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Ultrasound Enhanced Delivery of Macromolecular Agents in Brain Tumor Rat Model

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Abstract—**The purpose of this study was to evaluate the permeability of the blood-brain barrier (BBB) after focused ultrasound (FUS) exposure and to investigate if such an approach increases the tumor-to-ipsilateral brain permeability ratio. Normal rats and F98 glioma-bearing rats were injected intravenously with Evans blue (EB); these treatments took place with or without BBB disruption induced by transcranial FUS of one hemisphere of the brain. Sonication was applied at an ultrasound frequency of 1 MHz with a 5% duty cycle, and a repetition frequency of 1 Hz. The permeability of the BBB was quantitatively assessed by means of the extravasation of EB. Contrast-enhanced MR images were used to monitor the gadolinium deposition path associated with transcranial FUS and the influence of size and location was also investigated. Furthermore, whole brain histological analysis was performed. The results were compared by two-tailed unpaired *t* test. The accumulation of EB in brains and the tumor-to-ipsilateral brain permeability ratio of EB were significantly increased after FUS exposure. EB injection followed by sonication showed an increase in the tumor-to-ipsilateral brain ratio of the target tumors of about two-fold compared with the control tumors on day 8 after tumor implantation. MR images showed that FUS locally enhances the permeability of the BBB in the glioma-bearing rats. The BBB can be locally disrupted with FUS in the presence of microbubbles. This technology may offer new opportunities that will allow enhanced synergistic effects with respect to other brain tumor treatment regimens.**

I. Introduction

The blood-brain barrier (BBB) is a specialized vascular system consisting of endothelial cell tight junctions, basal lamina and glial processes [1]. Many therapeutic agents are difficult to be delivered to the brain due to BBB. Therefore, the BBB is the major factor in brain to limit drug delivery [2]. The main weakness of traditional chemotherapy is that insufficient drug enters the tumor tissue [3]. To allow selective delivery of a specific amount of drug to brain tumors without harming the normal functioning of the brain, it is necessary that the BBB should be disrupted; in such circumstances, a drug will work better and the side effects would be minimized.

Although the blood-tumor barrier (BTB) is in itself more permeable than the BBB, malignancies of the brain remain hard to treat with chemotherapy because the selective permeability of the BTB still blocks many potent agents from reaching their target [4]. Magnetic resonance imaging (MRI)-guided focused ultrasound (FUS) has been shown to locally and reversibly increase the permeability of the BBB, and it has been found that these changes to the BBB are affected by the applied pressure amplitude and the concentration of ultrasound contrast agent (UCA) [5-7]. In addition, previous studies have shown that enhanced delivery of various chemotherapeutic agents to tumors occurs after FUS and that this improves their antitumor effects [8]. Intracarotid infusion of mannitol has also been used to open the BBB in animal studies allowing increased drug delivery into the brain [9-12]. However, FUS may allows better control in terms of the selective delivery of a relatively high amount of drugs to the tumor cells, while at the same time the drug concentration in the normal tissue cells is kept low to minimize side effects on the normal tissue.

The aim of this study was to explore the performance when using FUS to enhance delivery of a relatively high amount of drugs to gliomas during tumor progression and determine if such an approach can also improve the tumor-to-ipsilateral brain ratio.

II. Methodology

A. Brain Tumor Animal Model

All animal experiments were performed according to the appropriate guidelines and approved by our Animal Care and Use Committee. Male Fischer 344 rats (9-12 weeks, about 290-340g) were anesthetized by an intraperitoneal administration of pentobarbital at a dose of 40 mg/kg of body weight. Then 1×10^5 F98 rat glioma cells in 10 μL Hanks' balanced salt solution without Mg^{2+} and Ca^{2+} were injected into the brains of the rats. The glioma cells were stereotactically injected into one location in each hemisphere of rat at a depth of 5.0 mm from the brain surface by a Hamilton syringe (26 G cannula). Next, the hole in the skull was sealed with bone wax and the wound was flushed with iodinated alcohol.

B. Ultrasound equipment

The FUS was generated by a 1.0 MHz single-element focused transducer (Panametrics, Waltham, MA, USA) which was mounted with a removable cone. The cone was

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filled with deionized and degassed water, and its tip was capped by a polyurethane membrane. The transducer with cone was fixed on a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA) that permitting submilimeter placement of the ultrasound focus at the target location in the brain. A function generator (33220A, Agilent Inc., Palo Alto, USA) was connected to a power amplifier (500-009, Advanced Surgical Systems, Tucson, AZ) to drive the FUS transducer and a power meter/sensor module (Bird 4421, Ohio, USA) was used to measure the input electrical power.

