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Multiple sessions of liposomal doxorubicin delivery via focused ultrasound mediated blood-brain barrier disruption: a safety study

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Abstract

Transcranial MRI-guided focused ultrasound is a rapidly advancing method for delivering therapeutic and imaging agents to the brain. It has the ability to facilitate the passage of therapeutics from the vasculature to the brain parenchyma, which is normally protected by the blood-brain barrier (BBB). The method's main advantages are that it is both targeted and noninvasive, and that it can be easily repeated. Studies have shown that liposomal doxorubicin (Lipo-DOX), a chemotherapy agent with promise for tumors in the central nervous system, can be delivered into the brain across BBB. However, prior studies have suggested that doxorubicin can be significantly neurotoxic, even at small concentrations. Here, we studied whether multiple sessions of Lipo-DOX administered after FUS-induced BBB disruption (FUS-BBBD) induces severe adverse events in the normal brain tissues. First, we used fluorometry to measure the doxorubicin concentrations in the brain after FUS-BBBD to ensure that a clinically relevant doxorubicin concentration was achieved in the brain. Next, we performed three weekly sessions with FUS-BBBD $\pm$ Lipo-DOX administration. Five to twelve targets were sonicated each week, following a schedule described previously in a survival study in glioma-bearing rats (Aryal et al., 2013). Five rats received three weekly sessions where i.v. injected Lipo-DOX was combined with FUS-BBBD; an additional four rats received FUS-BBBD only. Animals were euthanized 70 days from the first session and brains were examined in histology. We found that clinically-relevant concentrations of doxorubicin ($4.8 \pm 0.5 \mu\text{g/g}$) were delivered to the brain with the sonication parameters (0.69 MHz; 0.55–0.81 MPa; 10 ms bursts; 1 Hz PRF; 60s duration), microbubble concentration (Definity, 10 $\mu\text{l/kg}$), and the administered Lipo-DOX dose (5.67 mg/kg) used. The resulting concentration of Lipo-DOX was reduced by 32% when it was injected 10 minutes after the last sonication compared to cases where the agent was delivered before sonication. In histology, the severe neurotoxicity observed in some previous studies with doxorubicin by other investigators was not observed here. However, four of the five rats who received FUS-BBBD and Lipo-DOX had regions (dimensions: 0.5–2 mm) at the focal targets with evidence of minor prior damage, either a small scar (n=4) and a small cyst (n=1). The focal targets were unaffected in rats

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who received FUS-BBBD alone. The result indicates that while delivery of Lipo-DOX to the rat brain might result in minor damage, the severe neurotoxicity seen in earlier works does not appear to occur with delivery via FUS-BBB disruption. The damage may be related to capillary damage produced by inertial cavitation, which might have resulted in excessive doxorubicin concentrations in some areas.

Introduction

The blood-brain barrier (BBB) is one of the most challenging factors for effective diagnosis and treatment of brain diseases. It prevents the extravasation of most circulating therapeutics and imaging agents into the brain because of its selective permeability to only a small subset of molecules that have the correct size, charge and lipid solubility [1,2]. Invasive approaches such as direct injection, infusion, and implanted biocompatible devices have been used to achieve local high drug concentration [3–6]. Others have had promising results with biopharmaceutical approaches such as the modification of drugs to cross the barrier through endogenous transport mechanisms [7–9]. However, all current methods are either invasive, non-targeted, or require the expense of developing new drugs. A drug targeting technology that could noninvasively achieve controlled delivery of therapeutics across the BBB would be highly beneficial.

Over a decade ago, Hynynen et al. [10] discovered that the BBB can be temporarily disrupted with low-intensity bursts of focused ultrasound combined with circulating microbubbles. This method has several potential advantages over other approaches tested to overcome the BBB [11]. It is a noninvasive procedure, and effect can be localized to only desired volumes in the brain. Since that work was published, the method has been investigated in numerous animal studies as a noninvasive targeted drug delivery method [12]. These studies have demonstrated the delivery of a wide range of imaging and therapeutic agents including large agents such as antibodies, nanoparticles, and liposomally-encapsulated drugs [13–16]. They have also demonstrated that the BBB can be consistently disrupted without apparent neuronal damage [10,17–22]. The circulating microbubbles appear to concentrate the ultrasound effects to the blood vessel walls, causing BBB disruption through widening of tight junctions and activation of transcellular mechanisms, with little apparent effect on the surrounding parenchyma [23]. The use of injected microbubbles also makes the method more predictable than prior studies that used ultrasound alone [24–26] and reduces the acoustic power needed for BBB disruption by orders of magnitude, making FUS-BBBD substantially easier to apply through the intact skull without overheating the bone.

