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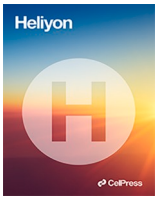

Research article

An easy-to-perform method for microvessel isolation and primary brain endothelial cell culture to study Alzheimer's disease

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ABSTRACT

Dysfunction of the blood-brain barrier (BBB) has been increasingly recognised as a critical early event in Alzheimer's disease (AD) pathophysiology. Central to this mechanism is the impaired function of brain endothelial cells (BECs), the primary structural constituents of the BBB, the study of which is imperative for understanding AD pathophysiology. However, the published methods to isolate BECs are time-consuming and have a low success rate. Here, we developed a rapid and streamlined protocol for BEC isolation without using transgenic reporters, flow cytometry, and magnetic beads, which are essential for existing methods. Using this novel protocol, we isolated high-purity BECs from cell clusters of cortical microvessels from wild-type and $APP_{swe}/PS1_{dE9}$ (APP/PS1, a classical AD model) mice at 2, 4 and 9 months of age. Reduced levels of tight junction proteins Claudin-5 and Zonula Occludens-1, as well as glucose transporter 1, were observed in the isolated cortical microvessels from APP/PS1 mice and amyloid-β (Aβ) oligomer-treated BECs from wild-type mice. *Trans*-well permeability assay showed increased FITC-dextran leakage in BECs treated with Aβ, suggesting impaired BBB permeability. BECs obtained using our novel protocol can undergo various experimental analyses, including immunofluorescence staining, western blotting, real-time PCR, and *trans*-well permeability assay. In conclusion, our novel protocol represents a reliable and valuable tool for *in vitro* modelling BBB to study AD-related mechanisms and develop targeted therapeutic strategies.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder characterised by progressive cognitive decline and memory loss [1,2]. The hallmarks of AD are the deposition of amyloid-β (Aβ) plaques and tau hyperphosphorylation caused neurofibrillary tangles in affected brains [3–5]. Notably, the blood-brain barrier (BBB) dysfunction has been increasingly recognised as an early event in AD pathogenesis [6–9]. The BBB, a highly selective semipermeable barrier between peripheral blood and the central nervous system (CNS), plays a key role in maintaining the environmental homeostasis of the CNS by preventing pathogen invasion and regulating substance transportation [10,11]. BBB dysfunction can increase its permeability and lose its selectivity, which results in oxidative

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stress and neuroinflammation due to the entry of substances that otherwise would not access the CNS. All these stress responses can promote Aβ accumulation, leading to AD pathogenesis [12–16]. The elimination of Aβ from brain parenchyma can also be impaired due to decreased low-density lipoprotein receptor-related protein on the brain endothelial cells (BECs) lining the BBB [17–19]. Therefore, understanding the mechanisms underlying BBB dysfunction holds the promise for developing novel therapeutic approaches for treating and preventing AD.

In the literature, investigations into BBB permeability have traditionally been facilitated by endogenous biomarkers and exogenous indicators [20]. Moreover, isolating viable microvessels from the rodent brain has made *in vitro* BBB modelling feasible, allowing controlled and reproducible conditions to study the molecular mechanisms and explore potential therapeutic strategies for CNS conditions [21,22]. BECs are one of the principal architectural elements of the BBB. They are elaborately connected by tight junctions and adherens junctions, enveloped by parenchymal basement membranes, pericytes, and astrocytic endfeet to form a barrier between blood and CNS [23,24]. BECs possess a distinctive cytoplasm of uniform thickness, with a limited number of pinocytotic vesicles and a notable absence of fenestrations. They contain a significant amount of mitochondria, providing ample energy potential, and exhibit an enzymatic barrier with luminal and abluminal polarisation. These unique structural and functional properties set BECs apart from peripheral endothelium, enabling them to actively manage the transportation and metabolism of nutrients and drugs [25–27].

Dysfunctional BECs, particularly those with abnormal levels of tight junction proteins, such as Claudin (Cldn)5, Cldn12, occludin, and Zonula Occludens (Zo)1, can disrupt BBB integrity, which has been implicated in the pathological progression of AD [28,29]. Decreased Cldn5 and Cldn12 mRNA expression in the choroid plexus and elevated BBB permeability in the hippocampus have been linked to cognitive impairment in a rat model of AD [30]. Conversely, in a mouse model of AD, increasing occludin levels can improve BBB integrity [31]. Therefore, tight junction integrity, regulated by Zo1, plays a key role in maintaining BBB permeability [32,33]. The insulin-independent glucose transporter (Glut)1 is responsible for glucose uptake in BECs. In the brains of AD mice, Glut1 levels are reduced, which is associated with reduced cellular glucose supply, BBB dysfunction, and early-stage cerebral microvessel degeneration [34]. The activation of the Wnt/β-catenin signalling pathway can increase the levels of tight junction protein and Glut1 in BECs, which plays a key role in initiating BBB development during embryonic and postnatal development and later maintaining BBB characteristics [35,36]. Additionally, the Wnt/Planar Cell Polarity pathway regulates tight junction integrity and apicobasal polarity of BECs [37].

