

In vitro model of the blood-brain barrier established by co-culture of primary cerebral microvascular endothelial and astrocyte cells

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Abstract

Drugs for the treatment and prevention of nervous system diseases must permeate the blood-brain barrier to take effect. *In vitro* models of the blood-brain barrier are therefore important in the investigation of drug permeation mechanisms. However, to date, no unified method has been described for establishing a blood-brain barrier model. Here, we modified an *in vitro* model of the blood-brain barrier by seeding brain microvascular endothelial cells and astrocytes from newborn rats on a polyester Transwell cell culture membrane with 0.4- μ m pores, and conducted transepithelial electrical resistance measurements, leakage tests and assays for specific blood-brain barrier enzymes. We show that the permeability of our model is as low as that of the blood-brain barrier *in vivo*. Our model will be a valuable tool in the study of the mechanisms of action of neuroprotective drugs.

Key Words: nerve regeneration; blood-brain barrier; astrocytes; brain microvascular endothelial cells; permeability; co-culture; Transwell chamber; neural regeneration

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Introduction

The blood-brain barrier (BBB) is a low-permeability cell system between the central nervous system and circulating blood (Hou et al., 2014). It mainly comprises brain microvascular endothelial cells (BMECs), basement membrane, and astrocytic foot, processes. The specific structure and function of the BBB mean it is difficult to replicate in an *in vitro* model. It is therefore important to establish a model that is as close to the *in vivo* environment as possible, and can be easily replicated; however, primary culture of brain cells can be difficult. Closely-spaced low-permeability cell lines, such as the umbilical vein endothelial (ECV-304) and C6 glioma cell lines, have previously been used to simulate BBB function (Kuhlmann et al., 2008; Wang et al., 2011b). The BBB skeleton is made up of BMECs (Wang et al., 2011a), so a compact monolayer of BMECs has also been used as a model (Winter et al., 2008; Bernas et al., 2010; Patabendige et al., 2013; Stephan et al., 2013). However, although these models simulate some characteristics of the BBB, *in vivo* the BBB contains a wide variety of cell types from different sources. For example, although ECV-304 cells simulate the low permeability of the BBB, this cell type is not found in the BBB *in vivo*; similarly, the BMEC model contains only a single cell type, which inadequately

represents the structure of the BBB. Furthermore, BMECs and astrocytes in the BBB are not in direct contact, but have a space between them (Abbott, 2005; Daneman and Barres, 2005). For this reason, Transwell chambers have been developed for the culture of BMECs and astrocytes and the construction of low-permeability BBB models (Kang et al., 2013; Sun et al., 2013). In the present study, we sought to establish a simple and effective method by which to model the BBB *in vitro* using modified cell culture techniques.

Materials and Methods

Experimental animals

Newborn male and female Sprague-Dawley rats, aged 1–3 days, were obtained from the Animal Center of Anhui Medical University, China (Certification No. SXCK (Wan) 2011-002). All rats were housed at 18–26°C and 40–70% humidity under a 12 hour light-dark cycle. All animal procedures were overseen and approved by the Animal Center of Anhui University of Chinese Medicine in China. Every effort was made to minimize animal suffering.

Primary culture and identification of astrocytes

Five rat pups were decapitated and disinfected in 75% ethanol

for 3–5 minutes. The whole brain was dissected out and put into precooled D-Hanks' solution, and the meninges and blood vessels were carefully removed on ice. The cerebral cortex was separated and washed three times in D-Hanks' solution before being cut into blocks (1 mm³) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA). The tissue was digested in 0.25% trypsin (Sigma, St. Louis, MO, USA) and filtered through a 200-mesh screen. The filtrate was centrifuged at 140 × g for 10 minutes and the supernatant discarded. The cells were resuspended in DMEM, and placed in a 75-cm² disposable culture bottle. To aid in the removal of fibroblasts by the differential adhesion method (Ki et al., 2010; Aremu et al., 2011; Tanahashi et al., 2012), the cell suspension was removed and placed in another bottle, and cultured at 37°C and 5% CO₂. The DMEM was then replaced every 3 days, and the cells were observed under an inverted microscope (Olympus, Tokyo, Japan) (Anderson and Swanson, 2000; Schmuck et al., 2002; Schousboe and Waagepetersen, 2005; Jayakumar et al., 2006; Sun et al., 2007; Pan et al., 2012; Yin et al., 2012; Zeng et al., 2013).

