Biochemical and Biophysical Research Communications 525 (2020) 192–196

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Disrupted blood-brain barrier in 5×FAD mouse model of Alzheimer's disease can be mimicked and repaired *in vitro* with neural stem cell-derived exosomes

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ARTICLE INFO

Article history:
Received 17 January 2020
Accepted 10 February 2020
Available online 18 February 2020

Keywords:
Alzheimer's disease
Blood-brain barrier
5×FAD
Exosomes
Neural stem cells
iPSC

ABSTRACT

Alzheimer's disease (AD) is a devastating neurodegenerative disease and is associated with blood-brain barrier (BBB) disruption. AD mice and cell culture models play an essential role in understanding AD pathogenesis and validation of therapeutic reagents. One of the commonly used AD mice is the 5 × FAD mouse and previous studies have shown that BBB leakage occurs at 9 months of age in the mice. However, it remains unknown whether disrupted BBB also occurs in young animals and whether AD-caused BBB impairment can be replicated and further corrected in a cell culture model. Here, we examine BBB breakdown in the 5 × FAD mouse model at different ages including both pre-symptomatic and post-symptomatic ages and test an *in vitro* BBB model established with the 5 × FAD primary cerebral endothelial cells. Moreover, with the BBB *in vitro* model, we also examined the therapeutic effect of human neural stem cells (NSCs)-derived exosomes on AD-caused BBB leakage. Our result indicated that BBB breakdown in the 5 × FAD mice occurred at 4 months of age, which could be mimicked with an *in vitro* BBB model. Importantly, we further demonstrated that treatment of the *in vitro* BBB model with NSCs-derived exosomes reversed AD-caused BBB deficiency. The information should be useful for researchers to determine which ages of the AD mice should be employed in specific *in vivo* and *in vitro* studies and the data also suggest that AD-caused BBB disruption can be corrected at least by NSC-derived exosomes.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia. AD is associated with impaired blood-brain barrier (BBB) [1], increased oxidative stress and neuroinflammation [2], mitochondria dysfunction [3] and impaired proteostasis [4], resulting in accumulation of misfolded proteins including senile (Aβ) plaques and neurofibrillary tangles in the brain. Indeed, BBB dysfunction has been causally linked to AD pathogenesis [5]. However, the mechanisms underlying early BBB disruption in AD remain elusive, and to date, there are few therapeutic options to treat AD-induced BBB leakage effectively.

The BBB is a highly specialized endothelial cell (EC) membrane that lines cerebral microvessels, representing the interface between neural cells and circulating cells of the immune system [1]. The intact barrier of EC is essential for normal blood vessel and brain function. However, accumulating data have suggested that BBB is impaired in both AD patients and AD mouse models. In AD mouse studies, impaired BBB has been shown by capillary leakages of blood-derived fibrinogen, IgG, albumin and intravenously administrated substances, such as Evans blue [6].

The 5 × FAD mouse model has increasingly been using in various AD studies due to its relatively earlier appearance of AD related cognitive dysfunction and neuropathological alterations [7], when compared to other AD mouse models. Previous data have shown that BBB is disrupted in this mouse model at least at 9–10 months of age [8], while the neurodegenerative and behavioral changes occur at 4–5 months in 5 × FAD mice [7]. It remains unknown whether BBB leakage in the AD mouse occur at an early age, such as before 9 months. In this study, we examine BBB breakdown in 5 × FAD mice at both pre-symptomatic and post-symptomatic

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https://doi.org/10.1016/j.bbrc.2020.02.074
0006-291X/

ages and also test an *in vitro* BBB model derived from the 5 × FAD primary ECs. Moreover, with the BBB *in vitro* model, we examined the therapeutic effect of human neural stem cells (NSCs)-derived exosomes on treating AD-caused BBB leakage.

2. Materials and methods

2.1. Animals

The 5 × FAD mouse and the wild-type (WT) mouse breeding pairs were on a B6SJLF1/J genetic background and were purchased from Jackson laboratory. Mice were housed in a standard light/dark cycle. All experimental procedures involved in using animals were approved by the Institutional Animal Care and Use Committee at the University of South Dakota and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Analysis of BBB permeability in vivo

