Gastrodin represses hydrogen peroxide-induced oxidative stress in retinal pigment epithelial cells through p38MAPK/iNOS pathway

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ORIGINAL ARTICLE

Abstract

Elevated reactive oxygen species (ROS) induce oxidative damage in retinal pigment epithelium (RPE) and contribute to the development of age-related macular degeneration (AMD). Gastrodin plays an antioxidant role in distinct diseases, such as epilepsy, cerebral ischemia, Alzheimer's disease, and cardiovascular diseases. However, the function of gastrodin in AMD remains unclear. Human RPE (ARPE-19) cells were incubated with 300 μM hydrogen peroxide (H_2O_2) for 24 hours. The results showed that H_2O_2 decreased cell viability and promoted the cell apoptosis of ARPE-19 cells. H_2O_2-induced ARPE-19 cells were then treated with different concentrations of gastrodin. Gastrodin increased cell viability of H_2O_2-induced ARPE-19 cells, suppressed the cell apoptosis of H_2O_2-induced ARPE-19 cells with reduced B-cell lymphoma (Bcl)-2 like protein (Bax), and enhanced Bcl-2. The levels of ROS were enhanced, malondialdehyde (MDA) was up-regulated, and superoxide dismutase (SOD) and glutathione (GSH) were down-regulated in H_2O_2-induced ARPE-19 cells. However, gastrodin reduced the levels of ROS and MDA and elevated SOD and GSH in H_2O_2-induced ARPE-19 cells. Furthermore, H_2O_2-induced increase of inducible nitric oxide synthase (iNOS) and p-p38 proteins in ARPE-19 was reversed by gastrodin. In conclusion, gastrodin exerted antiapoptotic and antioxidant capacities to protect against H_2O_2-induced oxidative stress in RPE, thereby acting as a potential agent for managing AMD.

Keywords: age-related macular degeneration; apoptosis; gastrodin; hydrogen peroxide; inducible nitric oxide synthase; oxidative stress; retinal pigment epithelium

Introduction

Age-related macular degeneration (AMD) is a progressive and degenerative eye disease that affects the macular area of the retina (Brown *et al*., 2018). It is the leading cause of permanent visual impairment and severe blindness in an aging world (Brown *et al*., 2018). Although the pathogenesis of AMD is not fully understood, dysfunction of retinal pigment epithelium (RPE) regulates retinal integrity and viability, thereby playing a central role in the progression of AMD (Somasundaran *et al*., 2020). Pathological events, including cell polarity and interactions, apoptosis and autophagy, and oxidative stress and inflammation, are implicated in the abnormal function of RPE and induce the progression of AMD (Yang *et al*., 2021). Cell, gene, and drug therapies that protect RPE against damages are beneficial for the prevention of AMD (Yang *et al*., 2021). For example, preclinically stem cell-derived RPE is used for human retinal degenerative diseases treatment (Yang *et al*., 2021). Drugs induced

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expression of nuclear factor erythroid 2–related factor 2 (Nrf2) to ameliorate retinal degeneration (Campello *et al*., 2020).

Increasing evidence has shown that an elevated level of reactive oxygen species (ROS) is associated with retinal diseases, including AMD, diabetic retinopathy, and glaucoma (Ruan *et al*., 2020). Accumulation of ROS results in inflammation, neuron degeneration, and vascular endothelial dysfunction in the retina (Ruan *et al*., 2020). Repression of oxidative stress might be effective in AMD treatment (Zhou *et al*., 2020).

Gastrodin is the bioactive constituent of the orchid plant *Gastrodia elata* Blume and has been widely used as a traditional Chinese medicine with anxiolytic, sedative, analgesic, anti-inflammatory, anticonvulsant, and antioxidant properties (Yan *et al*., 2019). For example, gastrodin stimulated osseointegration and suppressed osteoclastogenesis to prevent osteolytic diseases (Zhou *et al*., 2017). It reduced chronic ischemia-induced oxidative stress and improved cognitive impairment in rats with vascular dementia (Li and Zhang, 2015). Moreover, 14-3-3 η-induced anoxia/reoxygenation injury in cardiomyocytes was repressed by gastrodin (Zhu *et al*., 2018). Gastrodin retarded retinal microglia activation and reduced the loss of retinal ganglion cells to exert neuroprotective effect against retinal neurodegenerative diseases (Wang *et al*., 2017). Gastrodin repressed the high glucose-induced cell apoptosis of human retinal endothelial cell (Zhang *et al*., 2018) and it attenuated hydrogen peroxide (H_2O_2) and N-methyl-D-aspartate. Leading to decreased cell viability and increased ROS levels in retinal ganglion cells, thus ameliorating glaucoma-related retinal degeneration (Molinari *et al*., 2021). However, the role and related mechanism of gastrodin in AMD is still unclear.