One area that will likely benefit the most from transient BBB disruption is the use of chemotherapy for the treatment of brain tumors. BBB disruption in conjunction with chemotherapy has been investigated intensively for several decades using intra-arterial injection of hyperosmotic solutions such as mannitol. This procedure causes shrinkage of endothelial cells and consequent stretching of tight junctions [27] through which drugs may pass. The method has been tested clinically with promising results [28–33]. The use of focused ultrasound to disrupt the BBB has the potential to replicate these findings without

requiring an invasive procedure and at the same time targeting the chemotherapy delivery to only desired regions.

The chemotherapy agent doxorubicin (molecular weight: 580 Da) has been shown to be effective against glioma cells in vitro [34], but not in patients [35]. The poor clinical outcomes were presumably the result of the BBB and other challenges inherent in tumor drug delivery [11,36]. This agent is often used in a liposomal formulation, which reduces cardiotoxicity and other side effects but also makes drug delivery even more challenging due to the large size (~100 nm) after encapsulation. Several studies have shown that FUS-BBBD can enable the delivery of doxorubicin, either alone or encapsulated in a liposome, across the BBB and enhance its delivery across “blood-tumor barrier” [37–40]. Other works have demonstrated improvements in survival and decreased tumor growth in animal tumor models [38,41]. Recently, we investigated three weekly sessions of FUS-BBBD to enhance the delivery of liposomal doxorubicin (Lipo-DOX) to a rat glioma model and to enable its delivery across the BBB in the surrounding brain [42]. A pronounced improvement was observed: the median survival time was increased by 100% and 72% compared to controls and animals who received only Lipo-DOX, respectively; approximately 75% of the tumors appeared almost completely resolved. However, some adverse events were observed, including tissue loss at the tumor site, damage (infarct) in neighboring tissue, and intratumoral hemorrhage in one animal. We could not determine whether these effects were due to the sonications, the chemotherapy, or the tumors themselves, which in some cases reached a substantial volume before beginning to resolve.

In considering clinical translation, it will be critical to understand if these side effects were due to doxorubicin neurotoxicity. An effective drug treatment for an invasive brain tumor such as glioma will require chemotherapy delivery to the normal tissues at the tumor margin, where the BBB protects infiltrating tumor cells, in addition to the semipermeable solid tumor. In a patient with a glioma, this infiltrative margin can extend several centimeters [43]. While an earlier study of FUS-BBBD and Lipo-DOX found that a single drug delivery session did not result in the normal brain tissue damage in rats [44], it is possible that multiple treatments could result in the side effects observed in our prior tumor study [42]. Furthermore, early studies with mannitol BBB disruption and free doxorubicin suggested that this drug is significantly neurotoxic, even at small concentrations [45,46]. Others have observed concentration-dependent neurotoxicity when free doxorubicin or Lipo-DOX was infused into the brain via convection-enhanced delivery [47].

For these reasons, we tested whether multiple sessions of Lipo-DOX administration and FUS-mediated BBB disruption (FUS-BBBD) can induce severe adverse events in the normal brain tissue. The present study had two objectives. First, we aimed to confirm that the sonication parameters used in our prior study with FUS-BBBD and Lipo-DOX [42] can deliver clinically-relevant concentrations of doxorubicin and to test whether injecting the agent before, during, or after the sonications influences the resulting drug concentrations. Next, sonicating multiple targets in the normal brain over three weeks, we evaluated whether multiple sessions of FUS-BBBD and Lipo-DOX produced significant brain tissue damage. For these experiments, we aimed to recreate the sonications used in our earlier tumor study. We sonicated multiple overlapping brain targets to induce BBB disruption in regions that

increased in volume over the three weeks. The tissue effects were compared in histology to animals who received FUS-BBBD or Lipo- DOX alone.

