Combined therapy of focused ultrasound and aducanumab induces neurogenesis and decreases of beta-amyloid plaques in a mouse model of Alzheimer's disease

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Directed by Professor Jin Woo Chang

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Chanho Kong

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Combined therapy of focused ultrasound and aducanumab induces neurogenesis and decreases of beta-amyloid plaques in a mouse model of Alzheimer's disease

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Focused ultrasound (FUS) with microbubbles (MBs) can noninvasively open the blood–brain barrier (BBB) for drug delivery into the parenchyma. In preclinical studies, it is known that FUS-induced BBB opening not only enhances the penetration of therapeutic agents but also modulates neurogenesis and immunity. Recently, human clinical trials in Alzheimer's disease (AD) with FUS treatment have been initiated. Beta-amyloid (Aβ) deposition is a typical pathological phenomenon of AD. Although its mechanism remains questionable and controversial as a target for AD treatment strategies, numerous studies have focused on targeting Aβ. Meanwhile, numerous studies have reported that FUS-mediated BBB opening reduces Aβ levels in the brain. As previously used drugs for AD are limited to relieve symptoms, studies are more inclined to develop drugs, and there is a trend to develop drugs that can solve the cause of the disease. Aducanumab is a human monoclonal antibody that

reduces Aβ plaques in a dose-dependent manner. First, establishing FUS parameters to avoid blood vessel damage is an essential step to address safety issues and needs to be effective enough to open the BBB as well. Thus, the safety and efficacy of FUS-mediated BBB opening in the 5xFAD AD mouse model were examined, and whether FUS-mediated BBB opening contributes to the reduction of Aβ and improved cognitive function was investigated. As a result, a safe and efficient parameter of FUS was developed (1-Hz burst repetition frequency, 10-ms bursts, 120s in total, and average peak pressure 0.25 MPa). It did not only cause any hemorrhages or damage in the brain, but also proved its effectiveness. FUS-mediated BBB opening decreased Aβ in the hippocampus of 5xFAD mice and rescued cognitive function. Second, it was hypothesized that the combined therapy of FUS and aducanumab would be more effective than either FUS or aducanumab treatment alone. The effectiveness could be maximized depending on the number of treatments; FUS was targeted at four regions of the hippocampus where newly generated neurons occur, and aducanumab treatment was conducted three times biweekly and dose dependent. Combined therapy increased both proliferation and neurogenesis in the hippocampus. In line with this result, it was shown that the combined therapy significantly decreased Aβ plaques in the hippocampus and restored cognitive function. In addition, its effectiveness was more statistically significant when treated multiple times. In conclusion, the combined therapy of FUS and aducanumab for AD reduced amyloid aggregation, restored spatial memory, and increased neurogenesis. Furthermore, the remarkable aspect of this study is that the combined therapy was shown to be effective even with the minimal dose of the drug by increasing the BBB penetration rate with FUS, while excluding the possibility of side effects. This study provides a better insight into establishing a solid therapeutic strategy for the treatment of AD as well

as other neurodegenerative diseases.

Key words: Alzheimer's disease, focused ultrasound, blood–brain barrier, beta-amyloid, aducanumab

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I. INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease that progressively deteriorates cognitive functions, including memory. Similar to other neurodegenerative diseases, as the pathogenesis is complex and affected by various factors, its underlying mechanism has not been elucidated clearly. Pathological features of AD include extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles, leading to neurodegeneration and cell death. Because Aβ deposition contributes to the initiation of AD pathogenesis, therapeutic strategies that reduce or eliminate the production of Aβ plaques have long been the basis for AD treatment.

However, a fundamental treatment method for the treatment of AD has not yet been developed. Instead, in clinical studies, the current first-line treatment for AD is administration of drugs, such as donepezil and galantamine, which alleviate symptoms and delay progression.[1] According to the recently reported Alzheimer's

drug development pipeline, recent treatment development tends to progress toward the fundamental treatment of the disease rather than merely focusing on symptom relief. It also suggests that amyloid-related therapeutics account for the largest proportion of the current pipeline of agents.[2,3] Although the correlation between Aβ decrease and recovery in cognitive function is not well understood, reducing Aβ aggregates associated with the onset of AD would be a promising target to establish a solid therapeutic strategy for AD.[2,4-7]

Aducanumab is a medication designed to treat AD and an Aβ-directed human monoclonal antibody that selectively binds to the Aβ protein. In a clinical trial of aducanumab in patients with a clinical diagnosis of prodromal or mild AD, Aβ was decreased dose (3–10 mg/kg) dependently.[8] Consistent with the aforementioned outcome of clinical trials, an animal study using transgenic mice (Tg2576) showed that Aβ was significantly reduced in the group administered with a dose of 10 mg/kg or higher.[8] Aducanumab has been approved for the treatment of AD and AD-derived mild cognitive impairment in the United States Food and Drug Administration in June 2021. It is the first new drug treatment for dementia to reach the United States market in almost 20 years. However, the approval is still controversial due to ambiguous clinical trial results regarding its efficacy.[9,10] Aducanumab is currently the most promising drug for the treatment of AD, as research results have reported reducing Aβ even though its efficacy has not been fully demonstrated due to vague clinical trial results.[8]

The blood–brain barrier (BBB) is a unique structure that tightly regulates the transport of cells and molecules to restrict the penetration of noxious substances from the blood into the brain. Therefore, its permeability is highly selective to ensure optimal condition to maintain homeostasis of the central nervous system.[11] However,

its function has long been a challenge when potentially effective therapeutic agents are applied, as the BBB prevents about 98% of drug compounds from penetrating into the parenchyma.[12] Focused ultrasound (FUS) with microbubbles (MBs) is a minimally invasive or noninvasive approach for reversible and safe BBB opening.[13] Concentrating the acoustic pressure on the target area in the brain causes the cavitation effect of circulating MBs and temporarily opens the BBB.[14] As FUS is developed, research on drug delivery with FUS has been steadily progressing through the delivery of various therapeutic agents, such as chemotherapeutics,[15-17] cells,[18,19] and antibodies.[20] Furthermore, multiple evidence have reported that Aβ decreased by only opening the BBB without delivering therapeutic agents in the AD mouse model.[21] In line with previous research, it has been also proposed that BBB opening by FUS induced adult hippocampal neurogenesis in rodents, implying that FUS has a potential to be an effective therapeutic strategy for AD.[22,23]

However, to date, whether aducanumab with FUS would be more effective than applying a drug alone and to what extent FUS can modulate Aβ plaques and enhance cognitive functions remain unclear. To address these questions, this study aimed to demonstrate the effects of FUS, aducanumab, and the combined therapy of FUS and aducanumab in a 5xFAD AD mouse model in terms of Aβ reduction, neurogenesis, and cognitive function.

II. MATERIALS AND METHODS

Part 1. Safety and efficiency of FUS in a 5xFAD mouse model of AD

1. Animals

The 5xFAD mouse is a transgenic mouse with five familial mutations. These mice express high levels of both mutant human amyloid precursor protein (APP695) with Swedish mutation (K670N, M671L), London mutation (V717I), Florida mutation (I716V), and human presenilin 1 (PS1) with two mutations (M146L and L286V). 5xFAD mice were purchased from Jackson Laboratory (Sacramento, CA, USA) and maintained by crossing hemizygous transgenic mice with B6SJL F1 mice. Transgenic mice were identified by polymerase chain reaction (PCR), and non-transgenic littermates served as controls. Offspring were genotyped by PCR amplification using the APP and PSEN1 alleles (Table 1). The PCR products were separated on a 1% agarose gel. The APP and PSEN1 allele products were 377 bp and 608 bp, respectively (Figure 1). Extracellular amyloid deposition and gliosis beginning around 2 months are the main characteristics of 5xFAD.[24] Moreover, its phenotype can be defined as spontaneous alternation in the Y maze in that the impairment in spatial working memory begins at approximately 4 to 5 months of age.[25] Neuron loss has been observed in multiple brain regions in this model and begins at approximately 6 months of age.[24,26] All mice were housed in groups of 1–2 per cage with ad libitum access to food and water, in a humidity- and temperature-controlled, specific pathogen-free environment (12-h light cycle; lights on at 8 am) at the Yonsei University College of Medicine Animal Care Facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All

experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the Yonsei University Health System, Republic of Korea (IACUC no.: 2019-0213).

