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Oxyresveratrol reduces lipopolysaccharide-induced inflammation and oxidative stress through inactivation of MAPK and NF-κB signaling in brain endothelial cells

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ABSTRACT

Inflammatory responses and oxidative stress damage the integrity of the blood-brain barrier (BBB), which is a primary pathological modulator of neurodegenerative diseases. Brain endothelial cells are crucial components of BBB. In the present study, the effect of oxyresveratrol on lipopolysaccharide (LPS)-induced brain endothelial (bEnd.3) cells was assessed. Our results showed that oxyresveratrol diminished protein expressions of inducible nitric oxide synthase (iNOS) and adhesion molecules including intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), nitric oxide (NO) production, and proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) in LPS-elicited bEnd.3 cells. These anti-inflammatory effects were mediated through suppressing nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. In addition, we found that oxyresveratrol reduced reactive oxygen species (ROS) levels. To conclude, the current results demonstrated the protective role of oxyresveratrol against LPS-induced inflammation and oxidative stress in bEnd.3 cells, suggesting its potential effect for mitigating neurodegenerative and cerebrovascular diseases.

1. Introduction

Inflammation and oxidative stress are widely considered to contribute to various diseases, including neurodegenerative diseases [1], cardiovascular diseases [2], and metabolic disorders [3]. Blood-brain barrier (BBB) is an important barrier that separates the brain from the blood, protecting the central nervous system (CNS) from toxins and pathogens in the blood [4]. BBB is mainly composed of endothelial cells, pericytes, and astrocytic end-feet [5]. Inflammation leads to BBB disruption and thereby increases the risk of brain diseases such as Alzheimer's disease, stroke, multiple sclerosis, and post-traumatic brain injury [6]. Immortalized mouse brain endothelial cells (bEnd.3 cells) together with lipopolysaccharide (LPS) induction, are often used to study inflammation related to brain diseases in vitro. LPS, a major component of the gram-negative bacterial cell wall, induces inflammation and oxidative stress through activating classic nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways [7].

Oxyresveratrol (*trans*-2,3′,4,5′-tetrahydroxystilbene) (Fig. 1) is a natural stilbene found in grapes, peanuts, and mulberries [8]. It is an isomer of hydroxylated resveratrol (*trans*-3,4′,5-trihydroxystilbene) with better water solubility [9] and stronger antioxidant effects [10]. Oxyresveratrol exhibits various biological activities, including antioxidant, anti-inflammatory [8], antineoplastic, tyrosinase inhibitory, and immune-boosting properties [11]. Previous studies have shown the anti-inflammatory properties of oxyresveratrol, reducing the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as the production of inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in macrophages [12], human keratinocytes [13] and BV-2 microglial cells [14]. In addition, oxyresveratrol prevents the upregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) induced by TNF-α in endothelial progenitor cells [15]. However, no research has focused on the anti-inflammatory effect of

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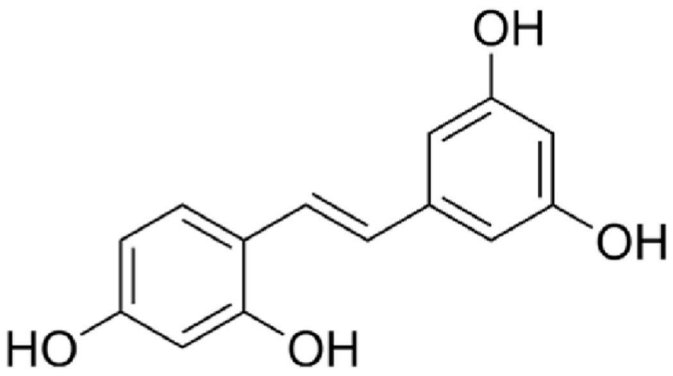

Fig. 1. Chemical structure of oxyresveratrol.

oxyresveratrol on brain endothelial cells. Therefore, our study aims to explore whether oxyresveratrol has anti-inflammatory and antioxidative effects on an LPS-induced bEnd.3 cells model and investigate the underlying mechanisms.

2. Materials and methods

2.1. Cell culture

The bEnd.3 cells (ATCC, USA) were cultured in DMEM/High Glucose medium (HyClone, USA) with 10 % (V/V) fetal bovine serum (FBS, Gibco, USA) and 1 % (V/V) penicillin streptomycin (P/S, Gibco, USA) and maintained in the atmosphere of 95 % O_2 and 5 % CO_2 at 37 °C. The cells were passed when they reached 80 % confluent in flask (SPL, Korea). Medium was changed to DMEM/High Glucose with 1 % FBS (V/V) and 1 % (V/V) P/S before treating with oxyresveratrol (TCI, Japan). Cells were divided into four groups: control, model (1 μg/mL LPS), low dose (1 μg/mL LPS and 10 μM oxyresveratrol) and high dose groups (1 μg/mL LPS and 50 μM oxyresveratrol). Oxyresveratrol was dissolved by DMSO (Sigma-Aldrich, USA). LPS was acquired from Sigma-Aldrich, USA.

2.2. Cell viability assay

The bEnd.3 cells were treated with different concentrations of oxyresveratrol (0, 5, 10, 25, 50 μM) for 24 h in 96-well plates. 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution (J&K Scientific, China) was added into cells after removing the medium. Cells were incubated at 37 °C for 4 h. DMSO was added after removing MTT solution. Absorbance of each well was detected at a wavelength of 490 nm by SpectraMax iD5 Multi-Mode microplate reader (Molecular Devices, USA).

2.3. Measurement of nitric oxide (NO)

The bEnd.3 cells were pre-treated with oxyresveratrol for 4 h (low dose group and high dose group) and stimulated by 1 μg/mL LPS for 24 h in 6-well plates. The cultured medium was collected and added to 96-well plate at a volume of 50 μl/well. Griess Reagent I and Griess Reagent II from NO test kit (Beyotime, China) at room temperature were successively added to each well at a volume of 50 μl/well. Absorbance of each well was detected at a wavelength of 540 nm by SpectraMax iD5 Multi-Mode microplate reader.

2.4. Measurement of inflammatory cytokines

The bEnd.3 cells were seeded in 6-well plates (6×10^5 cells/well) for culture overnight and had the same drug treatments as mentioned above. The conditioned cultured medium was collected for determination of inflammatory cytokines, IL-6 and TNF-α by ELISA kits (Milbio, China) according to the manufacturers' instructions.

