hyperintense voxels were present, indicating that spots of potential edema did not substantially affect behavioral responses. Decision making responses on sonication and non-sonication days were also not significantly different. Behavioral tests were conducted several hours after FUS+MB treatments, one hour after anesthesia ended [116]. Using exposure conditions but in alert macaques, the same group found significant decreases in touch error in responding to cue stimuli after treatment, but varied results in reaction time [117].

Behavioral effects of FUS+MB treatments in a natural canine model of aging have also been evaluated using a battery of neurological tests. Motor function, cranial nerve function, postural reactions, and alertness were unaffected after single and repeated weekly treatments [51]. These studies in large animal models suggest that FUS+MB-mediated increases in BBB permeability can be achieved without detectable changes in behaviour. Consistent with these large animal studies, published results from clinical trials have revealed no adverse behavioural events related to FUS+MB treatments [53,62].

3.4 Inflammation

Recently, much attention has been directed at discerning the effect of FUS+MBs on the production of inflammatory mediators, as well as characterizing the impact of the resulting acute inflammatory response on tissue health. To date, all data published on the topic indicate that some degree of inflammatory response follows FUS+MB-mediated increases in BBB permeability; however, there are variations in the reported magnitude, duration, and impact of this response.

At the level of transcription, evidence of an acute inflammatory response has been demonstrated in isolated microvessels 6 hrs following sonication, with an upregulation of *Ccl2, Ccl3, Ccl7, C3, Il1b, Il6, Sele,* etc. By 24 hrs, expression levels of these genes largely return to baseline or are reduced relative to the 6 hr time point [122]. In whole brain tissue, transcription of Nfkb2 pathway-related genes has been investigated following FUS+MBs in two independent studies. The Nfkb2 pathway is involved in a wide range of biological processes, including innate and adaptive immunity, inflammation, and stress responses. The first study demonstrated a significant upregulation of several genes involved in acute inflammation, including *Il1a, Il1b, Selp, Tnf,* and *Icam1*, at 0.5, 6, and 12 hrs following FUS +MBs. A number of other genes displayed increased expression only at the two later time points, including *Mmp9, Ccl5, Sele*, and *Birc3*. These changes in gene expression were largely mirrored at the protein level with elevated levels of Mcp1, Icam1, Tnfa, and Mmp9, persisting to the latest time point investigated, 24 hrs post-FUS+MBs [63]. A subsequent study replicated the magnitude of this response at 6 hrs following sonication and, notably, showed that the expression level of several key genes involved in acute inflammation, including *Tnf, Icam1, Ccl5, Birc3, Il1a,* and *Il1b*, were strongly correlated to the degree of BBB permeability. Additionally, MB dose appeared to significantly influence BBB permeability [64].

Changes in the expression level of glial fibrillary acidic protein (Gfap) and ionized calcium-binding adapter molecule 1 (Iba1), markers for astrocytes and microglia respectively, have been investigated as indicators of glial cell activation following FUS+MBs. In wild-type mice, Iba1 expression has been shown to increase in sonicated cortex, relative to non-

sonicated contralateral cortex, at 4 hours and 4 days following sonication, with no significant differences present at 15 days. Gfap expression in these mice was found to be increased at 4 days post-FUS+MBs, but not at 4 hrs or 15 days [32]. Conversely, with higher exposure levels, significantly elevated Gfap and Iba1 immunoreactivity has been reported 7 weeks after a single sonication and 7 days after 6 weekly sonications, with morphological changes indicative of glial scar formation [73]. In mouse models of Alzheimer's disease, increases in the number [48] and size [32] of beta-amyloid plaque-associated microglia has also been reported after FUS+MB treatments. Together, these studies indicate that some degree of glial cell activation follows FUS+MB-mediated increases in BBB permeability. It is also apparent that these change can be transient, normalizing within 2 weeks, or can persist for at least 7 weeks, depending on exposure conditions.

