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Research Article
Mechanism of Quercetin Mediating PI3K/Akt/mTOR Pathway in Alleviating Cerebral Vascular Stenosis Caused by Vascular Endothelial Cell Injury

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Abstract

Background and Objective: Venous stenosis can be accelerated by damage to vascular endothelial cells, which in turn leads to the ischemic death of brain cells such as cerebrovascular endothelial cells. This study explored the impact of Quercetin (Que) on the physiological responses of human brain microvascular endothelial cells (HBVECs) to oxygen-glucose deprivation (OGD). **Materials and Methods:** The HBVECs were randomly divided into Control group (CG, conventional culture), OGD group (OGDG), Que-5, 10 and 20 (supplemented with 5, 10 or 20 μmol/L Que). Cell proliferation, apoptosis, inflammation and oxidative damage factors and the expression of PI3K/Akt/mTOR pathway proteins were detected. The SNK-q test and One-way Analysis of Variance (ANOVA) were adopted (p-value below 0.05). **Results:** With the increase of Que concentration, DPPH and ABTS free radical (FR) scavenging rate (SR) increased. As against the CG, in the OGDG, the cell survival rate (CSR) dropped while the apoptosis rate (AR) rose; TNF-α, IL-6 and IL-1β, oxidative stress (OS) factors ROS, MDA and 8-OHdG were increased and SOD was decreased; damage genes VCAM-1, ICAM-1 and EDN-1 was increased; Bax, Caspase-3, p-PI3K, p-Akt and p-mTOR rose visibly and Bcl-2 dropped. However, when the Que group (QG) was compared with the OGDG, a reverse trend was observed (all $p<0.05$). The changes in cell biological behavior after Que treatment were concentration-dependent. **Conclusion:** The Que has antioxidant properties and can promote proliferation, inhibit apoptosis, improve inflammation and OS and alleviate cell damage in HBVECs cell injury model induced by OGD.

Key words: Cerebral vascular stenosis, human brain microvascular endothelial cells, Que, PI3K/Akt/mTOR pathway, oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cerebrovascular stenosis (CVS) involves the narrowing of intracranial or extracranial arteries by 50-99%, significantly increasing the risk of ischemic cerebrovascular diseases (ICVD) like cerebral insufficiency and stroke[1]. The CVS can lead to blood flow reduction in cerebral vessels, which leads to the ischemic death of brain cells such as cerebrovascular endothelial cells. Cerebral vascular endothelial cell injury under hypoxic conditions can further damage neurological function[2]. Consequently, improving the prognosis of patients with ICVD following CVS requires minimizing the damage to cerebrovascular endothelial cells. The control of cell metabolism, growth, migration, proliferation and other physiological activities is mediated[3,4], which regulates vascular endothelial cells' regular biological processes and influences vascular damage[5].

The Que is a natural flavonoid present in many plant foods, such as onions, apples, grapes and tea[6,7]. Especially in the cardiovascular field, Que has a series of positive effects on endothelial cells. At present, there are more and more studies on the efficacy of Que[8]. It has a variety of biological functions, including antihypertensive, oxygen FR scavenging, anti-adhesion and immune regulation outcomes. It can also delay aging and prevent cardiovascular diseases[9]. Due to its various biological activities, it has the functions of vasodilation and hypotension, coronary heart disease prevention and treatment, anti-thrombosis and so on. These effects can play a role in vascular repair so it has broad clinical application prospects[10]. The Que has a specific molecular structure and is recognized by the European pharmacopeia. Numerous researches have thus far shown Que's biological activity as an antioxidant, which may help to prevent and heal cerebral vascular ischemia lesions[11]. Related studies have confirmed that Que can effectively resist the apoptosis of endothelial cells caused by OS, which is helpful to maintain the physiological functional integrity of vascular endothelial cells and the repair of pathological state and can effectively reduce various adverse reactions of endothelial cells stimulated by OS[12]. Some studies have also shown that Que can promote the proliferation of vascular endothelial progenitor cells, but the specific mechanism of action is not very clear[13]. At present, the antioxidant mechanism of Que includes that it can freeze the cell cycle at the regulatory point of cell cycle and inhibit glycolysis and the activity of several enzymes. The interaction with estrogen II binding sites, these mechanisms of action can reduce the expression of genes required for cell proliferation[14].

At present, the mechanism of Que in vascular endothelial cells under OS is not clear. Therefore, this work analyzed the mechanism of action of different concentrations of Que on HBVECs injury induced by OGD. It offers a novel theoretical foundation for the use of Que in treatment applications.

MATERIALS AND METHODS

Study area: The study was performed in The First College of Clinical Medical Science, China Three Gorges University from November, 2022 to November, 2023.