This study investigates the effects of gastrodin on cell viability, apoptosis, and oxidative stress of H_2O_2-induced ARPE-19.

Materials and Methods

Cell culture and treatment

Human RPE cells (ARPE-19) were acquired from Procell Life Science & Technology (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, Shanghai, China). Cells were treated with 10, 50, or 100 μM gastrodin (Sigma-Aldrich, St Louis, MO, USA) or exposed to 300 μM H_2O_2 (Sigma-Aldrich) for 24 hours. Later the cells treated with H_2O_2 were reincubated with 10 or 50 μM gastrodin for 24 hours, and cells with 80% confluence were used for the functional experiments.

Measurement of oxidative stress

The ARPE-19 cells after H_2O_2 and gastrodin treatment were incubated with dichlorofluorescein-diacetate (0.5 μM; DCFH-DA; Sigma-Aldrich) for 0.5 hours, and then analyzed by FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) to determine fluorescence intensity of dichlorodihydrofluorescein. The enzyme-linked immunosorbent assay kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were used to measure the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH).

Cell viability and apoptosis assays

The ARPE-19 cells after H_2O_2 and gastrodin treatment were seeded in a 96-well plate for 24 hours and treated with cell counting kit-8 solution (10 μL; Beyotime, Beijing, China) for 2 hours. Absorbance at 450 nm was measured via a microplate reader (Sigma-Aldrich). ARPE-19 cells were harvested and resuspended in the binding buffer from Annexin V-FITC/ propidium iodide (PI) apoptosis detection kit (Becton Dickinson Biosciences). Following labeling with PI and annexin V-FITC, cells were analyzed under the flow cytometer.

Western blot

ARPE-19 cells were lysed in Radioimmunoprecipitation assay lysis buffer (Beyotime), and the protein samples were isolated and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were then transferred onto a nitrocellulose membrane. The membranes were blocked and probed with specific antibodies: anti-B-cell lymphoma (Bcl)-2 like protein (Bax) and anti-Bcl-2 (1:2000, Abcam, Cambridge, UK), anti-p38 and anti-p-p38 (1:3000, Abcam), and anti- inducible nitric oxide synthase (iNOS) and anti- glyceraldehyde 3-phosphate dehydrogenase i (1:4000, Abcam). The protein bands were visualized using chemiluminescence (Sigma-Aldrich) after incubation with horseradish peroxidase-conjugated secondary antibody (1:5000, Abcam) and tetramethylbenzidine.

Statistical analysis

All the data with at least triple replicates were expressed as mean ± standard error of the mean and analyzed by student's t-test or one-way analysis of variance in SPSS software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Gastrodin increased cell viability of H_2O_2-induced ARPE-19 cells

The ARPE-19 cells were incubated with different concentrations of gastrodin to investigate the cytotoxic effects of gastrodin on RPE. Gastrodin lower than 50 μM had no significant effect on cell viability of ARPE-19 cells (Figure 1A), and100 μM gastrodin reduced 20% of cell viability in ARPE-19 (Figure 1A). H_2O_2-induced ARPE-19 cells decreased the cell viability (Figure 1B), whereas a gastrodin enhanced cell viability of H_2O_2-induced ARPE-19 cells in a dosage-dependent manner (Figure 1B). These results suggested the protective effect of gastrodin against H_2O_2-induced cytotoxicity in ARPE-19.

Gastrodin decreased oxidative stress of H_2O_2-induced ARPE-19 cells

The H_2O_2-induced ARPE-19 cells showed ROS accumulation by the the presence of increased dichlorodihydrofluorescein fluorescence intensity. But the addition of gastrodin deteriorated the ROS levels in these cells in a dosage-dependent manner (Figure 2A and 2B). H_2O_2-treatment increased the levels of MDA (Figure 2C), decreased SOD (Figure 2D) and GSH (Figure 2E) in ARPE-19 cells, and this was reversed by gastrodin, demonstrating its antioxidant effect on H_2O_2-induced ARPE-19 cells.