Materials and Methods

Sonication System

An air-backed, single element, 690 kHz focused piezoelectric transducer (diameter/radius of curvature: 100/80 mm) generated the ultrasound field. It was driven by an arbitrary waveform generator (model 395, Wavetek) and RF amplifier (240L, ENI); electric power was measured with a power meter (E4419B, Agilent,) and dual-directional coupler (C5948-10, Werlatone). Reported exposure levels are absolute peak negative pressure amplitudes measured in water with a membrane hydrophone (Marconi; 0.5 mm diameter). Attenuation by the brain and rat skull is expected to reduce the pressure amplitude by ~30% at this frequency [48] with additional uncertainty arising from standing waves within the skull and increases in skull thickness as the animal ages [48]. The pressure distribution of the transducer was mapped using a 0.2 mm needle hydrophone (Onda, Sunnyvale, CA); its half-maximum diameter and length were 2.3 and 12 mm, respectively. The transducer efficiency was measured using a radiation force-balance.

Acoustic parameters were the same as in our previous study [42]. The sonications consisted of 10 ms bursts applied at a frequency 1 Hz for 60s at a pressure amplitude of 0.55 MPa. This pressure amplitude was initially set based on a prior study in rats with this device [17] and was increased on the basis of the animal age and weight to achieve a consistent level of BBB disruption. This observation was made in our initial treatments and is similar to previous reports [48]. Each sonication was combined with an intravenous injection of a microbubble-based ultrasound contrast agent (Definity; Lantheus) administered at the dose recommended for human ultrasound imaging (10 μ l/kg). Each milliliter of Definity contains 1.2×10^{10} microbubbles that consist of perfluorocarbon gas-filled lipid shells with a mean diameter of 1.1–3.3 μ m. To facilitate the injections of such a small volume, the agent was diluted in PBS to 0.1 times its normal concentration. It was injected as a bolus approximately 9 s before each sonication, followed by a 0.2 ml saline flush.

Experimental Setup

The sonication system was operated within a clinical 3T MRI scanner (Signa; GE Healthcare). The transducer was immersed in a small tank of degassed, deionized water and attached to an MRI-compatible, manually-operated positioning system (Figure 1). The animal was laid supine on a tray above this tank, with a water bag providing an acoustic path to the dorsal surface of the head. Images were obtained with a 7.5 cm-diameter transmit/receive MRI surface coil. The animal's body temperature was maintained with a heated water pad. Before the rat experiments, we visualized heating in a silicone phantom using temperature-sensitive MRI to localize the acoustic focal point in the MRI space. Accurate targeting in vivo was confirmed before the sonications in select animals (typically the first animal sonicated each week) by verifying that the resulting MRI contrast extravasation appeared at the desired target after one sonication.

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committees of Harvard Medical School. Tests were performed in 18 Male Sprague-Dawley rats (Charles River Laboratories; ~250 g) (Table 1). Before each procedure, the animals were anesthetized via intraperitoneal injections of ketamine (80 ml/kg/h) and xylazine (10 ml/kg/h). A catheter was placed in the tail vein, and the hair on the scalp was removed with clippers and depilatory cream. The body temperature was maintained throughout the experiment with a heated water pad. To avoid skin infections that were observed in animals who received Lipo- DOX in a previous study [42], animals receiving three weekly sessions with FUS-BBBD and chemotherapy were treated prophylactically with an antibiotic (Baytril®, Bayer; 2.5mg/kg).

Optimization of Lipo-DOX delivery

Nine rats were used to verify that clinically-relevant doxorubicin concentrations could be achieved in the brain with FUS-BBBD and the administration of Lipo-DOX. We aimed to achieve a doxorubicin concentration in the brain of at least 0.819 µg/g, which was reported to correlate with a clinical response rate in human tumors [49]. After determining the coordinates of the focal point within the MRI space, treatment planning MRI was acquired, and the focal region was positioned at 2 mm lateral to the midline and 4 mm deep from the dorsal brain surface. The sonicated volumes were centered 2.5 mm anterior to the bregma in the striatum. The striatum was of interest to us because that was the location of the implanted 9L-rat glioma tumors in our previous study [42]. Sonications were performed in a 3×3 grid pattern with 1–1.5 mm spacing. Doxorubicin hydrochloride encapsulated in long-circulating pegylated liposomes (Avanti Polar Lipids, INC), was administered intravenously at a total dosage of 5.67 mg/kg. That dosage was selected on the basis of prior work testing this agent in rats [50].