For evaluation of BBB permeability, the intraperitoneal injections of sodium fluorescein (NaFl, Sigma Aldrich) 10 mg in 0.1 ml sterile saline and Evans blue (Sigma Aldrich) 800 μl of 1% (w/v) were used [9]. Cardiac blood was collected immediately before perfusion into heparin coated tubes. The serum was stored at −80 °C until processing. Mice were perfused with PBS and the brain were collected and stored at −80 °C until processing. For NaFl analysis, protein was precipitated from serum samples with 1:3 trichloroacetic acid (20% TCA). Brain samples were first centrifuged at 1000×*g* for 5 min, after which the resulting supernatant was diluted 1:10 in 20% TCA. All samples were centrifuged at 10,000×*g* for 20 min to remove precipitated protein. The supernatant was removed and diluted with equal volumes of borate buffer (0.05 M, pH 10), resulting in a final concentration of 10% TCA and 0.025 M borate buffer. Samples were analyzed on a 2030 multilabel reader (PerkinElmer Life and Analytical Sciences) using an excitation wavelength of 485 nm, and emission wavelength at 535 nm. Evans blue test was performed according to published methodology [10]. Briefly, both brain and serum samples were precipitated in 50% TCA and the clarified lysates diluted 1:3 in 95% ethanol with readings at 620 nm excitation/680 nm emission.

2.3. Primary culture of murine brain microvascular ECs

Isolation of primary brain microvascular ECs was performed according to the published protocol with little modification [11]. Briefly, 10 adult AD or WT mice at either 2–3 months or 5 months of age were sacrificed. The meninges-free forebrains were transferred to a 50 ml Falcon tube filled with 13.5 ml of DMEM. The tissues were digested with a mixture of 0.6 ml collagenase CLS2 (10 mg/ml in DMEM) and supplement with 0.2 mg DNAse for 1 h at 37 °C on an orbital shaker at 180 rpm. Tissue suspensions were centrifuged at 1000×*g* for 10 min at 4 °C. BSA-DMEM (20% w/v) was used to remove myelin. The pellet was add in 9 ml DMEM and 1 ml collagenase/dispase mixed with 0.1 mg DNAse and then digested for 1 h at 37 °C on an orbital shaker at 180 rpm. The digested cell suspensions were centrifuged at 1000×*g* for 10 min at 4 °C and resuspended the pellet in 2 ml of DMEM followed by Percoll gradient centrifugation. Collecting about 12 ml of the interphase with cells was made, which was then centrifuged at 1000×*g* for 10 min at 4 °C. This was followed by resuspending cells in endothelial cell medium and seeding the cells in Collagen IV coated cell culture plates (0.2 ml of medium per 1 cm^2 surface) or in permeable cell culture inserts (0.8 × 10^3/well, CELLTREAT Scientific Products). Cells were incubated at 37 °C and 5% CO_2 incubator. Medium were changed every two to three days.

2.4. Isolation of exosomes from iPSCs-derived neural stem cell cultures

WT NSCs derived from human iPSCs [12] were cultured in neural progenitor cell medium (STEMCell Technologies). The cell culture media were changed every other day. Exosomes from culture medium were collected according to published protocol [13]. Briefly, the culture medium was collected by centrifugation at 300×*g* for 5min. Supernatants were collected and centrifuged first at 1500 rpm (300×*g*) for 15 min and then at 2500 rpm (1000×*g*) for 15 min to remove cellular debris. Exosomes were collected by ultracentrifugation at 100,000×*g* for 70 min at 4 °C using a Beckman SW41Ti Rotor. Pellets were washed with PBS and subjected to an additional centrifugation at 100,000×*g* for 1 h at 4 °C. Exosomes were aliquoted and stored at −80 °C until use.

2.5. EC treatments and in vitro BBB permeability assay

The culture inserts were incubated with 30 μg/ml exosomes for 48 h. The permeability was assessed using a fluorescent substance, sodium fluorescein (NaFl, MW = 376.27 g/mol, Sigma-Aldrich), according to published methodology [14] with little modification. Briefly, Hank's balanced salt sodium (HBSS) transporter buffer supplemented with 10 mM HEPES containing 10 μg/mL of Na-Fl was loaded onto the apical side of the insert and incubated at 37 °C for 1h. Fluorescence was measured with a multilabel plate reader (PerkinElmer Life and Analytical Sciences) with 485 nm excitation and 535 nm emission wavelengths.

3. Results

3.1. 5 × FAD mice showed impaired BBB at 4 months of age

Previous data have demonstrated that 5 × FAD mice show cognitive deficiency at 4–5 months of age [7]. To determine whether BBB impairment in the disease animals occurs at an early age, such as before 4 months, we examined BBB permeability in AD mice at 3 months and compared with their wild-type (WT) littermates. BBB permeability in AD mice did not differ from their WT littermates at 3 months (Fig. 1A and B). At 4 months, however, AD mouse BBB permeability showed a significant increase when compared to their littermates (Fig. 1C and D). Disrupted BBB in AD mice persisted at 5 months (Fig. 1E and F). These data suggest that BBB leakage likely occurs at a pre-symptomatic age in the mouse model.