2.5. Western blot analysis

The bEnd.3 cells in 6-well plates had the same drug treatments as mentioned above. 70 μL RIPA buffer (Beyotime, China) with 1 % protease inhibitor cocktail (Thermo Scientific, USA) and 1 % phenylmethylsulfonyl fluoride (PMSF, Thermo Scientific, USA) were added to cells on ice after the medium was removed and washed twice with PBS. The supernatants were collected after centrifugation at 4 °C for 30 min at 15000 rpm. BCA protein assay kit (Beyotime, China) was used to detect the concentrations of total protein of collected supernatants. Proteins were denatured by SDS/PAGE loading buffer (5X, Beyotime, China) and boiled for 8 min at 99 °C. The same amounts of protein (15 μg) were isolated by 10 % SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF, Bio-Rad, USA) membranes. The membranes were blocked by 5 % de-fatted milk (Bio-Rad, USA) in Tris-Buffered Saline/Tween 20 (TBST) for 2 h and probed with primary antibodies: GAPDH (Proteintech, 60004-1-Ig), IκBα (Cell Signaling Technology, 4814S), *p*-IκBα (Cell Signaling Technology, 2859S), p38 (Cell Signaling Technology, 8690S), p-p38 (Cell Signaling Technology, 4511S), NF-κB p65 (Proteintech, 66535), p–NF–κB p65 (Cell Signaling Technology, 3033S), JNK (Cell Signaling Technology, 9252S), *p*-JNK (Proteintech, 80024-1-RR), ICAM-1 (Proteintech, 10831-1-AP), VCAM-1 (Cell Signaling Technology, 14694S), IKKα (Cell Signaling Technology, 61294S), IKKβ (Cell Signaling Technology, 8943S), *p*-IKKα/β (Cell Signaling Technology, 2697S) and iNOS (Proteintech, 18985-1-AP) diluted with TBST overnight at 4 °C. The membranes were washed with TBST for 30 min and incubated with the secondary antibodies (anti-rabbit, Beyotime, A0208; anti-mouse, Beyotime, A02616) diluted with TBST at 1:3000 for 1.5 h at room temperature. After washing with TBST for 15 min, the protein bands were visualized by Supersignal™ West Femto Highest Sensitivity Substrate (Thermo Scientific, USA). The bands were photographed by ChemiDocTM MP Imaging System (BIO-RAD, USA) and quantified by Image Lab software.

2.6. Immunofluorescence assay

The bEnd.3 cells were seeded in PhenoPlate-96 TC + lid/case 2 × 20B (PerkinElmer, Canada) and cultured for one day. Cells had the same drug treatments as mentioned above. After removing the medium, cells were washed three times with PBS and fixed with 4 % polyformaldehyde (Beyotime, P0099) for 20 min. After washing by PBS three times, cells were permeabilized with 0.1 % Triton X-100 (Beyotime, T8787) dissolved in PBS for 10 min. Cells were blocked for 30 min by blocking solution: 3 % bovine serum albumin (BSA, Sigma-Aldrich USA) containing 0.3 % glycine (Sigma-Aldrich, USA) and 0.1 % tween-20 (Sigma-Aldrich, USA). Subsequently, cells were incubated with p–NF–κB p65 primary antibody diluted with blocking solution at 1:800 overnight at 4 °C. Cells were washed with PBS for 3 times and incubated with the Alexa Fluor 488-labeled secondary antibody (Beyotime, A0423) diluted with blocking solution at 1:500 for 1 h at room temperature. Nucleus was stained by DAPI (beyotime, C1006) for 5 min at room temperature. Finally, fluorescence images were photographed by Opera Phenix Plus High-Content Screening System (PerkinElmer, Canada).

2.7. Detection of intracellular reactive oxygen species (ROS) production

Dihydroethidium (DHE, Invitrogen, D11347) and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Invitrogen, D6827) staining measurements of ROS production were conducted. The bEnd.3 cells were pre-treated with oxyresveratrol for 4 h and stimulated by LPS for 24 h in 24-well plates as mentioned above. After removing the medium, cells were stained by DHE or CM-H_2DCFDA according to the instructions. The fluorescence of DHE staining was detected by Incucyte S3 Live-Cell Analysis System (BD, USA). The

fluorescence of CM-H_2DCFDA staining was detected by Leica-DMi8 Inverted fluorescent microscope (Leica, Germany).

2.8. Statistical analysis

All data were analyzed by GraphPad Prism 10 software, Image Lab software, ImageJ software, FlowJo software (BD, America) and shown as mean ± SEM. Significant differences between the experimental groups were determined using one-way ANOVA, followed by post hoc analysis Bonferroni's test. $P < 0.05$ was regarded as statistically significant difference. Every experiment was conducted for at least three times.

3. Results

3.1. Oxyresveratrol reduces adhesion molecules in bEnd.3 cells stimulated with LPS

We firstly evaluated whether oxyresveratrol with various concentrations influenced the cell viability of bEnd.3 cells by MTT analysis. The results showed that there was no significant effect in the cell viability after 24 h of treatment with oxyresveratrol at 5 μM, 10 μM, 25 μM and 50 μM in bEnd.3 cells (Fig. 2A). Among the safe concentrations, we selected two, 10 and 50 μM to investigate the effects of oxyresveratrol on bEnd.3 cells in the following experiments.

We evaluated the anti-inflammatory effect of oxyresveratrol using the LPS-induced bEnd.3 endothelial cell model. As shown in Fig. 2B–D, LPS stimulation (1 μg/mL) for 24 h led to enhanced protein expressions of adhesion molecules including ICAM-1 and VCAM-1 in bEnd.3 cells. Oxyresveratrol used at 10 and 50 μM significantly reversed the increase of the protein expressions of ICAM-1 and VCAM-1 upon LPS exposure, implicating that oxyresveratrol could reduce adhesion molecules on the endothelial surface of BBB during inflammation.

3.2. Oxyresveratrol diminishes production of NO and inflammatory cytokines in LPS-treated bEnd.3 cells

In bEnd.3 cells, stimulation with LPS at 1 μg/mL for 24 h increased protein level of iNOS, causing an outbreak of NO production, which could lead to the development of inflammation. The present study demonstrated that the increase of iNOS expression stimulated with LPS was diminished by higher concentration 50 μM of oxyresveratrol (Fig. 3A). Meanwhile, oxyresveratrol markedly alleviated NO generation at 10 μM and 50 μM (Fig. 3B). Furthermore, oxyresveratrol pretreatment inhibited the LPS-triggered elevations of IL-6 and TNF-α in bEnd.3 cells (Fig. 3C and D). These results indicated that the treatment of oxyresveratrol could ameliorate LPS-mediated proinflammatory responses.

3.3. Oxyresveratrol mitigates MAPK signaling in bEnd.3 cells stimulated with LPS

To investigate the mechanisms of LPS-activated inflammation, we measured MAPK signaling pathway by western blots. In bEnd.3 cells, 24-h LPS (1 μg/mL) induction elevated the phosphorylation expressions of p38 MAPK at Thr180/Tyr182 (Fig. 4A) and c-Jun N-terminal kinase (JNK) at Thr183/Tyr185 (Fig. 4B), which were changed by oxyresveratrol in concentration-dependent manner. These results demonstrated that phosphorylation of p38 and JNK in MAPKs involved in LPS-triggered inflammatory responses could be reversed by oxyresveratrol in bEnd.3 cells.