Lipo-DOX was injected either before (n=5; group A) or 10 min after (n=4; group B) the sonications. In group A, both hemispheres were sonicated. In the right hemisphere, Lipo-DOX was administered in 9 fractions, one before each sonication, mimicking the scheme from the previous rat tumor studies [42,44]. Since Lipo-DOX has a long plasma half-life (2–3 days) [51], we assumed the entire dose was still present in the circulation, and we sonicated the left hemisphere without administering more drug. In group B, the sonications were performed only in one hemisphere and the total dose of Lipo-DOX was injected as a bolus 10 min later. The contralateral hemisphere served as a control. The sonication time was approximately 30 min for the 9 targets. Trypan blue (ICN Biomedical, Aurora, OH; 80 mg/kg) was administered to the animals in Group A and group B through the tail vein after the completion of the sonications and MR imaging. This dye was administered to confirm successful BBB disruption and to mark the sonication targets *post mortem* for tissue harvesting. Four hours after sonication, the rats were sacrificed, samples of sonicated and control volumes (~30 mg) were collected from the brain, and doxorubicin concentrations were measured with a fluorometer.

Extraction and quantification of doxorubicin

Four hours after the last sonication, each animal was put into a state of deep anesthesia with an overdose of ketamine and xylazine. To flush unabsorbed Lipo-DOX from the cerebral vasculature, the brain was perfused by transcardiac method with normal saline. The brain was cut into 2–3 mm slabs using a brain matrix (ASI-instruments; Warren, MI). Right and left regions of BBB disruption, identifiable by the trypan blue staining, were harvested along with non-sonicated control regions. The doxorubicin concentrations were quantified by linear regression and a standard curve derived from eight serial concentrations. The concentration of doxorubicin in each tissue sample was determined by taking the average of at least three fluorometric readings on a benchtop fluorometer (VersaFluor; Bio-Red Laboratories, Hercules, CA). Measurements were normalized to those obtained from a control animal that did not receive doxorubicin to correct for autofluorescence.

A preparation of acidified alcohol (0.3 N HCl in 50% EtOH) was used to extract doxorubicin from the harvested tissue samples for fluorometric quantification. We assumed a tissue density of 1 g/cm³, so the mass of each sample was assumed to be equal to its volume. Each sample was measured and cut down until its mass was approximately 0.03 g. Each sample was put into a 1.5-mL centrifuge tube with 20 volumes (~600 µL) of acidified alcohol, then lysed with a tissue homogenizer (Bullet Blender; Next Advance, Averill Park, NY) and refrigerate at 4°C for 24 h. Samples were then centrifuged at 4,000 × g for 20 min at 4 deg C. The supernatant was extracted for immediate fluorescence intensity measurement (Excitation/Emission wavelengths: 480/590 nm).

Three weekly sessions

Nine rats were assigned to one of two groups: (1) three weekly sessions with FUS-BBBD and concurrent chemotherapy (FUS+DOX) (N=5), (2) three weekly sessions with FUS-BBBD only (FUS-Only) (N = 4). The sonication parameters and brain regions selected for FUS-BBBD were the same as described above. Sonications were performed in a grid pattern at 5, 9, and 12 targets, respectively, which mimicked the three weekly treatments we administered in our previous study in tumor-bearing rats [42]. The spacing between sonications was 1–1.5 mm. Lipo-DOX was administered intravenously in equal fractions over multiple slow injections just before each sonication. The total Lipo-DOX dose was 5.67 mg/kg in each FUS-BBBD session.

Histology

All of the animals from the FUS-Only and FUS+DOX groups were sacrificed 70 days after the first session to evaluate the histological effects. The animals were deeply anesthetized with ketamine/xylazine, sacrificed, and the brains fixed via transcardial perfusion (0.9% NaCl, 100 mL; 10% buffered formalin phosphate, 250 mL). The brains were then removed, embedded in paraffin, and serially sectioned at 5 µm in the axial plane (perpendicular to the direction of ultrasound beam propagation). Every 50th section (250 µm apart) was stained with hematoxylin and eosin (H&E). In each animal, the sections with the greatest tissue effects were identified and examined in details under high magnification; the dimensions of any damaged areas were measured.

Magnetic resonance imaging

The experiments were performed under MRI guidance. T2-weighted imaging was used to plan the treatments. BBB disruption was evaluated using T1-weighted imaging acquired before and after administration of the MRI contrast agent, gadopentetate dimeglumine (Magnevist Gd-DTPA; Bayer Healthcare; 0.25 mL/kg). T2*-weighted imaging was used to examine whether petechiae, which are produced by excessive FUS exposures [10], did or did not occur. Detailed imaging parameters are listed in Table 2. For the experiments that examined three weekly sessions of FUS-BBBD and Lipo-DOX, the health of the nine animals was monitored regularly, and MRI was acquired 53 (n=1) or 67 (n=8) days after the first session to evaluate the treatment effects.