Local inflammatory processes have also been investigated by assessing macrophage infiltration. Kobus *et al.* reported that 24 hrs following the last of six weekly FUS+MB treatments, rats sonicated with the lowest PNPs (0.66 and 0.73 MPa measured in water; driving frequency = 690 kHz) displayed no or few instances of parenchymal macrophages, as assessed by H&E staining. With a higher PNP (0.80 MPa), the number of parenchymal macrophages increased [123]. Others have similarly observed FUS+MB-induced extravasation of macrophages using H&E staining [18,124,125], CD68 immunodetection [46,63,73], and MRI of superparamagnetic iron oxide nanoparticle-labelled cells [126]. In total, these studies suggest that the extent of macrophage extravasation following FUS+MBs is largely influenced by sonication parameters.

Acute inflammation in the brain has a number of downstream effects, some of which have been observed following treatment with FUS in combination with MBs. While none have been causally linked to inflammation and all have a number of mechanisms that can drive their progression, it may be hypothesized that increases in hippocampal neurogenesis [43,44], angiogenesis [127], and β-amyloid plaque clearance [32,45–48] may be linked to acute inflammation. Future work should focus on determining if any causal relationships exist. Such relationships may be especially relevant in the case of plaque clearance, as determining the relative contributions of any driving mechanisms may inform modifications to FUS+MB treatment that can increase its efficacy.

Given the goal of FUS+MBs in this context is to increase BBB permeability, it is unsurprising that some level of inflammatory response has been observed. The combined impact of mechanical stress on vascular walls and the transit of plasma proteins from systemic circulation into the brain would be expected to disrupt homeostatic conditions, inducing a cascade of responses which may lead to some degree of inflammation. Examples of this response can be seen in a wide range of pathological conditions in which BBB permeability is increased, such as multiple sclerosis [128,129], traumatic brain injury [130], stroke [131–133], and epilepsy [134–136]. Important to note in this discussion, however, is that the magnitude of change in BBB permeability induced by sonication can be controlled and that the physical stimulus driving this change (i.e. ultrasound-stimulated MBs) is transient. This is contrasted by the pathologies listed above, where the causal relationships between inflammation and altered BBB permeability are not always clear. Additionally, the

underlying mechanisms driving both inflammation and permeability changes in these pathologies are often not transient.

Acute inflammation in the brain is a protective response that is initiated to return physiological functions to naive levels when homeostatic control pathways are insufficient [137]. However, if conditions are pushed too far from the setpoint, the magnitude or duration of the inflammatory response can lead to substantial detriment [138–141]. While it is apparent that the magnitude of inflammation that follows FUS+MBs is at least in part related to the degree to which BBB permeability has been increased [64], questions remain regarding the relative contribution of other factors (eg. magnitude of stress on vascular walls). Future work should focus on fully characterising the impact of FUS+MB-induced inflammation on short and long term tissue health. It is paramount, however, that continuing work in this arena is conducted with parameters that reflect current best practices, including the use of validated acoustic feedback control methods.

4. Clinical trials

The first use of FUS+MBs in humans for the purpose of increasing BBB permeability came as part of a clinical trial in July of 2014. This trial involved the implantation of a single element ultrasound device system into the skulls of 17 patients with recurrent glioblastoma, avoiding the complications of transcranial ultrasound propagation, but necessitating an invasive surgical procedure. Patients were sonicated at fixed pressures to induce increased BBB permeability, after which carboplatin, a chemotherapeutic agent, was administered. Treatments were repeated two to four times, monthly. Authors reported that patients tolerated the procedure well, with no evidence of acute hemorrhage, ischemia, or edema on susceptibility-weighted angiography, diffusion, or FLAIR sequences. Clinical symptoms relating to the FUS+MB procedure were not present in any patients in the subsequent hours or days, including the 11 epileptic patients that participated. Two adverse events occurred during the trial but were deemed unrelated to the procedure [53].