3.4. Oxyresveratrol diminishes NF-κB inflammatory signaling in bEnd.3 cells infected with LPS

Generally, BBB dysfunction caused by the increase of adhesion molecules is due to activation of NF-κB signaling pathway in neuroinflammation. Upon stimulation to LPS (1 μg/mL, 24 h), the phosphorylation of IKKα/β at Ser176/180, IκBα at Ser32 and p65 at Ser536 in NF-κB pathway in bEnd.3 cells were noticeably enhanced (Fig. 5). Oxyresveratrol at 50 μM effectively downregulated the phosphorylation of inhibitory κB kinase (IKK)α/β at Ser176/180 (Fig. 5A–C). The enhanced phosphorylation of IκBα at Ser32 and p65 at Ser536 were normalized by co-treatment of oxyresveratrol at both concentrations 10 μM and 50 μM (Fig. 5D–E), whereas the total protein levels of IKKα/β, IκBα and p65 were unchanged. In addition, the NF-κB p65 phosphorylation and nuclear translocation were simultaneously assessed by immunofluorescence. The results showed that LPS-induced bEnd.3 cells

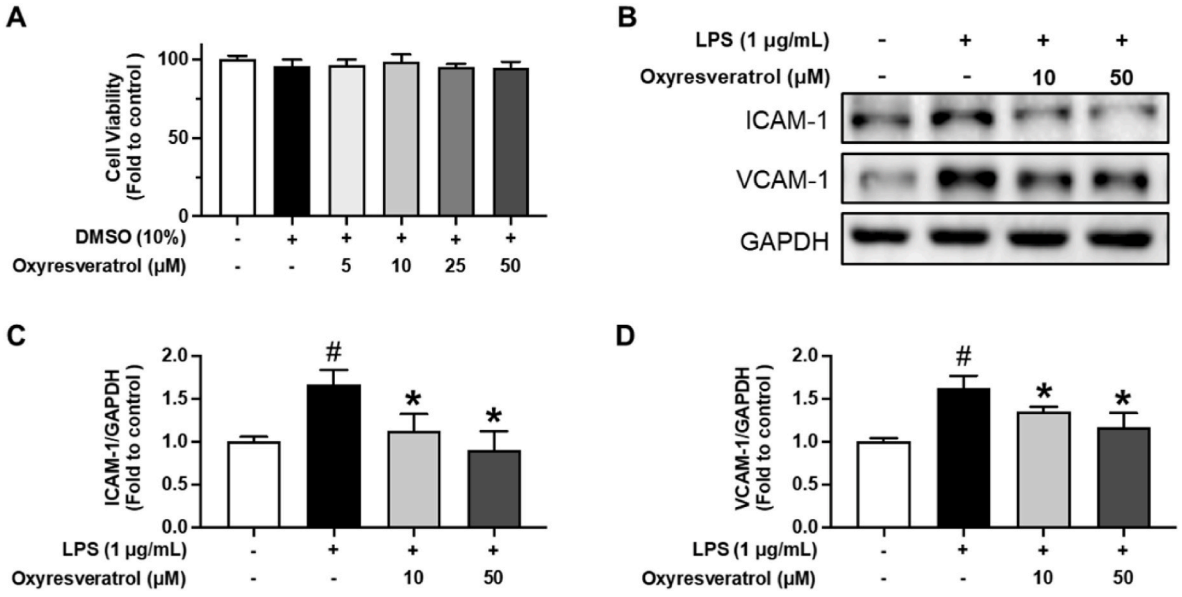

Fig. 2. Effect of oxyresveratrol on cell viability and on LPS-stimulated adhesion molecules in bEnd.3 cells. (A) Cells were cultured with the indicated concentration (5–50 μM) of oxyresveratrol for 24 h, and the numbers of viable cells were determined by MTT assay. **(B–D)** Cells were pretreated with 10 μM and 50 μM of oxyresveratrol for 4 h followed by the presence of 1 μg/mL LPS or with LPS alone for 24 h for determination of ICAM-1 and VCAM-1 protein expressions by western blots. All data are mean ± SEM (n = 6). #$p < 0.05$ vs. control, *$p < 0.05$ vs. LPS.

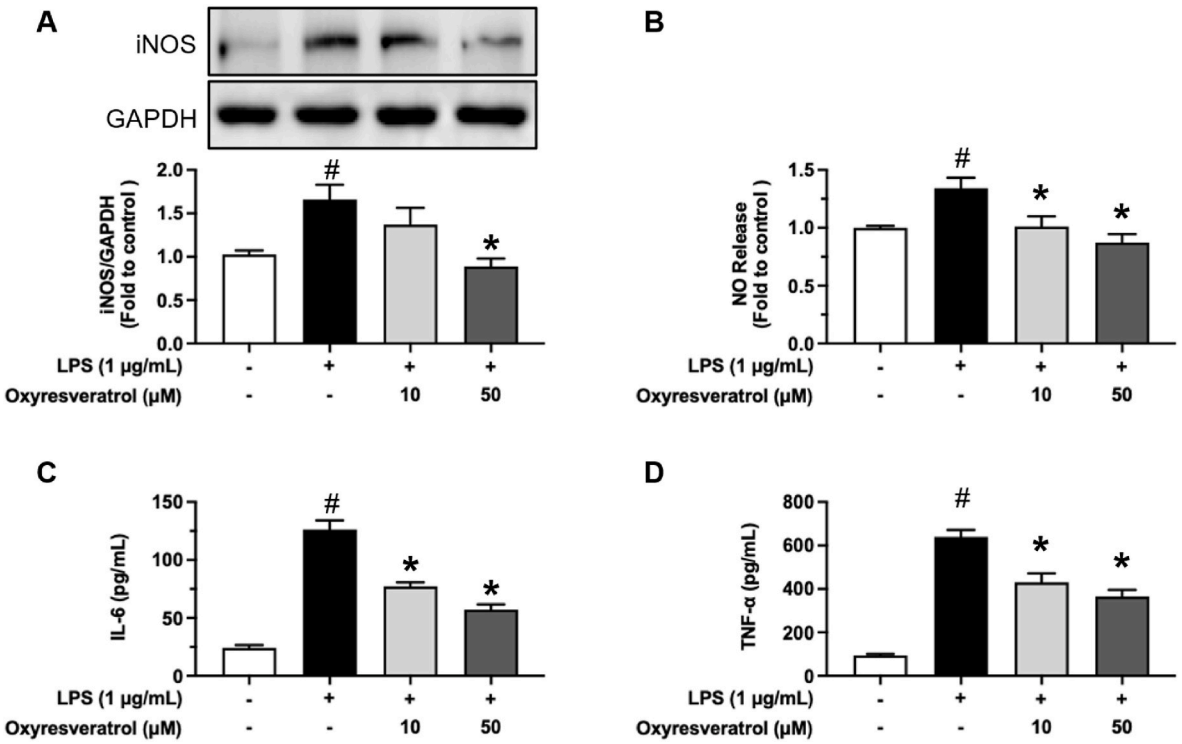

Fig. 3. Effect of oxyresveratrol on LPS-induced iNOS expression, NO production and inflammatory cytokines in bEnd.3 cells. Cells were pretreated with 10 and 50 μM of oxyresveratrol for 4 h followed by the presence of 1 μg/mL LPS or with LPS alone for 24 h. **(A)** Western blot analysis of iNOS protein expression (130 kDa). **(B)** The levels of NO generation were examined by Griess reagent assay. **(C, D)** The proinflammatory cytokines levels of IL-6 and TNF-α were tested by ELISA. All data are mean ± SEM (n = 6). #$p < 0.05$ vs. Control, *$p < 0.05$ vs. LPS.