Statistical Analysis

The mean doxorubicin concentration in the brain and the standard deviation were calculated for the non-sonicated hemispheres and for the cases where the Lipo-DOX was administered before, during, and 10 minutes after the sonications. The concentrations among the different experimental groups were compared using two-tailed paired student's t-tests. Values of $p < 0.05$ were considered statistically significant.

Results

BBB permeabilization

BBB permeabilization and the presence or lack of petechiae were confirmed using contrast-enhanced and T2*-weighted MRI, respectively (Figure 2). A higher pressure amplitude (0.81 vs. 0.55 MPa) was needed for the third session to induce consistent BBB permeabilization, presumably because of an increase in skull or dura thickness as the rats grew [48]. All animals demonstrated hyperintense regions in contrast enhanced T1-weighted images after each session, confirming BBB permeabilization. In several cases, one or more hypointense spots were observed in T2*-weighted MRI in the focal plane or at the brain surface in the cortex, demonstrating the presence of petechiae. Such spots were observed in both hemispheres near the brain surface in 4 of the 5 animals in the animals where doxorubicin concentrations were measured and that received Lipo-DOX before or during after sonication. They were not observed in animals who received Lipo-DOX after sonication. In the animals where histological effects were examined, such spots were observed after the second or third session in 4 of the 5 animals who received both FUS-BBBD and Lipo-DOX and in 3 of the 4 animals who received FUS-BBBD alone. They were not seen in MRI at day 53 or 67. In addition to these hypointense spots in the focal plane, hypointense spots were observed in the cortex at the brain surface in every animal except one who received FUS-BBBD alone. In addition to the examples shown Fig. 2, additional MRI findings are shown below along with histology.

Doxorubicin concentrations

The doxorubicin concentration in the brain tissue was compared between the experimental groups in which Lipo-DOX was injected before, during, or after the sonications (Figure 3). In group A, both hemispheres were sonicated in each animal. In the right hemisphere, Lipo-

DOX was administered in 9 fractions, one fraction before each sonication. In the left hemisphere, the sonications were performed without additional Lipo-DOX administration. In group B, the sonications were performed only in one hemisphere, and the total dose of Lipo-DOX was administered in a single, slow injection 10 minutes later. The contralateral hemisphere in these animals served as controls. The total sonication time for 9 targets was approximately 30 min.

In group A, the mean doxorubicin concentrations in the right and left hemispheres were 4.7 ± 0.5 and 4.8 ± 0.5 $\mu\text{g/g}$, respectively; they were not significantly different ($p > 0.05$). In group B, the doxorubicin concentration was significantly less ($p < 0.001$) than in both hemispheres in group A. The mean concentration in these animals was 3.2 ± 0.3 $\mu\text{g/g}$, a 38% less compared to the volumes in group A. The doxorubicin concentrations were significantly enhanced in all cases ($p < 0.0001$) compared to the control tissue. In addition, in every case the doxorubicin concentrations in the sonicated areas exceeded our goal of 0.819 ± 0.482 $\mu\text{g/g}$, a value reported to correlate with a clinical response for human tumors [49].

Histological findings

All animals that received FUS-BBBD alone were found to be unaffected in the targeted plane in the striatum (Figure 4). However, four of the five rats who received FUS-BBBD and Lipo-DOX had small regions with evidence of prior damage in the focal plane (Figure 5). In three of these animals, this was evidenced by the presence of small scars with largest dimensions of 0.5–2.0 mm. These scars consisted of infiltrating macrophages and activated astroglial cells. In the fourth animal, a small (1.0×2.0 mm) cyst formation was observed. No changes were observed in the control hemisphere that received Lipo-DOX only (black boxes in Figure 5).

Evidence of more severe prior damage was observed in the cortex near the brain surface in four of the five animals who received FUS-BBBD and Lipo-DOX and in one of the four animals who received FUS-BBBD alone. The effects were similar in appearance to those in the striatum, but the dimensions of the affected areas, which ranged from 0.5–2.5 mm, were larger. Examples of these effects are shown in Figure 6.