Table 1. Primer sequences

Gene	**Forward sequence (5' – 3')**
APP	AGG ACT GAC CAC TCG ACC AG
PSEN1	AAT AGA GAA CGG CAG GAG CA
GAPDH	AAT AGA GAA CGG CAC GAG CA
Gene	**Reverse sequence (3' – 5')**
APP	CGG GGG TCT AGT TCT GCA T
PSEN1	GCC ATG AGG GCA CTA ATC AT
GAPDH	GCC ATG AGG AGCA CTA ATC AT

Figure 1. Photograph of the PCR gene products for APP and PS1 in 5xFAD mice. Mutations in the genes for amyloid precursor protein (APP) and presenilins (PS1, PS2) increased the production of β-amyloid 42 (Aβ42) and caused familial Alzheimer's disease.

2. Focused ultrasound system

A 0.5-MHz single-element focused transducer (H-107MR; Sonic Concepts, Bothell, WA, USA) was used, with a transducer diameter of 51.7 mm and a radius of curvature of 63.2 mm. The transducer was used with a conical container that could be filled with degassed water to efficiently transfer the acoustic energy. A waveform generator (33220A, Agilent, Palo Alto, CA, USA) was connected to a 40-dB radio frequency power amplifier (210 L, ENI Inc., Rochester, NY, USA) to drive the FUS transducer, and a power meter (E4419B, Agilent) was used to measure the input electrical power (Figure 2).

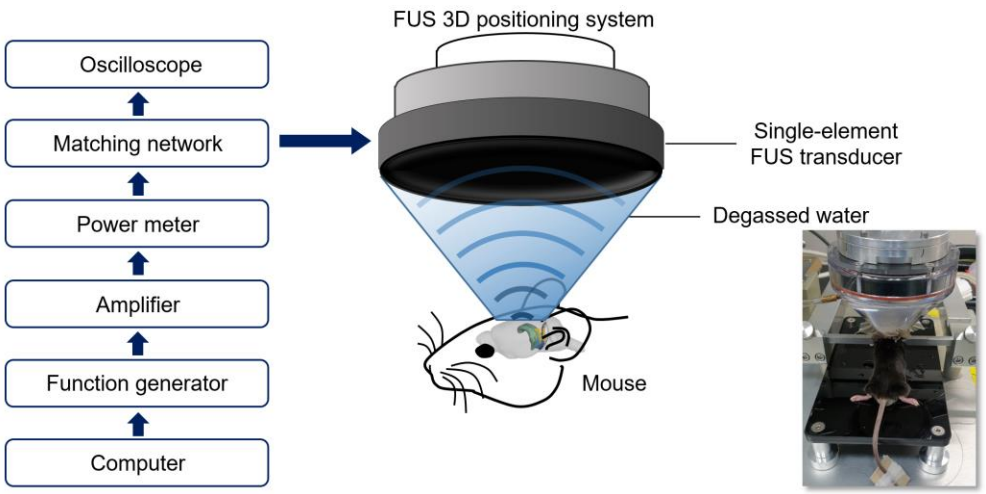


Figure 2. Scheme of the FUS system setup for BBB opening in mice.

3. Blood–brain barrier opening and safety

To determine the FUS-mediated BBB opening efficiency and safety according to changes in sonication acoustic pressure amplitude, both Evans blue (EB) and histological tissue damage were confirmed in response to 0.22-, 0.23-, 0.24-, and 0.25-MPa acoustic pressures. EB, an albumin marker used to measure BBB permeability, was intravenously injected (2%, 100 mg/kg) immediately after FUS sonication. Hematoxylin and eosin (H&E) staining was performed to confirm brain damage.

4. Focused ultrasound treatment

To confirm the changes in Aβ followed by FUS treatment, sonication was performed on the hippocampus unilaterally (coordinates from bregma: AP − 2.0, ML + 1.2 mm) of 5xFAD (n = 3), and animals were sacrificed a week later (Figure 3A). To confirm the cognitive improvement of 5xFAD by FUS treatment, 6-month-old male 5xFAD mice were divided into four groups: sham (n = 13), sham + FUS (n = 11), TG (n = 7), and TG + FUS (n = 7) groups. During surgery, mice were anesthetized with 5% isoflurane in oxygen, and animal heads were fixed on a stereotaxic frame (Narishige, Tokyo, Japan). A medical sterile ultrasound gel (ProGel; Dayo Medical Co., Seoul, South Korea) was used to fill the space between a coupling cone filled with degassed water and the skull for energy transfer efficiency. The FUS was targeted to four focal spots (coordinates from bregma: AP − 1.6, ML ± 1.2 mm/AP − 2.6, ML ± 2.4 mm) on the hippocampus bilaterally (Figure 3B). DEFINITY MBs (0.04 mL/kg; Lantheus Medical Imaging, North Billerica, MA, USA) were injected intravenously after confirming the coordinates of the target point on the skull, and sonication (1-Hz pulse repetition frequency (PRF), 10-ms bursts, 120 s in total, and average peak pressure 0.25 MPa) was started at the same time (Figure 3C).

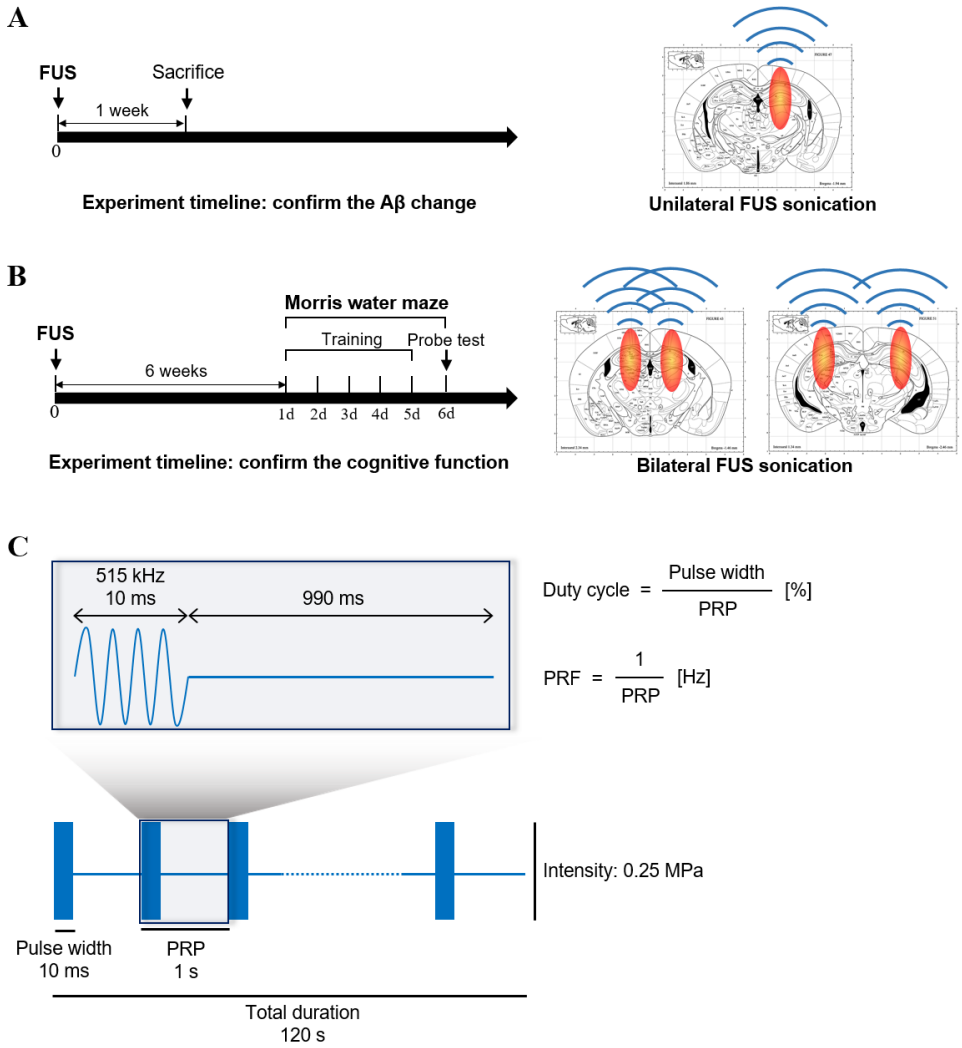


Figure 3. Scheme of the experimental procedure.