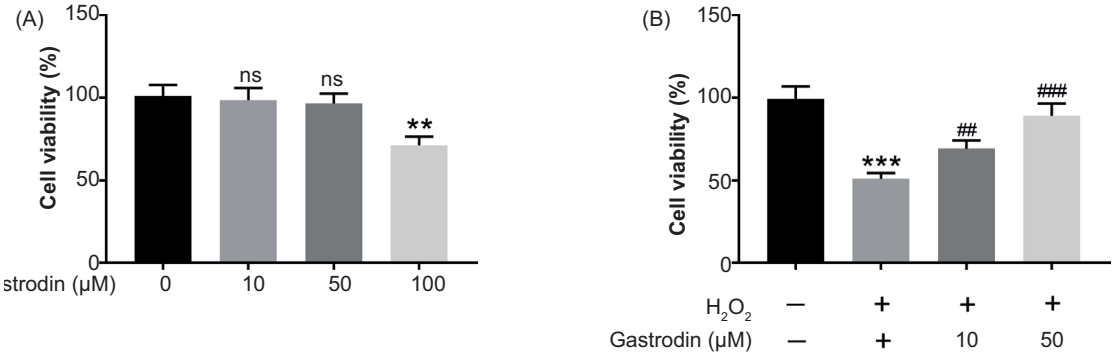

Figure 1. Gastrodin increased cell viability of hydrogen peroxide-induced ARPE-19 cells. (A) Gastrodin lower than 50 μM had no significant effect on cell viability and 100 μM gastrodin reduced 20% of cell viability in ARPE-19 cells. (B) Gastrodin enhanced cell viability of hydrogen peroxide-induced ARPE-19 cells in a dosage dependent manner. **P < 0.01, ***P < 0.001. @P < 0.05, ##P < 0.01, and ###P < 0.001.

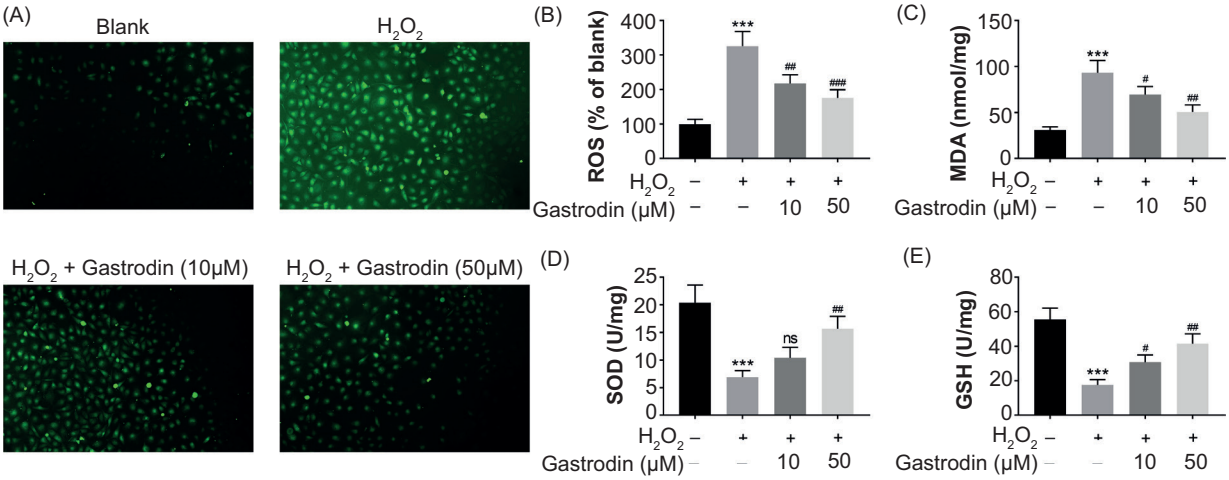

Figure 2. Gastrodin decreased oxidative stress of hydrogen peroxide (H2O2)-induced ARPE-19 cells. (A) Gastrodin reduced reactive oxygen species of H_2O_2-induced ARPE-19 in a dosage dependent manner. (B) The relative levels of reactive oxygen species in each group are shown. Gastrodin reduced (C) malondialdehyde of H_2O_2-induced ARPE-19 in a dosage dependent manner, and enhanced (D) superoxide dismutase and (E) glutathione of H_2O_2-induced ARPE-19 in a dosage dependent way. ***P < 0.001, #P < 0.05, ##P < 0.01, and ###P < 0.001.