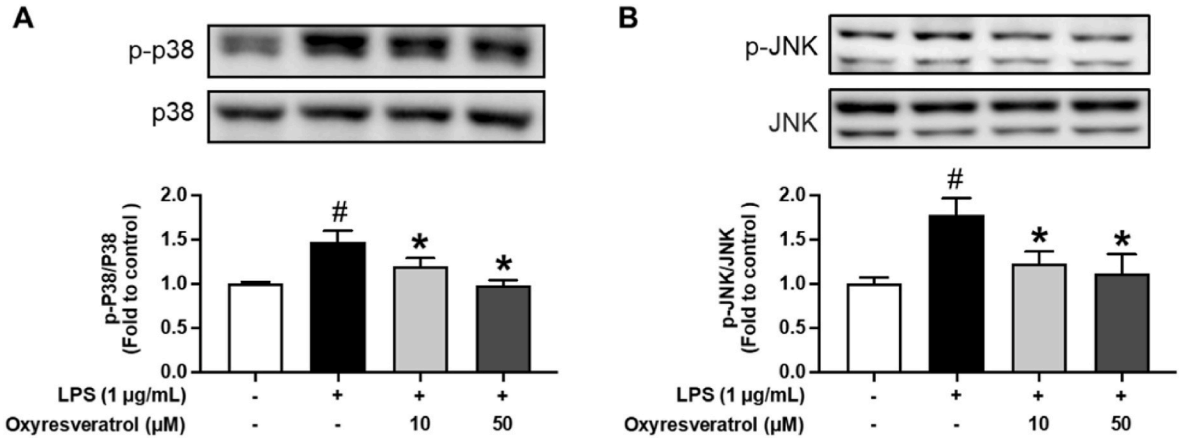

Fig. 4. Effect of oxyresveratrol on LPS-stimulated JNK and p38 MAPK in bEnd.3 cells. Cells were incubation with 10 μM and 50 μM of oxyresveratrol for 4 h and then co-treated the presence of 1 μg/mL LPS or with LPS alone for 24 h. Representative western blots and their analysis of the phosphorylation of **(A)** p38 at Thr180/Tyr182 (40 kDa) and **(B)** JNK at Thr183/Tyr185 (46 kDa, 54 kDa) in bEnd.3 cells. All data are mean ± SEM (n = 6). #$p < 0.05$ vs. Control, *$p < 0.05$ vs. LPS.

(1 μg/mL) had upregulation of NF-κB p65 phosphorylation and nuclear translocation, while co-incubation of oxyresveratrol diminished such translocation (Fig. 6). These data illustrated that oxyresveratrol could abate the inflammatory responses via NF-κB signaling pathway in bEnd.3 cells infected with LPS.

3.5. Oxyresveratrol suppresses oxidative stress in bEnd.3 cells insulted by LPS

To examine the antioxidant capacity of oxyresveratrol, ROS levels in bEnd.3 cells were measured using two probes including DHE and CM-H_2DCFDA. As shown in Fig. 7, after incubation with LPS concentration of 1 μg/mL for 24 h, the fluorescence intensity remarkably increased, suggesting an excessive level of intracellular ROS. Pretreatment of oxyresveratrol (10 and 50 μM, 4 h) significantly suppressed the level of ROS in bEnd.3 cells imposed by LPS. Our results indicated that oxyresveratrol could attenuate ROS generation in bEnd.3 cells.

4. Discussion

In our present study, we explored the suppressive effects of oxyresveratrol on inflammation and oxidative stress in brain endothelial cells and investigated the underlying mechanisms. Our results suggested that treatment with oxyresveratrol following LPS stimulation strongly

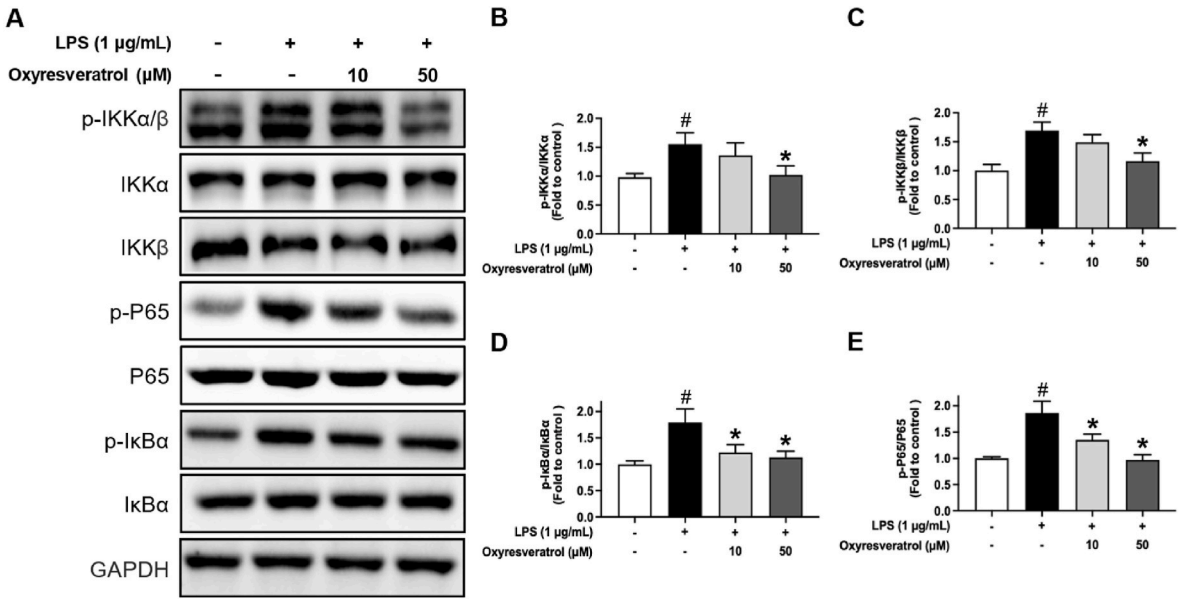

Fig. 5. Effect of oxyresveratrol on LPS-infected NF-κB pathway in bEnd.3 cells. Cells were incubation with 10 μM and 50 μM of oxyresveratrol for 4 h and then co-treated the presence of 1 μg/mL LPS or with LPS alone for 24 h. **(A)** Representative western blots and **(B–E)** their quantifications of the phosphorylation of IKKα/β at Ser176/180 (85 kDa, 87 kDa), IκBα at Ser32 (39 kDa) and p65 at Ser536 (65 kDa) compared to their respective total protein with GAPDH (37 kDa) as housekeeping protein. All data are mean ± SEM (n = 6). #$p < 0.05$ vs. Control, *$p < 0.05$ vs. LPS.

attenuated inflammatory responses and oxidative stress in bEnd.3 cells. This effect was achieved by suppressing MAPK and NF-κB signaling pathways.