(A) To confirm the changes in Aβ, the animals were sacrificed 1 week after sonication. FUS was sonicated unilaterally, and the contralateral brain was used as a control. (B) To confirm the cognitive function, sonication was performed on four spots of the hippocampus, and the Morris water maze test was performed 6 weeks after sonication. (C) The sonication parameters included an amplitude of 0.25 MPa, PRF of 1 Hz, pulse repetitive period (PRP) of 1 s, duty cycle of 1 %, and total sonication time of 2 min.

5. Behavioral test: Morris water maze

A water maze test was performed 6 weeks after FUS treatment to verify the improvement in cognitive function by FUS. A circular water pool (12 cm in diameter) was filled with water (23 ± 2°C temperature), and the water was mixed with edible dye (Bright White Liqua-Gel; Chefmaster, CA, USA) to make the water opaque, so the platform (10 cm in diameter) was not visible. Different-shaped visual cues were marked on the walls of the four quadrants: north (N), west (W), south (S), and east (E) of the pool. Water was filled up to 1 cm above the platform, and the platform was placed in the center of one (SW) of the four quadrants. Learning training, a process for animals to remember the platform, was performed for 5 consecutive days, and probe tests were performed on the sixth day. All mice were trained to remember the platform for 5 days (four times a day), and the probe test was performed on the sixth day. The animals were placed in four quadrants facing the wall and were given 1 min to find the platform. Once they found the platform within 1 min, they were left on the platform for 10 s for learning. Meanwhile, animals that were unable to find the platform were placed on the platform for 10 s at the end of the session. The probe test was performed without the platform. Animals started on the wall of the quadrant zone opposite the platform, and their movements for 1 min were recorded using a tracking system (Harvard Apparatus, Holliston, MA, USA).

Part 2. The combined therapy of FUS and aducanumab for AD

1. Animals

As in Part 1, the 5xFAD AD mouse model was used. All mice were housed in groups of 2–5 per cage with ad libitum access to food and water, in a humidity- and temperature-controlled, specific pathogen-free environment (12-h light cycle; lights on at 8 am) at the Seoul National University College of Medicine Animal Care Facility. The animal cages were changed once a week by experimenters. All animal experiments were approved by the Animal Care Committee of Seoul National University College of Medicine using the Animal Care and Use Guidelines. Animal treatment and maintenance were approved and performed according to the Animal Care Committee of Seoul National University, Seoul, Republic of Korea (approval no.: SNU-201005-2-1).

2. Drug: aducanumab

The human aducanumab antibody was produced using the Expi293 expression system by transient transfection with a vector encoding variable regions cloned into a human IgG1 framework and purified using protein A/G chromatography. Heavy-chain and light-chain sequences were identified in Biogen Idec's patent submission for WO2014089500A1.

3. Combined therapy of FUS and aducanumab

Six-month-old male 5xFAD mice were divided into five groups: 5xFAD, 5xFAD + aducanumab, 5xFAD + FUS, 5xFAD + FUS + aducanumab, and sham groups (n = 10–15 per group). The FUS parameters and procedures were the same as those described in Part 1. The first treatment was performed when mice were 6months old. Each group underwent FUS treatment three times at an interval of 2 weeks (Figure 4). Aducanumab (3 mg/kg in saline) was injected intravenously at the end of FUS sonication.

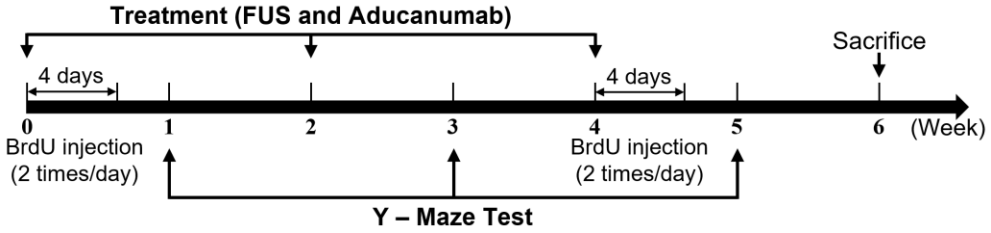


Figure 4. Experimental timeline for combined therapy of FUS and aducanumab. Three FUS treatments were performed at an interval of 2 weeks. Y-maze tests were performed 1 week after each treatment.

4. Magnetic resonance imaging

To confirm the permeability of the BBB, post-T1-weighted images with gadolinium were compared to the pre-T1-weighted images without gadolinium (Figure 5A–F). T2-weighted images confirmed the safety of FUS in the absence of microbleeding and edema (Figure 5G–H). Magnetic resonance imaging (MRI) was performed immediately followed by sonication with a Bruker 9.4 T 20-cm bore MRI system (Biospec 94/20 USR; Bruker, Ettlingen, Germany) and mouse head coil (Table 2). A gadolinium-based MRI contrast agent, gadobutrol (Gd; Gadovist; 0.2 mL/kg), was injected intravenously.

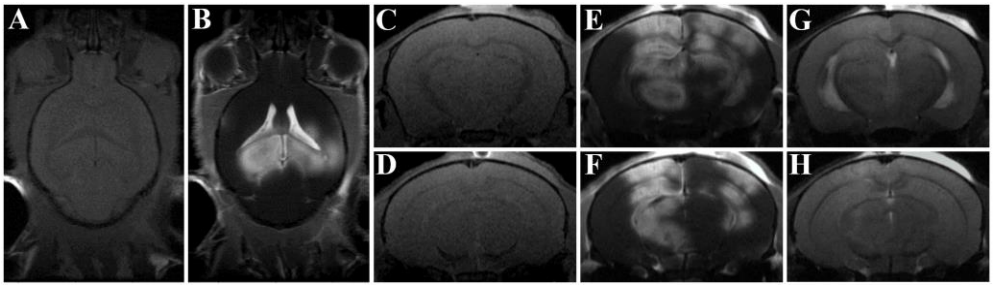


Figure 5. Confirmation the FUS-mediated BBB opening.

Four regions were targeted per sonication. (A–B) Transverse T1-weighted pre-/post-gadolinium MR images were taken to confirm the increase in BBB permeability. (C–D) Coronal T1-weighted pre-gadolinium MR images after FUS. (E–F) Coronal T1-weighted post-gadolinium MR images after FUS. (G–H) No clear hemorrhage or edema was observed in the coronal T2-weighted images.

Table 2. MRI sequences and parameters

	T1-weighted imaging	**T2-weighted imaging**
Echo	1	1
TR (ms)	500	2500
TE (ms)	8.1	33
FA (deg)	90	90
NEX	5	2
FOV (cm)	2 × 2	2 × 2
Matrix	256 × 256	256 × 256

5. Behavioral test: spontaneous alteration in the Y-maze test

To investigate the impairment of spatial working memory, spontaneous alternation in the Y-maze was observed. The Y-maze test was performed three times 1 week after each treatment. The alternation performance was tested using a symmetrical Y-maze, consisting of three equal arms (40 × 15 × 9 cm) and constructed using black acrylic plastic. All mice were placed at the center of the Y-maze and allowed to explore freely for 8 min. All movements were recorded using a video camera and were analyzed to determine the alternation ratio by manually evaluating the number of triads containing entries into all three arms.

6. BrdU labeling

To determine whether FUS induced neurogenesis, 5-bromo-2′-deoxyuridine (BrdU) was injected intraperitoneally twice a day for 4 days 24 h after treatment. BrdU, a marker of newborn cells, is a thymidine analog that incorporates into dividing cells during the S-phase of the cell cycle.[27] To investigate the effects of repeated FUS treatment, differences in neurogenesis were compared between single treatment and multiple treatment groups. Half of the group was injected after the first treatment, and the remaining half was injected after the third treatment.