Astrocytes were selected at different growth stages, rinsed with PBS three times for 5 minutes each, fixed in 4% paraformaldehyde for 20 minutes, rinsed in PBS as before, and blocked in normal goat serum for 20 minutes at 37°C. The serum was discarded, and residual moisture was absorbed using filter paper. Mouse anti-rat glial fibrillary acidic protein (GFAP) monoclonal antibody (1:100; Beyotime Institute of Biotechnology, Shanghai, China) was added to the cells and incubated for 30 minutes at room temperature. After three further PBS washes, fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG (1:100; Beyotime Institute of Biotechnology) was added and the cells were incubated for 30 minutes at room temperature. Samples were observed and photographed under a fluorescence microscope (Olympus) (Anderson and Swanson, 2000; Schmuck et al., 2002; Schousboe and Waagepetersen, 2005; Jayakumar et al., 2006; Sun et al., 2007; Pan et al., 2012; Yin et al., 2012; Zeng et al., 2013).

Primary culture and identification of BMECs

Ten rat pups were selected for cell isolation. The separation procedure was the same as that described for astrocytes. The meninx and meningeal vessels were absorbed by the filter paper. The isolated brain tissue was cut into 1-mm³ blocks in DMEM, and digested in 10 mL 0.1% type II collagenase containing 30 U/mL DNase I (Invitrogen, Carlsbad, CA, USA) for 15 minutes, and centrifuged at 140 × g for 10 minutes. The supernatant was discarded. The tissue was blocked with 20% bovine serum albumin (Sigma) and centrifuged at 1,000 × g at 4°C for 20 minutes. After removal of nerve tissue and blood vessels in the upper layer, the isolated tissue was digested with 2-mL 0.1% collagenase containing 20 U/mL DNase I (Roche, Basel, Switzerland) for 10 minutes, and centrifuged at 140 × g for 10 minutes before the supernatant was discarded. Cells were incubated with 800 mL DMEM/nutrient mixture F12 (DMEM/F12) (Hyclone, Logan, UT, USA), 4 mM L-gluta-

mine, 0.05 g heparin sodium, 1 g NaHCO₃, 75 mg endothelial cell growth supplement, and 200 mL fetal bovine serum in a 75 cm² disposable culture bottle prepared by coating with 1% gelatin at 37°C and 5% CO₂. The medium was replaced every 3 days (Fischer et al., 1999; Gaillard et al., 2000; Gomez and Reich, 2003; Shen et al., 2004; Calabria and Shusta, 2008; Demarest et al., 2012; Hsieh et al., 2012; Chen et al., 2013).

The identification method was the same as that described for astrocytes, except the primary and secondary antibodies were rabbit anti-factor VIII heavy chain polyclonal antibody (H-140) (1:500; Santa Cruz Biotechnology, Shanghai, China) and FITC-labeled goat anti-rabbit IgG (1:200; Beijing ZhongShan JinQiao Company, Beijing, China), respectively (Fischer et al., 1999; Gaillard et al., 2000; Gomez and Reich, 2003; Shen et al., 2004; Calabria and Shusta, 2008; Demarest et al., 2012; Hsieh et al., 2012; Chen et al., 2013).

Construction of *in vitro* BBB model

Transwell chamber inserts with a 0.4-μm aperture (Corning, New York, USA) were placed into volumetric flasks. Astrocytes (1 mL at 1 × 10⁶/mL) were added to the underside of the Transwell (Figure 1A) and incubated at 37°C and 5% CO₂ for 24 hours. The chambers were then placed carefully into a 6-well plate (Corning) (Figure 1B). When the astrocytes reached 60% confluence under an inverted microscope, BMECs (1 × 10⁷/mL) were seeded on top of the Transwell (Figure 1C) at 37°C and 5% CO₂. The cells were observed under an inverted microscope until the astrocytes and BMECs reached a high-density co-culture (Figure 1D) (Abbott et al., 2006; Ribatti et al., 2006; Kuo and Lu, 2011; Takata et al., 2013; Zhou et al., 2013).