Given the compelling evidence linking abnormal changes in tight junction protein to BBB function and AD pathophysiology, isolated microvessels (containing BECs clusters) and primary BEC from the microvessels are ideal option for performing functional and mechanistic studies. This approach is challenging with $APP_{swe}/PS1_{dE9}$ (APP/PS1) mice, especially those with aged brains, due to reduced tissue integrity compared with young and healthy brains. Therefore, here we aimed to develop a new protocol, which can make it easier to isolate microvessels and obtain BECs from both wild-type and APP/PS1 mice in order to aid the *in vitro* investigation of the complex role of tight junction proteins in increased BBB permeability during AD pathogenesis. It may pave the way for novel therapeutic interventions targeting BBB dysfunction in this devastating neurodegenerative disorder.

2. Materials and methods

2.1. Animals

The animal study was approved by the Institutional Animal Care and Use Committee, Shenzhen Bay Laboratory (Approval# IACUC- AEYCJ202202) and followed the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Mixed-sex C57BL/6J (wild type) and transgenic APP/PS1 mice (2, 4 and 9 months of age, Nanjing Junke Bioengineering Co., Ltd, China) were housed in individually ventilated cages (20 ± 2 °C, 12 h light, 12 h dark cycle, lights on at 06:00 h) with *ad libitum* access to standard laboratory rodent chow and drinking water in a PC2 facility.

2.2. Cortical microvessel isolation and primary BEC culture

Mice were humanely sacrificed via cervical dislocation in a sterile workstation, followed by the sterilisation of the neck area with 80 % ethanol. The skull was exposed, and the brain was carefully dissected and collected in ice-cold Dulbecco's PBS (D-PBS, containing no calcium or magnesium ions) supplemented with penicillin-streptomycin. The cortex was isolated and minced finely after removing the leptomeninges and undesirable brain regions, such as the cerebellum and brainstem. The minced cortex was then digested using 10 mL DMEM medium (containing 1 mg/mL collagenase and 0.1 mg/mL DNase I) via aspiration using a 10 mL pipet for at least 25 times, followed by incubation at 37 °C and shaking at 250 rpm for 1 h to facilitate tissue digestion (orbital shaker-incubator, Labgic, Beijing, China). After stopping the digestion using 10 mL DMEM, microvessels containing BECs were isolated by centrifugation (1000 g for 8 min at room temperature). The pellet was suspended in 25 mL of DMEM medium containing 20 % BSA and centrifugated at 1000 g for 20 min at 4 °C, resulting in a layered solution containing myelin, BSA, small vessels and big vessels arranged from top to bottom. The resulting whitish-red microvessel pellet from mice was at the bottom of the tube, which can be used for protein and mRNA analysis. To further obtain BECs, the microvessel pellet was suspended with 5 mL DMEM (for up to 5 brains) containing 1 mg/mL collagenase-dispase and 0.1 mg/mL DNase I and shaken at 250 rpm for 45 min at 37 °C. An additional 10 mL DMEM medium was added to the raspberry-coloured homogenate and centrifuged (1000 g) at 4 °C for 6 min. The resulting pellet was resuspended in endothelial cell culture medium (80 % DMEM + 20 % FBS + 1 % Pen-strep + basic fibroblast growth factor (bFGF, 10 ng/mL final concentration, add freshly during medium change)) into BEC clusters (consisting of 5–7 BECs) and seeded on collagen type IV coated plates. Additionally, the initial culture medium was supplemented with puromycin to enhance BEC purity, which was subsequently replaced with a regular endothelial cell medium after 24 h. BECs started to proliferate from these cell clusters after 24 h. The details of reagent sources and

maintenance methods are provided in Table 1.

This comprehensive method facilitated the successful isolation of microvessels, especially aged mice, and the acquisition of high-purity primary BECs from these microvessel BEC clusters for *in vitro* studies. The high purity and optimal cell status of BECs enable us to conduct several experiments, including immunofluorescence staining, western blotting, real-time PCR, Aβ oligomer treatment, as well as *in vitro trans*-well permeability assay. Additionally, BECs exhibit characteristics akin to the BBB, rendering them suitable for functional and mechanistic investigations into BBB dysfunction in the pathological processes of AD [38]. Therefore, these BECs served as an *in vitro* model to verify the factors underlying dysfunction during AD pathogenesis, addressing the objectives of this study.