Detection of antioxidant activity of Que

DPPH FR scavenging test: As 1, 2, 4, 8, 16, 32, 64 and 128 μmol/L Que standards (Sigma-Aldrich Company, USA) were applied to the colorimetric tube, 0.5 mL of 0.6 mmol/L DPPH-methanol solution was applied and the volume was fixed to 5 mL with ethanol. After mixing, the combination spent 30 min being incubated without exposure to light. The absorbance (OD) was subjected to 3 times measurements at 517 nm by V-5800 ultraviolet spectrophotometer (Shanghai Metash Instruments Co., Ltd., China). The clearance rate (CR) of DPPH FR was computed.

ABTS FR scavenging test: An ABTS stock solution was formulated by blending a 7 mmol/L ABTS in water with potassium persulfate at 2.45 mmol/L, followed by incubation at 25°C for 16 hrs. There was a 5 mL ethanol dilution of the solution and 1, 2, 4, 8, 16, 32, 64 and 128 μmol/L Que standards were applied in turn, mixing and incubation without exposure to light for 5 min. The OD was subjected to measurement at 734 nm 3 times. The CR of ABTS FR was computed.

Grouping: The HBVECs (ATCC, USA) were cultivated in a CellXpert C170i cell incubator (Eppendorf, Germany) with 5% CO_2 at 37°C. They were subcultured once every 48 to 72 hrs in DMEM complete media with 10% fetal bovine serum and 1% double antibody (Gibco, USA). The HBVECs in the log phase in the plate with 6 wells at 1×10^4/well, following 24 hrs of adherent culture, were grouped and treated:

- HBVECs in CG was cultured routinely without other treatment
- In OGDG, HBVECs was replaced with glucose-free DMEM medium, culture in a cell incubator having 95% N_2 and 5% CO_2 for 6 hrs to construct an OGD-damaged cell model
- HBVECs in QG were subjected to culture in complete medium with 5, 10 and 20 μmol/L Que based on the treatment in OGDG

MTT assay for detecting cell viability: After HBVECs were cultured for 24 hrs, 2 hrs were spent incubating a 5 mg/mL MTT solution at 20 μL with 5% CO_2 at 37°C. Following discarding the original growth media and dissolution of the crystals with 150 μL of DMSO, the mixture was agitated for 10 min. The OD value was subjected to measurement at 490 nm 3 times by HBS-1096A microplate reader (Nanjing Detie Experimental Equipment Co., Ltd., China).

Apoptosis detected by FC: Following trypsin was adopted to break down the cells, that were gathered. To resuspend the cells, 200 μL of binding buffer was applied by the guidance of Annexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich, USA). Subsequently, 5 μL Annexin V-FITC and PI working solution were applied and incubation in the dark for 20 min at 25°C. The Attune CytPix flow cytometer (Thermo Fisher Scientific, China) was adopted for detecting the AR, repeating 3 times.

Detection of inflammatory and oxidative damage factors: A phosphate buffer solution (PBS) was adopted to rinse the cells following discarding the original culture medium. To extract proteins from the cells, RIPA cell lysate (Shanghai Beyotime Biotechnology Co., Ltd., China) was adopted. The technique of bicinchoninic acid (BCA) was employed to ascertain the concentration. Following the ELISA kit instruction, inflammatory factors were determined (NanJing JianCheng Bioengineering Institute, China). The OS factors related to oxidative damage were subjected to detection 3 times.

RT-qPCR detection: The cells were rinsed with PBS following discarding the original culture medium and Trizol lysate (Thermo Fisher Scientific, China) was applied for lysing the cells to carry out the extraction of total RNA and the density and purity were measured. First-strand cDNA was synthesized by reverse transcription based on the introduction of SuperScript IV kit (Thermo Fisher Scientific, USA). It was adopted as a template and the SYBR® Green RT-qPCR kit (Sigma-Aldrich, China) was adopted for quantitatively detecting VCAM-1, ICAM-1 and ELAM-1. Quantitative primer information:

- **Upstream VCAM-1:** 5'-GGGAAGATGGTCGTGATCCTT-3'
- **Downstream:** 5'-TCTGGGGTGGTCTCGATTTTA-3'
- **Upstream ICAM-1:** 5'-ATGCCCAGACATCTGTGTCC-3'
- **Downstream:** 5′-GGGGTCTCTATGCCCAACAA-3'
- **Upstream ELAM-1:** 5'-GTCGGCCATCTCCTTACAGAA-3'
- **Downstream:** 5'-ACTCGAATCAGGACCCTCTTG-3'
- **Upstream GAPDH:** 5'-TGTGGGCATCAATGGATTTGG-3'
- **Downstream:** 5'-ACACCATGTATTCCGGGTCAAT-3'

The GAPDH as the reference and $2^{-\Delta\Delta Ct}$ as the approach was employed to compute the relative expression (RE) 3 times.

