Basic Fibroblast Growth Factor Reduces Permeability and Apoptosis of Human Brain Microvascular Endothelial Cells in Response to Oxygen and Glucose Deprivation Followed by Reoxygenation via the Fibroblast Growth Factor Receptor 1 (FGFR1)/ERK Pathway

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Source of support: The study was supported by the Foundation of the Program of Technology of Education Committee of Chongqing (No. KJQN201800122) and the Program of Technology of Science and Technology Committee of Yuzhong District in Chongqing (No. 20180131)

Background: Disruption of the blood–brain barrier (BBB) is a mechanism in the pathogenesis of traumatic brain injury. Basic fibroblast growth factor (bFGF) is expressed in angiogenesis, neurogenesis, and neuronal survival. This study aimed to investigate the role of bFGF *in vitro* in human brain microvascular endothelial cells (HBMECs) challenged by oxygen-glucose deprivation/reperfusion (OGD/R).

Material/Methods: HBMECs were cultured in glucose-free medium and an environment with <0.5% oxygen in an anaerobic chamber. Immunocytochemistry, Western blot, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were used to measure the protein and mRNA expression levels of bFGF, tight junction, adherens junction, apoptotic proteins, and matrix metalloproteinases (MMPs). The effects of bFGF on the viability of HBMECs was evaluated using the cell counting kit-8 (CCK-8) assay. Cell apoptosis was evaluated using the TUNEL assay, and endothelial permeability was quantified using a transwell migration assay with fluorescein isothiocyanate (FITC) conjugated with dextran. The effects of bFGF were evaluated following inhibition of fibroblast growth factor receptor 1 (FGFR1) with PD173074 and inhibition of ERK with PD98059.

Results: Following OGD/R of HBMECs, bFGF significantly reduced cell permeability and apoptosis and significantly inhibited the down-regulation of the expressions of proteins associated with tight junctions, adherens junctions, apoptosis and matrix metalloproteinases (MMPs). The effects of bFGF were mediated by the activation of FGFR1 and ERK, as they were blocked by FGFR1 and ERK inhibitors.

Conclusions: Permeability and apoptosis of HBMECs challenged by OGD/R were reduced by bFGF by activation of the FGFR1 and the ERK pathway.

MeSH Keywords: Blood–Brain Barrier • Brain Injuries • Receptor, Fibroblast Growth Factor, Type 2

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/918626

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Background

Traumatic brain injury is an increasing public health problem and is a leading cause of long-term disability in children and young adults in some parts of the world [1]. Worldwide, more than 50 million people are affected annually by traumatic brain injury, with a global economic burden estimated at $400 billion [2]. Traumatic brain injury can result in disability, long-term neurological dysfunction, or death. However, there are no effective treatments to reverse or reduce the pathological changes resulting from brain trauma. Therefore, it is necessary to study and develop innovative treatments for patients with traumatic brain injury.

The blood–brain barrier (BBB) is a physiological barrier consisting mainly of brain microvascular endothelial cells (BMECs), which can regulate the micro-environment of the brain by regulating the traffic of substances between the blood and the brain. Following traumatic brain injury, at the level of the BBB, microvascular hyperpermeability results in vasogenic brain edema and increased intracranial pressure [3]. Preclinical and clinical studies have shown that disruption of the BBB is an important pathophysiological feature associated with the neurological sequelae of traumatic brain injury [4,5]. Dysfunction of the BBB results in increased microvascular permeability and decreased expression of genes encoding proteins for tight junctions and adherens junctions [4,5].

Basic fibroblast growth factor (bFGF), or fibroblast growth factor 2 (FGF2), is a member of the fibroblast growth factor (FGF) superfamily, and its functions include regulation of angiogenesis [6], development of neuronal networks [7,8] and the growth of axons [9]. In 1997, Kawamata et al. showed that in a rat model of focal cerebral infarction, administration of bFGF promoted axonal growth and the formation of new synapses, reduced infarct size, and promoted the recovery of sensation and motor function [10]. Also, in a mouse model of cerebral hemorrhage, treatment with bFGF increased the expression of endothelial cell tight junction proteins, reduced the release of inflammatory factors, and protected against cerebral damage [11]. However, the specific mechanisms involved in the effects of bFGF on microvascular endothelial cells remain unclear.

Previous studies have shown that bFGF has an important biological role in binding to fibroblast growth factor receptors (FGFRs), mainly to the high-affinity fibroblast growth factor receptor 1 (FGFR1) to activate downstream signaling pathways in the central nervous system (CNS) [8,12,13]. Also, binding of bFGF to receptors after brain injury mediates downstream activation of pathways involving mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and other signaling pathways and plays an important role in changes affecting the BBB [8,12,13]. In human cultured CNS pericytes *in vitro*, bFGF was found to upregulate platelet-derived growth factor receptor beta (PDGFRβ) and via the ERK pathway, enhancing their activity [14]. In a previous study using an *in vitro* model of human immunodeficiency virus (HIV)-infected vascular endothelial cells, we showed that bFGF mediated changes in the ERK, PI3K/AKT, and PKC pathways and had a protective effect [15].

This study aimed to investigate the role of bFGF in human brain microvascular endothelial cells (HBMECs) cultured *in vitro* and challenged by oxygen-glucose deprivation/reperfusion (OGD/R), including permeability, apoptosis, and cell viability.

Material and Methods

Reagents and antibodies

Primary human brain microvascular endothelial cells (HBMECs) were obtained from Angio-Proteomie (Boston, MA, USA). Recombinant human basic fibroblast growth factor (bFGF) was purchased from RayBiotech (Atlanta, GA, USA). The fibroblast growth factor receptor 1 (FGFR1) inhibitor, PD173074, and the ERK inhibitor, PD98059, were purchased from APExBIO (Houston, TX, USA). Antibodies to fibroblast growth factor receptor 1 (FGFR1), ERK, p-ERK, and β-catenin were purchased from Cell Signaling Technology (CST) (Danvers, MA, USA). Antibodies to p-FGFR1, p-FGFR2, FGFR2, occludin, matrix metalloproteinase 2 (MMP2), MMP9, BAX, Bcl-2, and caspase-3 were purchased from Abcam (Cambridge, UK). The antibody to claudin-5 was purchased from GeneTex (Irvine, CA, USA), and the antibody to zonula occludens-1 (ZO-1) was purchased from Novus Biologicals (Centennial, Colorado, USA). The cell counting kit-8 (CCK8) assay was purchased from Dojindo Laboratories (Kyushu, Japan), and TUNEL C1090 was obtained from Beyotime Institute of Biotechnology (Shanghai, China). The fluorescein isothiocyanate conjugated to dextran (FITC-dextran) was purchased from Xi'an Ruixi Biological Technology Co. Ltd. (Changzhou, China).