2.3. Immunofluorescence staining

After fixation with 4 % paraformaldehyde for 15 min, BECs on coverslips were incubated with a blocking buffer (PBS containing 2 % BSA and 0.5 % Triton-X100) for 30 min, followed by primary antibodies overnight at 4 °C and corresponding Alexa Fluor conjugated secondary IgG antibodies (1:1000, Thermo Fisher Scientific) for 1 h at room temperature. After washing and mounting, fluorescence images were captured using a slice scanner VS200 (Olympus). Three independent experiments were performed in triplicate for the quantitative analysis. Five non-overlapping regions of interest were analysed and averaged for each coverslip. The fluorescence intensity was analysed by Fiji software, standardised by DAPI positive cell number in each region, and then normalised to the control group. Primary antibodies included: CD31 (1:200, rat, 553370, BD), Cldn5 (1:100, rabbit, 35–2500, Invitrogen), Glut1 (1:200, rabbit, HPA031345, Sigma), CD13 (1:200, rabbit, ab108310, Abcam), IBA1 (1:1000, goat, ab5076, Abcam), GFAP (1:400, rabbit, ab7260, Abcam), and Zo1 (1:200, mouse, 339100, Invitrogen).

2.4. Protein extraction and western blotting

Sodium dodecyl sulphate (SDS) lysis buffer containing a protease inhibitor cocktail (Nacalai Tesque) and phenylmethylsulphonyl fluoride (2 mM) was used to lysate cells for protein extraction. Enhanced BCA Protein Assay Kit (Beyotime) was used to quantify protein concentration. Then, the protein samples were denatured at 95 °C for 10 min, separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked and incubated overnight with primary antibodies (Cldn5 (1:1000, rabbit, AF5216, Affinity), Glut1 (1:1000, rabbit, ab115730, Abcam) and HRP-Conjugated GAPDH (1:10000, HRP-60004, Proteintech)) at 4 °C. After incubation with secondary antibodies, the bands of interest were detected with SuperSignal West Atto Ultimate Sensitivity Substrate (Thermo Fisher Scientific, A38555) by ChemiDoc MP Imaging System (Bio-Rad, USA). The band densities were measured using Fiji software. The densities of proteins were normalised to GAPDH and standardised by the control group.

2.5. RNA extraction and real-time PCR

Total RNA was isolated by RNAzol reagent (R4533, Sigma-Aldrich). Reverse transcription was performed to obtain cDNA using the Evo M-MLV reverse transcriptase kit (AG11706, Accurate Biology) according to the manufacturer's protocol. Real-time PCR was performed using SYBR Green Premix Pro Taq HS qPCR Kit (AG11701, Accurate Biology) and SYBR Green primers (Table 2). The mRNA expression of genes of interest was calculated using the 2ˆ−ΔΔCt method with *Gapdh* as a housekeeping gene and standardised by the control group.

Table 1
Reagents and preparation used in this study.

Reagents	Company	Cat. No.	Final concentration	Storage	Preparation media
Dulbecco's PBS (D-PBS)	Hyclone	SH30028.01B	1 ×	15–30 °C	–
DMEM	Thermo Fisher Scientific	C11995500BT	–	4 °C	–
penicillin-streptomycin	Shanghai BasalMedia	S110JV	1 %	−30 ~ −5 °C	D-PBS/DMEM
collagenase	Sigma	C6885-100 MG	1 mg/mL	In 1 mL DMEM and 10 mg BSA at −20 °C	DMEM
DNase I	Sigma	DN25-1G	0.1 mg/mL	−20 °C	DMEM
BSA	Sangon Biotech	A600332-0100	20 %	In DMEM at −20 °C	–
Collagenase-dispase	Sigma	10269638001	1 mg/mL	In DMEM	DMEM
FBS	bio-channel	BC-SE-FBS01C	20 %	−20 °C	DMEM
Basic fibroblast growth factor (bFGF)	Sangon Biotech	C610029	10 ng/mL	In sterile ddH2O containing 0.1 % BSA at −20 °C	D-20
collagen type IV	Sigma	C5533	6~10 μg/cm^2	In sterilized ddH2O at −20 °C	ddH2O
puromycin	Sangon Biotech	A610593	3 μg/mL	−20 °C	D-20
D-20	–	–	80 % DMEM + 20 % FBS + 1% Pen-strep	4 °C	–

Table 2
SYBR green primers used for real-time PCR.