The BBB is composed of three types of cells in the cerebral microvascular system, including endothelial cells, astrocytes, and pericytes [16]. Among them, endothelial cells of brain microvessels are the most important structure for BBB formation, as they control the entry and exit of blood-derived substances and leukocytes under normal physiological conditions [17]. In pathological conditions, most leukocytes are activated and migrated, thereby disrupting the integrity of BBB, which could cause neurological disorders, including neuroinflammation, stroke, and neurodegenerative diseases [18–20]. In our study, we chose bEnd.3 cells to investigate the effect of oxyresveratrol on inflammation and oxidative stress related to BBB dysfunction. The BBB damage is closely related to the upregulation of adhesion molecules in bEnd.3 cells [21]. ICAM-1 and VCAM-1 are essential receptors that maintain intercellular adhesion and affect leukocyte migration, which are significantly increased in multiple sclerosis, dementia, cognitive impairment, and Alzheimer's diseases associated with neuroinflammatory conditions [20,22]. Our data agreed with prior studies showing that LPS-induced bEnd.3 cells exhibited enhanced expressions of ICAM-1 and VCAM-1, leading to leukocyte adhesion to brain microvascular endothelial cells. This disruption compromised the tight junction structure and increased vascular wall permeability, resulting in BBB dysfunction. These findings highlight the relevance of adhesion molecules expressed by brain endothelial cells to BBB damage and brain injury [23]. Interestingly, our results demonstrated that oxyresveratrol mitigated the upregulation of ICAM-1 and VCAM-1 imposed by LPS, suggesting a potential protective effect of oxyresveratrol against adhesion molecule-mediated BBB dysfunction.

LPS is commonly used to induce cells to secrete cytokines and other inflammatory components, such as IL-6 and TNF-α, thereby increasing the permeability of cerebral microvasculature [24,25]. When LPS triggers an inflammatory response, the upregulations of ICAM-1 and VCAM-1 in brain endothelial cells disrupt the integrity of BBB and exacerbate cell permeability [26]. Given that LPS-mediated inflammation can contribute to BBB disruption, we selected LPS as an inducer to stimulate bEnd.3 cells. In a rat model of bacterial meningitis, the activation of the inflammatory cytokine TNF-α induces subarachnoid inflammation and brain edema, resulting in neuronal damage [27]. Increased levels of NO, IL-6 and TNF-α in the brain of ischemia and hypoxia model cause the destruction of tight junction and adhesion molecules, resulting in BBB dysfunction [28]. It has been well established that LPS stimulation triggers iNOS expression and thereby NO generation, which is implicated in neurological disorders and other pathological processes [29]. Moreover, LPS can elevate ROS levels in cerebral vascular endothelial cells, causing oxidative stress [30]. Oxidative stress is a key mediator in the development of neurodegeneration and vascular disorder and has been proven to be a major factor contributing to endothelial dysfunction [31,32]. In certain situations like neuroinflammation and cerebral hypoxia, excessive accumulation of ROS beyond antioxidant capacity can exacerbate oxidative stress, which enhances the protein expressions of adhesion molecules and disrupts the integrity of BBB [33]. These findings are consistent with our current research that LPS exposure increased expressions of adhesion molecules and iNOS, elevated levels of inflammatory factors including IL-6 and TNF-α, and enhanced ROS and NO productions. However, little is known about the effects of oxyresveratrol in brain endothelial cells. Here, we applied LPS-cultured bEnd.3 cells model, combined with Western blot analysis, ELISA detection, Griess reagent assay and immunofluorescence staining to demonstrate the anti-inflammatory and antioxidant activity of oxyresveratrol.

Studies on the ameliorative effects of natural Chinese herbs on cerebral ischemia, brain injury and neuronal damage have garnered significant attention. The use of Chinese herbal preparations in the treatment of cerebrovascular diseases has been documented since the Han Dynasty [34]. Oxyresveratrol, a natural product and an isomer of resveratrol, possesses strong biological activities including anti-inflammatory and antioxidant properties [13,35]. Oxyresveratrol has been shown to have a protective effect on the nervous system, alleviating the occurrence and progression of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [36]. It combats neuroinflammation by attenuating the PI3K/Akt/p70S6K pathway in IL-1β-induced human microglia cells [37]. Oxyresveratrol is also an effective free radical scavenger and antioxidant, exhibiting lower cytotoxicity compared to resveratrol. In microglia cells, oxyresveratrol is discovered to be more effective than resveratrol in reducing the levels of NO and ROS, demonstrating its neuroprotective effects [38].