Western blot (WB) detection: The cells were rinsed with PBS following discarding the original culture medium, RIPA cell lysate to lyse the cells to extract proteins. The BCA method was employed to determine the concentration. Proteins were resolved by SDS-PAGE electrophoresis and subsequently blotted onto a PVDF membrane, which was treated with a 5% skim milk solution to prevent non-specific binding. First antibodies specific for Bax, Bcl-2, Caspase-3, p-PI3K, PI3K, p-Akt, Akt, p-mTOR and mTOR were adopted, all sourced from Abcam (UK). They were applied at a dilution of 1:1000 and incubation was carried out at 4°C for an extended period. The lgG secondary antibody containing horseradish peroxidase conjugate diluted at 1:2000 (Abcam, United Kingdom) was applied and incubation was carried out for 2 hrs at 25°C. Color development was performed using enhanced chemiluminescence and a WD-9423BC type system (Beijing Liuyi Biotechnology Co., Ltd., China) was adopted for presenting and photographing. The relative expression (RE) was quantified 3 times using lmageJ 1.8.0.345 software (National Institutes of Health, America), with β-actin used as the internal reference.

Statistical processing: The SPSS 19.0 was employed for data analysis and Mean±SD was how measurement data were expressed. The SNK-q test and One-way Analysis of Variance (ANOVA) were adopted. Statistical meaning was defined by a p-value below 0.05.

RESULTS

Antioxidant capacity of Que: The SR of DPPH and ABTS FR in Que is illustrated in Fig. 1a-b, respectively. With the increase of Que density, the SR of DPPH and ABTS FR also gradually enhanced. When Que concentration was $\geq$32 μmol/L, SR of DPPH FR was $\geq$86.4% and the SR of ABTS FR was $\geq$95.4%.

Impact of Que on OGD-induced multiplication of HBVECs: The MTT assay was employed (Fig. 2). In contrast to the CG, the CSR in the OGDG was reduced, as against the OGDG, the CSR in the QG was noticeably enhanced in a dose-dependent way (all $p<0.05$).