Gene Symbol	Forward primer (5->3)	Reverse primer (5->3)
Cldn5	ATGTCGTGCGTGGTGCAGAGT	GCGCCGGTCAAGGTAACAAAG
Slc2a1(*Glut1*)	CACTGGTGTCATCAACGCCC	CACGGAGAGAGACCAAAGCG
Tjp1(*Zo1*)	CGCTAAGAGCACAGCAATGG	TGGAGGTTTCCCCACTCTGA
CD31	ACGCTGGTGCTCTATGCAAG	TCAGTTGCTGCCCATTCATCA
Gapdh	CCCCAGCAAGGACACTGAGCAA	GTGGGTGCAGCGAACTTTATTGATG

2.6. Aβ oligomer treatment of the primary BECs

Synthetic $A\beta_{25-35}$ peptides (A4559, Sigma-Aldrich) were reconstituted in dimethyl sulphoxide (D2650, Sigma-Aldrich), lyophilised overnight, and stored at −80 °C for long-term storage or processed for oligomer formation. The lyophilised stock was resuspended in the cell culture medium to the desired concentration and incubated at 37 °C for 24 h to form the oligomers before the experiments, as described previously [39]. BECs were treated with $A\beta_{25-35}$ (20 μM) for 24 h. Each experiment was repeated at least three times independently.

2.7. In vitro trans-well permeability assay

BECs were seeded onto 24-well plates with a 0.4 μm pore polycarbonate membrane inserted in a transwell configuration. Following the desired treatment for each experimental group, BECs underwent a permeability assay. In the upper chamber, 150 μL of 1 mg/mL FITC-dextran (40 kDa) was added, while the bottom well was replenished with 600 μL of fresh BECs culture media. Samples were

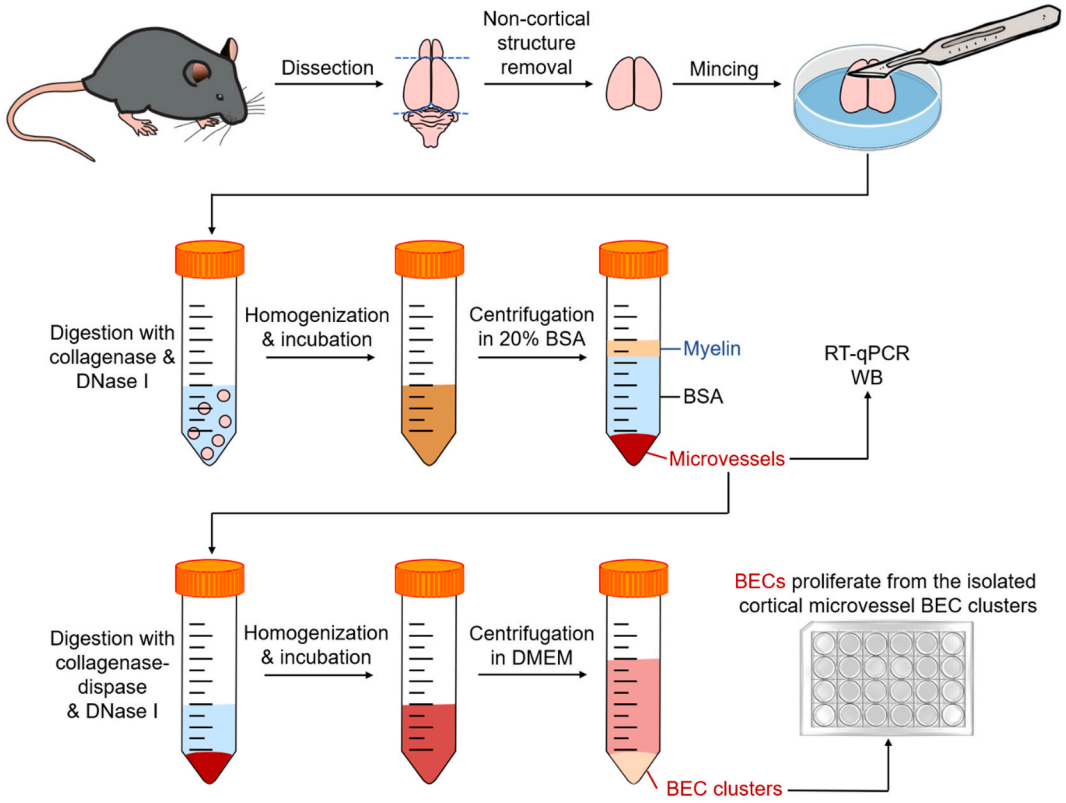

Fig. 1. Schematic overview of cortical microvessel and BEC isolation protocol. Mouse brains were dissected and rinsed in ice-cold Dulbecco's phosphate-buffered saline. The cortices were dissected, minced, and homogenised in DMEM supplemented with 1 mg/mL collagenase and 0.1 mg/mL DNase I. After incubation with shaking at 37 °C, 250 rpm for 1 h, the digestion was terminated, and the homogenate was centrifuged to obtain a pellet. This pellet was resuspended and centrifuged (1000 g) in DMEM containing 20 % BSA at 4 °C for 20 min. The bottom layer, which is whitish-red, contains microvessels. Then, the microvessel pellet was homogenised in DMEM containing 1 mg/mL collagenase-dispase and 0.1 mg/mL DNase I, followed by shaking at 37 °C, 250 rpm for 45 min. After termination of digestion, the homogenate was centrifuged, and the cell pellet was resuspended as BEC clusters and seeded onto collagen IV-coated plates for further BEC proliferation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

collected from the bottom well after 30 min, and fluorescence intensities were measured using a microplate reader at excitation and emission wavelengths of 485 nm and 535 nm, respectively. The data were normalised to the control group.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The differences between the two groups were analysed by a two-tailed Student's *t*-test (GraphPad Prism 10). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Schematic overview of cortical microvessel and BEC isolation protocol