C. *Sonications*

The rat's head was mounted on the stereotaxic apparatus with the nose bar positioned 3.3 mm below the interaural line. UCA (SonoVue, Bracco International, Amsterdam, The Netherlands) was injected into the femoral vein of the rats about 15 s before each sonication. The UCA contains phospholipid-coated microbubbles with a mean diameter = 2.5 μm, and at a concentration of 1 x 10^8 to 5 x 10^8 bubbles/ml. Sonication was pulsed with a burst length of 50 ms at a 5% duty cycle and a repetition frequency of 1 Hz. The duration of the sonication was 60 s. FUS was delivered to one location in the right hemisphere brain at the location of tumor cell implantation. For all of the animal experiments, the rats were sonicated after an injection of 300 μL/kg UCA at an acoustic power of 5.72 W.

D. *Evaluation of blood-brain barrier permeability*

The integrity of the BBB was examined using Evans Blue extravasation. Evans Blue (EB) (Sigma, St. Louis, MO) (100mg/kg) was injected intravenously about 5 minutes before FUS exposure. The animals were sacrificed approximately 4 hours after the EB injection. Rats were perfused with saline through the left ventricle until colorless perfusion fluid was obtained from the right atrium. After perfusion and brain removal, the hemispheres of the brain were dissected into tumor tissue and normal brain tissue before measuring the amount of EB extravasation. The left unsonicated brains acted as the controls. Samples were weighed and then soaked in 50% trichloroacetic acid solution. After homogenization and centrifugation, the extracted dye was diluted with ethanol (1:3), and the amount of dye present measured using a spectrophotometer (PowerWave 340, BioTek, USA) at 620 nm. The EB present in the tissue samples was quantified using a linear regression standard curve derived from seven concentrations of the dye; the amount of dye was denoted in absorbance per gram of tissue. Results are typically expressed as means ± SEM. Any differences in EB concentration were analyzed by *t* test. Statistical significance was defined as a p value ≤ 0.05.

E. *MR imaging*

MR imaging of the glioma-bearing rats was performed using a 3T MRI system (TRIO 3-T MRI, Siemens MAGNETOM, Germany). A loop coil (Loop Flex Coil, approximately 4 cm in diameter) for RF reception was used. Each rat was injected intravenously with 1 mmol/kg of gadolinium (Gd-DTPA, Omniscan, GE Healthcare, Cork, Ireland) immediately after sonication. The rats were anesthetized with 1.5 % isoflurane mixed with O_2, and maintained on 1% isoflurane during the imaging procedure. A multi-slice spin echo sequence was performed to obtain 20 slices of the T1-weighted MR image; this covered the whole brain in order to depict the BBB disruption (repetition time/echo time = 500/13 ms; matrix = 243 × 512; section thickness = 1.0 mm). The imaging plane was located across the tumor at the depth of tumor center. In addition, tumor volumes were assessed from T2-weighted images by summing up the tumor area measured from each slice and multiplying by the slice thickness (0.1 cm). MRI contrast enhancement was analyzed 30 min post-gadolinium injection. The contour maps describing the spatial distribution of the contrast enhancement were quantified in a second group of experiments. For each rat, the regions of contrast enhancement above 4, 8, 12 and 16 standard deviations of the averaged spatial normal brain regions were color-coded, allowing the distinguishable features to be easily observed.

F. *Histology*

After the MRI scanning, the second group of rats was prepared for histological evaluation. The rat was perfused with saline and 10% neutral buffered formalin. The brain was removed and embedded in paraffin and then serially sectioned into 6 μm thick slices. The slices were stained with hematoxylin-eosin (H&E) in order to confirm tumor progression. The histological evaluation was carried out by light microscopy (Olympus BX61, Olympus, Shinjuku-Ku, Tokyo, Japan).

III. RESULTS

The BBB opening was observed in the focal zone with Evans Blue extravasation. Figure 1 shows the EB extravasation visible at the front (Fig. 1A) and back (Fig. 1B) of axial normal brain slices at an acoustic power of 5.72 W in the presence of UCA at 300 μL/kg.