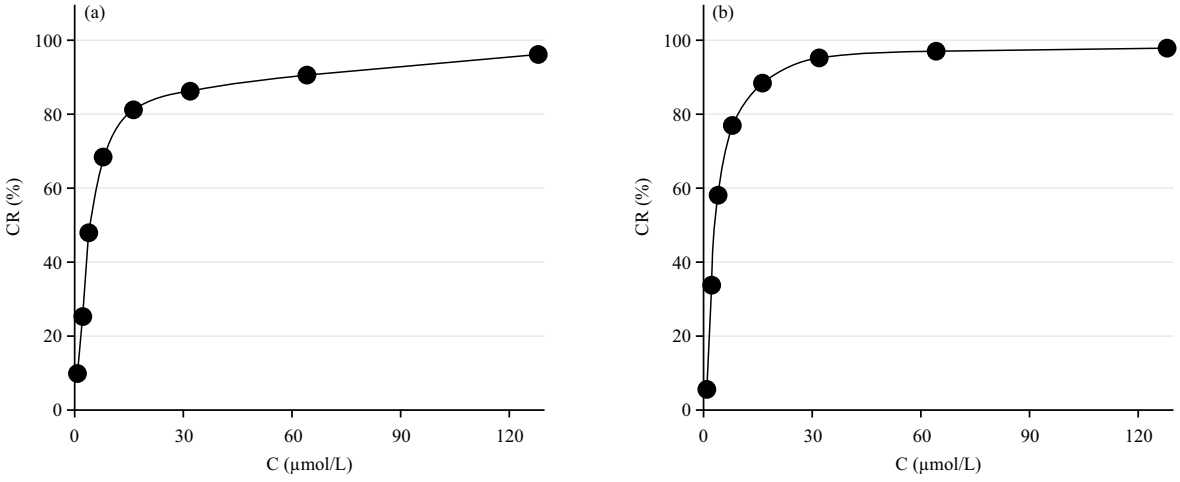

Fig. 1(a-b): Curve of FR and SR in Que, (a) DPPH FR and (b) ABTS FR

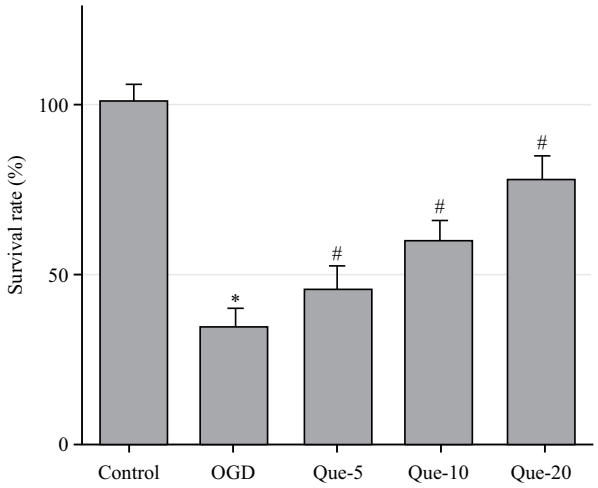

Fig. 2: Contrast of cell viability

*As against CG, #as against OGDG and p<0.05

Impact of Que on apoptosis of HBVECs induced by OGD: Figure 3a illustrates the impact of Que on the AR of HBVECs. As against the CG, the AR of the OGDG was visibly enhanced, as against the OGDG, the ARs of the QG were visibly decreased (all $p<0.05$), showing dose-dependent characteristics. Figure 3b-e depicts the alterations in the expression levels of apoptosis-related proteins in HBVECs. The results suggested that as against the CG, Bax and Caspase-3 were visibly enhanced and Bcl-2 dropped clearly in the OGDG, however, when the QG was compared with the OGDG, a reverse trend was observed in a density-dependent manner (all $p<0.05$).

Impact of Que on OGD-induced inflammation and OS in HBVECs: The levels of TNF-α (Fig. 4a), IL-6 (Fig. 4b), IL-1β (Fig. 4c), ROS (Fig. 4d), MDA (Fig. 4e), 8-OHdG (Fig. 4f) and SOD (Fig. 4g) were detected by ELISA. As against the CG, TNF-α, IL-6, IL-1β, ROS, MDA and 8-OHdG were visibly raised and SOD was markedly decreased in the OGDG, however, when the QG was compared with the OGDG, a reverse trend was observed based on density (all $p<0.05$).

Impact of Que on OGD-induced HBVECs cell damage: The expression of VCAM-1 (Fig.5a), ICAM-1 (Fig. 5b) and ELAM-1 (Fig. 5c) were detected by RT-qPCR. As against the CG, the RE of cellular adhesion molecules, ELAM-1 in the OGDG was markedly raised, as against the OGDG, that was markedly decreased in the QG (all $p<0.05$), suggesting density-dependent characteristics.