Phase 1 clinical trials conducted at Sunnybrook Research Institute in Toronto, Canada were the first to utilize transcranial ultrasound exposures with a multi-element hemispherical phased array. The first published results from these trials come from a study in which a presumed non-eloquent region, the superior frontal gyrus white matter of the dorsolateral prefrontal cortex, was targeted in five patients with mild to moderate Alzheimer’s disease. Two stages of sonications, separated by one month, were performed, with the volume of targeted tissue doubling in the second stage. No participant presented with clinical symptoms believed to be related to the FUS+MB procedure during this study, nor displayed persistent increases in BBB permeability on CE-T1w imaging 24 hrs following the procedure. Two participants displayed hypointensities on T2*w images immediately following sonication that resolved within 24 hrs. Tests interrogating cognition and daily functioning revealed no clinically significant changes between pre- and three months post-treatment [62].

Currently, there are eight clinical trials recruiting participants around the world (ClinicalTrials.gov Identifiers: NCT03321487, NCT03119961, NCT02343991,

NCT03608553, NCT03626896, NCT03616860, NCT03671889, NCT03712293). Thus far, studies have demonstrated the ability to increase BBB permeability with minimal short-term, and no evidence of long-term side-effects in human participants. Ongoing trials are focused on determining the safety of using FUS+MB treatments in a variety of pathological contexts, including glioblastoma, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Demonstrating a high safety profile in these studies will enable future work to explore the use of FUS+MBs to deliver therapeutic agents and the treatment of larger volumes.

5. Conclusion

FUS+MB treatments offer a transient and minimally invasive method of increasing BBB permeability, allowing therapeutic agents to be delivered to targeted locations in the brain by systemic administration. For the past two decades, preclinical research using FUS+MBs has demonstrated successful delivery of a variety of therapeutics, along with efficacious outcomes in a number of preclinical models. Continuing efforts are being directed at developing methods to improve the safety profile of FUS+MB treatments, the most important of which being acoustic feedback control. Currently, the safety of using FUS +MBs in the context of various pathologies, from focal brain tumours to widespread neurodegenerative diseases, is being evaluated in phase 1 clinical trials. The field continues to evaluate how the brain responds to a variety of FUS+MB exposure conditions, however, there remain several avenues of investigation that are underexplored. In the next section, *Expert Opinion*, we offer our outlook on research topics that require greater focus, with the goal of maximizing patient safety and treatment success in clinical trials.

6. Expert opinion

The BBB is recognized by many as the single most important factor limiting drug delivery to the brain [1]. The administration of FUS, in conjunction with circulating MBs, presents a tuneable and reliable tool for minimally invasive therapeutic agent delivery to targeted locations in the brain. It is our opinion that the continued characterization of biological responses following FUS+MB-mediated increases in BBB permeability, with and without drug delivery, along with technological advancements, will lead to widespread clinical implementation and have substantial impact on treatment strategies for several neuropathologies.

From the perspective of assessing treatment safety, further work is needed to fully characterise the relationship between exposure conditions and FUS+MB-induced inflammation, as well as the impact of this response on short- and long-term tissue health. Given the prevalence of chronic inflammation in a variety of neuropathologies for which FUS+MB-based treatment strategies may be employed, it is also important for work to be directed at exploring how FUS+MB-induced acute inflammatory processes interact with existing inflammation. Other areas of safety characterization that require increased attention include: (1) thoroughly evaluating the degree to which target volume impacts the associated risks of FUS+MB treatment, (2) characterizing the accumulated detriment associated with treatment frequency and number (with and without therapeutic agent delivery), and (3)

analysing dose response and toxicity associated with systemically delivering therapeutic agents while BBB permeability is elevated above baseline. As iterated above, it is essential that continuing work aimed at characterizing the safety profile of FUS+MBs is conducted with parameters that reflect current best practices, including the use of validated acoustic feedback control methods.