3.2. Mimicking BBB condition with the primary ECs in vitro

To determine whether a BBB cell culture model mimics the *in vivo* condition, we isolated the primary ECs from either 2–3 months or 5 months of AD and WT brains and cultured them in hanging cell culture inserts (Fig. 2A). After 5 days, the permeability of the EC monolayer on the apical side of the insert was assessed by measurement of the translocation of sodium fluorescein (NaFl) from the apical side to the basolateral side. Our results indicated that the permeability of AD EC monolayer isolated from 2 to 3 months of mouse brains did not differ from their WT littermate ECs (Fig. 2B). However, the monolayer of the ECs isolated from 5 months of AD brains revealed significantly increased permeability compared to their WT counterparts (Fig. 2C). Thus, our results indicate that the BBB cell culture model utilized here can recapitulate the *in vivo* condition, in which the ECs are derived from.

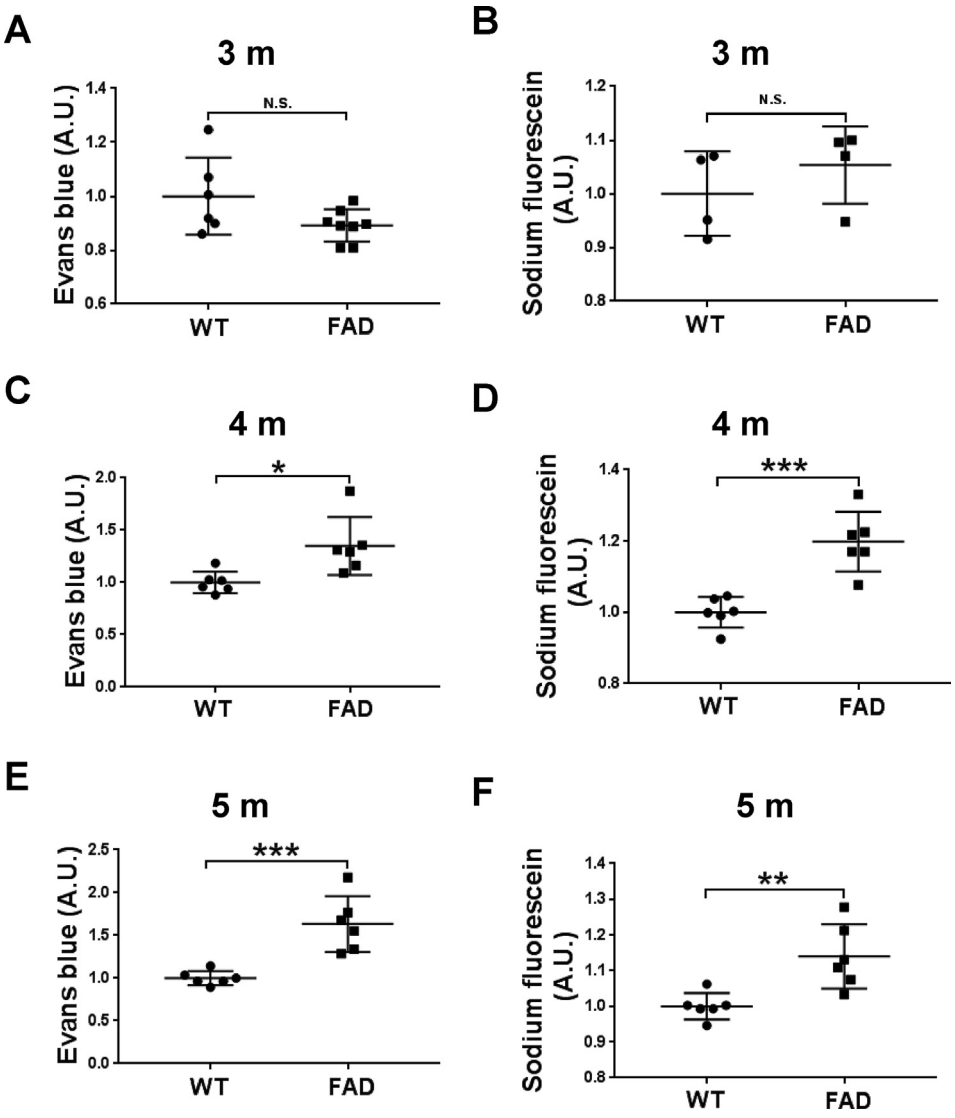

Fig. 1. 5 × FAD mice showed increased BBB permeability at 4 months. Measured Evan Blue levels in the brain at 3 (**A**), 4 (**C**) and 5 months (**E**) and NaFl levels in the brain at 3 (**B**), 4 (**D**) and 5 months (**F**). N = 4–8. Data are shown as mean ± SD. N.S., no significant difference, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3. Human NSC-derived exosomes reverse AD induced BBB deficiency in vitro

To further examine whether treatment of AD BBB with a specific reagent reduces AD induced BBB leakage, we tested the therapeutic efficacy of the exosomes isolated from the human NSC cultures induced from the WT iPSCs [12]. Incubation of ECs with NSC-derived exosomes in the BBB cell culture system did not alter the permeability of AD ECs isolated from young mouse (2–3 months) brains (Fig. 3A). For the EC monolayer isolated from those 5 months of mice, AD cells showed the similar permeability as their WT counterparts in the *in vitro* BBB permeability assay (Fig. 3B), suggesting reversal of AD-caused BBB deficiency by the NSC-derived exosome treatment.