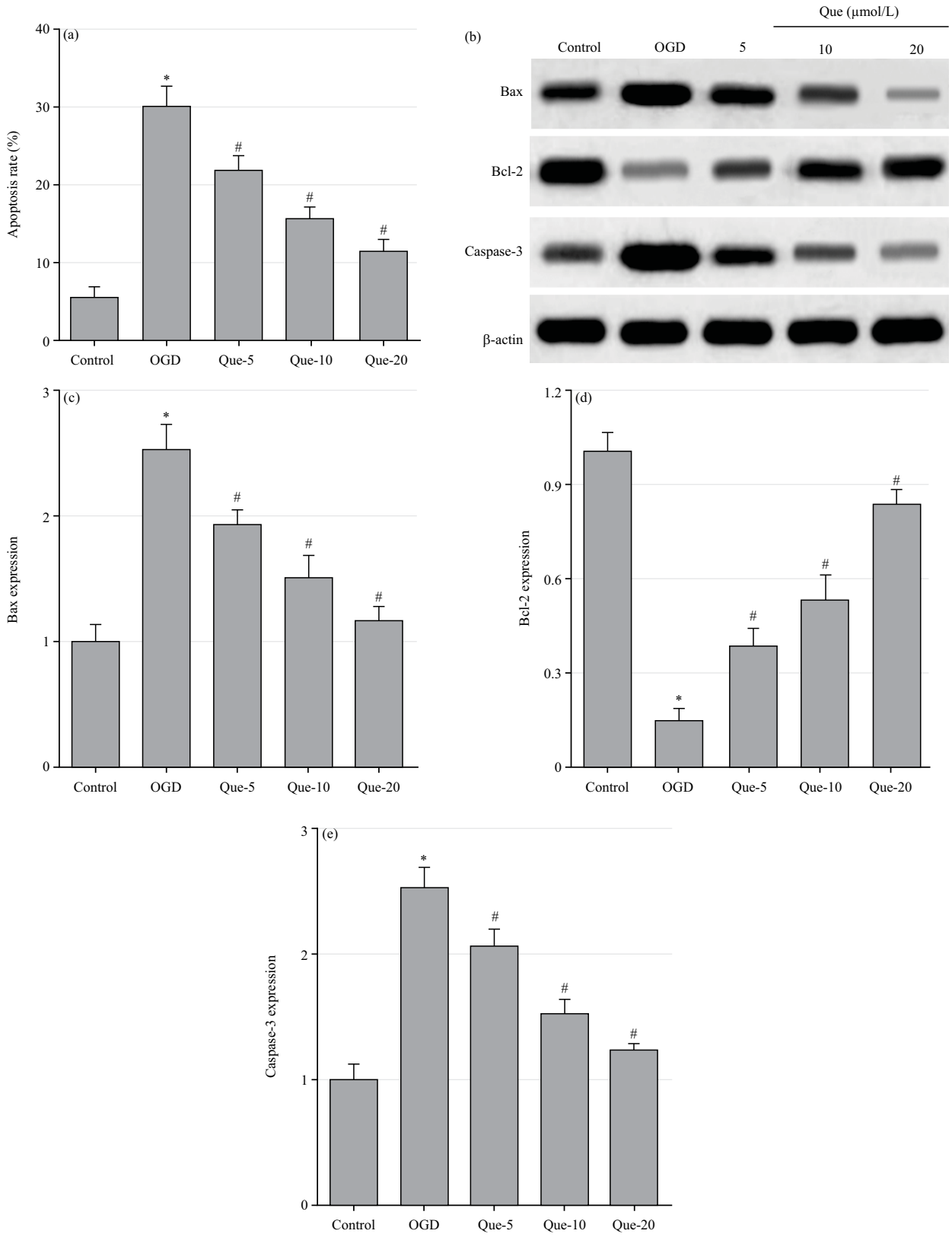

Fig. 3(a-d): Contrast of apoptosis of HBVECs, (a) AR, (b) WB, (c) Bax, (d) Bcl-2 and (e) Caspase-3