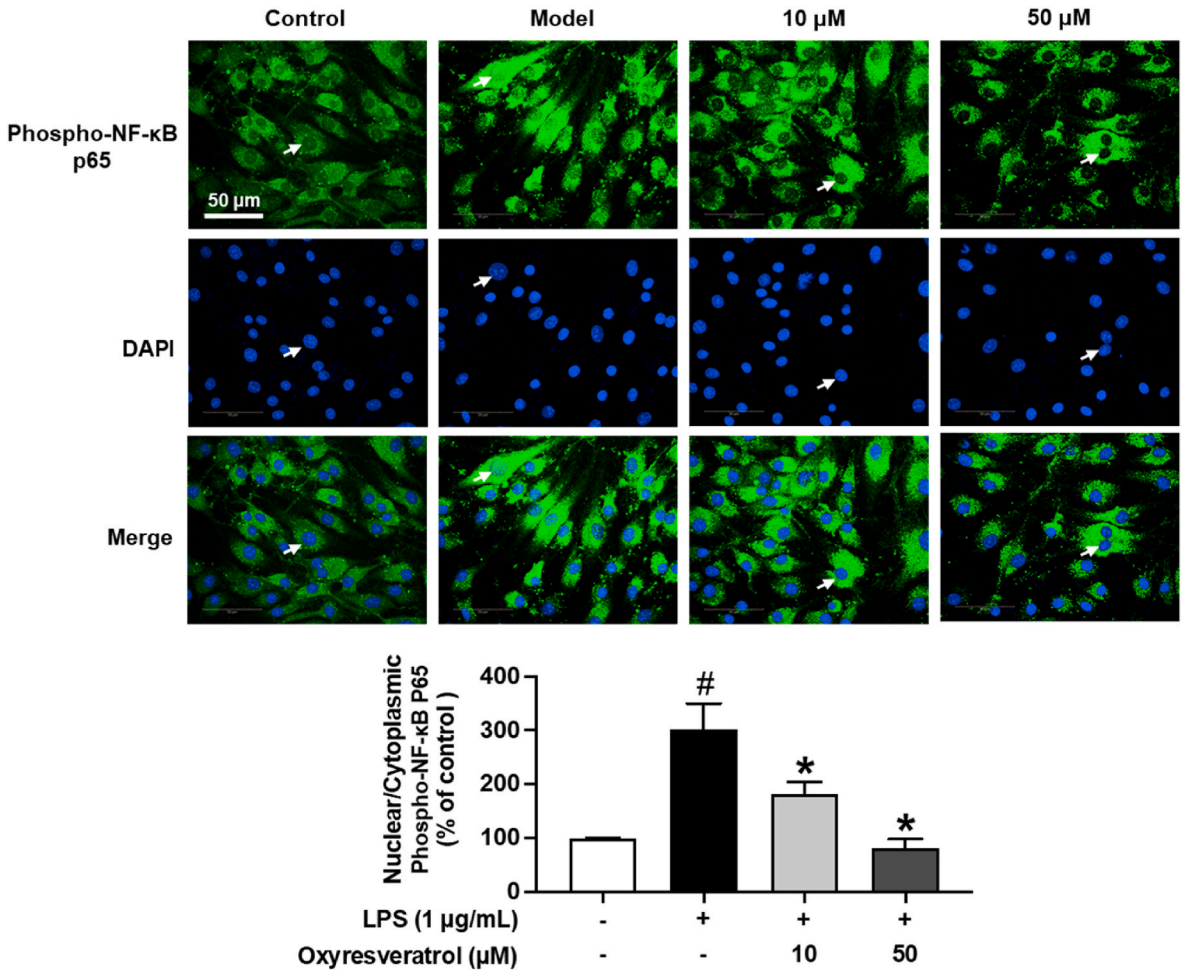

Fig. 6. Effect of oxyresveratrol on LPS-infected NF-κB P65 nuclear translocation in bEnd.3 cells. Cells were incubation with 10 μM and 50 μM of oxyresveratrol for 4 h and then stimulation with the presence of 1 μg/mL LPS or with LPS alone for 24 h. The immunofluorescence staining and quantification of phospho–NF–κB p65 at Ser536 (65 kDa) with DAPI stained for nucleus in bEnd.3 cells. Arrows indicated the nuclei with varied levels of phospho–NF–κB p65 at Ser536. All data are mean ± SEM (n = 6). #$p < 0.05$ vs. Control, *$p < 0.05$ vs. LPS.

Nevertheless, early studies only disclosed the potential therapeutic effect of oxyresveratrol on neurological disorders, while the underlying molecular mechanisms remain to be explored.

Regarding the protective mechanism of oxyresveratrol against LPS-mediated damage in our cell model, we investigated the MAPK and NF-κB signaling pathways that are the most important hallmarks of chronic inflammation. MAPK signaling pathway is well known to promote inflammation when activated by LPS and is widely expressed in different tissues, such as the central nervous system [39]. JNK and p38 are among the most crucial MAPKs involved in various cellular processes. Dysregulation of MAPKs has been implicated in regulating neurotoxicity such as inflammation, oxidative stress, and apoptosis [40]. Zhu et al. have reported that hypoxia induces inflammation in mouse brain microvascular endothelial cells through the regulation of p38 and JNK pathways [41]. Consistent with the previous reports, this study indicated the proinflammatory effects of p38 and JNK MAPKs in bEnd.3 cells stimulated with LPS. In addition, many researchers have confirmed that the inflammatory response after exposure to extracellular stimuli is highly dependent on the upregulation of NF-κB transcription factor [42]. The degradation of IκBα and translocation of NF-κB are classical pathways of NF-κB activation. When LPS stimulates bEnd.3 cells, the phosphorylation of IκBα at serine residues and subsequent ubiquitination lead to protein degradation [43]. Meanwhile, LPS-triggered NF-κB p65 nuclear translocation has been demonstrated to be mediated by activation of three MAPK cascades [44]. Activated MAPKs can regulate the expression of iNOS protein by promoting the phosphorylation and activation of NF-κB [45]. Upregulated ICAM-1 and VCAM-1 expressions in bEnd.3 cells upon LPS insult are also mediated by activation of the regulatory factor NF-κB [46]. In this study, we observed the upregulation of the MAPK and NF-κB signaling pathways in bEnd.3 cells following LPS exposure. This eventually led to the phosphorylation of p65 and its translocation to the nucleus to induce the secretion of proinflammatory factors such as IL-6 and TNF-α. These factors also contributed to the upregulation of immune molecules, including iNOS, and NO generation. Results from the present study indicated that oxyresveratrol implied an inhibitory effect on the NF-κB pathway by suppressing LPS-induced phosphorylation of IKKα/β, IκBα and p65. At the same time, oxyresveratrol inhibited nuclear translocation of phospho–NF–κB p65. In addition, LPS-induced phosphorylations of JNK and p38 were inhibited by oxyresveratrol. Our data revealed that oxyresveratrol effectively inhibited the MAPK and NF-κB signaling pathways in bEnd.3 cells upon LPS stimulation. Notably, CM-H_2DCFDA and DHE are common fluorescent probes used to assess ROS levels in cells. The fluorescence imaging using these two probes indicated that pretreatment with oxyresveratrol effectively prevented LPS-stimulated ROS formation in bEnd.3 cells.