Detection of transepithelial electrical resistance (TEER)

Permeability changes in the BBB can be detected by changes in TEER, which directly reflects the structural integrity of the BBB (Czupalla et al., 2014). We measured TEER in the co-culture model and single-layer BMEC groups at 2, 4, 8, 12, 16, 20, 24, 48, 72, and 96 hours after BMEC seeding. Measurements were adjusted for insertion of the Millicell ERS-2 Volt-Ohm Meter (Millipore, Boston, MA, USA) into 70% ethanol for 15 minutes. The samples were air-dried for 15 seconds, and washed in sterilized electrolyte solution. The short and long electrode probes were inserted vertically into the medium inside and outside the Transwell chamber, respectively, and the values were recorded (Rubin et al., 1991; Nakagawa et al., 2009; Ribeiro et al., 2010).

Liquid surface leakage test

Once the TEER value was constant, DMEM was added to the interior of the Transwell chamber so that the difference between the depth of the medium inside and outside the Transwell was 0.5 cm. The depths of the media above and below the Transwell were measured again after culturing for 4 hours at 37°C and 5% CO₂. If the difference between the liquids remained at 0.4–0.5 cm, this showed that the cells had formed a dense barrier (Steiner et al., 2011; Burek et al., 2012; Wuest and Lee, 2012; Zhou et al., 2013; Hanada et al.,

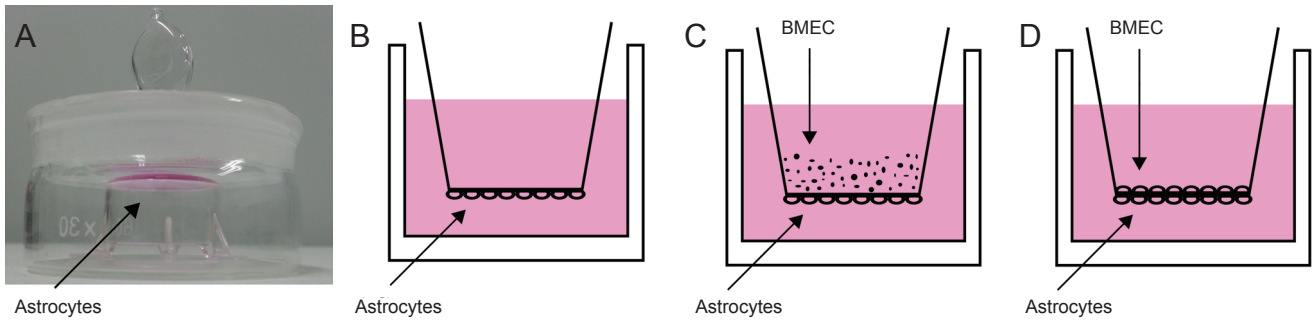


Figure 1 Establishing the *in vitro* blood-brain barrier model.

(A) Astrocytes were seeded (1 mL at 1×10^6 /mL) on the underside of a Transwell chamber, which was placed upside-down in a weighing bottle. (B) Astrocytes were allowed to adhere to the wall for 24 hours, and the Transwell chamber was carefully turned up-right and placed in a 6-well plate. (C) When astrocytes reached 60% confluence, BMECs (1×10^7 /mL) were seeded on the top of the Transwell chamber. (D) Astrocytes and BMECs were co-cultured to a high density. BMECs: Brain microvascular endothelial cells.

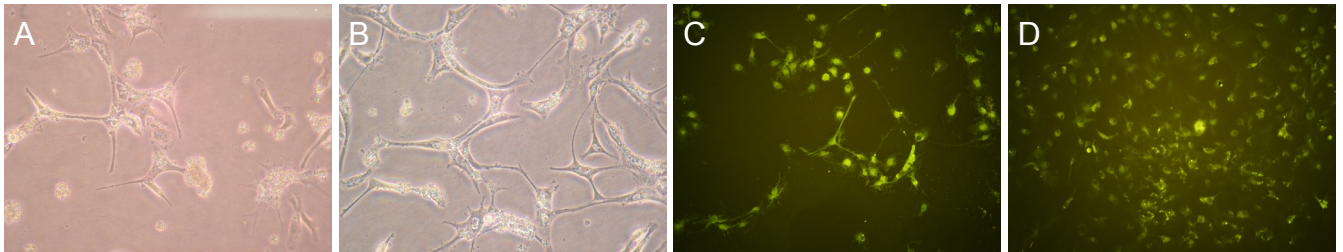


Figure 2 Morphology of primary cultured astrocytes.