7. Immunohistochemistry

Brains were fixed in 4% paraformaldehyde for 24 h, transferred to 30% sucrose for 3 days for immunohistochemistry, and stored at −20°C in a cryoprotectant storage solution until use. Brains were cut into 30-μm coronal sections. Free-floating sections were washed in PBS and incubated in blocking solution (PBS, 5% normal goat serum, 0.2% Triton X100) for 3 h at room temperature. The sections were incubated with primary antibodies in blocking solution overnight at 4°C. The following primary antibodies were used for immunohistochemistry: BrdU (proliferation marker, Abcam, ab6326, rat, 1:250), NeuN (mature neuronal marker, Millipore, ABN78, rabbit, 1:500), and 6E10 (anti-human Aβ monoclonal antibody, Biolegend, SIG39320, mouse, 1:500). After the primary immunoreaction, sections were incubated with secondary antibodies conjugated with Alexa488 (Invitrogen, A11008, 1:500) and Alexa 594 (Abcam, A150156, 1:250). Immunostaining of the sections was visualized using an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany) or an Axio Imager M2 (Carl Zeiss) light microscope. The number and area of plaques detected by 6E10 were quantified using ImageJ software (version 1.52a, Wayne Rasband, NIH, MD, USA).

8. Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Data were calculated using one-way and two-way analysis of variance (ANOVA) followed by Tukey's and least significant difference post hoc analysis. The p-value < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS (version 25, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA).

III. RESULTS

Part 1. Safety and efficiency of FUS in a 5xFAD mouse model of AD

1. Examination of safety for FUS-mediated noninvasive BBB opening

To determine BBB opening efficiency and safety depending on the changes in acoustic pressure amplitude, both EB extravasation and histological tissue damage were confirmed in response to 0.22-, 0.23-, 0.24-, 0.25-MPa acoustic pressures. As a result of the BBB opening according to acoustic pressures, the transmission rate of EB was the highest at 0.25-MPa acoustic pressures (Figure 6A). Evaluation of the brain region through H&E staining revealed no damage, such as microbleeding or visible structural injuries at the targeted regions (Figure 6B).

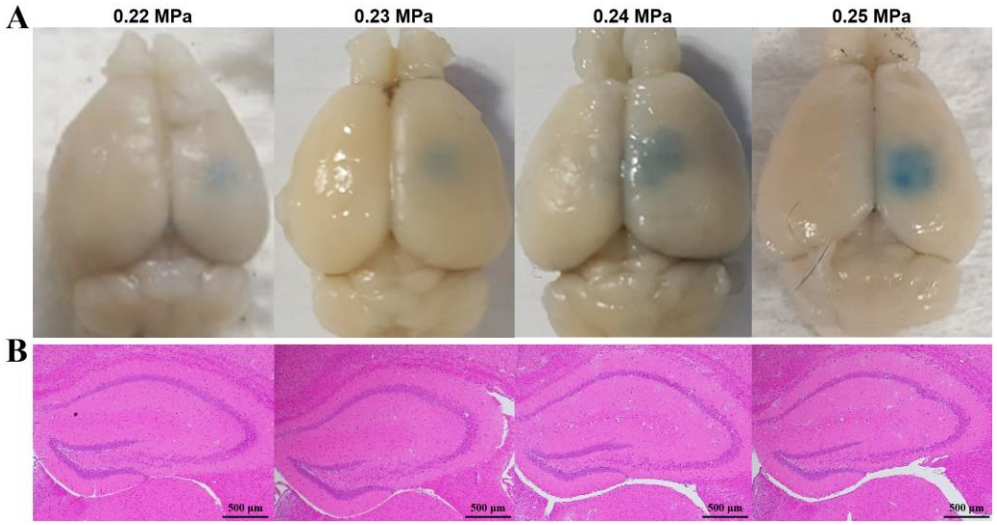


Figure 6. Confirmation of BBB opening and evaluation of tissue damage.

(A) Evans blue extravasation in the sonicated regions of the brain. BBB opening with increasing acoustic pressure amplitude from 0.22 MPa to 0.25 MPa. (B) The targeted brain regions were histologically evaluated by H&E staining.

2. FUS-induced BBB opening decreased amyloid plaques in the hippocampus.

To determine whether FUS-mediated BBB opening affects Aβ reduction, mice (n = 3) were sacrificed 1 week after FUS treatment. The total number of Aβ in the dentate gyrus (DG) of the hippocampus in the FUS-treated hemisphere was lower than that in the contralateral hippocampus (Figure 7A). The number of Aβ plaques in the ipsilateral hippocampus (50.00 ± 8.185) was significantly lower than that in the contralateral hippocampus (91.00 ± 9.849); $p < 0.05$ (Figure 7B). Additionally, the total area of Aβ plaques in the ipsilateral hippocampus (1,028 ± 163) was significantly lower than that in the contralateral hippocampus (2,796.00 ± 210.70); $p < 0.01$ (Figure 7C).

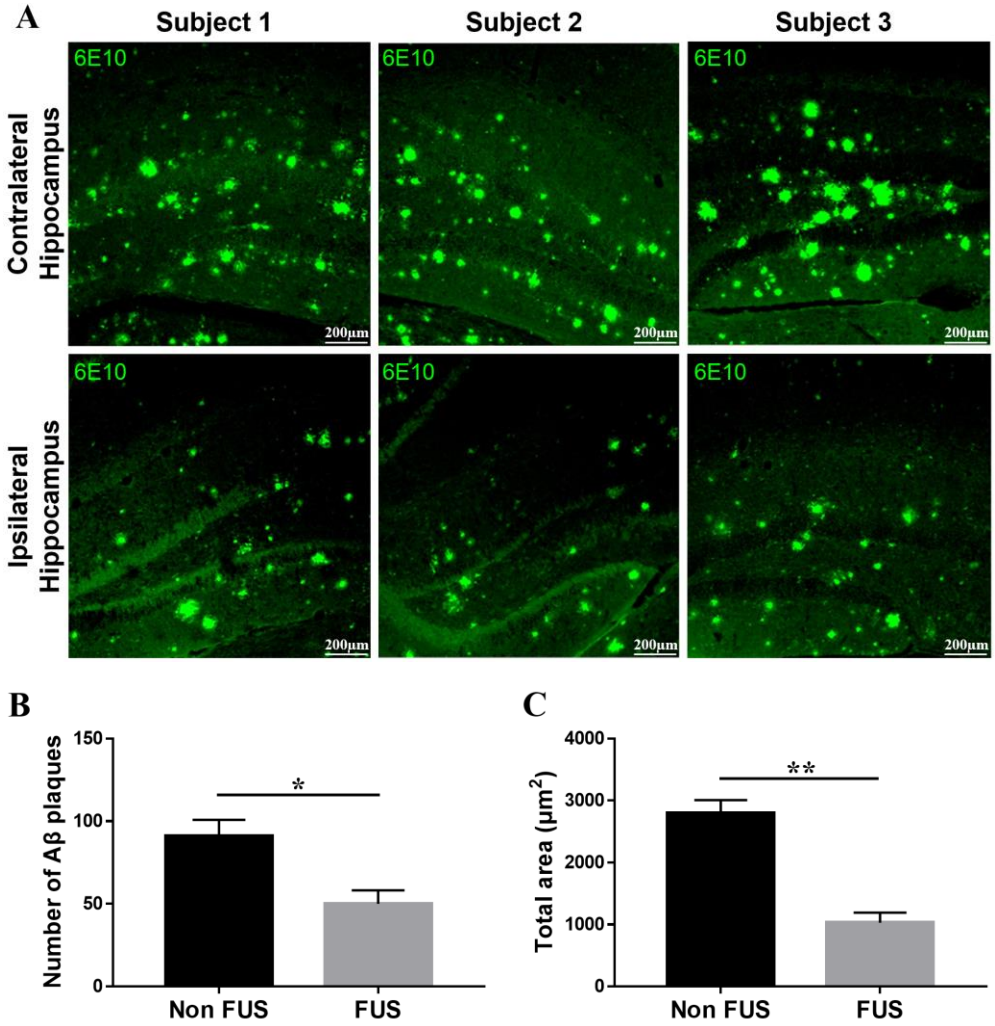


Figure 7. FUS-mediated BBB opening reduced Aβ plaques. (A) M2 images (10×) of Aβ-stained 6E10 antibody in the dentate gyrus (DG) of the hippocampus. (B–C) Bar graph shows the number of total Aβ plaques and total area in the DG of the hippocampus. *$p < 0.05$, **$p < 0.01$, n = 3; Unpaired t-test (two-tailed) was used for the statistical analysis.