5. Conclusions

In summary, our results suggested for the first time that

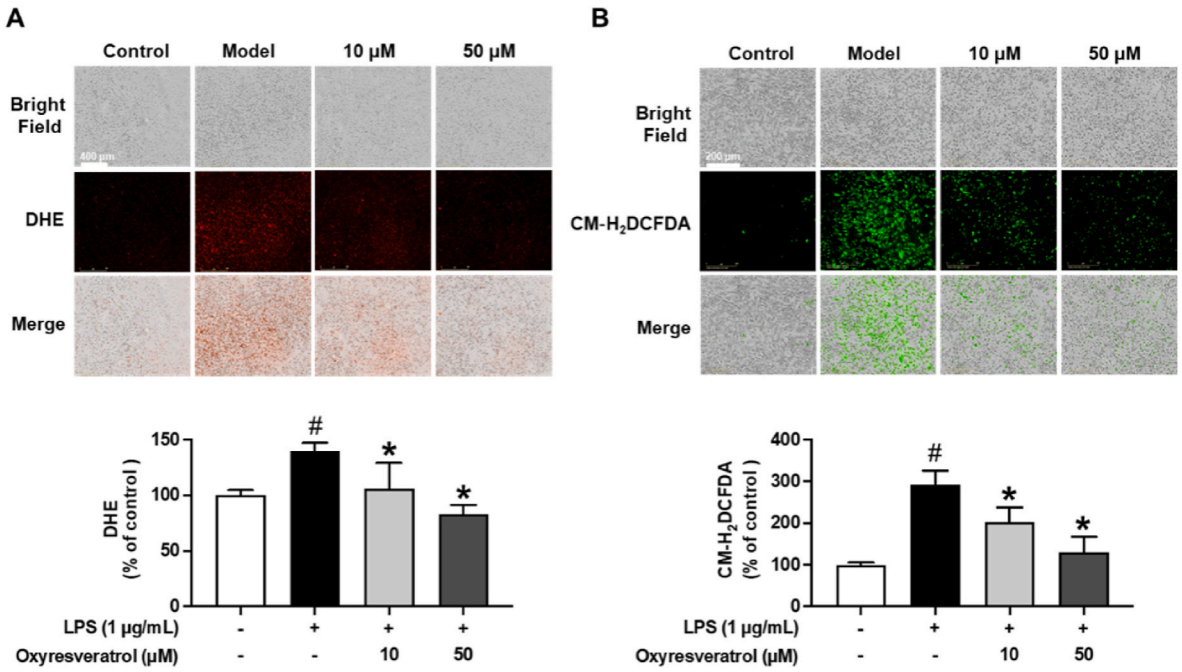

Fig. 7. Effect of oxyresveratrol on LPS-insulted oxidative stress in bEnd.3 cells. Cells were incubation with 10 μM and 50 μM of oxyresveratrol for 4 h followed by the presence of 1 μg/mL LPS or with LPS alone for 24 h. Immunofluorescence and their qualitative analysis of intracellular ROS measured with **(A)** DHE and **(B)** CM-H_2DCFDA probes. All data are mean ± SEM (n = 6). #$p < 0.05$ vs. Control, *$p < 0.05$ vs. LPS.

oxyresveratrol possesses remarkable properties in mitigating inflammatory responses and oxidative stress by suppressing MAPK and NF-κB pathways in LPS-induced brain microvascular endothelial cells. Thus, this study contributes to the understanding that oxyresveratrol has a protective effect against brain endothelial dysfunction. However, the current research data are limited to in vitro experiments, and *in vivo* explorations are necessary to study the effects of oxyresveratrol to imply its therapeutic potential against neurological and cerebrovascular diseases.

CRediT authorship contribution statement

Yan Zhou: Writing – original draft, Methodology, Investigation, Formal analysis. **Qiaowen Deng:** Writing – original draft, Methodology, Investigation, Formal analysis. **Chi Teng Vong:** Writing – review & editing, Supervision. **Haroon Khan:** Writing – review & editing, Supervision. **Wai San Cheang:** Writing – review & editing, Supervision, 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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Appendix A. Supplementary data

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References

[1] J.W. Kinney, S.M. Bemiller, A.S. Murtishaw, A.M. Leisgang, A.M. Salazar, B. T. Lamb, Inflammation as a central mechanism in Alzheimer's disease, Alzheimer's Dementia: Translational Research & Clinical Interventions 4 (2018) 575–590.
[2] P. Libby, Inflammation in atherosclerosis, Nature 420 (6917) (2002) 868–874.
[3] E. Lontchi-Yimagou, E. Sobngwi, T.E. Matsha, A.P. Kengne, Diabetes Mellitus and inflammation, Curr. Diabetes Rep. 13 (3) (2013) 435–444.
[4] D. Wu, Q. Chen, X. Chen, F. Han, Z. Chen, Y. Wang, The blood–brain barrier: structure, regulation, and drug delivery, Signal Transduct. Targeted Ther. 8 (1) (2023) 217.
[5] I. Galea, The blood–brain barrier in systemic infection and inflammation, Cell. Mol. Immunol. 18 (11) (2021) 2489–2501.
[6] J. Yang, M. Ran, H. Li, Y. Lin, K. Ma, Y. Yang, et al., New insight into neurological degeneration: inflammatory cytokines and blood-brain barrier, Front. Mol. Neurosci. 15 (2022) 1013933.
[7] H.J. Kim, H.S. Lee, Y.H. Chong, J.L. Kang, p38 mitogen-activated protein kinase up-regulates LPS-induced NF-κB activation in the development of lung injury and RAW 264.7 macrophages, Toxicology 225 (1) (2006) 36–47.
[8] K. Likhitwitayawuid, Oxyresveratrol: sources, productions, biological activities, pharmacokinetics, and delivery systems, Molecules 26 (14) (2021) 4212.
[9] W. Chen, S.C.M. Yeo, M.G.A.A. Elhennawy, H.-S. Lin, Oxyresveratrol: a bioavailable dietary polyphenol, J. Funct.Foods 22 (2016) 122–131.
[10] O.G. Agbadua, N. Kúsz, R. Berkecz, T. Gáti, G. Tóth, A. Hunyadi, Oxidized resveratrol metabolites as potent antioxidants and xanthine oxidase inhibitors, Antioxidants 11 (9) (2022) 1832.
[11] L. Xu, C. Liu, W. Xiang, H. Chen, X. Qin, X. Huang, Advances in the study of oxyresveratrol, Int. J. Pharmacol. 10 (2014) 44–54.
[12] S. Suriyaprom, P. Srisai, V. Intachaisri, T. Kaewkod, J. Pekkoh, M. Desvaux, Y. Tragoolpua, Antioxidant and anti-inflammatory activity on LPS-stimulated RAW 264.7 macrophage cells of white mulberry (morus alba L.) leaf extracts, Molecules 28 (11) (2023) 4395.
[13] H.G. Tran, A. Shuayprom, P. Kueanjinda, A. Leelahavanichkul, P. Wongsinkongman, S. Chaisomboonpan, et al., Oxyresveratrol attenuates inflammation in human keratinocyte via regulating NF-kB signaling and ameliorates eczematous lesion in DNCB-induced dermatitis mice, Pharmaceutics 15 (6) (2023).
[14] L. Wang, H. Zhao, L. Wang, Y. Tao, G. Du, W. Guan, et al., Effects of selected resveratrol analogues on activation and polarization of lipopolysaccharide-stimulated BV-2 microglial cells, J. Agric. Food Chem. 68 (12) (2020) 3750–3757.