(A, B) Astrocytes under an inverted microscope (200 \times magnification). At 3 days of primary culture (A), astrocyte bodies were observed with a small number of short processes. At 7 days (B), large astrocyte bodies were observed, with several slender branching processes. (C, D) Immunofluorescence staining for glial fibrillary acidic protein, an astrocyte marker (100 \times magnification). Between 3 (C) and 7 days of primary culture (D), the cell bodies swelled markedly and formed a distinct cell configuration.

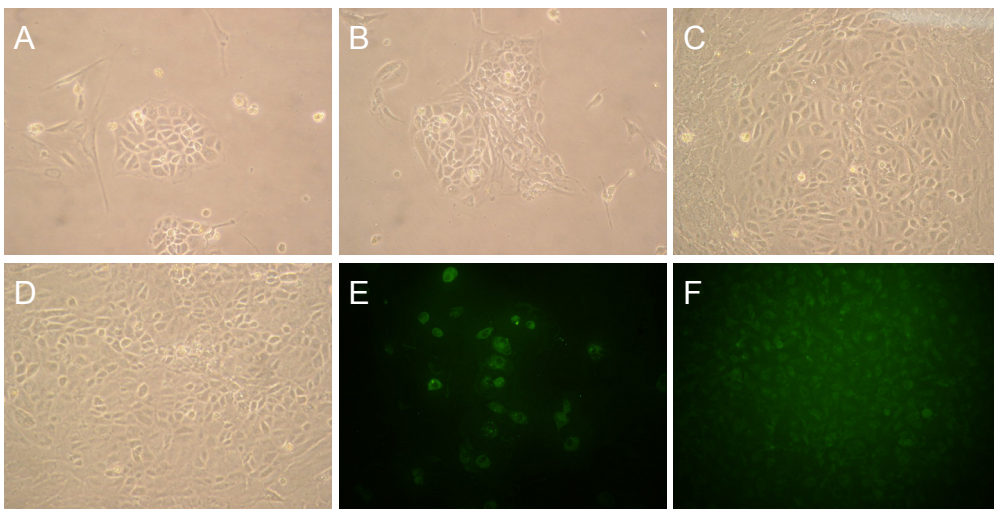


Figure 3 Morphology of primary cultured BMECs.

(A–D) BMECs under an inverted microscope (100 \times magnification). At 3 days of primary culture (A), BMECs showed single-layer growth. At 7 days (B), the area of growth had expanded. At 14 days (C), cells were polygonal or fusiform, pale, with nuclear membranes; spiral distribution was observed. At 16 days (D), cells formed a tight single layer with a typical cobblestone-like appearance. (E, F) Immunofluorescence staining for the BMEC marker factor VIII (200 \times magnification). At 7 days of primary culture (E), green fluorescence was observed in the cytoplasm of several cells. At 14 days (F), cells were densely arranged and showed marked green fluorescence. BMECs: Brain microvascular endothelial cells.

2014).

Quantification of specific BBB enzymes

Gamma-glutamyl transpeptidase (γ -GT)

BMECs in the co-culture model and single layer BMEC groups were washed three times in cold PBS, collected gently using a cell scraper (Corning), centrifuged at $700 \times g$ or 5 minutes, and the supernatant was discarded. Cells in each well were lysed with 150 μ L cell lysate (YuanYe Biological Technology Co., Ltd., Shanghai, China) on ice for 30 minutes (shaken every 10 minutes), and centrifuged at $1,104 \times g$ or 30 minutes at 4°C . The filtrate was aliquoted and stored. γ -GT was measured using an enzyme linked immunosorbent assay kit (YuanYe Biological Technology Co., Ltd.) according to the manufacturer's instructions. The optical density of each well was measured with a microplate reader (Awareness Technology, Palm City, FL, USA) at a wavelength of 450 nm.