Cell culture and oxygen-glucose deprivation/reoxygenation (OGD/R) injury

The HBMECs were cultured and maintained in complete endothelial cell medium (ECM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA), and incubated at 37°C and 5% CO_2. When the HBMECs reached 80–90% confluence, glucose-free medium was used to replace the ECM, and the cell cultures were incubated for 4 h in <0.5% oxygen in an anaerobic chamber (Billups-Rothenberg Inc., San Diego, CA, USA). Finally, the medium was replaced with complete ECM, and the cells were incubated at 37°C and 5% CO_2.

In the bFGF treatment group, 2.5 µM of recombinant bFGF was added to the medium 1 h before OGD/R was applied, as described above. In the PD173074 group, the HBMECs were treated with the FGFR1 antagonist, PD173074 (100 nM), 2 min before bFGF administration. In the PD98059 group, the HBMECs were treated with the ERK antagonist, PD98059 (25 µM), 2 min before bFGF administration. Cell culture monolayers not subjected to OGD/R treatment were used as controls. After reoxygenation for 24 h, the cells were harvested for further analysis. All experiments were performed in triplicate.

Endothelial permeability assay

Endothelial permeability assays were performed using FITC-dextran (70 kDa) extravasation for quantification across HBMECs seeded in transwell chambers, as previously described [16]. Briefly, HBMECs were seeded at 1×10^5 cells/well in 200 µL of complete ECM in 24-well polycarbonate transwell chambers with 0.4 µm pores. After 48 h, the cells were cultured until they reached confluence and were treated with or without OGD/R, bFGF, PD173074, and PD98059. The medium was replaced with medium containing 1% FITC-dextran (1 mg/mL), and the cells were incubated for 4 h. Finally, the relative fluorescence was measured that crossed the lower chamber using a multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at wavelengths of 485 nm for excitation and 520 nm for emission.

Cell viability assay

The cell counting kit-8 (CCK-8) assay was used to investigate the viability of the cultured endothelial cells. Briefly, HBMECs were cultured in complete ECM as a cell suspension of 100 µl per well, in a 96-well plate. After OGD/R treatment, the CCK8 reagent (10 µl) was added to each well before the cells were incubated for 2 h at 37°C. Absorbency at 450 nm was measured against a blank background control and a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

Apoptosis assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to evaluate cell apoptosis after OGD/R treatment, as previously described [17]. Briefly, the cultured cells were collected following OGD/R and were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by treatment with 3% H_2O_2 for 30 min at 37°C. After being rinsed with PBS, the cells were incubated with the TUNEL reaction mixture and 0.3% Triton X-100 in a dark room for 2 h at 37°C. For quantitative measurement, the immunopositive cells were counted in five randomly selected fields at a magnification of ×200, using a Zeiss Imager A2 microscope (Zeiss, Oberkochen, Germany).

Immunocytochemistry

The cultured HBMECs were washed with PBS (pH 7.4) 24 h following OGD/R and fixed for 30 min with 4% paraformaldehyde. After a second wash with PBS, the cells were incubated with 5% FBS for 1 h and s incubated at 4°C overnight with primary antibodies to zonula occludens-1 (ZO-1) (1: 100), occludin (1: 100), claudin-5 (1: 50), or β-catenin (1: 200). After washing with PBS, the final incubation was at 37°C for 1 h with goat anti-rabbit or goat anti-mouse secondary FITC-labelled antibody (1: 50) (ZhongShan Golden Bridge Biotechnology, Beijing, China). The cell nuclei were counterstained with Hoechst 33258 (Beyotime-Bio, Shanghai, China). The results were analyzed at a magnification of ×200 using a Zeiss Imager A2 fluorescence microscope (Zeiss, Oberkochen, Germany).

Western blot

Twenty-four hours following OGD/R, the HBMECs were extracted with buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, and 0.1% sodium dodecyl sulfate (SDS) supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland). Equal amounts of protein were fractionated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 µm polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Burlington, MA, USA). The blotted proteins were probed with antibodies to ZO-1 (1: 1000), occludin (1: 1000), claudin-5 (1: 1000), β-catenin (1: 1000), Bcl-2 (1: 500), BAX (1: 500), caspase-3 (1: 500), MMP2 (1: 500), or MMP9 (1: 500), and β-actin was used as the internal control. The proteins were visualized using a Tannon-5200 enhanced chemiluminescence detection system (Tanon, Shanghai, China). Quantification was performed by densitometry using a TU 1900 ultraviolet spectrophotometer (Purkinje General Instrument Co., Beijing, China).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The amount of mRNA in the cultured HBMECs was measured using quantitative real-time PCR (qRT-PCR), according to standardized procedures. Twenty-four hours after the treatment, the total RNA was extracted from the cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's guidelines. After DNase treatment, the RNA was reverse transcribed to cDNA using the ReverTra AceH kit (Toyobo Co Ltd., Osaka, Japan) according to the manufacturer's instructions. The cDNA underwent PCR using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The PCR primer sequences are shown in Table 1. The level of each gene was normalized to the β-actin values, and the reactions were performed in triplicate.