*As against CG, #as against OGDG and p<0.05

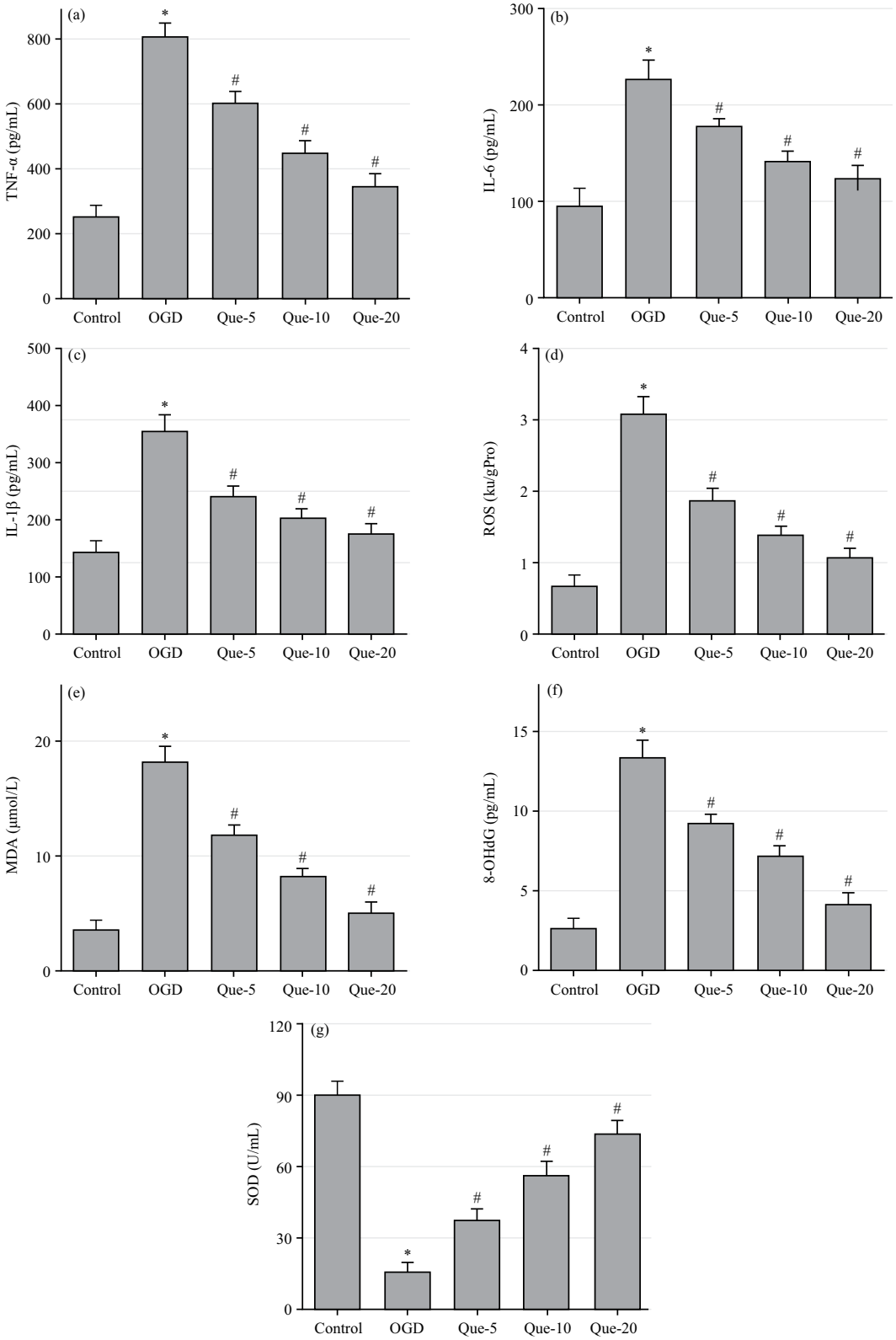

Fig. 4(a-g): Contrast of inflammation and oxidation of HBVECs, (a) TNF-α, (b) IL-6, (c) IL-1β, (d) ROS, (e) MDA, (f) 8-OHdG and (g) SOD

*As against CG, #as against OGDG and p<0.05

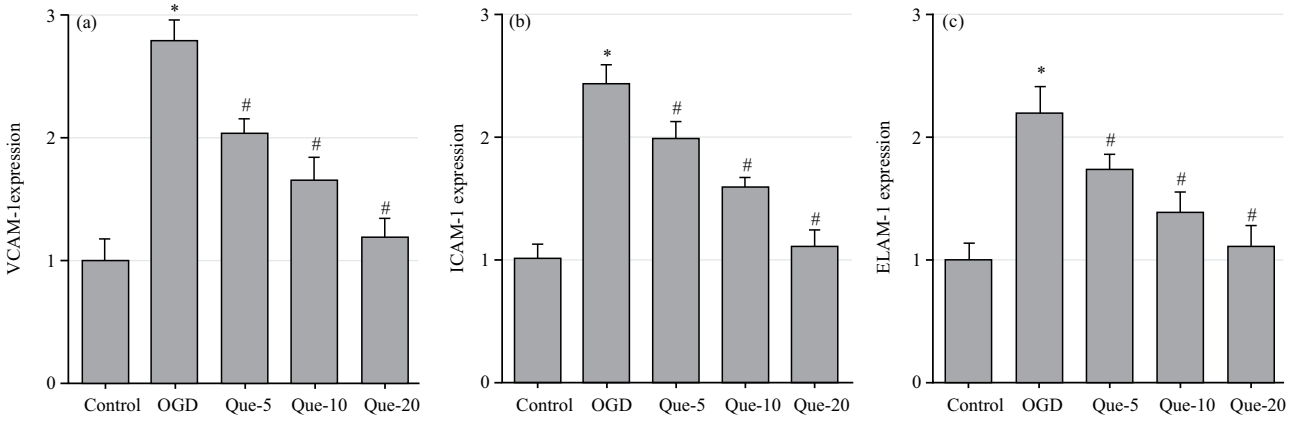

Fig. 5(a-c): Contrast of HBVECs injury, (a) VCAM-1, (b) ICAM-1 and (c) EDN-1
*As against CG, #as against OGDG and p<0.05