Gastrodin repressed cell apoptosis of H_2O_2-induced ARPE-19 cells

Cell apoptosis of ARPE-19 cells was promoted by H_2O_2 treatment (Figure 3A), whereas gastrodin reduced cell apoptosis of H_2O_2-induced ARPE-19 cells in a dosage-dependent manner (Figure 3A). Gastrodin also attenuated H_2O_2-induced increase in Bax and Bcl-2 decrease in ARPE-19 (Figure 3B), indicating the antiapoptotic effect of gastrodin on H_2O_2-induced ARPE-19.

Gastrodin suppressed activation of p38MAPK/iNOS in H_2O_2-induced ARPE-19 cells

Protein expression of p38 was not affected either by H_2O_2 or gastrodin treatment in ARPE-19 cells (Figure 4). However, p-p38 was upregulated in H_2O_2-induced ARPE-19 cells and down-regulated post gastrodin treatment (Figure 4). Moreover, the increased expression of iNOS in H_2O_2-induced ARPE-19 cells was restored by gastrodin (Figure 4), revealing the suppressive effect of

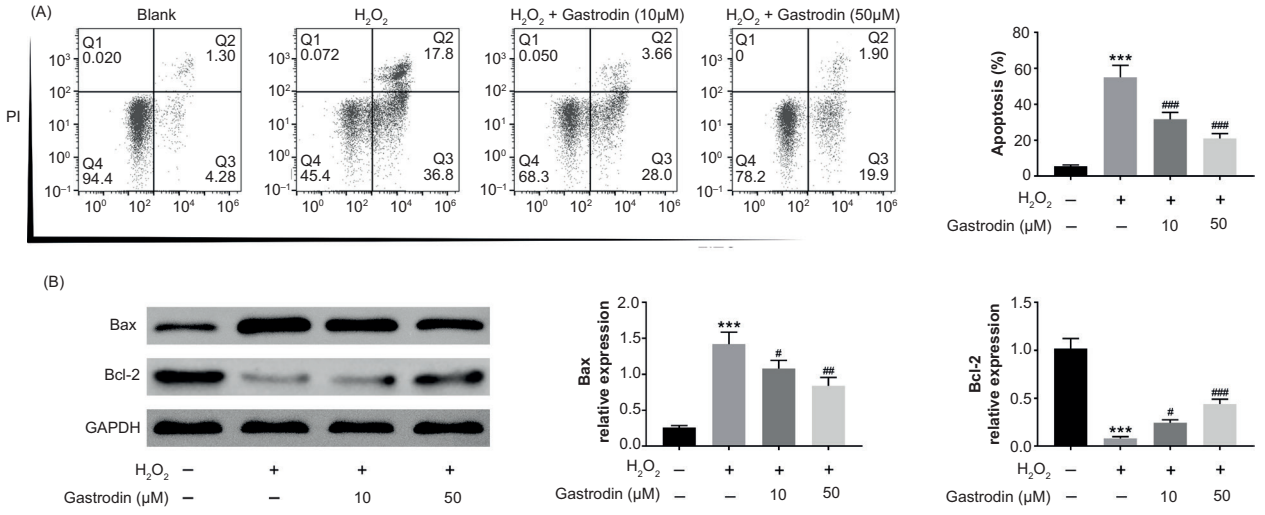

Figure 3. Gastrodin repressed cell apoptosis of hydrogen peroxide (H_2O_2)-induced ARPE-19 cells. (A) Gastrodin suppressed cell apoptosis of H_2O_2-induced ARPE-19 in a dosage dependent manner. (B) Gastrodin enhanced B-cell lymphoma (Bcl)-2 expression and reduced B-cell lymphoma (Bcl)-2 like protein (Bax) of H_2O_2-induced ARPE-19 cells in a dosage dependent manner. ***P < 0.001, #P < 0.05, ##P < 0.01, and ###P < 0.001.