Table 1. Primer sequences for the polymerase chain reaction (PCR).

| Gene	Sense primer (forward)	Antisense primer (reverse)
ZO-1	5'GGGGCTGTCTCAACTCCTGTAAAG3'	5'TGGGCATACACAGGCTTTGGTT3'
Occludin	5'ACTCGAAGAAAGATGGACAGGTATG3'	5'CGGCGTGGATTTATAGGAAGACTC3'
Claudin-5	5'ACCGCCTTCCTGGACCACAAC3'	5'CCGCCAGGGTCACGAAGAG3'
β-catenin	5'AGCCGACACCAAGAAGCAGCGATG 3'	5'CGGCGCTGGGTATCCTGATG3'
BCL-2	5'TCGCCCTGTGGATGACTGAGTAC3'	5'GGCAGGCATGTTGACTTCACTTG3'
BAX	5'GCCGCCGTGGACACAGACTC3'	5'AGGGCCTTGAGCACCAGTTTTG3'
β-actin	5'ACCCCGTGCTGCTGACCGAG3'	5'TCCCGGCCAGCCAGGTCCA3'
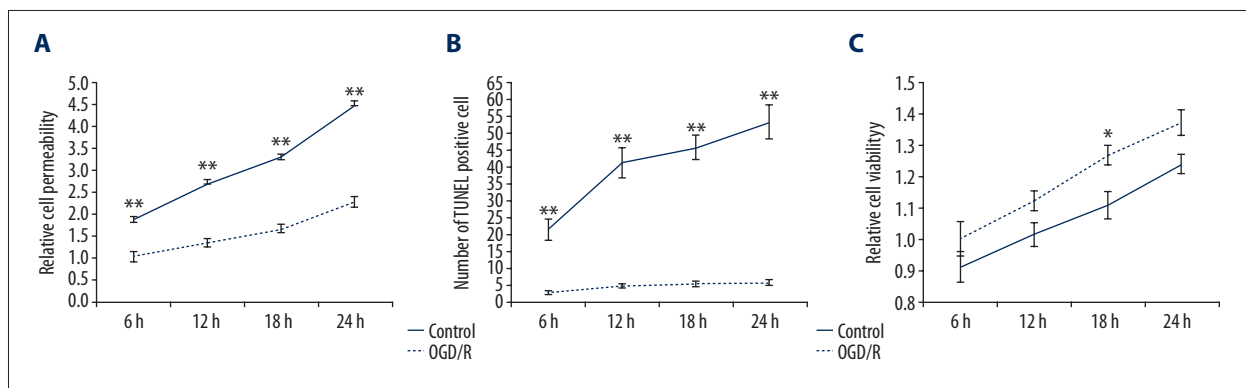
Figure 1. Oxygen-glucose deprivation/reperfusion (OGD/R) resulted in increased human brain microvascular endothelial cell (HBMEC) permeability and apoptosis. (**A**) Fluorescein isothiocyanate (FITC) conjugated with dextran quantified endothelial permeability by analyzing the integrity of the cell monolayer. The human brain microvascular endothelial cells (HBMECs) were examined at 6, 12, 18, and 24 h after OGD/R treatment. (**B**) Quantification of apoptotic cells at 6, 12, 18, and 24 h following OGD/R, detected by the TUNEL assay. (**C**) Endothelial cell viability was measured by the cell counting kit-8 (CCK-8) assay at 6, 12, 18, and 24 h after OGD/R treatment. Control group *vs.* the OGD/R group (* P<0.05, ** P<0.01). N=6 in each group at each timepoint. N=3 independent experiments.

Statistical analysis

Six samples were analyzed in each group for Western blot, PCR assays, endothelial monolayer permeability, cell viability, and apoptosis. Data were reported as the mean±standard deviation (SD). Data were analyzed using SPSS version 19.0 software (IBM, Chicago, IL, USA). Differences between the groups were determined by one-way analysis of variance (ANOVA) or Student's t-test. A P-value <0.05 was considered to be statistically significant.

Results

The permeability of human brain microvascular endothelial cells (HBMECs) challenged by oxygen-glucose deprivation/reperfusion (OGD/R) and treated with basic fibroblast growth factor (bFGF)