Discussion

Doxorubicin is commonly used for the treatment of a wide range of cancers excluding those in the brain. It belongs to the family of anthracycline antibiotics and was formerly known as adriamycin. While its effectiveness against glioma has been shown in vitro [34] and in vivo when injected directly into the tumor [52,53], systemic administration has not been effective clinically [35], presumably reflecting insufficient delivery [54]. A number of drug formulations have been developed to enhance the delivery of doxorubicin in animal glioma models [55–59] and at least one clinical trial is ongoing with such an agent [60,61]. Here, we demonstrated that focused ultrasound and microbubbles can enhance the delivery of Lipo-DOX to the rat brain parenchyma, removing the need to develop and test new drug formulations. The method is noninvasive and restricts the drug delivery only to desired brain regions.

While a high doxorubicin concentration was achieved in each group, higher concentrations were achieved when we injected Lipo-DOX before sonication (group A) rather than afterwards (group B). This finding may have been due to partial BBB restoration in group B. With the acoustic parameters used in this study, Park et al. reported an exponential decay in permeability to Gd-DTPA (molecular weight: 938 Da) after FUS-BBBD with half-life of 2.2 hours [62]. With this time, the barrier permeability would be reduced by approximately 20% for the first target that was sonicated 40 min before Lipo-DOX administration. Others have shown that this restoration is faster for larger agents and suggested that an agent the size of Lipo-DOX (100 nm) would have a half-life of only a few minutes [63].

This finding might also reflect transient effects occurring during sonication that actively transported Lipo-DOX out of the blood vessels. A number of studies on “sonoporation” have shown that sonication with microbubbles can drive drugs across cell membranes through the creation of pores that are present for a short time [64–69]. It may also be possible that the microbubble-enhanced sonications lysed the ~100 nm liposomes, releasing free doxorubicin which was readily transported through the BBB disruption. Others have shown that microbubble collapse can cause such a release when the liposomes are attached to the microbubbles [70] or in lesser amounts when co-injected with a microbubble agent [71]. However, it is challenging to relate such results to the in vivo environment, where the microbubbles are circulating in tiny capillaries that cannot be reproduced in vitro. Additional experiments would be necessary to determine whether the exposure levels used here could release doxorubicin, perhaps using in vivo microscopy [72]. It would also be interesting to repeat these experiments using a smaller drug or a drug with a short plasma half-life. The dependence on the order of the sonication and the drug administration on the delivered drug concentrations may be different in such cases.

We do not know whether the doxorubicin detected in the brain samples was still encapsulated or how far from the blood vessels it penetrated into the brain parenchyma. Doxorubicin is a substrate for the efflux pump P-glycoprotein, which is present on normal blood vessels in the brain and in some cancer cells [73]. One might expect P-glycoprotein to rapidly pump doxorubicin out of the brain parenchyma after its release from the liposomes. However, given that earlier work with free doxorubicin found significant drug concentrations in the brain 16 hours after delivery via FUS-BBBD [62], we do not think that necessarily is the case. Future work is needed to evaluate the dynamics of the liposome delivery and drug release after delivery across the BBB. For example, it would be interesting to fluorescently tag Lipo-DOX and examine in microscopy the penetration of intact liposomes released doxorubicin into the brain after FUS-BBBD.

Regardless of the order of the sonication, it is clear that the sonication parameters used here effectively resulted in a clinically-relevant doxorubicin concentration in the normal brain. However, the severe neurotoxicity that was observed in earlier studies was not observed here. Early work on the toxicity of intracarotid administration of free doxorubicin in rats and dogs following osmotic BBB modification revealed that this agent can be significantly neurotoxic, even at small concentrations [46,74]. In that study, the animals developed neurological deficits including seizures, with corresponding necrosis and hemorrhagic infarcts observed in histopathology. Other experiments using convection-enhanced delivery

have also observed extensive necrosis when high concentrations of doxorubicin or Lipo-DOX were infused into the brain [47]. These studies, along with effects we observed previously at the tumor margins study [42], prompted the current study to investigate whether FUS-BBBD and Lipo-DOX can induce such extensive damage. Such effects were not observed.