3. FUS-induced BBB opening improved the cognitive function in 5xFAD

The Morris water maze test was performed to investigate the changes in cognitive function and memory following FUS treatment. Throughout the 5-day training, the time to find the platform gradually decreased in the sham, sham + FUS and TG + FUS groups (Figure 8A). In contrast, the TG group spent more time searching for and reaching the platform, indicating that they had difficulties with learning and memory. The sham ($p < 0.0001$) and sham + FUS ($p < 0.0001$) groups displayed significantly better memory and learning abilities than that in the TG group (Figure 8A). In the probe test, the TG group (0.42 ± 0.20) had a significantly fewer number of crossings than that in the sham (3.07 ± 0.62; $p < 0.05$) and sham + FUS (3.72 ± 0.54; $p < 0.01$) groups (Figure 8B). The TG + FUS group (1.02 ± 0.24) showed a significantly longer time in the platform area compared with that in the TG group (0.10 ± 0.05); $p < 0.01$ (Figure 8C). The amount of time spent in the quadrant zone was significantly decreased in the TG group (7.04 ± 1.85) compared to that in the sham (17.00 ± 1.71; $p < 0.01$), sham + FUS (24.21 ± 1.73; $p < 0.0001$), and TG + FUS groups (22.15 ± 3.33; $p < 0.001$) (Figure 8D). Additionally, the mean speed in the pool was significantly slower in the TG group (13.62 ± 2.52) than that in the sham group (19.79 ± 1.12); $p < 0.05$ (Figure 8E).

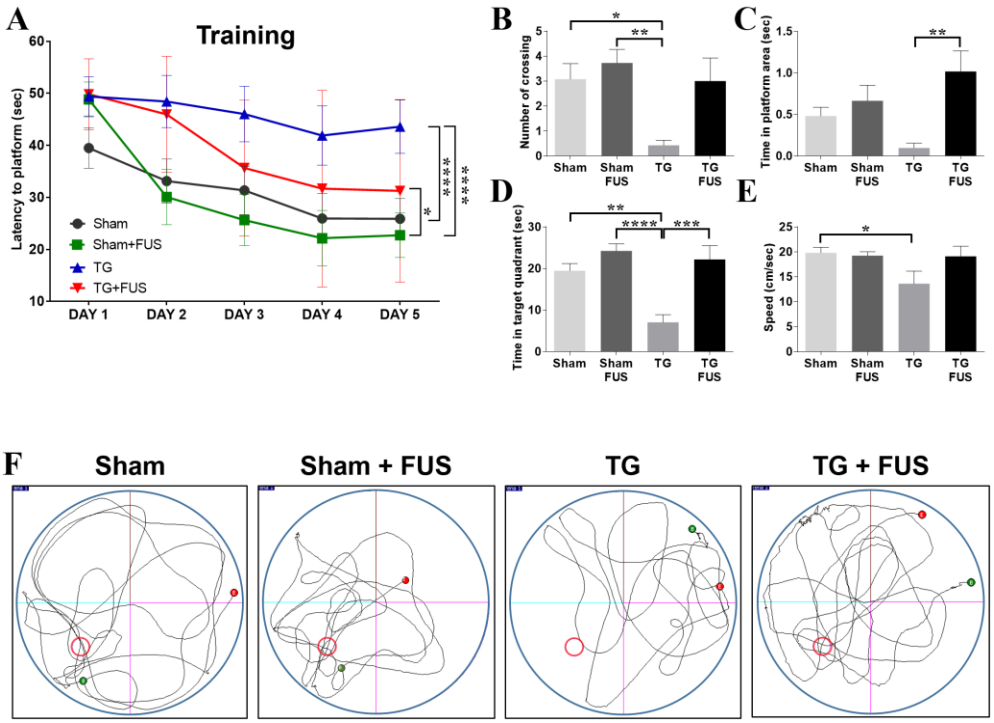


Figure 8. FUS improves cognitive function and spatial memory.

(A) Learning curves of the 5 consecutive days. Latency to platform means the time required for mice to find the escape platform during training trials. Data are presented means ± standard error of the mean (SEM). *p < 0.05, ****p < 0.0001; Two-way ANOVA with Tukey's multiple comparison test. (B–E) Results of the probe test one day after the end of the training for 1 days. Data are presented means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; One-way ANOVA with Tukey's multiple comparison test. (F) Trajectory maps of animals in the probe test. Groups: sham (n = 13), sham + FUS (n = 11), TG (n = 7), TG + FUS (n = 7).

Part 2. The combined therapy of FUS and aducanumab for AD

4. The combined therapy of FUS and aducanumab rescued the cognitive impairments in 5xFAD

The combined effect of FUS and aducanumab on spatial memory was evaluated using the Y-maze test. FUS treatment was performed three times at 2-week intervals, and the Y-maze test was performed 1 week after each treatment. As a result, compared with the 5xFAD group, alteration rates in the combined treatment group were all recovered regardless of the number of FUS treatments. In the first treatment, the combined treatment group (0.59 ± 0.03) featured significantly recovered alteration rates compared with that in the 5xFAD group (0.47 ± 0.02); $p < 0.05$ (Figure 9). In the repeated treatment, the combined treatment group (0.66 ± 0.02) exhibited even higher recovered alteration rates compared with that in the 5xFAD group (0.48 ± 0.05); $p < 0.001$ (Figure 9). Moreover, the combined treatment group featured significantly recovered alteration rates compared with the only aducanumab (0.55 ± 0.03; $p < 0.04$) and only FUS groups (0.54 ± 0.03; $p < 0.01$). Thus, the repeated combined treatment contributed to a more significant improvement in cognitive function than a single treatment.

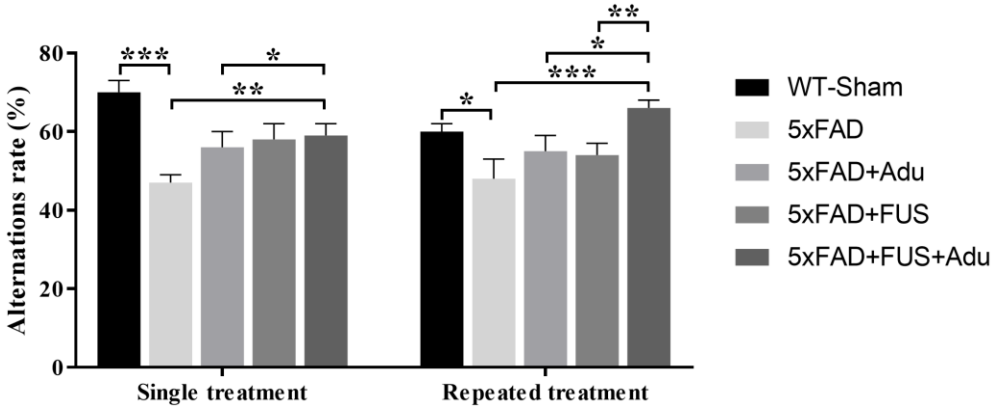
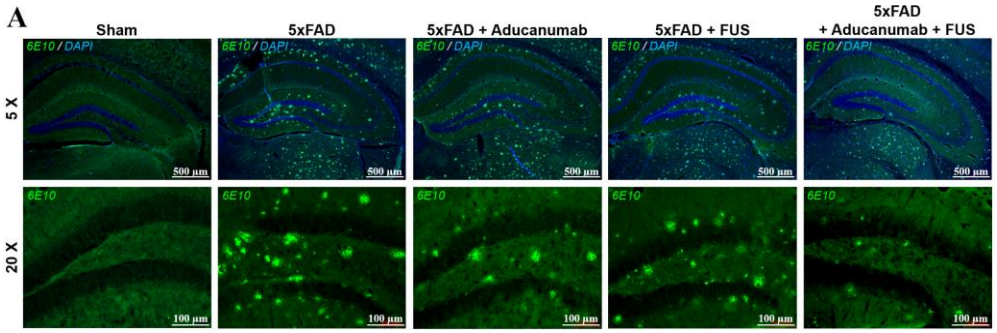
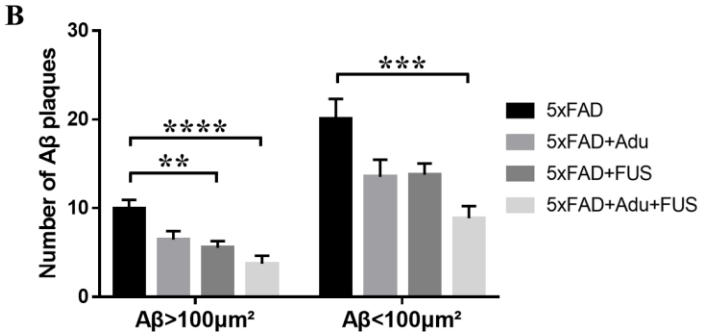


Figure 9. The combined therapy of FUS and aducanumab restored cognitive impairments. Bar graphs show the spontaneous alternation rate in 5xFAD mice 1 week after the first treatments (left) and 1 week after repeated treatment (right). Data were expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Statistical analysis was performed using one-way ANOVA followed by least significant difference analysis. Groups of single treatment: sham (n = 20), 5xFAD (n = 15), 5xFAD + Adu (n = 14), 5xFAD + FUS (n = 15), and 5xFAD + Adu + FUS (n = 22) groups. Groups of repeated treatment: sham (n = 13), 5xFAD (n = 13), 5xFAD + Adu (n = 12), 5xFAD + FUS (n = 17), and 5xFAD + Adu + FUS (n = 17) groups.