The permeability of HBMEC decreased, and apoptosis increased, after 24 hr of oxygen-glucose deprivation/reperfusion (OGD/R), but cell viability did not change significantly after 24 h. The monolayer of HBMEC showed a significantly increased rate of migration, shown by fluorescein isothiocyanate conjugated with dextran (FITC-dextran), after OGD/R treatment compared with controls (P<0.01) (Figure 1A), indicating that the permeability of the HBMECs was significantly increased. The findings from the TUNEL assay showed that endothelial cell apoptosis was significantly increased after 24 hr of OGD/R treatment compared with controls (P<0.01) (Figure 1B), indicating that OGD/R induced apoptosis of HBMECs. However, there was no
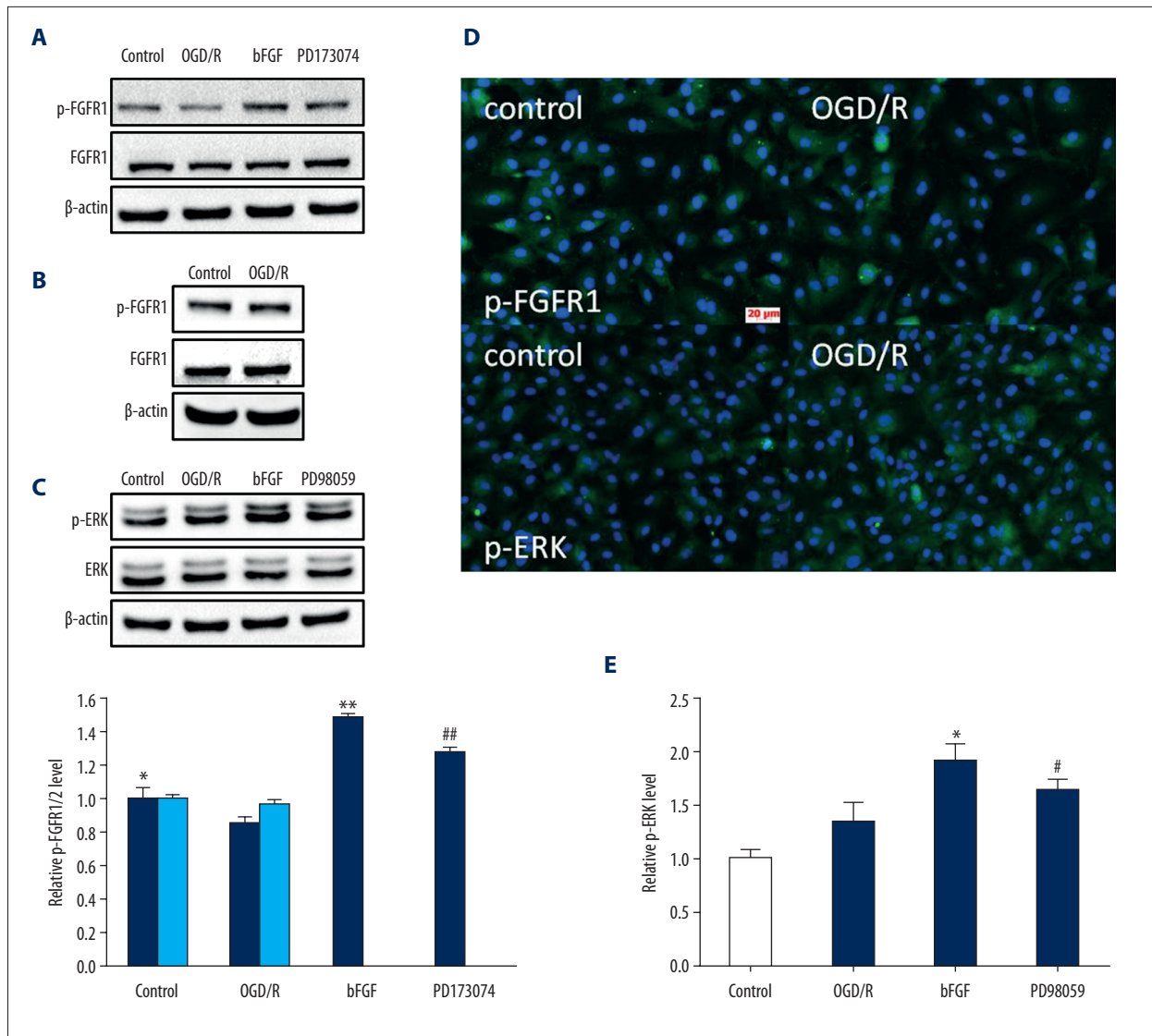
Figure 2. Changes in phospho-fibroblast growth factor receptor 1 (p-FGFR1) and p-ERK expression in human brain microvascular endothelial cells (HBMECs). (**A**) Representative Western blot images for FGFR1 and p-FGFR1. The expression of p-FGFR1 was affected by the use of the agonist, bFGF, and inhibition of FGFR1 with PD173074. (**B**) Representative Western blot images for FGFR2 and p-FGFR2. The expression of the FGFR2 and p-FGFR2 proteins did not change after oxygen-glucose deprivation/reperfusion (OGD/R) treatment. (**C**) Representative Western blot images for ERK and p-ERK. The expression of p-FGFR1 was affected by an agonist, bFGF, and inhibition of ERK with PD98059. (**D**) Immunofluorescence staining of p-FGFR1 and p-ERK in HBMECs shows that the expression levels were changed after OGD/R treatment. (**E**) Quantification of p-FGFR1/2 and p-ERK protein levels. The control group or the bFGF group *vs.* the OGD/R group (* $P<0.05$, ** $P<0.01$). PD173074 or PD98059 treatment *vs.* the bFGF group (# $P<0.05$, ## $P<0.01$). N=6 in each group. N=3 independent experiments.

significant change in the viability of the HBMECs following OGD/R treatment (Figure 1C), indicating that increased permeability of the endothelial cell monolayer induced by OGD/R was not due to decreased cell viability.

OGD/R treatment of HBMECs and the expression of fibroblast growth factor receptor 1 (FGFR1) and ERK

HBMECs that underwent OGD/R showed increased expression of FGFR1 and ERK phosphorylation, shown by Western blot, indicating that OGD/R induced decreased endothelial monolayer permeability by regulating the activity of the FGFR1 and the ERK signaling pathway.
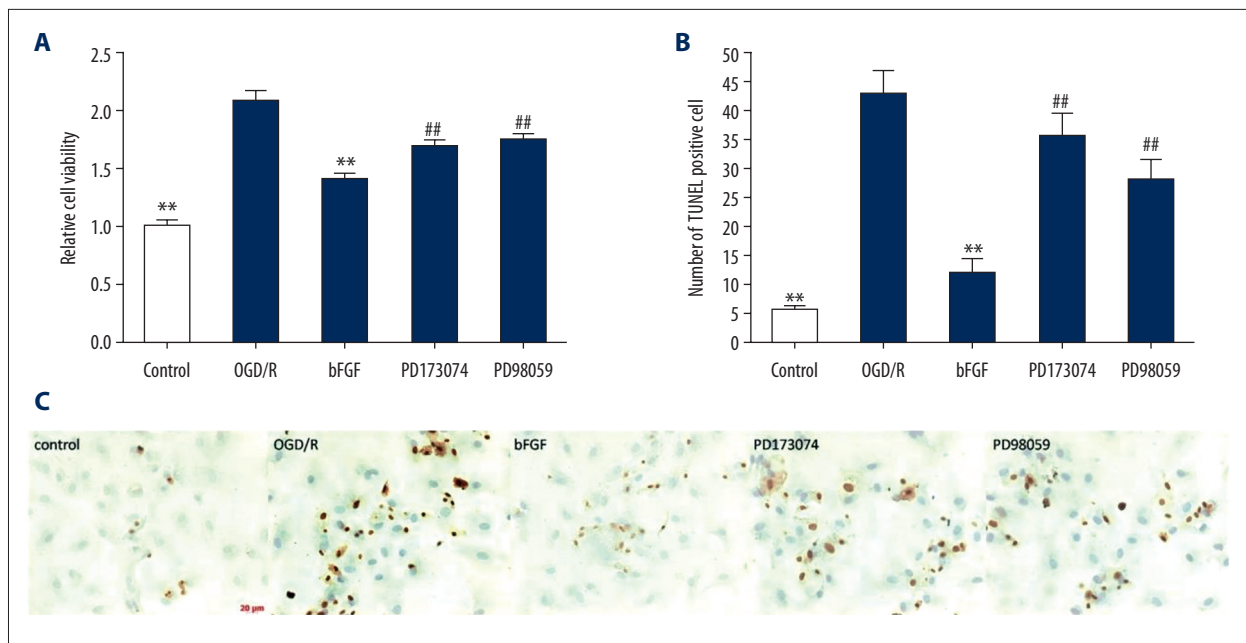
Figure 3. Basic fibroblast growth factor (bFGF) reduced the increase in endothelial monolayer permeability and apoptosis induced by oxygen-glucose deprivation/reperfusion (OGD/R). (**A**) Treatment with bFGF significantly decreased the permeability of HBMECs 12 h after OGD/R treatment. (**B**) Quantification and (**C**) immunostaining of apoptotic cells 12 h post-OGD/R, as detected by the TUNEL assay. The number of immunoreactive cells was reduced by bFGF, indicating that it suppressed OGD/R-induced HBMEC apoptosis. The control or bFGF group *vs.* the OGD/R group (** $P<0.01$). Inhibition of FGFR1 with PD173074 or inhibition of ERK with PD98059 compared with the bFGF group (## $P<0.01$). N=6 in each group. N=3 independent experiments.