Given the range of exposure conditions that will produce some degree of increased BBB permeability, it is unsurprising that a range of biological responses have been reported following FUS+MB treatments. There is a need for strategies that enable direct comparison between treatments. This should consist of both quantitative analysis of BBB permeability at specific time points following sonication, as well as analysis of acoustic emissions. In regards to assessing BBB permeability, dynamic contrast enhanced (DCE) MRI presents a means of more precisely quantifying the diffusion of a contrast agent from systemic circulation into the brain compared to signal intensity changes measured in CE-T1w images. To aid in comparing results across studies, it would be necessary to have consensus on both the type of data collected, as well as the methods of collection. For DCE-MRI, this would include standardizing parameters such as type and dose of contrast agent, method of calculating arterial input function, time points of data collection following sonication, and pharmacokinetic model employed. The use of standardized methods would improve both the precision and accuracy of the data collected, and enable a more direct comparison across studies. From this approach, the field would gain a more comprehensive understanding of how the initial impact on BBB permeability influences downstream biological responses.

Ultimately it would be optimal to quantify not only the increases in BBB permeability to contrast agents, but to the drug being delivered. For this purpose, therapeutic agents labelled with molecules that can be non-invasively imaged is one possible approach. For example, chemotherapeutics could be labeled with a positron emission tomography agent, then imaged to quantify the amount and distribution of the therapeutic agent in the tumor.

From the perspective of technological advancement, acoustic monitoring strategies continue to progress, providing more detailed information that can be used to control MB-mediated FUS treatments. For example, the incorporation of multi-element receiver arrays within FUS brain systems has enabled three-dimensional (3D) spatial localization of MB activity during sonication [104,142–144]. The information provided from 3D acoustic imaging should enable more consistent effects on BBB permeability over large volumes, controlling for spatial variance in vasculature and MB concentration. Additionally, 3D localization of MB activity enables the detection of threshold events outside of the focal volume and subsequent treatment adjustment, reducing the risk of off-target bioeffects. Controlling FUS+MB treatments with four-dimensional reconstruction of acoustic emissions will further increase the safety profile of this technique and should provide a greater ability to predict biological impacts of treatment.

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Article highlights:

- Focused ultrasound and microbubble treatment is a minimally invasive method to transiently increase blood-brain barrier permeability in targeted locations.
- For hours after sonication, therapeutic agents can be administered systemically and extravasate in targeted brain areas.
- The magnitude of increased blood-brain permeability following focused ultrasound and microbubble treatment ranges greatly between studies - largely due to differences in exposure conditions - leading to the observation of a range of biological responses following sonication.
- With appropriately selected parameters, blood-brain barrier permeability can be transiently increased without evidence of behavioral deficits, structural damage, or lasting histopathological changes; however, substantial, long-lasting damage can be induced with parameters that are suboptimal.
- Real-time acoustic feedback control has greatly increased the safety profile of focused ultrasound and microbubble treatments, and continues to develop.
- Phase 1 clinical trials are underway at several institutes, with positive early results.