Our protocol (schematic overview in Fig. 1) can successfully isolate high purity BECs from mouse brains and is easy for any experimenter to operate. It still needs to be acknowledged that the BECs cultured *in vitro* are removed from the endogenous brain microenvironment, including blood supply and cell-to-cell communications. Deprivation of the physiological microenvironment can potentially affect the phenotype and molecular signature of BECs, thus compromising the outcomes in both physiological and pathological conditions [40,41]. Several protocols have been developed to isolate BECs from the microvasculature [42,43]. However, one caveat of such protocols is repeated enzymatic digestion to achieve single cells, which can adversely affect RNA and protein quality, hindering the detection of changes in low-abundance transcriptions, gene expression, and signalling pathway activation [44]. Therefore, it is important to assess the quality and phenotype of cortical microvessels and BECs obtained using our novel protocol. Here, we verified the changes in tight junction proteins which are a known mechanism in AD pathogenesis [45] in our isolated cortical microvessels from APP/PS1 mice of different ages and $A\beta_{25\text{-}35}$ treated BECs proliferated from cortical microvessel cell clusters of 3-month-old wild-type mice.

3.2. The high purity of BECs cultured from the isolated cortical microvessel cell clusters

The composition of cells proliferated from cortical microvessel cell clusters isolated from 3-month-old wild-type mice was validated by immunofluorescence staining of CD31, GFAP, IBA1, and CD13, which are markers for endothelium, astrocyte, microglia, and pericyte, respectively. The composition of CD31-positive BECs proliferated from cortical microvessel cell clusters was 99.46 ± 0.13 %, suggesting high purity (Fig. 2A and B). The existence of other brain cell components, including pericytes, astrocytes and microglia, are too minor (0.54 %) to require additional isolation steps, which are usually performed by enzymatic digestion methods followed by flowcytometry or fluorescence-activated cell sorting (FACS) using strain-specific reporters [46]. Therefore, our protocol can obtain BECs with good purity.

3.3. Destruction of the cortical microvessel integrity in AD model mice

The tight junctional protein Cldn5 and glucose transporter Glut1 are vital to the stable structure and function of BBB [29,34]. The protein levels of Cldn5 and Glut1 were significantly reduced in cortical microvessels from APP/PS1 mice at the age of 4 months and further reduced at the age of 9 months, but the Cldn5 and Glut1 protein levels in cortical microvessels from 2-month-old APP/PS1 mice remained similar to age-matched wild type mice (Fig. 3A–C). Zo1 is important for maintaining BBB permeability by regulating the tight junction integrity of the endothelium, which was measured by its surface marker CD31 [32, 33]. The mRNA expression of Cldn5, Glut1, and Zo1 was significantly reduced in the cortical microvessels from APP/PS1 mice at the age of 4 months and further reduced at the age of 9 months, while the CD31 mRNA expression remained similar between the WT and APP/PS1 mice at the age of 2, 4, and 9 months (Fig. 3D–G). These results indicated that the integrity of the cortical microvessels from AD mice is impaired, which is consistent with the results of the *in vivo* study [45].

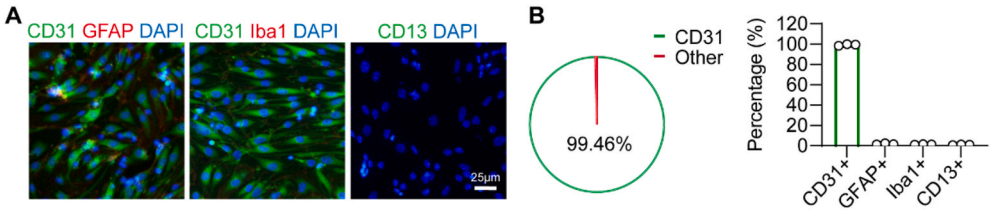

Fig. 2. High purity of BECs proliferated from the isolated cortical microvessel BEC clusters. (A) Representative immunostaining images of the endothelium (CD31), astrocyte (GFAP), microglia (Iba1) and pericyte (CD13) in the primary BECs from 3-month-old wild-type mice. Scale bar = 25 μm. (B) Percentage of CD31, GFAP, IBA1, and CD13 positive cells in the primary BECs. Data are expressed as mean ± SEM, $n = 3$ independent experiments.