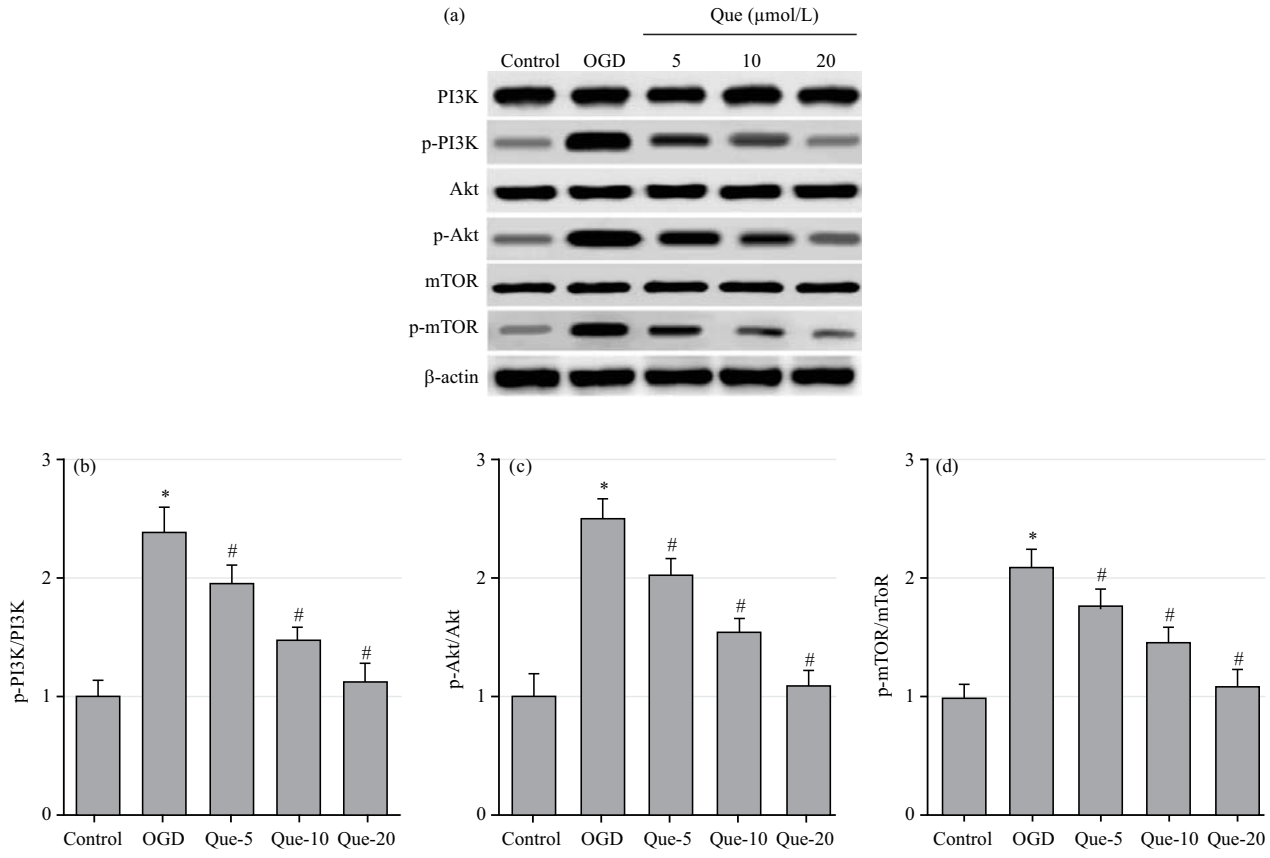

Fig. 6(a-d): Contrast of PI3K/Akt/mTOR in HBVECs, (a) WB, (b) p-PI3K/PI3K, (c) p-Akt/Akt and (d) p-mTOR/mTOR
*As against CG, #as against OGDG and p<0.05

Impact of Que on PI3K/Akt/mTOR induced by OGD in HBVECs: The WB (Fig. 6a) was adopted to detect the phosphorylation levels of PI3K (Fig. 6b), Akt (Fig. 6c) and mTOR (Fig. 6d). It was found that as against the CG, PI3K, Akt and mTOR proteins phosphorylation in the OGDG was markedly raised, as against the OGDG, those had a marked decrease based on density in the QG (all p<0.05).

DISCUSSION

This study first detected that quercetin can significantly scavenge DPPH and ABTS free radicals, indicating that quercetin has strong antioxidant properties. However, the mechanism by which quercetin improves cerebrovascular endothelial cell damage and treats cerebrovascular stenosis requires further confirmation. To this end, this study analyzed the effects of different concentrations of quercetin on the biological behavior of human brain vascular endothelial cells (HBVECs) after OGD-induced damage.

Cerebral vascular stenosis can cause blood and oxygen supply disorders in the brain and is the main cause of ICVD[15]. Cerebrovascular endothelial cell damage is an important pathological feature in the development and progression of ischemic cerebrovascular disease. Ischemia/hypoxia can cause cerebrovascular endothelial cell damage, leading to excessive vascular constriction and inhibition of collateral circulation around the ischemic focus, ultimately exacerbating ischemia/hypoxia-induced neuronal damage[16,17]. Deepika *et al.*[18] demonstrated that quercetin has a wide range of physiological and pharmacological activities, can cross the blood-brain barrier and is used in the treatment of neurodegenerative diseases, cancer and inflammation. The results of this study are similar to those of Wangsawangrung *et al.*[19], who prepared quercetin/HP-β-CD hydrogels and found that they can scavenge DPPH free radicals, indicating that quercetin has strong antioxidant capabilities.

The OGD is an experimental method used to simulate hypoxic-ischemic injury in cells *in vitro*. This study found that after OGD induction, the survival rate of HBVECs decreased and the apoptosis rate increased. This was similar to the results of Yang *et al.*[20], who used OGD to induce ischemic injury in HUVEC cells, indicating that hypoxia and ischemia conditions cause vascular endothelial cell damage, characterized by inhibited proliferation and increased apoptosis. Furthermore, this study found that after quercetin intervention, the survival rate of the OGD-induced HBVECs damage model increased and the apoptosis rate decreased, indicating that quercetin can significantly alleviate hypoxia/ischemia-induced human cerebrovascular endothelial cell damage. Brain injury induces oxidative stress, further promoting neuronal apoptosis[21]. During oxidative stress, a large amount of ROS with strong oxidizing properties is generated, attacking intracellular lipids and nucleic acids, causing cell damage and generating oxidative products such as 8-OHdG and MDA, while reducing the activity of antioxidant enzyme SOD[22,23]. Excessive oxidative stress promotes the expression of adhesion molecules such as VCAM-1, ICAM-1 and ELAM-1, leading to the activation and further damage of vascular endothelial cells[24].