[15] Y. Zhang, H. Liu, W. Tang, Q. Qiu, J. Peng, Resveratrol prevents TNF-α-induced VCAM-1 and ICAM-1 upregulation in endothelial progenitor cells via reduction of NF-κB activation, J. Int. Med. Res. 48 (9) (2020) 300060520945131.
[16] P. Ballabh, A. Braun, M. Nedergaard, The blood-brain barrier: an overview: structure, regulation, and clinical implications, Neurobiol. Dis. 16 (1) (2004) 1–13.
[17] V.H. Perry, D.C. Anthony, S.J. Bolton, H.C. Brown, The blood-brain barrier and the inflammatory response, Mol. Med. Today 3 (8) (1997) 335–341.
[18] M.A. Petty, E.H. Lo, Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation, Prog Neurobiol 68 (5) (2002) 311–323.
[19] J.C.S. Chaves, S.J. Dando, A.R. White, L.E. Oikari, Blood-brain barrier transporters: an overview of function, dysfunction in Alzheimer's disease and strategies for treatment, Biochim. Biophys. Acta, Mol. Basis Dis. 1870 (2) (2023) 166967.
[20] I.J. Biose, S. Ismael, B. Ouvrier, A.L. White, G.J. Bix, The potential role of integrin signaling in memory and cognitive impairment, Biomolecules 13 (1) (2023).
[21] L. Huang, Y. Chen, R. Liu, B. Li, X. Fei, X. Li, et al., P-glycoprotein aggravates blood brain barrier dysfunction in experimental ischemic stroke by inhibiting endothelial autophagy, Aging Dis 13 (5) (2022) 1546–1561.
[22] F. Wang, Z. Zou, Y. Gong, D. Yuan, X. Chen, T. Sun, Regulation of human brain microvascular endothelial cell adhesion and barrier functions by memantine, J. Mol. Neurosci. 62 (1) (2017) 123–129.
[23] Y. Zhou, H. Khan, M.P.M. Hoi, W.S. Cheang, Piceatannol protects brain endothelial cell line (bEnd.3) against lipopolysaccharide-induced inflammation and oxidative stress, Molecules 27 (4) (2022).
[24] N. Gan, Y. Zhou, J. Li, A. Wang, Y. Cao, Propofol suppresses LPS-induced BBB damage by regulating miR-130a-5p/ZO-1 Axis, Mol. Biotechnol. 66 (2023) 2007–2015.
[25] X. Lou, H. Li, Alharbi S. Ali, T. Rengarajan, J. Wang, Natural pigment zeaxanthin ameliorates lipopolysaccharides induced acute lung inflammation in both in vitro and in vivo models, Arab. J. Chem. 17 (3) (2024) 105569.
[26] G. Wang, Z. Chen, Y. Song, H. Wu, M. Chen, S. Lai, X. Wu, Xueshuantong injection alleviates cerebral microcirculation disorder in middle cerebral artery occlusion/reperfusion rats by suppressing inflammation via JNK mediated JAK2/STAT3 and NF-kappaB signaling pathways, J. Ethnopharmacol. 298 (2022) 115592.
[27] S.L. Leib, D. Leppert, J. Clements, M.G. Tauber, Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis, Infect. Immun. 68 (2) (2000) 615–620.
[28] G.Z. Feuerstein, T. Liu, F.C. Barone, Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha, Cerebrovasc. Brain Metab. Rev. 6 (4) (1994) 341–360.
[29] H.S. Kim, S.K. Ye, I.H. Cho, J.E. Jung, D.H. Kim, S. Choi, et al., 8-hydroxydeoxyguanosine suppresses NO production and COX-2 activity via Rac1/STATs signaling in LPS-induced brain microglia, Free Radic. Biol. Med. 41 (9) (2006) 1392–1403.
[30] L. Huang, D. Zhan, Y. Xing, Y. Yan, Q. Li, J. Zhang, et al., FGL2 deficiency alleviates maternal inflammation-induced blood-brain barrier damage by blocking PI3K/NF-kappaB mediated endothelial oxidative stress, Front. Immunol. 14 (2023) 1157027.
[31] N. Payal, L. Sharma, A. Sharma, Y.H. Hobanii, M. Ahmed Hakami, N. Ali, et al., Understanding the therapeutic approaches for neuroprotection, Curr Pharm Des (2023).
[32] Y. Tan, M.S. Cheong, W.S. Cheang, Roles of reactive oxygen species in vascular complications of diabetes: therapeutic properties of medicinal plants and food 2 (3) (2022) 246–268.
[33] E.I. Chukanova, A.S. Chukanova, D.M. Rodionova, [Hypoxia and oxidative stress in cerebral circulation insufficiency - effective ways of correction], Zh. Nevrol. Psikhiatr. Im. S S Korsakova 122 (8) (2022) 35–40.
[34] K. Sun, J. Fan, J. Han, Ameliorating effects of traditional Chinese medicine preparation, Chinese materia medica and active compounds on ischemia/reperfusion-induced cerebral microcirculatory disturbances and neuron damage, Acta Pharm. Sin. B 5 (1) (2015) 8–24.
[35] J.E. Lee, J. Oh, D. Song, M. Lee, D. Hahn, Y.C. Boo, N.J. Kang, Acetylated resveratrol and oxyresveratrol suppress UVB-induced MMP-1 expression in human dermal fibroblasts, Antioxidants 10 (8) (2021).
[36] N. Mahamud, P. Songvut, C. Muangnoi, R. Rodsiri, W. Dahlan, R. Tansawat, Untargeted metabolomics reveal pathways associated with neuroprotective effect of oxyresveratrol in SH-SY5Y cells, Sci. Rep. 13 (1) (2023) 20385.
[37] P. Hankittichai, H.J. Lou, N. Wikan, D.R. Smith, S. Potikanond, W. Nimlamool, Oxyresveratrol inhibits IL-1beta-induced inflammation via suppressing AKT and ERK1/2 activation in human microglia, HMC3, Int. J. Mol. Sci. 21 (17) (2020).
[38] P. Lorenz, S. Roychowdhury, M. Engelmann, G. Wolf, T.F. Horn, Oxyresveratrol and resveratrol are potent antioxidants and free radical scavengers: effect on nitrosative and oxidative stress derived from microglial cells, Nitric Oxide 9 (2) (2003) 64–76.
[39] C.C. Lin, H.L. Hsieh, R.H. Shih, P.L. Chi, S.E. Cheng, C.M. Yang, Up-regulation of COX-2/PGE2 by endothelin-1 via MAPK-dependent NF-kappaB pathway in mouse brain microvascular endothelial cells, Cell Commun. Signal. 11 (1) (2013) 8.
[40] O.M. Ijomone, J.D. Iroegbu, M. Aschner, J. Bornhorst, Impact of environmental toxicants on p38- and ERK-MAPK signaling pathways in the central nervous system, Neurotoxicology 86 (2021) 166–171.
[41] Y. Zhu, Y. Sun, L. Xie, K. Jin, N. Sheibani, D.A. Greenberg, Hypoxic induction of endoglin via mitogen-activated protein kinases in mouse brain microvascular endothelial cells, Stroke 34 (10) (2003) 2483–2488.
[42] P.J. Barnes, M. Karin, Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases, N. Engl. J. Med. 336 (15) (1997) 1066–1071.
[43] S. Grimm, P.A. Baeuerle, The inducible transcription factor NF-kappa B: structure-function relationship of its protein subunits, Biochem. J. 290 (Pt 2) (1993) 297–308. Pt 2.
[44] J.H. Je, J.Y. Lee, K.J. Jung, B. Sung, E.K. Go, B.P. Yu, H.Y. Chung, NF-kappaB activation mechanism of 4-hydroxyhexenal via NIK/IKK and p38 MAPK pathway, FEBS Lett. 566 (1–3) (2004) 183–189.
[45] S.U. Kim, Y.H. Park, J.S. Min, H.N. Sun, Y.H. Han, J.M. Hua, et al., Peroxiredoxin I is a ROS/p38 MAPK-dependent inducible antioxidant that regulates NF-kappaB-mediated iNOS induction and microglial activation, J. Neuroimmunol. 259 (1–2) (2013) 26–36.
[46] B.K. Lee, W.J. Lee, Y.S. Jung, Chrysin attenuates VCAM-1 expression and monocyte adhesion in lipopolysaccharide-stimulated brain endothelial cells by preventing NF-kappaB signaling, Int. J. Mol. Sci. 18 (7) (2017).