Western blot of proteins from the endothelial cells showed that following OGD/R treatment, there was significant down-regulation of p-FGFR1 expression when compared with the control ($P<0.05$) (Figure 2A, 2D, 2E) and significant upregulation of p-ERK expression ($P<0.05$) (Figure 2C–2E). After OGD/R treatment, the expression of FGFR1, ERK, FGFR2, and p-FGFR2 proteins did not significantly change (Figure 2A–2C, 2E). There was significant upregulation of the expression of p-FGFR1 and p-ERK in the bFGF group compared with the OGD/R group ($P<0.05$) (Figure 2A, 2C, 2E). Also, the expression of p-FGFR1 and p-ERK were significantly reduced following inhibition of FGFR1 with PD173074 and inhibition of ERK with PD98059 compared with bFGF ($P<0.05$) (Figure 2A, 2C, 2E).

Treatment of HBMECs with bFGF prevented OGD/R-induced HBMEC endothelial monolayer permeability and apoptosis

After the addition of exogenous bFGF, the osmotic disruption of the HBMECs was reduced 12 h after OGD/R treatment ($P<0.01$) (Figure 3A), and the number of apoptotic cells was significantly reduced following treatment with FGF compared with OGD/R ($P<0.01$) (Figure 3B, 3C). However, following inhibition of FGFR1 with PD173074 and inhibition of ERK with PD98059, these groups showed increased permeability and apoptosis, which were statistically significantly different compared with the bFGF group ($P<0.01$) (Figure 3A, 3B).

Treatment of HBMECs with bFGF reduced OGD/R-induced down-regulation of endothelial tight junction and adherens junction proteins

The expression of zonula occludens-1 (ZO-1), occludin, claudin-5, and β-catenin was significantly reduced after treatment with OGD/R, whereas the expression of tight junction and adherens junction proteins was increased by bFGF. Inhibition of fibroblast growth factor receptor 1 (FGFR1) with PD173074 and inhibition of ERK with PD98059 reversed the protective effect of bFGF (Figure 4A–4D).

Treatment of HBMECs with bFGF regulated the expression of matrix metalloproteinases (MMPs) and apoptotic proteins after OGD/R

Western blot showed that the expression of tight junction and adherens junction proteins and Bcl-2 were significantly reduced in the OGD/R group treated compared with the control group ($P<0.01$) (Figure 5A–5F, 5J). The expression of MMPs, BAX, and caspase-3 was significantly increased compared with control group after OGD/R treatment ($P<0.01$) (Figure 5B, 5G–5I, 5K), but bFGF upregulated the expression of tight junction and adherens junction proteins and Bcl-2 in the endothelial cells ($P<0.01$) (Figure 5A–5F, 5J), and significantly reduced the expression of
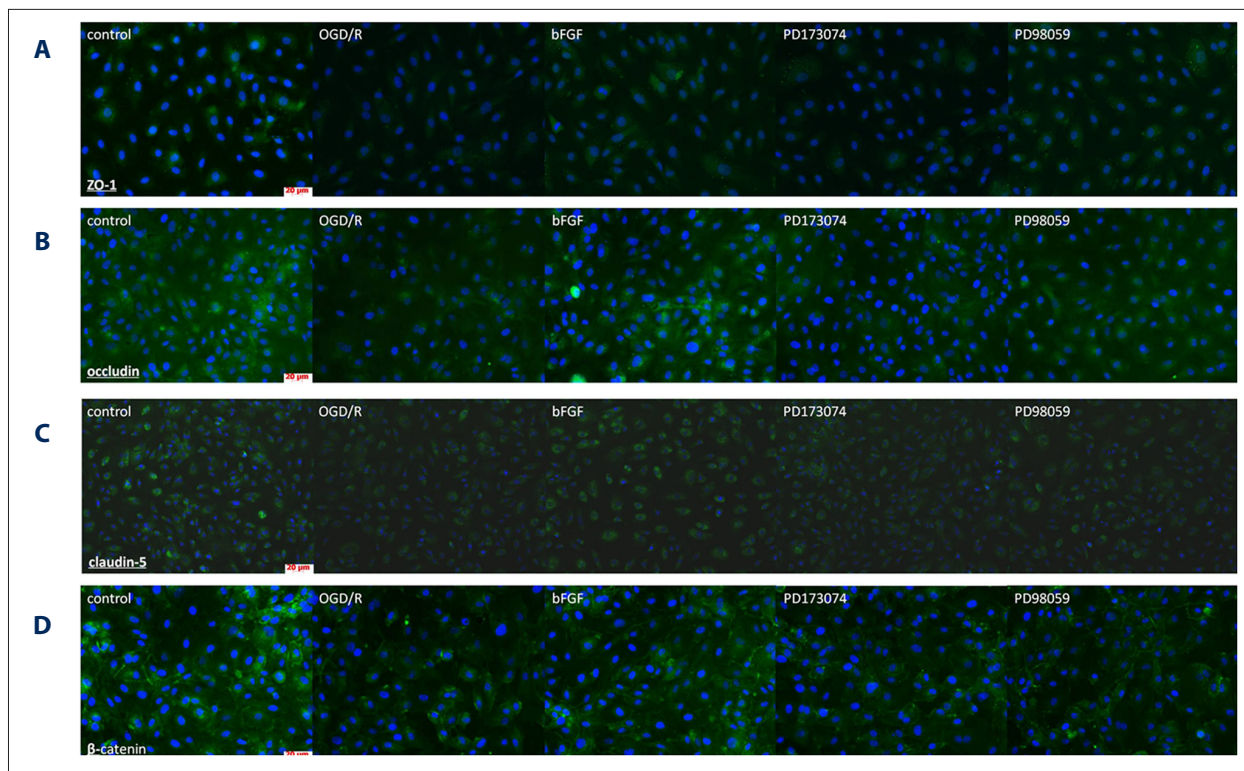
Figure 4. Following oxygen-glucose deprivation/reperfusion (OGD/R) of endothelial cells, basic fibroblast growth factor (bFGF) inhibited the decrease in tight junction and adherens junction proteins. (**A–D**) Treatment with bFGF reversed or reduced zonula occludens-1 (ZO-1), occludin, claudin-5, and β-catenin expression in HBMECs 24 h after OGD/R treatment. Inhibition of fibroblast growth factor receptor 1 (FGFR1) with PD173074 and inhibition of ERK with PD98059 reversed the protective effect of bFGF.