Alkaline phosphatase (ALP)

The Bradford protein concentration determination kit was used to measure the protein concentrations of each sample in the co-culture model and single layer BMEC groups. ALP was measured using an ALP determination kit (Jiancheng Biological Engineering Research Institute, Nanjing, Jingsu Province, China), according to the manufacturer's instructions. The optical density of each well was measured in a spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 520 nm.

Statistical analysis

Data are expressed as the mean \pm SD. Statistical analysis was performed using independent sample *t*-tests of variance in SPSS 17.0 software (SPSS, Chicago, IL, USA). $P = 0.05$ was considered significant.

Results

Morphology of primary cultured astrocytes

Most primary cultured astrocytes had adhered to the wall by the third day of culture, and a small number of short branching processes were observed between cell bodies (Figure 2A). At 7 days, all cells had adhered to the wall, and the number of cell bodies had markedly increased, with many slender branching processes stretching between neighboring cells (Figure 2B).

After immunofluorescence staining for GFAP, the cytoplasm and branches of cultured astrocytes at 3 and 7 days fluoresced green and showed distinct cellular contours (Figure 2C, D).

Morphology of primary cultured BMECs

Primary culture BMECs were short, polygonal, and spindle-like, showing regional monolayer growth at 3 days (Figure 3A). At 7 days, the medium was replaced. The number of cells had increased, and the area of growth had expanded (Figure 3B). As incubation time and number of medium changes increased, other cells disappeared while endothelial cells proliferated, appearing pale with a poly-

onal or fusiform shape, a nuclear membrane, and spiral distribution. By 14 days, the endothelial cells had integrated, forming a dense, cobblestone-like monolayer (Figure 3C, D).

Green immunofluorescence was observed in the cytoplasm of only a few BMECs at 7 days (Figure 3E). At 14 days, however, the cells were tightly connected to each other, and strong green fluorescence was observed (Figure 3F).

Characterization of the *in vitro* BBB model

Under an inverted phase contrast microscope, we observed good astrocyte growth, with interconnecting branches, underneath the Transwell chamber (Figure 4A). The BMECs seeded in the Transwell chamber were also well-formed, and were connected to the astrocytes after 24 hours (Figure 4B).

The TEER value in the co-culture model and single layer BMEC groups increased with time. No significant difference in TEER value was detected between the co-culture model group and the single-layer BMEC group ($P < 0.01$). TEER values plateaued by 72 hours in both groups (Table 1).

When the TEER value was constant, the difference between the levels of the internal and external media was ≥ 0.5 cm (Figure 5A). After 4 hours of culture, this difference remained ≥ 0.5 cm (Figure 5B), indicating that the cultured cells had formed a dense barrier.

γ -GT and ALP contents in the co-culture model group were greater than those in the single-layer BMEC group ($P < 0.01$; Figure 6).

Discussion

Primary culture of astrocytes

In the BBB, a small number of microglia and other cells are found on the astrocyte side and have no special growth requirements. The original method for BBB modeling is therefore simpler than BMEC culture. Here, we used five newborn rat brains to grow BMECs in a 75-cm² culture bottle after trypsin digestion and filtration purification. Removal of contaminating fibroblasts is important during BMEC culture. Because fibroblasts attach faster than astrocytes, we used a fresh cell culture bottle after 1 hour of culture, and used the differential adhesion method to remove the fibroblasts. DMEM containing 20% fetal bovine serum is nutritionally sufficient for astrocyte growth. Astrocyte density is particularly important, because when the density is low, astrocyte growth is slow and may even cease. It is therefore important to maintain high astrocyte density during co-culture generation.

Primary culture of BMECs

BMECs are difficult to culture and demand a strict growth environment. They are sensitive to hybrid cells, divide slowly, and require the addition of growth factors. A unified approach for the separation, purification and culture of BMECs has not yet been described. Here, we built a culture system by selecting experimental animals, specifying anatomic location, and separating and purifying brain

capillaries. We selected newborn rats (1–3 days), instead of those 3–7-weeks-old as previously described (Molino et al., 2014), because despite gray matter being easier to separate at 3–7 weeks, rats at that age should be killed by cervical dislocation, a time-consuming method requiring removal of meningeal blood vessels and multiple rounds of mechanical homogenization and centrifugation, which may result in tissue damage. In 1–3-day-old rats, however, the tissues are easy to separate and cell proliferation ability is strong. We were able to remove the pia mater and meningeal blood vessels carefully and accurately, effectively reducing contamination by hybrid cells.