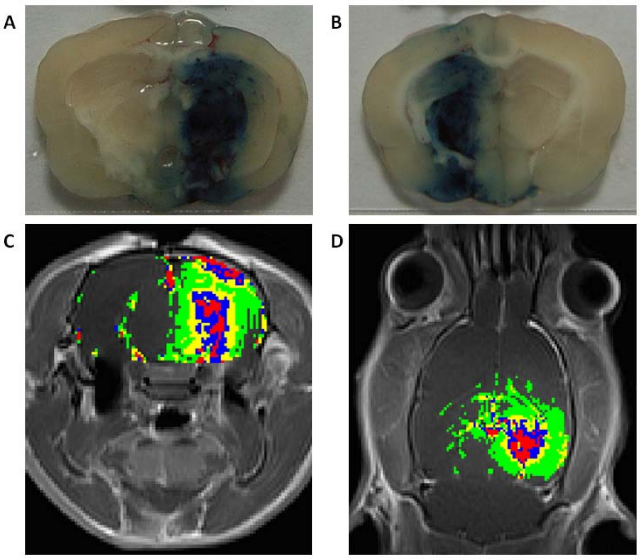

Fig. 1: Distribution of BBB disruption for the normal brain as indicated by EB extravasation on the front (A) and reverse

(B) sides of brain slices. Spatial distribution of gadolinium deposition for the axial (C) and coronal (D) views in normal brain at the sonicated site after FUS-induced BBB disruption. Regions of contrast enhancement that are >4 (green), >8 (yellow), >12 (blue) and >16 (red) standard deviations above the average MRI signal intensity of the left contralateral brain are highlighted.

The focal increase in contrast enhancement in the T1-weighted images caused by diffusion of the contrast agent into the brain was related to BBB permeability. Figures 1C and 1 D illustrate the spatial deposition of gadolinium in the sonicated site of the normal brain. In both figures, there is clearly a non-uniform distribution of gadolinium in the focal region of FUS beam.

Figure 2A illustrates the degree of EB staining in the right and left hemispheres with and without sonication on day 8 after tumor implantation. Both the size and color intensity of the EB staining increased with tumor progression and that of the sonicated right hemispheres was greater than the non-sonicated left hemispheres on day 8 following tumor implantation. Figure 2B shows the sonication pathway can be monitored using MR images in the right sonicated hemispheres. To better understand the extent of deposition of gadolinium, the contour maps of the spatial distribution of gadolinium for tumors with and without sonication are presented in Fig 2C and 2D. The contrast-enhanced regions in the right sonicated tumor were greater than in the left control tumor.

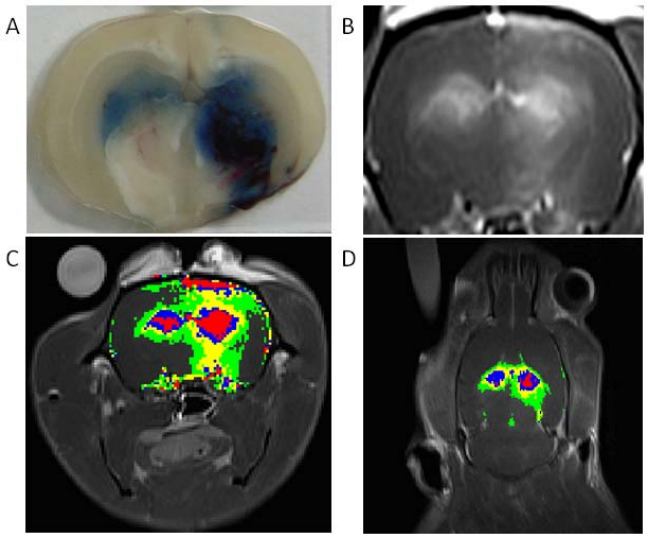

Fig. 2: Distribution of BBB disruption for brain tumors as evaluated by extravasation of EB (A) and Gd-DTPA (B) into the brain. Right brain: tumor with FUS exposure. Left brain: control tumor without FUS exposure. Magnetic resonance images of rats bearing F98 gliomas in the (C) axial view and (D) coronal view. The spatial distribution of brain tumor BBB disruption with and without sonication in the right and left hemispheres, respectively, is shown. The rat brains were analyzed 30 minutes post-gadolinium administration. Regions of contrast enhancement >4 (green), >8 (yellow), >12 (blue) and >16 (red) standard deviations above the average MRI signal intensity of the normal brain tissue.

Figure 3 shows the mean extravasation of EB per unit mass (in micrograms per gram of tissue) for the brain tumors and the neighboring normal brain tissues with or without FUS exposure. EB extravasation was quantified in each tumor-implanted hemisphere brain; both the sonicated tumor and contralateral unsonicated control tumor were examined. Not only was the permeability of the control tumor BBB significantly greater than that of the adjacent normal brain region, but it was also found that the BTB disruption was obviously greater at the tumor site after sonication than in the control tumor. FUS exposure administered after EB introduction increased the EB concentration in the tumor by 580%. As shown in Fig. 4, the derived tumor-to-ipsilateral brain ratios were greater after sonication than without sonication.