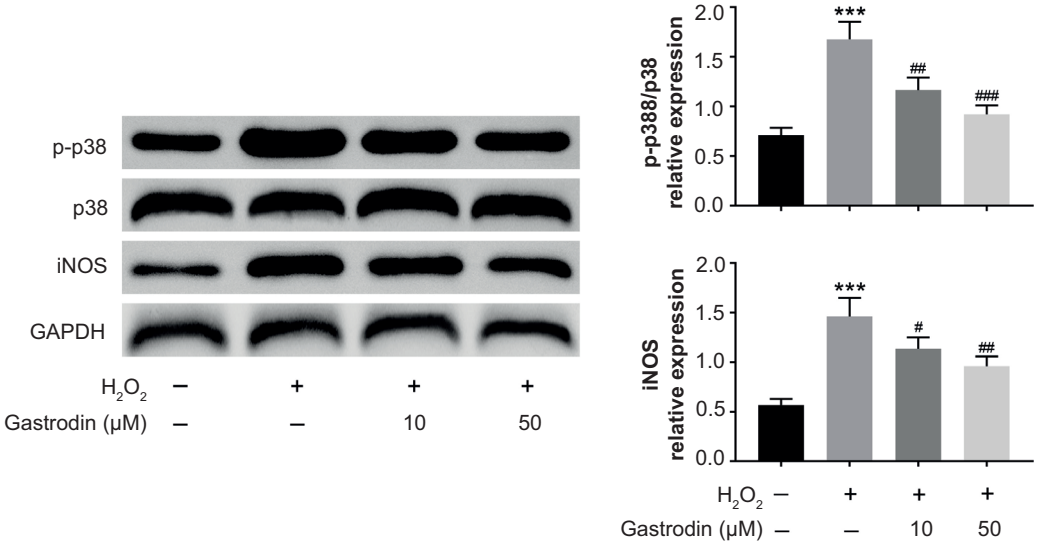

Figure 4. Gastrodin suppressed activation of p38MAPK/iNOS in hydrogen peroxide-induced ARPE-19 cells. Gastrodin reduced p-p38 and iNOS expression of hydrogen peroxide -induced ARPE-19 in a dosage dependent manner. The relative expression of p-p38/p38 and iNOS are shown on the right. ***P < 0.001, #P < 0.05, ##P < 0.01, and ###P < 0.001.

gastrodin on p38MAPK/iNOS signaling in H_2O_2-induced ARPE-19 cells.

Discussion

RPE is a postmitotic polarized epithelial cell. They are present between choroid and photoreceptors and play the role of a caretaker in the function and health of the photoreceptors (Taylor *et al*., 2021). Abnormal functioning of RPE underlies acquired and inherited diseases, such as AMD, which can lead to permanent blindness (Taylor *et al*., 2021). Diabetes or degenerative retinopathy induces excessive accumulation of ROS and reduces the antioxidant levels in RPE, thus leading to oxidative damage and retinal dysfunction (Yang *et al*., 2021). Administration of antioxidant nutrients reduced the risk for AMD (Raniga and Elder, 2009). Therefore, traditional Chinese herbs with antioxidant capacity are widely used in the clinical prevention of AMD (Jin *et al*., 2018; Li *et al*., 2021). Gastrodin, isolated from *Gastrodia elata* Blume, exerted antioxidant effect against retinal neurodegenerative diseases (Wang *et al*., 2017), high glucose-induced retinal endothelial cell apoptosis (Zhang *et al*., 2018), and glaucoma-related retinal degeneration (Molinari *et al*., 2021). This study investigated the role of gastrodin in AMD-associated RPE dysfunction.

H_2O_2 is generated in RPE through the photoreceptor outer segments phagocytosis, photoexcited pigment lipofuscin, or light irradiation of pigment melanin (Cia *et al*., 2014). The activity of catalases that neutralize $H_2O_2$was reduced in patients with AMD, and accumulation of H_2O_2in RPE during aging is strongly related to AMD (Cia *et al*., 2014). Therefore, H_2O_2-induced RPE damage was widely used as an *in-vitro* cell model of AMD (Cia *et al*., 2014). This study showed that H_2O_2 induced a decrease in cell viability of ARPE-19 cells and promoted cell apoptosis, demonstrating cytotoxicity effect on RPE. Catechin isolated from green tea is epigallocatechin gallate, suppresses H_2O_2-induced oxidative stress of RPE (Cia *et al*., 2014). This study results showed that gastrodin protected ARPE-19 against H_2O_2-induced cytotoxicity through increased cell viability and decreased cell apoptosis. The study by Yang *et al*. (2021) has shown that RPE in patients with AMD exhibited higher levels of endoplasmic reticulum stress, autophagy, and apoptosis than normal cells, leading to an increased number of autophagosomes and decreased mitochondrial activity in RPE cells isolated from AMD donors (Golestaneh *et al*., 2017). Strategy to reduce cell apoptosis of AMD-associated RPE might be effective for the prevention of AMD (Arumugam *et al*., 2019). Therefore, gastrodin that exerted an antiapoptotic effect against H_2O_2-induced RPE can be regarded as a potential strategy for AMD treatment.