We also used enzyme digestion combined with gradient centrifugation to separate and purify the capillaries in the brain. Cerebral microvascular tissue was completely separated from the brain by digestion with collagenase II. Nerve tissue and blood vessels were removed by gradient centrifugation after adding 20% bovine serum albumin, and hybrid cells were removed by a second digestion with collagenase and other enzymes. To ensure endothelial cell vitality, a digestion time of 10–15 minutes must be used. A previous study (Molino et al., 2014) reported a large cell loss with a 2–3-hour experiment. Our method avoided cell damage by mechanical homogenization and centrifugation, and only needed 1-hour experimental time; we thus obtained a high percentage of isolated cells.

Because BMECs are more fragile than cells of other tissues, it is important that their environment is suitable for their growth, and not that of other cells. We modified the DMEM/F12 culture medium by adding endothelial cell growth supplement to promote the growth of BMECs, and heparin sodium was used to inhibit that of smooth muscle cells. Because of the weak adherence of BMECs, we chose 1% gelatin to pre-coat the disposable culture bottles to facilitate adherence of capillaries and the growth of BMECs.

Evaluation index of the *in vitro* BBB model

Cells were observed through transparent polyester Transwell material (aperture 0.4 μm). The cells grew normally on both sides of the Transwell and a dense structure was observed under the inverted phase contrast microscope. TEER dynamic measurements showed that resistance on either side of the Transwell increased with cell growth. At 72 hours, TEER was constant, indicative of a dense structure. The difference in resistance was greater in the single-layer BMECs than in the co-culture model, indicating that the structure is more compact in the monolayer cells after astrocyte and BMEC co-culture. This may lead to cellular interaction, promote the expression of tight junctions, and make the structure more compact. When TEER values were constant, we performed a 4-hour leakage test to confirm the density of the structure and ensure experimental rigor; the results showed that the levels of medium inside and outside the Transwell chamber remained significantly different after 4 hours, indicating that the compact structure could withstand strong osmotic pressure.

We have also shown here that the γ -GT and ALP content of *in vitro* cultured endothelial cells is low; but when co-cul-

tured, endothelial cells showed high levels of γ -GT and ALP. Our results support those from previous studies comparing the activities of γ -GT and ALP after BMEC monoculture (Reichel et al., 2003; Tóth et al., 2011).

Conclusion

Our approach comprises the following: (1) selection of newborn rats, instead of 3–7-week-old rats as commonly used, for the primary culture of BMECs to ensure homology of astrocytes; (2) enzymatic digestion to avoid mechanical damage by centrifugation; (3) minimization of centrifugation during primary culture to avoid cell loss; (4) shortening of the time required for primary culture, resulting in improved cell yield; (5) use of transparent polyester Transwell inserts for real-time direct observation of cell growth on the membrane under the inverted microscope.

In conclusion, the modeling method we have presented here is simple and feasible, and consumables and instruments required are easy to obtain. However, it is a basic model, and we encourage researchers to make appropriate adjustments according to different research purposes.

Author contributions: YW, NW and BC conceived and designed the experiments. YW, BC, GYW and JL were responsible for sequencing, finishing and annotating the paper. YW, BC, GYW, JL, and XXP performed experiments and data analysis. NW, BC and GYW provided reagents/materials/analysis tools. YW, BC, and GYW wrote the paper. All authors read and approved the final version of the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using Cross-Check to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

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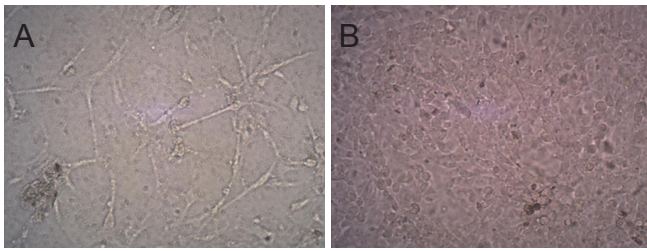


Figure 4 Cellular morphology of the simulated *in vitro* blood-brain barrier (inverted microscope, × 200).
 (A) Astrocyte lower layer showed good growth and adherence to the wall; branching processes connected the cells. (B) BMEC upper layer showed dense growth and defined morphology. BMEC: Brain microvascular endothelial cell.