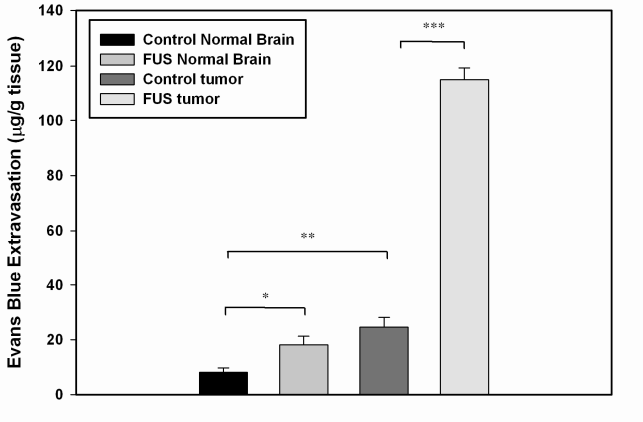

Fig. 3: Measurements of EB in the tumor and neighboring normal brain regions with and without sonication. The EB extravasation in the brain tumor with sonication was significantly higher than in brain tumor without sonication. Compared with the neighboring normal tissues of the control tumors, there was a significant difference for the control tumors and for the neighboring normal tissues of sonicated tumors on day 8 after tumor implantation. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

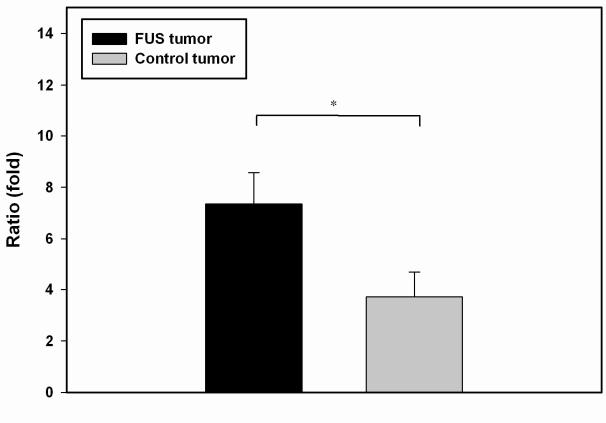

Fig. 4: The derived tumor-to-ipsilateral brain ratios after sonication and without sonication .

Furthermore, the corresponding H&E stained section was observed for tumor progression (Fig. 5). Based on the histological observation, tumor progression was consistent with the MR images.

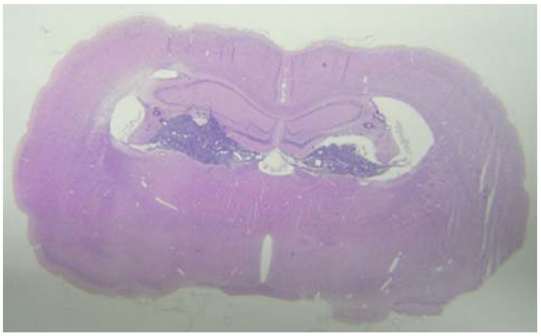

Fig. 5: Observations of the tumors with and without FUS exposure by hematoxylin-eosin-stained sections.

IV. DISCUSSION

Due to the presence of the BTB, only a limited amount of chemotherapeutic agent present in the circulation is able to be transported into a brain tumor without the assistance of a BTB delivery system. Several researches have reported that using infusion of hyperosmotic solution of mannitol, which disrupts the BBB, the drug uptake in brain tumor could be higher than in tumor without BBB disruption [13-15]. Our research here has shown that FUS can not only significantly increase the permeability of the BTB in brain tumors, but also significantly elevated the tumor-to-brain ratio in the focal region that was elicited by an ultrasound beam passing through the intact skull.

Experimental results showed that a combination of FUS and microbubbles increased the relative permeability of BTB. By using EB, we found this combination increased EB extravasation in brain tumors. The use of MRI contrast enhancement also revealed that this approach increased the level of gadolinium entering the brain tumor tissue. Gadolinium deposition and the pattern of contrast enhancement were monitored by signal intensity level. Figure 2C and 2D revealed that these are larger in size at high intensity level sites in the sonicated tumor. This is consistent with the EB extravasation results. The sonication pathway can be observed from brain surface to the bottom of the brain (Fig. 2B). Additionally, the pattern of contrast enhancement while the FUS beam is targeted over a non-homogeneous tumor tissue does not correspond to the circular pattern of the ultrasound beam on the cross section (Fig. 2D).

This technology, combined with recent advances in targeted microbubbles, may promote new approaches to be developed that make targeted brain tumor therapy possible. The results of this pilot study therefore suggest it would provide targeted access for chemotherapy and allow the use of recombinant pharmaceuticals for the brain diseases.

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