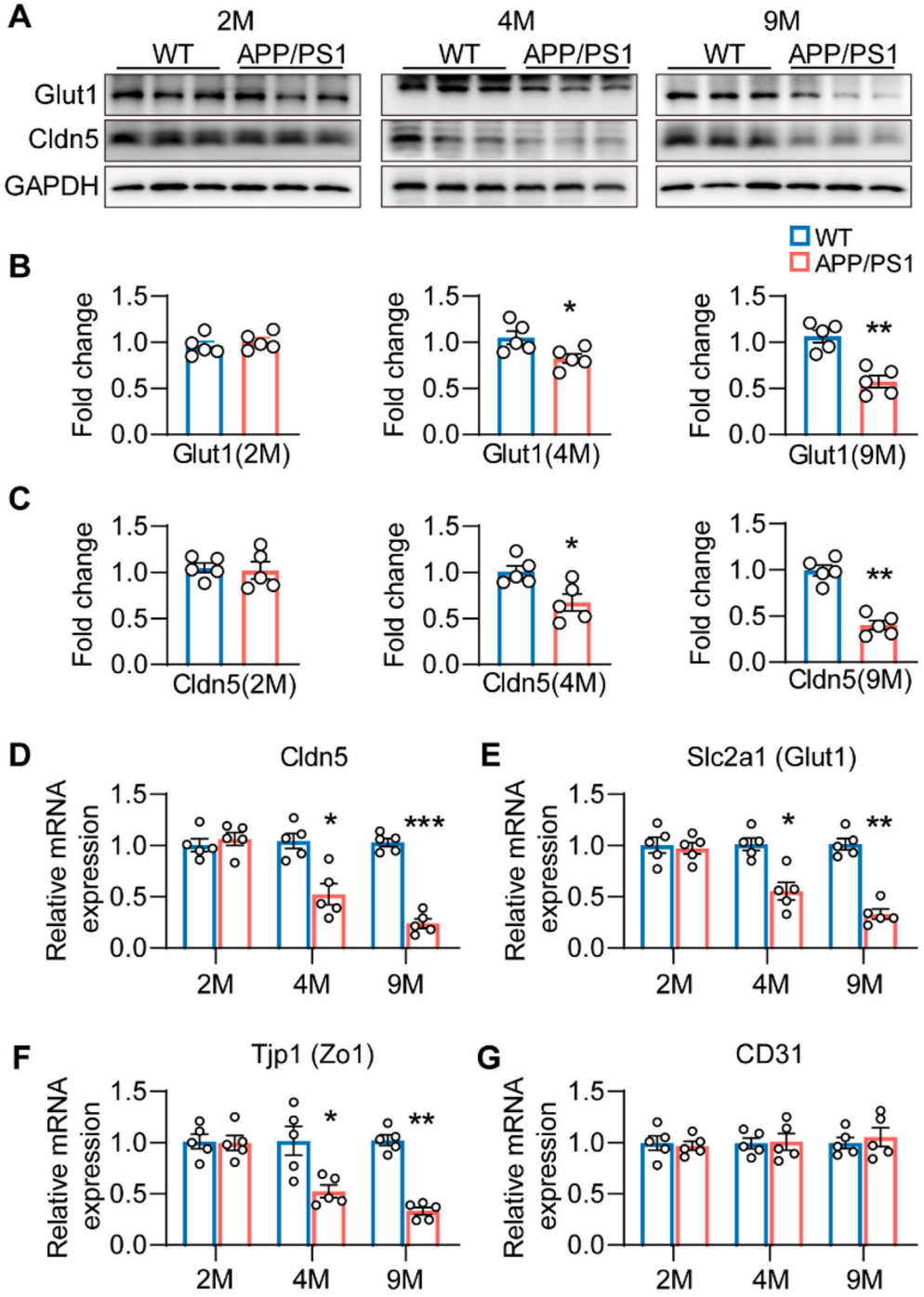

Fig. 3. Disruption of the cortical microvessel integrity in AD model mice. (A-C) Representative images and quantification of western blotting analysis of Cldn5, Glut1, and GAPDH proteins in isolated cortical microvessels from 2-, 4- and 9-month-old wild-type (WT) and APP/PS1 mice. (D–G) mRNA expression of Cldn5, Glut1, Zo1, and CD31 in isolated cortical microvessels from 2-, 4- and 9-month-old WT and APP/PS1 mice. Data are expressed as mean ± SEM, $n = 5$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs WT.