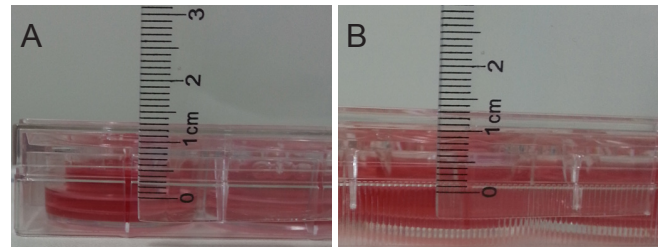


Figure 5 Leakage test of liquid surface in co-culture model group.
 (A) Measurement of the difference in levels between the medium on either side of the Transwell chamber (0.5 cm). (B) After 4 hours of culture, the difference in levels remained significant (0.4–0.5 cm, indicating that the co-cultured cells had formed a dense barrier).

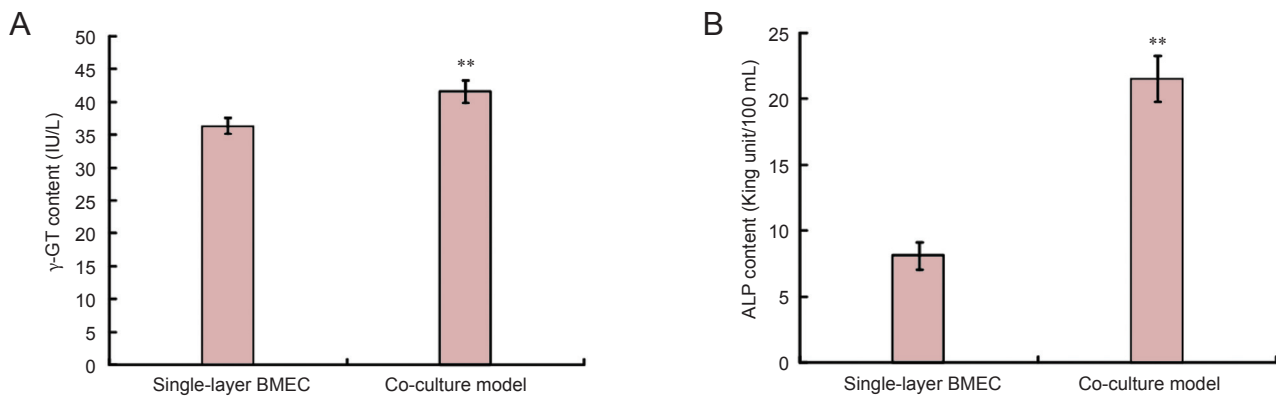


Figure 6 Quantification of specific blood-brain barrier enzymes in the co-culture model and single-layer BMEC groups.
 (A) γ -GT; (B) ALP. Data are presented as the mean \pm SD ($n = 6$). ** $P < 0.01$, vs. single-layer BMEC group (independent sample t -test). ALP: Alkaline phosphatase; γ -GT: gamma-glutamyl transpeptidase; BMEC: brain microvascular endothelial cell.

Table 1 TEER values (Ω /pore) in co-culture model and single-layer BMEC groups

Time (hour)	Single-layer BMEC group	Co-culture model group
2	34.50 \pm 3.93	54.50 \pm 4.23**
4	45.00 \pm 4.73	71.17 \pm 6.37**
8	54.83 \pm 5.46	86.17 \pm 4.92**
12	65.33 \pm 4.97	100.50 \pm 3.78**
16	72.83 \pm 4.31	118.33 \pm 9.73**
20	86.00 \pm 5.10	150.83 \pm 17.65**
24	98.50 \pm 5.32	222.00 \pm 20.82**
48	119.33 \pm 9.69	307.83 \pm 17.14**
72	141.83 \pm 12.01	428.67 \pm 33.03**
96	141.83 \pm 12.07	437.83 \pm 20.47**

Data are presented as the mean \pm SD ($n = 6$). ** $P < 0.01$, vs. single layer BMEC group (independent sample t -tests). BMEC: Brain microvascular endothelial cell; TEER: transepithelial electrical resistance.

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