4. Discussion

Mouse models of AD play an important role in understanding the pathogenesis of the disease and in validation of therapeutic strategies and reagents. Owing to their relatively early onset [7], 5 × FAD mice have been widely utilized in various studies. However, previous studies did not address when the mouse model starts to show disrupted BBB. Here, we demonstrated that 5 × FAD mice showed increased BBB permeability at 4 months, the earliest age when BBB impairment occurred. Moreover, we also showed that an *in vitro* BBB model established with the primary ECs isolated from the AD or WT mouse brains were able to mimic their *in vivo* permeability conditions. Importantly, we further demonstrated that treatment of the *in vitro* BBB model with NSCs-derived exosomes reverse AD-caused BBB deficiency. These data should be useful in guiding investigators to use appropriate ages of mice in specific experiments. Our results also suggest that AD-caused BBB disruption can be corrected at least by NSC-derived exosomes.

As 5 × FAD mice show reduced cognitive performance between 4 and 5 months [7] and our data reveal impaired BBB in the mice at 4 months, these data suggest that BBB leakage may precede cognitive decline in AD mice. This is in agreement with previous observations from a study with human subjects that disrupted BBB is associated with more rapid cognitive decline [15]. We cannot exclude the possibility that increased BBB permeability may occur between 3 and 4 months, which is associated with later cognitive decline and pathological alterations as previously described [7,8].

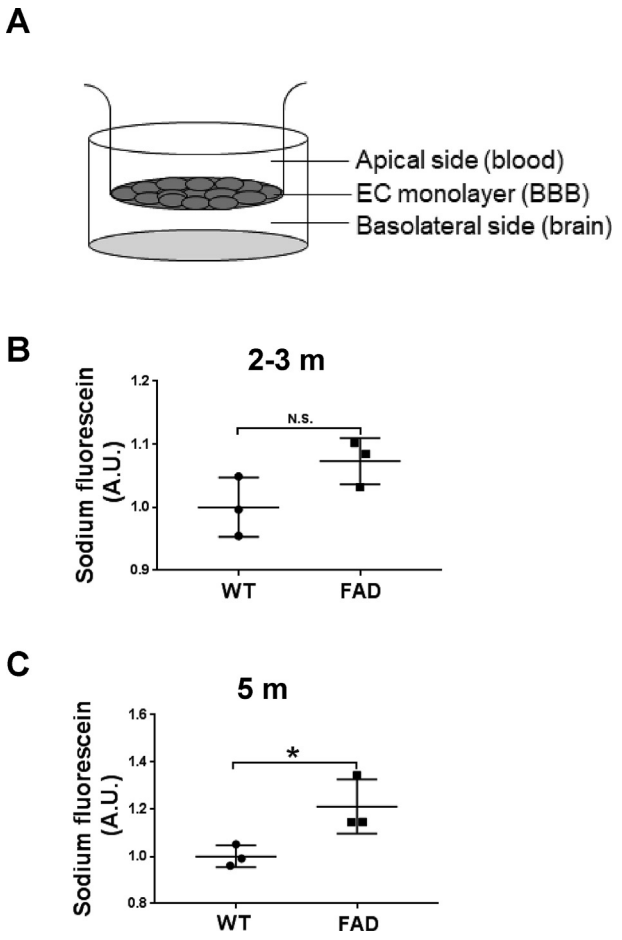

Fig. 2. Mimicking BBB permeability condition with the primary ECs *in vitro*. ***A.*** *Schematic illustration of an in vitro model of BBB with the primary cerebrovascular ECs of mice.* ***B & C.****Measured sodium fluorescein levels from the culture system derived from 2-3 (**B**) or 5 months (**C**) of mice. N = 3 for each group. Data are shown as mean ± SD. N.S., no significant difference, * p < 0.05.*