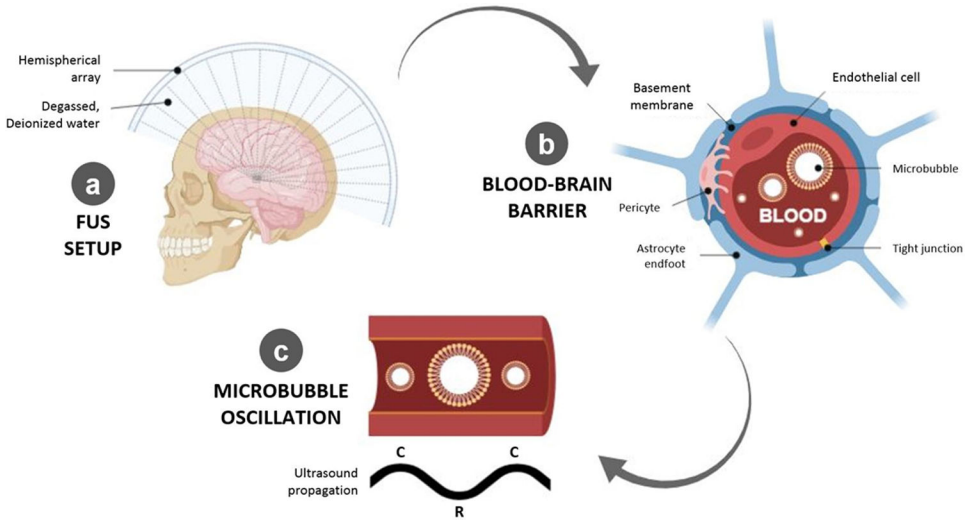

Figure 1. Overview of FUS+MBs treatment.

(a) Transcranial human clinical trials employ a hemispherical dome, containing a phased array of transducers, coupled to a water bath surrounding the shaved head. The skull is intact. (b) The blood-brain barrier consists of endothelial cells, their tight junctions, basement membrane, and other participating glial cells, such as pericytes and astrocytes. Prior to FUS exposure, MBs are intravenously injected into the systemic circulation. (c) During FUS exposure, microbubbles expand and contract with the cycles of rarefaction (R) and compression (C) of the ultrasound wave, generating forces in the surrounding blood and against vascular walls.

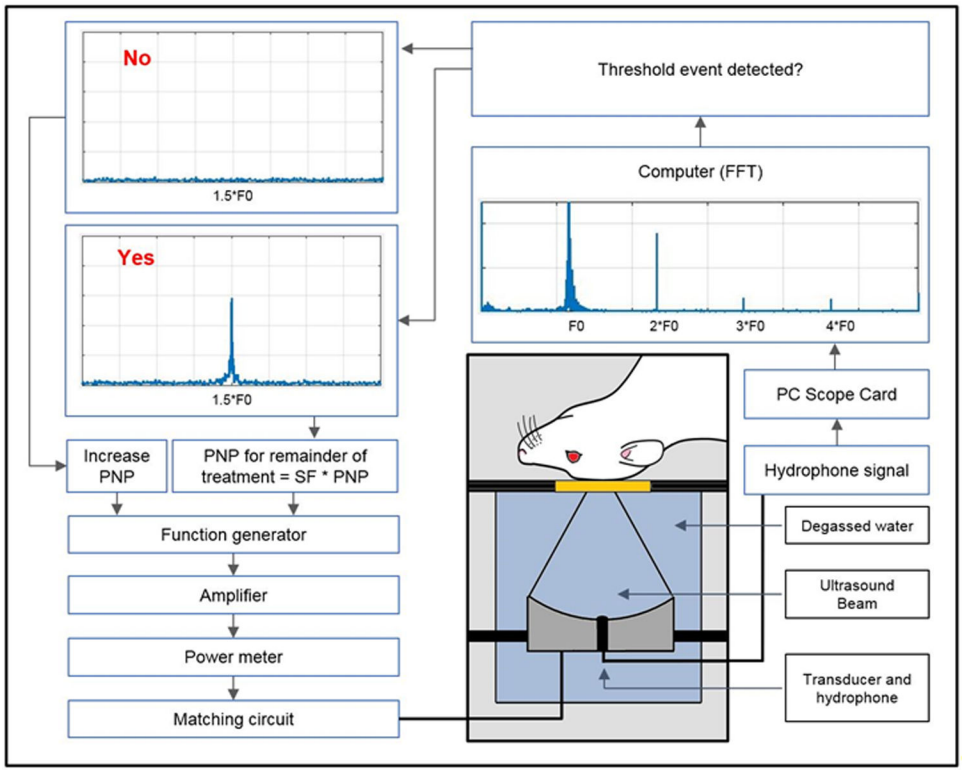

Figure 2. Pre-clinical FUS set-up with acoustic controller.