This study found that after OGD induction, the levels of ROS, MDA and 8-OHdG increased in HBVECs cells, the expression levels of VCAM-1, ICAM-1 and ELAM-1 increased and SOD activity decreased. This indicates that hypoxia/ischemia interferes with the secretion of intercellular adhesion molecules by cerebrovascular endothelial cells, causing oxidative stress and cell damage. Furthermore, this study found that after quercetin intervention, the levels of ROS, MDA and 8-OHdG decreased in the OGD-induced HBVECs damage model, the expression levels of VCAM-1, ICAM-1 and ELAM-1 decreased and SOD activity increased, showing a dose-dependent effect. This was similar to the results of Dong *et al.*[25], who found that quercetin can inhibit Nrf2 expression and MDA production in a type 1 diabetic rat model, exerting antioxidant effects. It indicates that quercetin can significantly improve the oxidative stress response induced by hypoxia/ischemia in human cerebrovascular endothelial cells, reduce the secretion of intercellular adhesion molecules and exert a protective effect on cells.

Inflammatory response is an important mechanism in the occurrence of cerebrovascular disease and inflammation and autophagy are the main mechanisms leading to ischemic cerebrovascular disease[26]. This study found that after OGD induction, the levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β increased in HBVECs cells, while quercetin intervention reduced the levels of TNF-α, IL-6 and IL-1β in the damage model, showing a dose-dependent effect. This is similar to the results of Beken *et al.*[27], who found that quercetin can reduce the levels of IL-6, IL-8 and IL-1β in an atopic dermatitis cell model and enhance SOD expression. It was also similar to the findings of Tang *et al.*[28], who found that quercetin can reduce the levels of TNF-α, IL-6 and IL-1β in RAW264.7 cells stimulated by lipopolysaccharides and reduce ROS levels. This indicates that quercetin can reduce the release of pro-inflammatory cytokines, thereby alleviating the inflammatory response induced by hypoxia/ischemia in human cerebrovascular endothelial cells. The PI3K/Akt/mTOR signaling pathway regulates the infiltration of innate immune cells and promotes their migration to the site of inflammation[29]. Activation of this pathway enhances NF-κB activation and promotes the expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and adhesion molecules (VCAM-1, ICAM-1 and ELAM-1), leading to an inflammatory response. This study found that after OGD induction, the phosphorylation levels of PI3K, Akt and mTOR increased in HBVECs cells, indicating that hypoxia/ischemia activates the PI3K/Akt/mTOR signaling pathway in cerebrovascular endothelial cells, thereby causing an inflammatory

response[30,31]. Furthermore, it found that after quercetin intervention, the phosphorylation levels of PI3K, Akt and mTOR decreased in the OGD-induced HBVECs damage model, showing a dose-dependent effect. This was similar to the findings of He *et al.*[32], who found that quercetin can inhibit the activation of the PI3K/Akt/mTOR pathway in high-glucose cultured SRA01/04 cells, regulating the early development of diabetic cataracts. It was also similar to the findings of Li *et al.*[33] who found that quercetin can regulate the PI3K/Akt/NF-κB pathway to promote M2 polarization of microglia/macrophages after ODG/R, thereby treating cerebral ischemia/reperfusion injury. This indicates that quercetin can mediate the activation of the PI3K/Akt/mTOR signaling pathway, thereby inhibiting hypoxia/ischemia-induced damage to human cerebrovascular endothelial cells.

CONCLUSION

The Que has antioxidant activity, which can reduce OGD-induced injury of HBVECs by promoting multiplication, inhibiting inflammation, OS response, apoptosis, etc. The discovery of this mechanism can provide new research ideas for ICVD results from CVS. It also provides a reference for a better understanding and application of Que in ICVD.

SIGNIFICANCE STATEMENT

This study aimed to explore the mechanism by which quercetin alleviates oxygen-glucose deprivation (OGD) induced damage to human brain microvascular endothelial cells through the PI3K/Akt/mTOR pathway. Key findings indicated that quercetin has antioxidant effects, can enhance cell survival rate, inhibit cell apoptosis, improve inflammatory response and oxidative stress and reduce cell damage. These findings are important as they reveal the potential application of quercetin in the treatment of cerebral vascular stenosis, providing a scientific basis for the development of new treatment methods.

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