Evidence for small regions of minor prior tissue damage, however, was detected in this work. We suspect these regions were due capillary damage induced during the sonications. The exposure levels used were the same as in our prior study in tumors and were somewhat aggressive, as evidenced by the presence of hypointense spots in T2*-weighted imaging in most animals. Prior work suggests that these dark spots are caused by the presence of iron-laden particles from petechiae [75] presumably caused by capillary damage caused by inertial cavitation [76]. Based on other studies examining long-term effects of FUS-BBBD, such minor capillary damage is not expected to result in significant long-term effects [77,78], and indeed this was the case here for the animals who did not receive Lipo-DOX. In contrast, evidence of small areas of prior tissue damage was observed in most of the animals who received FUS-BBBD and Lipo-DOX. The cause of this difference is not known, but it is possible that excessive doxorubicin concentrations resulted around capillaries damaged by inertial cavitation. It may also be possible that doxorubicin impaired repair or otherwise exacerbated such capillary damage that could have resolved over time without the drug. Based on these results we anticipate that while sonication with low-level inertial cavitation may be acceptable – or even desirable – when the focal region is inside a solid tumor, such aggressive exposure levels may not be appropriate for delivering Lipo-DOX to the surrounding normal brain tissue.

An unexpected finding of this work was that more severe damage was evident in both MRI and histology near the brain surface in the cortex. This damage could have been the result of the low frequency and a long focal region – longer than the thickness of the rat brain – that was used in this study. This frequency was chosen to be similar to the transcranial FUS system that is currently being used in clinical trials [79] and it is known that such a frequency can result in reflections and standing waves when sonicating transcranially in a rat [80]. However, the lack of tissue damage in the far-field close to the skull base, where one might expect the pressure amplitude to be highest due to reflection, suggests that perhaps something else caused these surface effects. Perhaps the microbubble concentration was sufficiently large in the highly vascular cortex to produce shielding, resulting in a lower pressure amplitude at the focal point. While future work is needed to verify this speculation, if it were correct, it may be possible that under some circumstances elevating the exposure level of the sonications can lead to diminishing returns in FUS-BBBD at the focal plane.

Another unexpected finding was that surface damage was observed in the animals in group A in the doxorubicin concentration study, but not in group B. This finding could suggest that the presence of Lipo-DOX, which was in circulation during the sonications in group A, somehow reduced the inertial cavitation threshold. The mechanism by which this lowering could have been produced is unknown. This finding needs to be verified.

This study had some limitations. The sample size was relatively small, and more work is needed to verify the link between the capillary damage produced during some of the sonications and the small areas of downstream tissue damage that were evident here with the delivery of Lipo-DOX. We also only considered a single dose of Lipo-DOX and a single set of sonication parameters, and we examined the tissue effects only at a single time point using standard light microscopy and H&E stained sections. A larger study using more sensitive methods may find minor tissue effects that were missed here. However, the relevance of such potential minor effects is questionable in the context of a glioma treatment.

Conclusions

This work demonstrates that FUS-BBBD combined with intravenous administration of Lipo-DOX can deliver clinically-relevant concentrations of doxorubicin to the normal rat brain. Higher concentrations were achieved when we injected the agent either before or during the sonications rather than afterwards. After multiple sessions with FUS-BBBD and Lipo-DOX, the neurotoxicity that was observed in the normal brain in an earlier tumor study with FUS-BBBD and in other works evaluating other doxorubicin delivery methods was not observed here. However, evidence of small areas of prior damage was observed in the targeted area that was not observed with FUS-BBBD or Lipo-DOX administration alone. This damage may have been related to capillary damage produced during the sonications at the exposure levels used here. Overall, this work suggests that FUS-BBBD can be used to deliver Lipo-DOX to the normal brain at the tumor margin with only minor damage. This finding is encouraging for the treatment of the normal brain tissue surrounding an infiltrative tumor such as a glioma. However, due to the presence of small areas of evident prior necrosis that were observed, these results suggest that it would be prudent to use a lower pressure amplitude when sonicating the margins to avoid inertial cavitation.

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Figure 1. Schematic of MRI-guided focused ultrasound system used in this work. The function generator, amplifier, and power meter were located outside the MRI room.

Figure 2.