5. The combined therapy of FUS and aducanumab decreased amyloid plaques in the dentate gyrus of the hippocampus

6E10, an Aβ marker, was stained to determine whether the combined therapy would be more effective in reducing Aβ than either FUS or aducanumab treatment alone (Figure 10A). The combined therapy group (2.5 ± 0.65) showed a significantly decreased number of Aβ plaques >100 μm^2 in the DG of the hippocampus compared with that in the 5xFAD group (10 ± 0.78); $p = 0.0003$ (Figure 10B). In Aβ plaques <100 μm^2, the combined therapy group (8.33 ± 0.93) showed a significantly decreased number of Aβ plaques compared with the 5xFAD group (19.9 ± 2.43; $p = 0.0037$) (Figure 10B). No significant differences were found in the number of Aβ plaques >100 μm^2 in the DG of the hippocampus in the FUS (6 ± 0.7) and aducanumab groups (6.57 ± 1.04) compared with that in the 5xFAD group (10 ± 0.78) (Figure 10B). No significant differences were found in the number of Aβ plaques <100 μm^2 in the DG of the hippocampus in the FUS (15.12 ± 1.56) and aducanumab groups (15.28 ± 2.12) compared with that in the 5xFAD group (19.9 ± 2.43) (Figure 10B). The total area of the Aβ plaques in the DG of the hippocampus significantly decreased in the combined treatment group (Aβ > 100 μm^2, 495.29 ± 172.99; $p < 0.0002$; Aβ < 100 μm^2, 298.58 ± 14.14; $p < 0.0064$) compared with that in the 5xFAD group (Aβ > 100 μm^2, 2841.02 ± 328.38; Aβ < 100 μm^2, 653.85 ± 91.60) (Figure 10C). A significant difference was not observed in the FUS (Aβ > 100 μm^2, 1421.71 ± 182.44; Aβ < 100 μm^2, 484.06 ± 50.73) and aducanumab groups (Aβ > 100 μm^2, 1754.28 ± 296.52; Aβ < 100 μm^2, 556.71 ± 74.25) compared with that in the 5xFAD group (Aβ > 100 μm^2, 2841.02 ± 328.38; Aβ < 100 μm^2, 653.85 ± 91.60) (Figure 10C).





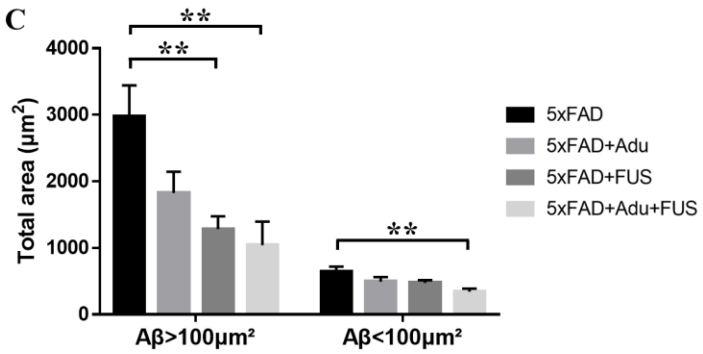


Figure 10. Combined therapy of FUS and aducanumab decreased Aβ accumulation in 5xFAD mice. (A) Above, representative M2 images (5×) of Aβ-stained 6E10 antibody and DAPI in the hippocampus. (A) Below, representative M2 images (20×) of Aβ-stained 6E10 antibody in the dentate gyrus (DG) of the hippocampus. (B) Bar graph shows the number of Aβ plaque >100 μm^2 and <100 μm^2 in the DG of the hippocampus. (C) Bar graph shows the total area of Aβ plaque >100 μm^2 and <100 μm^2 in the DG of the hippocampus. Data are presented means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; One-way ANOVA with Tukey's multiple comparison test. Groups: 5xFAD (n = 13), 5xFAD + Adu (n = 11), 5xFAD + FUS (n = 13), and 5xFAD + Adu + FUS (n = 15).

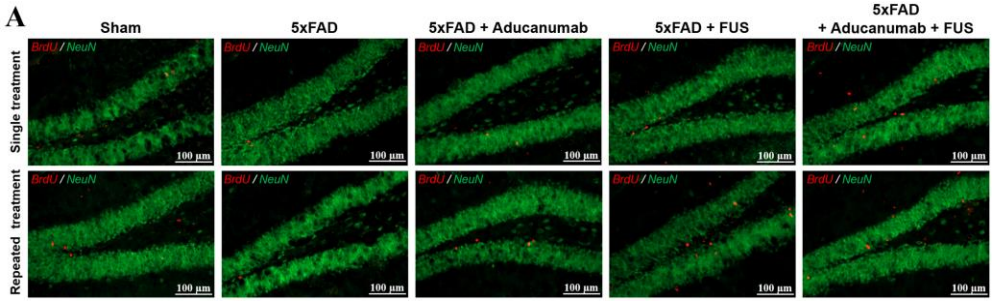
6. The combined therapy of FUS and aducanumab increased neurogenesis in 5xFAD mouse

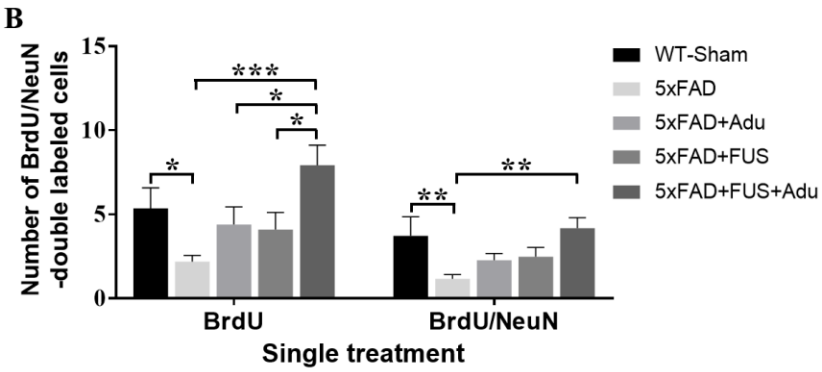
To confirm the effects of proliferation and neurogenesis, the stained BrdU and NeuN in the subgranular zone and granular cell layer of the DG were quantified (Figure 11A). The number of $BrdU^+$ and $BrdU^+$/NeuN represents the average of seven sections, serially sectioned at 30-μm intervals of the hippocampus.

In the single treatment, the number of BrdU-positive cells was significantly increased in the combined therapy group (7.92 ± 1.19) compared with that in the 5xFAD (2.19 ± 0.36; $p < 0.001$), 5xFAD + Adu (4.45 ± 0.87; $p < 0.05$), and 5xFAD + FUS (4.09 ± 1.02; $p < 0.05$) groups (Figure 11B). Meanwhile, the bar graphs show a significantly decreased number of BrdU-positive cells in the 5xFAD group (2.19 ± 0.36) compared with that in the sham group (5.35 ± 1.22); $p < 0.05$ (Figure 11B). For $BrdU^+$/NeuN double-labeled cells, the combined therapy group (4.18 ± 0.62) also exhibited a higher number of $BrdU^+$/NeuN double-labeled cells compared with that in the 5xFAD group (1.17 ± 0.24); $p < 0.01$ (Figure 11B), whereas the 5xFAD group (1.17 ± 0.24) had a significantly decreased number of $BrdU^+$/NeuN double-labeled cells compared with that in the sham group (3.71 ± 1.13); $p < 0.01$ (Figure 11B).