3.4. Aβ oligomer treatment impaired the barrier integrity of BECs

Our previous study showed that BBB and BECs were dysfunctional in APP/PS1 mice starting at 4 months of age when soluble Aβ oligomers start to assemble into fibrillar Aβ plaques [47]. $A\beta_{25\text{-}35}$ can also reduce BEC viability and increase the production of reactive oxygen species (ROS) and BBB permeability by altering functional proteins *in vitro* [48]. Here, after BEC proliferation from cortical

microvessel cell clusters reached 100 % confluency, they were treated with $A\beta_{25\text{-}35}$ for 24 h to model Aβ pathology. After $A\beta_{25\text{-}35}$ exposure, Cldn5, Zo1, and Glut1 were significantly decreased (Fig. 4A–B). mRNA levels of these proteins also followed a similar trend (Fig. 4C). FITC-dextran leakage in the bottom wells was increased in the $A\beta_{25\text{-}35}$ treated cells, suggesting that $A\beta_{25\text{-}35}$ impaired the barrier integrity of BECs (Fig. 4D). Collectively, our results suggested that Aβ oligomer treatment impaired the structural and functional integrity of isolated BECs *in vitro*.

4. Discussion

Endothelial cells comprise only ~ 1–2% of a tissue cell component, surrounded by various other vascular cell types and extracellular matrix, which make it challenging to isolate and purify BECs. Therefore, existing methods in the literature are time-consuming and require multiple steps of enzymatic digestions, centrifugation, and cell screening to improve the purity [49,50], which may affect the phenotype of isolated BECs. Here, we established a new protocol to isolate microvessels with BEC clusters from murine cortices, allowing further proliferation *in vitro*, which is easy to perform and can yield BECs with high purity and preserved physiological characteristics from both healthy and "diseased" mouse cortices.

While AD has been frequently modelled using toxic Aβ peptides on "healthy" BECs from young wild-type mice, directly using BECs from a mouse model of AD or wild-type rodents for *in vitro* studies has been reported previously, using different protocols from ours [51–58]; however, none has been performed using APP/PS1 mice. This may be due to the difficulties in getting abundant and viable microvessels and BECs from this strain using published protocols that do not agree with the atrophic, dysfunctional, and vulnerable BBB integrity in old mouse brains. However, BECs directly sourced from APP/PS1 mice with AD possess inherent value, as they bear the phenotype and molecular signatures of endothelial cells that are more authentic compared to those wild-type BECs treated with Aβ. We, therefore, developed and validated this protocol by modifying previously published methods [38,52], which consistently yield a substantial quantity of high-quality BECs and are straightforward to execute. The phenotype of BECs from the AD cortices is also consistent with that observed in our previous study [45], when we used tight junction proteins and Glut1 as surrogates for comparison. The isolation and culture of BECs facilitate the construction of an *in vitro* model of BBB. This model can be instrumental in the

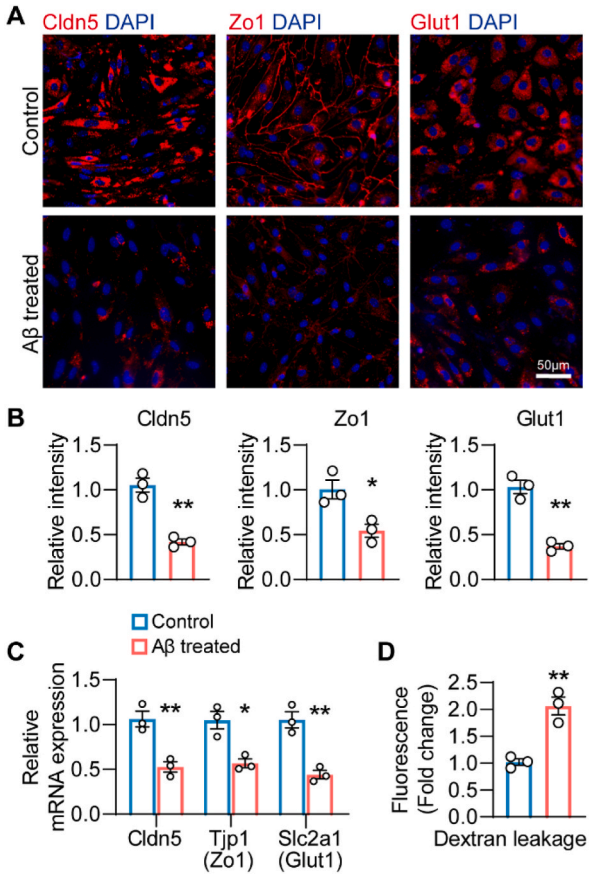

Fig. 4. Aβ oligomer treatment impaired the barrier integrity of BECs. (A–B) Representative immunostaining images and quantification of Cldn5, Zo1, and Glut1 in BECs treated by $A\beta_{25\text{-}30}$. Scale bar = 50 μm. (C) mRNA expression of *Cldn5*, *Zo1*, and *Glut1* in BECs treated by Aβ. (D) *In vitro trans*-well permeability assay in BECs treated by $A_{\beta 25\text{-}30}$. Data are shown as mean ± SEM, $n = 3$ independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group.

large-scale screening of anti-AD drugs and in conducting time-lapse studies at single-cell resolution. Such experiments are complex and expensive to carry out *in vivo*, making the *in vitro* model a valuable tool for drug discovery and mechanistic studies in AD research.