MMPs, BAX, and caspase-3 in the HBMECs after OGD/R treatment ($P<0.01$) (Figure 5B, 5G–5I, 5K). Inhibition of FGFR1 with PD173074 and inhibition of ERK with PD98059 inhibited the protective effect of bFGF ($P<0.05$) (Figure 5A–5K).

Treatment of HBMECs with bFGF regulated the expression of tight junction, adherens junction, and apoptosis-related genes after OGD/R

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed that tight junction, adherens junction, and apoptosis-related genes were significantly reduced in the OGD/R group compared with the control group ($P<0.01$) (Figure 6A–6E) and the expression of the BAX gene was significantly increased after OGD/R treatment ($P<0.01$) (Figure 6F). Treatment with bFGF significantly regulated the expression of these genes when compared with OGD/R treatment ($P<0.05$) (Figure 6A–6F), and inhibition of FGFR1 with PD173074 and inhibition of ERK with PD98059 inhibited gene expression, resulting in the loss of the protective effect of bFGF ($P<0.05$) (Figure 6A–6F).

Discussion

The aim of this study was to investigate the role of basic fibroblast growth factor (bFGF) *in vitro* in human brain microvascular endothelial cells (HBMECs) challenged by oxygen-glucose deprivation/reperfusion (OGD/R). Pathophysiological processes induced by damage to the blood–brain barrier (BBB) in conditions such as traumatic brain injury lead to neurological dysfunction [5,18–20]. Therefore, *in vitro* models of the microvasculature of the brain and breakdown of the BBB are important in research, bearing in mind that the BBB *in vivo* is composed predominantly of vascular endothelial cells, pericytes, astrocytes within the brain.