In the repeated treatment, the effectiveness was consistent with the results of the single treatment. The number of BrdU-positive cells was significantly increased in the combined therapy group (8.10 ± 0.29) compared with that in the 5xFAD (3.76 ± 0.71; $p < 0.05$) and sham (4.57 ± 0.44; $p < 0.05$) groups (Figure 11C). The bar graphs showed a significantly increased number of BrdU-positive cells in the 5xFAD + FUS group (9.27 ± 1.98) compared with that in the sham (4.57 ± 0.44; $p < 0.01$), 5xFAD (3.76 ± 0.71; $p < 0.01$), and 5xFAD + Adu (5.06 ± 0.99; $p < 0.05$) groups (Figure 11C). The combined therapy group (3.70 ± 0.34) also exhibited a higher number of

$BrdU^+$/NeuN double-labeled cells compared with that in the 5xFAD group (1.83 ± 0.27); $p < 0.05$ (Figure 11C). Moreover, a significantly increased number of $BrdU^+$/NeuN double-labeled cells was observed in the 5xFAD + FUS group (4.04 ± 0.93) compared with that in the sham (2.42 ± 0.29; $p < 0.01$), 5xFAD (1.83 ± 0.27; $p < 0.05$) groups (Figure 11C).





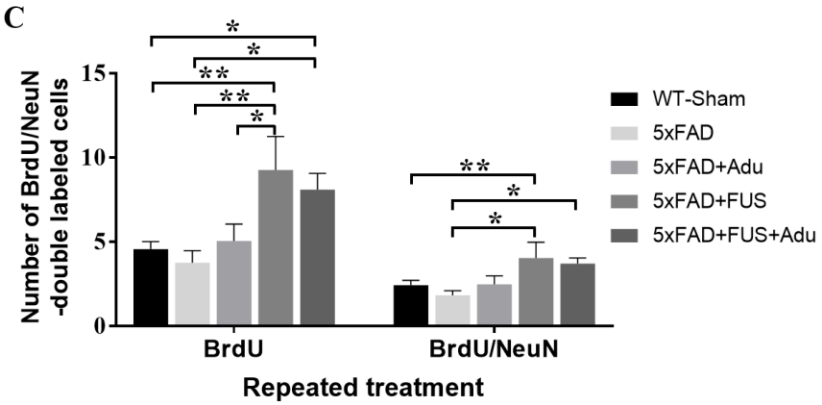


Figure 11. Analysis of cell proliferation and neurogenesis in the DG of the hippocampus. (A) Representative images showing immunofluorescence of neuronal nuclear antigen (NeuN, green), 5-bromo-2'-deoxyuridine (BrdU, red), or their colocalization (BrdU/NeuN). Scale bars: 100 μm. (B) A number of BrdU and BrdU/NeuN–positive cells in the hippocampal dentate gyrus (DG) with a single treatment. Groups: sham (n = 8), 5xFAD (n = 8), 5xFAD + Adu (n = 6), 5xFAD + FUS (n = 6), and 5xFAD + Adu + FUS (n = 7). (C) The number of BrdU and BrdU/NeuN–positive cells in the hippocampal DG with repeated treatments. Groups: sham (n = 10), 5xFAD (n = 6), 5xFAD + Adu (n = 5), 5xFAD + FUS (n = 8), and 5xFAD + Adu + FUS (n = 7). Data are expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; Statistical analysis was performed using one-way ANOVA followed by least significant difference post hoc analysis.

IV. DISCUSSION

This study aimed to demonstrate the fundamental AD treatment effects by reducing Aβ aggregation through the application of FUS with antibody therapy in a 5xFAD AD mouse model. In recent years, preclinical and clinical studies using FUS have been actively conducted around the world, and their use in clinical practice has gradually become increasingly stable and safe.[16,28,29] Although there have been several attempts to combine FUS with other therapeutic strategies, it has been challenging to secure safety as well as effectiveness at a time.[30-32] However, this study may provide some insight into the development of a novel therapeutic strategy for AD. For many of the treatments for neurodegenerative diseases, it is important that the drugs travel down to the target region, but the BBB mostly hinders the molecules from reaching the target in the brain, thereby making the potentially effective therapy ineffective. Commonly, only lipophilic molecules with a small molecular weight (under 400–600 Da) can cross the BBB.[33] Monoclonal antibodies have a therapeutic potential for treating neurodegenerative diseases, but their delivery in the brain is limited by the BBB due to its large molecular weight.[34] The weight of aducanumab used in this study is 146 kDa, which normally cannot pass through the BBB without any kind of modulation. Therefore, with regard to its effectiveness, treatment with antibody therapy alone is marginal or infeasible. However, it is believed that FUS could not only resolve these drawbacks of antibody therapy but also show the synergistic effects of FUS and antibody therapy.

To address these fundamental issues, the therapeutic effects were analyzed from three perspectives: Aβ accumulation, decline of cognitive function, and the occurrence of adult hippocampal neurogenesis. In this study, the combined therapy

of FUS and aducanumab was confirmed to have significant effects on the recovery of cognitive function, Aβ reduction, and neurogenesis. In AD, cognitive function is severely impaired and considered to be one of the most common symptoms. As shown in the behavioral tests, 5xFAD mice that did not undergo any treatment showed significantly lower alteration rates than that in other treatment groups. These results prove that even a single FUS treatment can enhance memory function, but multiple treatments greatly augment its effectiveness. Further, neurogenesis in the target area has increased where FUS was sonicated, stating that it may have served to increase cognitive function.

Since Hynynen et al. reported that FUS-mediated BBB opening induces hippocampal neurogenesis, numerous follow-up studies have been conducted.[22,23,35,36] However, the mechanism by which FUS induces neurogenesis has not yet been clearly elucidated. This study demonstrated that neurogenesis only occurs when FUS-mediated BBB opening occurs. Since neurogenesis is induced only when the BBB is opened, it is assumed that changes in the intravascular microenvironment or the components of the tight junction may have played a role in promoting neurogenesis. In addition, the brain-derived neurotrophic factor (BDNF) is one of the most important factors in neurogenesis,[37] and a report revealed that FUS-mediated BBB opening increases BDNF.[23] Therefore, these results could be in line with those of previous studies and contribute to understanding the mechanism of FUS and neurogenesis.

Doublecortin (DCX; marker of neurogenesis) was examined using immunohistochemistry, but this was not presented in this study. At the beginning of the experiment, mice were 6 months old, and the time point of sacrifice was when mice turned 8 months. The expression of DCX in all groups was hardly observed.[38]

In modern medicine, the development of new pharmaceuticals for numerous brain treatments, including AD, has been delayed because all large-molecule pharmaceuticals cannot penetrate the BBB. Aducanumab is also a large-molecule pharmaceutical that can hardly cross the BBB and has a dose-dependent therapeutic effect only when the BBB is functionally disrupted. For this reason, aducanumab has been administered at a high dose, causing side effects such as amyloid-related imaging abnormalities, headache, and urinary tract infection.[8] In another study using transgenic mice (Tg2576), Aβ was significantly reduced in the group given a dose of ≥10 mg/kg, and at the 0.3–3 mg/kg dose, Aβ did not decrease significantly.[8] When aducanumab is administered at a high dose, the effect of Aβ reduction has been shown to be evident. However, because the probability of side effects increases significantly, a strategy to reduce the dose and increase the effectiveness is needed.

When FUS was treated unilaterally with aducanumab, aducanumab was expressed only in the ipsilateral hemisphere, and the expression increased depending on the dose (Figure 12). Aducanumab is normally expressed at 146 kDa, as mentioned above. However, in vivo generation of the aducanumab antibody may vary slightly as glycosylation occurs.