There are several advantages of our method. Firstly, there is no need for multiple steps of enzymatic digestion for our new protocols. We used enzymatic digestion during the step of isolating small BEC clusters consisting of 5–7 cells from the microvessels, which can improve the viability as well as purity of BECs obtained. This step did not compromise the phenotype and physiological responses of BECs. Such BECs can also proliferate rapidly *in vitro* to obtain sufficient numbers for different assays. In the literature, multiple enzymatic digestions have been reported to aid in the purification of BECs. However, this method can lead to alterations in cellular metabolism and activities of certain signalling pathways due to compromised RNA and protein integrity and affect the measurement of low-abundance genes [42,43]. Such changes may also affect the response of tight junction proteins and gene expression to physiological and pathological conditions in isolated BECs. Therefore, a specific molecular signature of isolated BECs, e.g. CD31, has been proposed by analysing their transcriptome and proteome profiling, which can be used as a benchmark to verify "healthy" BECs [59–61] for research on signalling pathways and pre-clinic drug testing for ischemic or inflammatory conditions [62,63]. Secondly, our method has produced BECs proliferated from microvessels with a high purity without the need to use flow cytometry and magnetic-bead separation, which are essential for published protocols [64,65] to process brains in different species, including rat [66], mouse [67], porcine [68], bovine [69] and humans [20]. Flow cytometry can compromise the integrity and temperature of the cells, potentially inducing stress or injury responses, especially in endothelial cells and vascular smooth muscle cells [70,71]. It can also affect genes related to angiogenesis pathways [72]. Regarding the magnetic bead-based cell sorting method, anti-CD31 antibody-coupled Dynabeads are commonly used [73]. However, the efficiency of eliminating non-BECs is low, which is unsuitable for BECs-focused experiments, such as BBB permeability experiments. There is no need for reporter mice for BEC isolation. Utilising such reporter mice can be both labour-intensive and expensive, especially when researchers intend to crossbreed these mice with others with diverse genetic backgrounds of different diseases.

To study BBB function, it is important to isolate capillaries rather than small arteries or veins from mouse brains. This preference stems from variations in phenotypes and functions among endothelial cells in different segments of the microvasculature. Cerebral capillary endothelial cells, as shown in freeze-fracture images, exhibit stronger tight junctions characterised by more comprehensive tight junction strands [74,75], as well as elevated levels of solute transporters including efflux transporters and specific receptors for transcytosis, such as transferrin receptors, compared to arteriolar or venular endothelial cells [76–78]. Capillary endothelial cells occupy a significantly larger surface area of the cerebral microvasculature than arteriolar or venular endothelial cells. Therefore, isolating endothelial cells from cerebral capillary fragments can produce tight monolayers that closely resemble the properties of the BBB transporting endothelium [79]. This procedure can effectively eliminate most potential contamination of other cell types, such as fibroblast-like leptomeningeal cells or arterial and arteriolar smooth muscle cells, which exhibit a faster growth rate than endothelial cells.

We must acknowledge a limitation of our protocol. The isolated BECs cannot be maintained for an extended period due to the loss of phenotype observed in aged BECs after several passages. However, we are yet to determine whether freeze-thaw cycles can also influence the phenotype of the primary BECs in future studies. Immortalisation of BECs has been reported previously [80]. However, immortalisation often involves the introduction of exogenous immortalising genes, such as telomerase and SV40 large T antigen. This process can lead to alterations in the function and expression of numerous genes and disruptions to the normal cell cycle [81], which may change cellular response to stimuli. Nevertheless, BECs isolated using our protocol are suitable for most short-term *in vitro* experiments that can be completed within days.

5. Conclusion

We have developed a rapid and streamlined protocol for isolating cortical microvessels and BECs, especially from aged AD mice, which is highly reproducible and reliable. The phenotype of isolated microvessels and BECs is feasible for *in vitro* modelling BBB for AD research and useful for various experiments, including immunofluorescence staining, western blotting, real-time PCR, Aβ oligomer treatment and *in vitro trans*-well permeability assay. Our protocol can help researchers discover new therapeutic approaches targeting endothelial cells in cerebrovascular and neurodegenerative diseases.

Ethical statement

The animal study was approved by the Institutional Animal Care and Use Committee, Shenzhen Bay Laboratory (Approval# IACUC- AEYCJ202202) and followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Availability of data and materials

All datasets are presented in the main manuscript.

CRediT authorship contribution statement

Yang Chen: Methodology, Investigation. **Xiaomin Huang:** Writing – original draft, Methodology, Investigation. **Hui Chen:** Writing – review & editing, Writing – original draft. **Chenju Yi:** Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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