Axial MR-images acquired during three weekly treatments: T2-weighted imaging (T2WI) used for treatment planning; Contrast-Enhanced T1-weighted imaging (CE-T1WI) verified the BBB permeabilization; T2*-weighted imaging (T2*WI) detected tissue damage. The targeted spots are indicated in dots; the yellow curves outlined extent of MRI contrast enhancement due to BBB permeabilization. Sonications (0.69 MHz; 0.55–0.81 MPa; 10 ms bursts; 1 Hz PRF; 60s duration) were applied in a grid pattern to 5, 9, and 12 targets, respectively, mimicking the three weekly treatments which were administered in a prior tumor treatment study. The contrast enhancement in T2*-weighted imaging after the three treatments was due to injection of Gd-DTPA after. Bar: 5 mm

Figure 3.

DOX concentration corresponding to DOX injection protocol. DOX concentration was 3.2 ± 0.3 µg/g, when DOX was administered 10 minute after sonication. DOX concentration was 4.7 ± 0.5 and 4.8 ± 0.5 µg/g, when DOX was administered in fractions before each sonication and in full dose before sonications respectively; they were not significantly different ($p > 0.05$). Thus, DOX concentration was reduced by 32% when DOX was injected 10 minute after sonication ($p < 0.001$). However, DOX concentrations were significantly enhanced in all cases corresponding to control. DOX concentrations in all sonicated volumes were above the therapeutic range of 0.819 ± 0.482 µg/g tumor in vivo, which was reported to correlate with a 39% clinical response rate in patients with breast carcinoma [81]

Figure 4.

The T2*- weighted images and corresponding histological appearance of the brains treated with FUS-Only (red square in B). Untreated locations served as control (black square in B). Treated regions appeared unaffected in FUS-Only (C) and control (untreated) (D) locations. H&E; bars: 5 mm in A–B; 200 μ m in C–D

Figure 5.

The representative examples of T2*- weighted images in the focal planes acquired during three weekly treatments and at 67th day after the last treatment and corresponding histological appearance at day 70th (A–D) Treated regions appeared unaffected in FUS +DOX (red square in B) and DOX- only (black square in B) treated locations. (E–H) An example of affected brains treated by FUS+DOX: hypointense spots in T2*WI (arrows) and scar formation in H&E- stained section (G) indicated tissue damage; a small cluster of hemosiderin (inset) evidenced the earlier petechiae. No changes were observed in DOX- only treated location (H). Note, that damage of those brain tissue (scar) was evident in the area that was hypointense in T2*weighted imaging. (C–D, G–H), The magnified views of the areas shown in boxes in (B) and (F). H&E; Bars: 5 mm in A–B, E–F; 200 μ m in C–D, G–H)

Figure 6.

An example of adverse effect on the brain surface of FUS+DOX (n=4) and FUS-Only (n = 1) treated animals. Tissue necrosis (C) is evident in the area that appeared hypointense in T2*-weighted imaging (A). Hemosiderin (yellowish- brown granular pigments formed by breakdown of hemoglobin) can be seen in macrophages within the necrotic area (inset in C). No changes were found in the control (untreated) hemisphere and on the surface of the brain treated with DOX Only. These adverse effects resulted probably due to: (i) a longer focal length of the transducer (12 mm in the longitudinal plane) compared to thickness of the rat brain (10–12 mm); (ii) reflection of the ultrasonic beam from the rat's skull. H&E; Bars: 5 mm in A–B; 200 μ m in C–D)

Table 1

Summary of the different experiments

Group	Purpose	Lipo-DOX injected	N	N targets	Pressure amplitude (MPa)	Time between FUS and euthanasia
A	Doxorubicin concentration	During FUS	5	9	0.55	4 hours
B	Doxorubicin concentration	Before FUS	5	9	0.55	4 hours
C	Doxorubicin concentration	After FUS	4	9	0.55	4 hours
FUS+DOX	Safety study	During FUS	5	5, 9, 12	0.55–0.81	70 days
FUS-Only	Safety study	N/A	4	5, 9, 12	0.55–0.81	70 days

Table 2

MRI parameters used in this study

Sequence	Purpose	FOV (mm)	TR (ms)	TE (ms)	Matrix size	FA	ETL	Slice thickness (mm)	Bandwidth (±kHz)
T2-weighted fast spin echo (2D)	Anatomy	80	2000	85	256×256	90°	8	1	16
Contrast-enhanced T1-weighted fast spin echo (2D)	Confirm BBBD	80	500	17	256×256	90°	4	1	16
T2*-weighted spoiled gradient echo (3D)	Tissue damage	80	33	19	256×256	15°	1	0.7	16

(FOV: Field of view; TR: Repetition time; TE: echo time; FA: flip angle; ETL: echo train length)