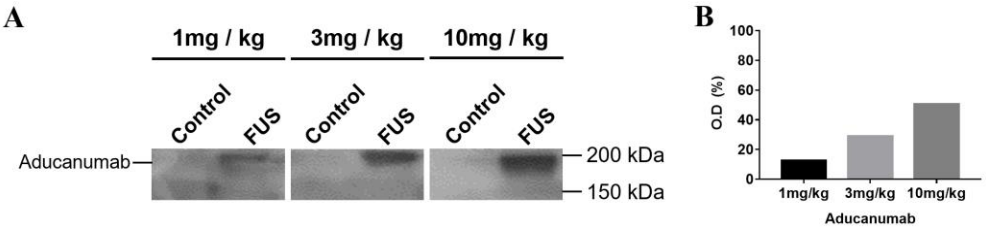


Figure 12. Dose dependent aducanumab expression level delivered by FUS.

(A) Representative images present dose-dependent aducanumab expression in the FUS-sonicated brain. Aducanumab was not expressed in the hemisphere not sonicated with FUS. (B) Expression rates (1 mg/kg, 12.52%; 3 mg/kg, 20.05%; 10 mg/kg, 50.63%) of dose-dependent aducanumab after FUS-mediated BBB opening.

FUS plays an important role in increasing the delivery rate of aducanumab into the brain to reduce amyloid plaques. The results of this study suggest that FUS not only contributes to the increase in BBB permeability, but also has the ability to directly reduce amyloid plaques. Accumulating evidence consistently reported that FUS reduced amyloid plaques, but its mechanism remained unclear.[39-43] Previous studies suggested increases in endogenous immunoglobulins binding to Aβ plaques with FUS-mediated BBB opening.[40] In addition, they reported that FUS increased microglia and astrocytes in relation to Aβ phagocytosis, which remains controversial.[40] As microglia are the main phagocytes in the central nervous system, it is more likely to rapidly decrease in Aβ plaques. It is also possible that FUS induces infiltration of systemic phagocytic immune cells, which may also aid in Aβ reduction. Furthermore, FUS has recently been shown to induce an increase in monocyte chemotactic protein-1 and migration of $CD68^+$ systemic macrophages.[44,45]

In this study, Aβ was not significantly decreased in the group treated with aducanumab alone, and Aβ was significantly decreased when administered with FUS. A positive treatment strategy of using a low dose of 3 mg/kg rather than a high dose of 10 mg/kg or more has been suggested, even by increasing the drug delivery rate to the target using FUS. Although the efficacy of aducanumab has been demonstrated in current clinical studies, it faced difficulties with disapproval in phase 3 clinical trials due to side effects caused by high doses. Recently, aducanumab has received FDA approval. Although it has shortcomings in terms of efficacy and side effects, it is believed to overcome barriers to FUS.

There are still uncertainties about the benefits of aducanumab, and additional clinical trials are needed to confirm the efficacy of the drug. However, this study is the first to demonstrate that combined treatment with FUS and antibody therapy can

directly reduce Aβ plaques and enhance cognitive functions as well as neurogenesis. Currently, AD patients desperately need treatment to delay disease progression, but this study presented the possibility of targeting the fundamental issues of AD.

V. CONCLUSION

This study aimed to demonstrate the effects of FUS and aducanumab in an AD mouse model and propose the potential of the combined therapy for AD treatment. In part 1, safe and efficient parameters of FUS in the 5xFAD AD mouse model were established. Consequently, a safe parameter that could maximize the effectiveness of FUS was secured. The FUS-treated group showed remarkably decreased levels of Aβ plaques without any hemorrhages or edema, and it was shown that FUS promoted cognitive function. Part 2 shows that the combined therapy significantly reduced Aβ plaques and restored cognitive function compared to either FUS or aducanumab treatment alone. Moreover, the combined therapy significantly induced proliferation and neurogenesis. Interestingly, even a low dose of aducanumab was shown to be effective, whereas previous studies reported that only a high dose of aducanumab was effective. These data indicate that FUS increased the permeability of the BBB and enabled drugs to pass through the barrier without any side effects, which is the most controversial issue and downside of drug delivery. Overall, the potential of the combined therapy would provide insight into establishing a novel therapeutic strategy for the treatment of AD as well as other neurodegenerative diseases.

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ABSTRACT (IN KOREAN)

알츠하이머 마우스 모델에서 집속초음파와 아두카누맙의 병합 처치를 통한 베타 아밀로이드 플라크 감소 및 신경발생 유도에 의한 치료효과 검증

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집속초음파는 미세기포와 함께 사용되어 비침습적으로 뇌혈관장벽을 제어하고 뇌 내로 약물을 전달할 수 있다. 집속초음파를 통한 뇌혈관 장벽의 제어는 뇌 내로 약물 전달이 가능하게 할 뿐만 아니라 신경발생이나 면역, 다양한 환경변화를 조절할 수 있다는 전임상 연구들이 보고되었으며, 최근 치매환자를 대상으로 집속초음파 임상시험이 시작되었다. 베타 아밀로이드 침착은 치매에서 나타나는 대표적인 병리학적 현상이다. 치매 치료 전략으로써 베타 아밀로이드가 적절한지에 대해서는 아직도 많은 논란과 의문이 남아있지만, 이를 타겟으로 많은 치료 연구들이 진행중에 있다. 최근 집속초음파를 통한 뇌혈관 장벽의 제어가 뇌 내 침착된 아밀로이드 플라크를 줄여준다는 보고가 있었다. 한편 치매치료제 개발에 있어 이전에는 증상을 완화시키고 지연시키는 목적에 국한되었다면, 최근에는 병의 근본적인 원인을 해결할 수 있는 치료제를 개발하는 추세이다. 아두카누맙은 인간

단일클론항체 치료제로써 약물의 용량에 따라 치매 환자의 뇌 내 아밀로이드 플라크를 줄여주는 치료 효과가 확인되어 근본적인 치매 치료제로 여겨진다. 하지만 아두카누맙 역시 큰 분자량으로 뇌혈관장벽을 투과하기 어렵기 때문에 임상에서는 고용량을 사용하며 이는 다양한 부작용을 일으킬 수 있다. 본 연구의 최종 목적은 집속초음파와 아두카누맙의 병합 치료를 통해 신개념 치매 치료효과를 검증하는 것이다.

본 연구의 1부에서는 5xFAD 치매 마우스 모델을 이용하여 집속초음파를 통한 뇌혈관 장벽 제어의 안전성과 효율을 확인하였다. H&E 염색을 통해 뇌 내 미세손상이 발생하지 않는 안전한 파라미터를 구축하였다. 집속초음파에 의해 아밀로이드 플라크가 감소함을 면역조직화학염색을 통해 확인하고, 모리스 수중 미로 실험을 통해 인지기능이 개선됨을 확인함으로써 집속초음파가 치매 치료 수단으로서의 가능성을 확인하였다.

2부에서는 앞서 확립한 집속초음파 파라미터를 기반으로, 아두카누맙 치료제와 병합 치료에 대해 연구하였다. 연구를 위해 5xFAD, 5xFAD + 집속초음파, 5xFAD + 아두카누맙, 5xFAD + 집속초음파 + 아두카누맙, 대조군 총 5개 그룹으로 나누어 진행하였다. 집속초음파는 뇌 내 해마 영역의 4개 부위를 조사하였으며, 치료는 2주 간격으로 총 3번 실시하였다. 인지기능 회복을 조사하기위해 Y자 미로 테스트를 수행하였으며, 아밀로이드 플라크와 신경발생 변화를 조사하기 위해 면역조직화학염색을 수행하였다. 실험결과 집속초음파와 아두카누맙을 병합치료한 그룹에서 유의하게 인지기능이 회복되고, 아밀로이드 플라크가 감소하며, 신경발생이 유도되는 효과를 확인하였다. 또한 단회 치료보다 반복 치료했을 경우 인지기능 회복이 더 증가하였다.

본 연구에서는 집속초음파를 통해 뇌 내 투과율을 증가시킴으로써

치료제를 적은 용량으로 효과를 극대화 시킬 수 있게 하며, 부작용을 최소화할 수 있다는 장점을 제시한다. 또한 뇌혈관장벽에 의해 사용이 제한되는 항체치료제의 가능성을 보여주었다는 점에서 매우 의미 있는 연구 결과라고 생각한다. 본 연구에서 제시하는 새로운 치료전략이 알츠하이머병 치료에 크게 기여할 수 있기를 기대하며, 또 더 나아가 다양한 퇴행성 뇌질환 치료에 확대 적용할 수 있을 것이라 생각한다.

핵심되는 말: 알츠하이머병, 집속초음파, 뇌혈관장벽, 베타아밀로이드, 아두카누맙

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