There have been several recent clinical studies that have shown the potential role of biomarkers for the breakdown of the BBB in traumatic brain injury. The findings from a recent study showed that microtubule-associated protein tau (MAPT) in postmortem samples of saliva and urine was a potential biomarker for disruption of the BBB in traumatic brain injury [21]. A retrospective study of elderly patients with traumatic brain injury showed that in samples of extracellular fluid sampled by microdialysis, glutamate, glycerol, and fibroblast growth
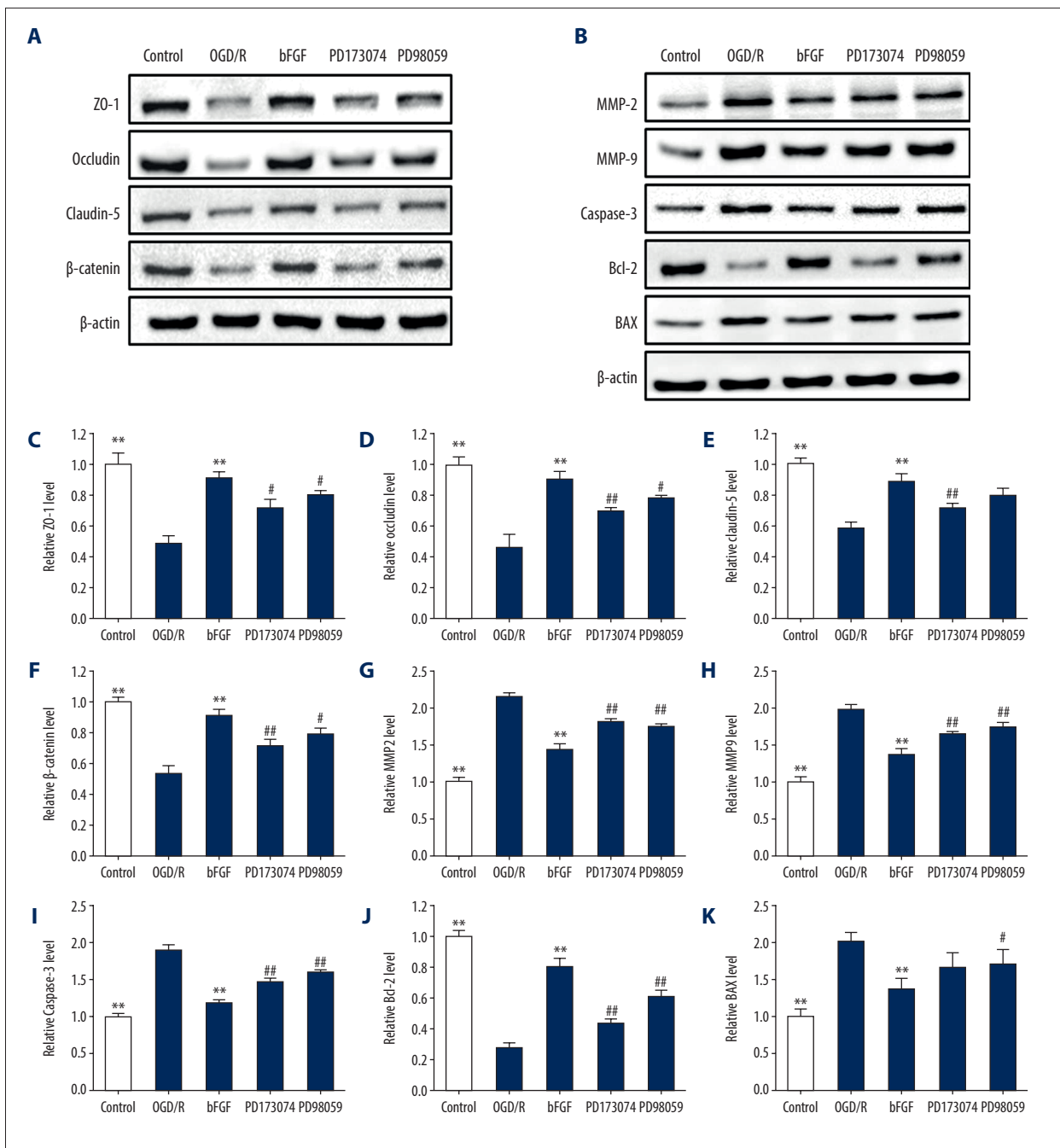
Figure 5. bFGF ameliorated the OGD/R-induced adverse effect of endothelial tight junction, adherens junction, MMPs and apoptosis proteins. (**A**) Representative Western blot image for tight junction and adherens junction proteins, zonula occludens-1 (ZO-1), occludin, claudin-5, and β-catenin. (**B**) Representative Western blot image for matrix metalloproteinases (MMPs) and apoptosis proteins, MMP2, MMP9, caspase-3, Bcl-2, and BAX. (**C–F**) Quantitative data for expression of tight junction and adherens junction proteins, zonula occludens-1 (ZO-1), occludin, claudin-5, and β-catenin in HBMECs 24 h following OGD/R. Note that bFGF promoted the expression of tight junction and adherens junction proteins, suggesting that it reversed or reduced the increased permeability after OGD/R treatment. (**G–K**) Quantitative data for expression of MMPs and apoptosis-related proteins, MMP2, MMP9, caspase-3, Bcl-2, and BAX in HBMECs 24 h following OGD/R. Note that bFGF promoted the expression of Bcl-2 and inhibited the expression of MMP2, MMP9, caspase-3, and BAX, suggesting that it reversed or reduced cellular apoptosis and integrity after OGD/R treatment. The control group or the bFGF group *vs.* the OGD/R group (** $P<0.01$). Inhibition of fibroblast growth factor receptor 1 (FGFR1) with PD173074 or inhibition of ERK with PD98059 *vs.* the bFGF group (# $P<0.05$, ## $P<0.01$). N=6 in each group. N=3 independent experiments.
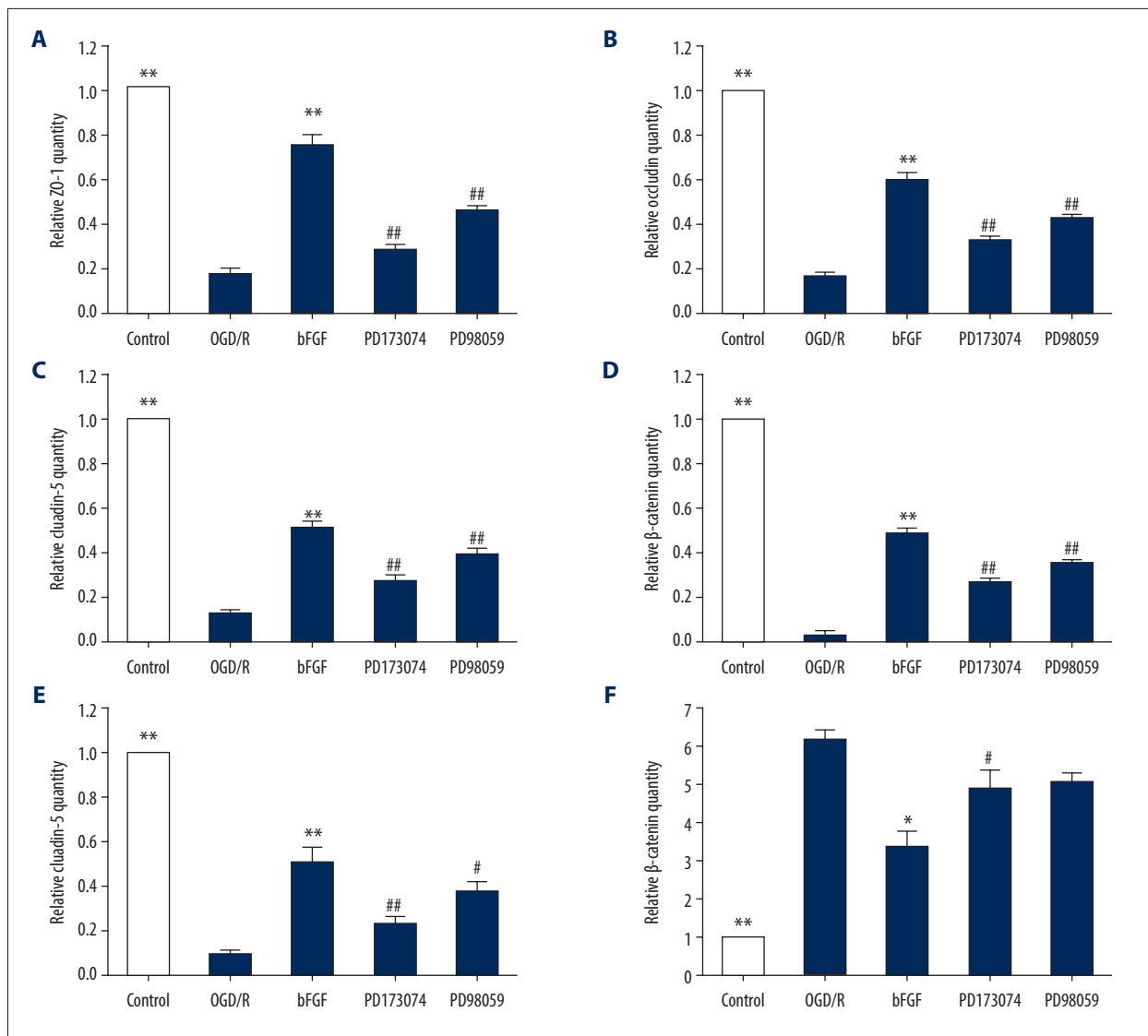
Figure 6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed that bFGF enhanced the expression of tight junction, adherens junction and apoptosis-related genes in HBMECs, while inhibition of fibroblast growth factor receptor 1 (FGFR1) with PD173074 and inhibition of ERK with PD98059 removed the protective effect of bFGF. (**A–F**) Expression of genes in HBMECs, 24 h following OGD/R, after treatment with bFGF, inhibition of FGFR1 with PD173074, or inhibition of ERK with PD98059. In the bFGF group, the relative expression levels of zonula occludens-1 (ZO-1), occludin, claudin-5, β-catenin, and Bcl-2 were significantly increased. The expression level of BAX was decreased in the bFGF group, but the protective effects of bFGF were reversed after PD173074 or PD98059 treatment. The control group or the bFGF group *vs.* the OGD/R group (* $P<0.05$, ** $P<0.01$). Inhibition of fibroblast growth factor receptor 1 (FGFR1) with PD173074 or inhibition of ERK with PD98059 *vs.* the bFGF group (# $P<0.05$, ## $P<0.01$). N=6 in each group. N=3 independent experiments.