A rodent is positioned supine on a sled with an acoustically transparent membrane. Hair on top of the skull shaved and coupled to a water bath containing the transducer. The computer initiates a signal being sent to the transducer through the function generator, amplifier, power meter, and matching circuit. The hydrophone receives acoustic signals and the computer processes the spectral data. If the FFT signal at the first ultraharmonic is indicative of a threshold event (eg. ultraharmonic and subharmonic emissions), then the pressure is reduced by a set scaling factor (SF; e.g. 50% of the PNP that induced the threshold event). If the amplitude of the signal does not meet threshold, then the pressure is increased by a predetermined step size.

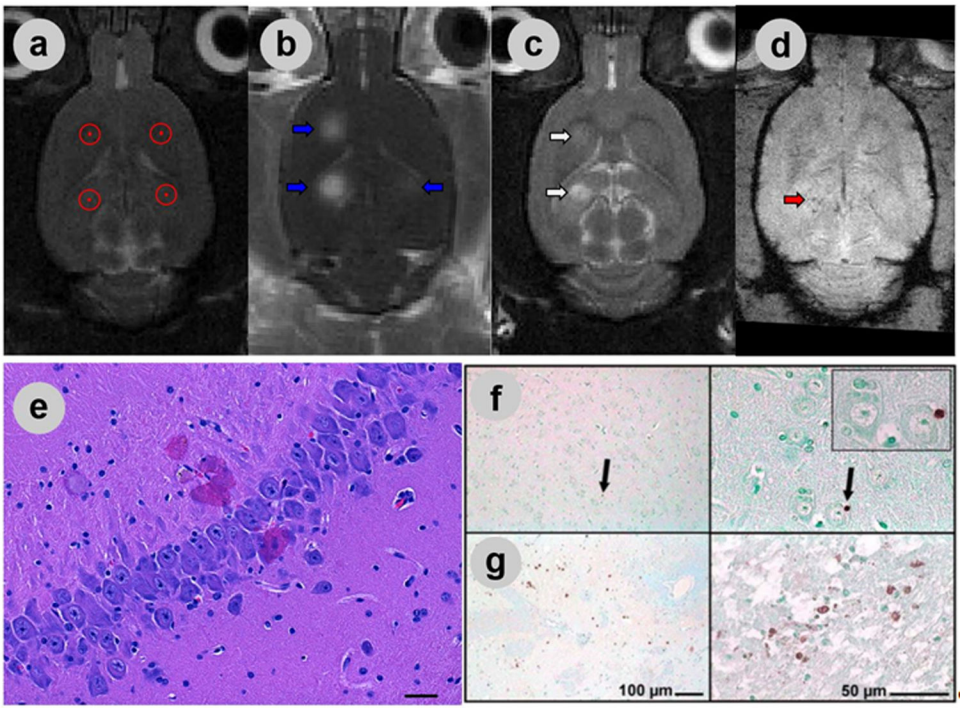

Figure 3. Range of MRI and histological findings investigating safety profile of various FUS+MB parameters.

(a) Pre-FUS+MBs T2w image with targets. (b) Post-FUS+MBs CE-T1w image with a range of treatment effects (15 min following sonication). (c) Post-FUS+MBs T2w image showing edema in two locations (4 hrs following sonication). (d) Post-FUS+MBs T2*w image showing hypointensities at one target (4 hrs following sonication). (e) H & E staining (20x objective) showing regions of RBC extravasation in porcine brain one day post-FUS+MBs (scale bar = 15 μm). (f-g) TUNEL staining. Brain sections after exposure to (f) 1.4 MPa (0.69 MHz), resulting in potentially one apoptotic cell, and (g) 3.1 MPa, resulting in a region of dark apoptotic cells. (a-d) Modified from McMahon and Hynynen 2017 [64]. (e) Reproduced with permission from Jones *et al.* 2017 [145]. (f-g) Reproduced with permission from Hynynen *et al.* 2005 [146].