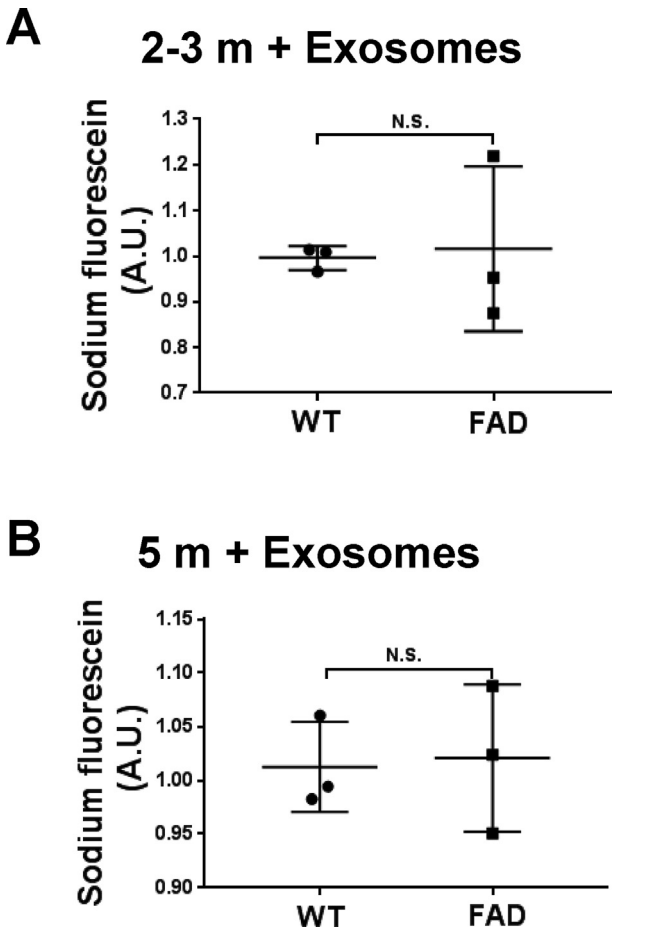

Fig. 3. Reversal of AD-caused BBB deficiency with NSC-derived exosomes *in vitro*. Measured sodium fluorescein levels from the culture system derived from 2 to 3 months (**B**) or 5 months (**C**) of mouse ECs. N = 3 in each group. Data are shown as mean ± SD. N.S., no significant difference.

Another interesting finding from this study was that the permeability of mouse primary EC cells in the BBB cell culture model reliably recapitulated the cells' *in vivo* permeability condition. Similar to the 3 months' AD mouse BBB, the permeability of the ECs derived from the 2–3 months of AD brains did not differ from that of their WT counterparts, whereas the cells derived from 5 months showed increased permeability compared to the control cells. More importantly, AD-caused BBB deficiency can be corrected following treatment of ECs with NSC-derived exosomes. This is in agreement with previous studies that NSC-derived exosomes enhance angiogenesis and functional recovery following spinal cord injury [16]. Thus, our data support that NSC-derived exosomes are a therapeutic reagent in effectively treating AD. Since the NSCs we used were differentiated from the iPSCs reprogramed from human dermal fibroblasts [12], this should have significant application in personalized medicine and regenerative medicine.

In summary, we defined BBB breakdown in the 5 × FAD mice occurring at 4 months of age, which can be mimicked with an *in vitro* BBB model using the primary ECs isolated from the AD or WT mouse brains. Importantly, we further demonstrated that the treatment of the *in vitro* BBB model with NSCs-derived exosomes reversed AD-caused BBB deficiency. These data provide useful information to determine which ages of AD mice should be selected in specific *in vivo* and *in vitro* studies, and they also suggest that AD-caused BBB disruption can be corrected at least by NSC-derived exosomes.

Declaration of competing interest

The authors declare no competing financial interests.

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