factor 2 (FGF2) were significantly increased [22]. Cerebral levels of fibroblast growth factor receptor 2 (FGFR2) following subarachnoid hemorrhage (SAH) and traumatic brain injury were identified as potential indicators to monitor brain injury [23].

The expression of chemokines following traumatic brain injury were reported to regulate neuroinflammatory responses that affect the permeability of the BBB by interacting with CX3C chemokine receptor 1 (CX3CR1) [24]. NOD-like receptors (NLRs),

which are a subset of pattern recognition receptors (PRRs), and the interferon-inducible protein, AIM2, were reported to be involved in inflammasome-mediated apoptosis, or pyroptosis, in brain microvascular endothelial cells, resulting in BBB leakage and cerebral edema [25]. In 2009, Sun et al. reported that bFGF could act as a stem cell mitogen, to affect the levels of injury-induced neurogenesis, contributing to cognitive recovery following traumatic brain injury [26]. Also, bFGF has been shown to promote the therapeutic effect of bone marrow stem

cells (BMSCs) transplanted in rats following traumatic brain injury [27]. Therefore, modification of the levels of bFGF may have a therapeutic role in traumatic brain injury, which may be initially investigated using an *in vitro* model using HBMECs with OGD/R treatment as a model of traumatic brain injury.

The biological roles of bFGF are effected through downstream signaling pathways by binding to fibroblast growth factor receptors (FGFRs). There have been few previous studies on the regulation of bFGF and FGFRs by the BBB, but FGFR1 has been shown to mediate the regulation of bFGF in the central nervous system. During the development of the brain cortex, bFGF plays an important role in cell proliferation and neurogenesis, and the regulation of bFGF is mainly achieved by binding to FGFR1 [28]. In cases of spinal cord injury, bFGF has been shown to maintain the integrity of the blood-spinal cord barrier, and the interaction of FGFR1 with cavolin-1 may be important in this process [29]. The findings from the present study showed that FGFR1 phosphorylation was altered after OGD/R-induced vascular endothelial cell injury, whereas FGFR2 phosphorylation did not change. Also, the protective effect of bFGF on endothelial cells was prevented by inhibition of FGFR1 with PD173074 and by the inhibition of ERK with PD98059.

Also, ERK has an important neuroprotective function as the major downstream signaling pathway activated by bFGF. Modified citrus pectin (MCP) has been shown to reduce brain damage in SAH by reducing damage to the BBB and may prevent damage to the BBB after SAH by activating the ERK pathway and inhibiting galectin-3 [30]. In a model of traumatic brain injury, Liu et al. showed that sesamin could reduce damage to the BBB by reducing the activity of the ERK pathway, with anti-oxidative and anti-apoptotic effects [4]. In 2009, Narasimhan et al. showed that in mouse vascular endothelial cells studied *in vitro*, oxygen-glucose deprivation (OGD) induced vascular endothelial growth factor (VEGF) and initiated oxidative stress through FLk-1 signaling that activated the ERK pathway [31].

In the present study, the interactions of the ERK pathway with the signaling mechanisms involved in damage to HBMECs were investigated using OGD/R. The use of the ERK inhibitor, PD98059, showed that bFGF inhibited the damage to the

OGD/R-induced HBMECs, and was further blocked by the inhibitor. Proteins associated permeability and apoptosis were measured after treatment with bFGF and PD98059. Western blot and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were used to show that treatment with PD98059 reversed the bFGF-mediated regulation of protein and gene expression. These findings showed that bFGF reduced damage to the HBMECs induced by OGD/R by regulating proteins associated with permeability and apoptosis via the ERK pathway.

This preliminary study had several limitations. This study involved the *in vitro* investigation of a single cell line of HBMECs grown in culture, and the effects of bFGF and the findings cannot be directly compared with changes that occur *in vivo* at the BBB. Also, changes associated with OGD/R were studied as an *in vitro* model of traumatic brain injury, which is a complex physiological response in the brain to a variety of injuries. Further studies should be conducted using *in vivo* models to study traumatic brain injury, to provide more information on the possible roles of bFGF on microvascular endothelial cells in the brain.

Conclusions

This study aimed to investigate the role of bFGF *in vitro* in human brain microvascular endothelial cells (HBMECs) challenged by oxygen-glucose deprivation/reperfusion (OGD/R). The findings from this *in vitro* study showed that permeability and apoptosis of HBMECs challenged by OGD/R were reduced by bFGF through activation of the FGFR1 and the ERK pathway. Future *in vivo* studies are needed to determine the possible roles of bFGF on microvascular endothelial cells in the brain in models of traumatic brain injury.

Acknowledgments

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