RPE with high metabolic activity enriches mitochondria and contributes to the primary production of ROS (Apte, 2021). Excessive accumulation of ROS results in structural damage and changes in RPE during the progression of AMD (Apte, 2021). Therefore, therapeutic strategies to reduce oxidative stress are effective in reducing retinal damage associated with AMD (Rohowetz *et al*., 2018). In this study, H_2O_2-induced ARPE-19 cells showed an increased ROS and MDA and reduced SOD and GSH, thus promoting oxidative stress. A previous study has shown that gastrodin attenuates cell viability and increases ROS levels in H_2O_2-induced retinal ganglion cells (Molinari *et al*., 2021). In this study, the levels of ROS and MDA in H_2O_2-induced ARPE-19 cells were down-regulated by gastrodin, whereas SOD and GSH were upregulated, indicating an antioxidant effect on AMD-associated RPE. Inflammatory response of RPE was also implicated in the pathogenesis of AMD (Hytti *et al*., 2021), and suppression of inflammation prevented the progression of AMD (Mao *et al*., 2017). Since gastrodin repressed spinal synaptic potentiation and protected against chronic inflammation (Xiao *et al*., 2016), it can also exert an anti-inflammatory effect against H_2O_2-induced RPE in AMD, which needs to be the topic of interest in future studies.

Dysregulated metabolic pathways involved in mitochondrial dysfunction and disintegration, cytoplasmic glycogen accumulation, ROS production, and autophagy function are reported to be the major contributors to AMD pathophysiology (Zhang *et al*., 2020). Mitogen-activated protein kinase (MAPK) signaling is associated with proliferation, apoptosis, and differentiation of RPE, and is regarded as the potential target for AMD (Kyosseva, 2016). Oxidative damage of RPE is seen to activate MAPK signaling pathways and contribute to RPE degeneration during the development of AMD (Chan *et al*., 2015). Activation of p38 MAPK was also involved in linking oxidative stress and the production of proinflammatory cytokines in RPE (Fernandes *et al*., 2008). Suppression of the MAPKs pathway protected against oxidative stress-induced damage in RPE (Chen *et al*., 2018). Gastrodin repressed oxidative stress in 1-methyl-4-phenylpyridinium-induced dopaminergic cells through inactivation of p38 MAPK (Jiang *et al*., 2014). Here, they also reduced protein expression of p-p38 in H_2O_2-induced RPE, thereby repressing activation of p38 MAPK. Moreover, iNOS is the pivotal enzyme for the synthesis of nitric oxide, and its excessive activity can lead to oxidative stress (Song *et al*., 2013). p38 MAPK/iNOS promoted the production of nitric oxide in high glucose-induced RPE (Yuan *et al*., 2008), and blocking of p38MAPK/iNOS pathway alleviated the oxidative stress (Song *et al*., 2013). Gastrodin has been reported to decrease the expression of iNOS by inactivation of MAPKs in lipopolysaccharide (LPS)-stimulated

microglia (Dai *et al*., 2011). Protein expression of iNOS in H_2O_2-induced RPE was down-regulated by gastrodin treatment, suggesting that gastrodin protected against oxidative stress in RPE through inactivation of p38 MAPK/iNOS pathway.

In summary, gastrodin suppressed oxidative stress and retarded cell apoptosis in H_2O_2-induced RPE. The inactivation of the p38 MAPK/iNOS pathway was involved in the gastrodin-mediated suppression of oxidative stress in RPE. Therefore, gastrodin might be a novel strategy for the management of AMD. However, the effect of gastrodin on the H_2O_2-injured animal model should be the topic of interest in further studies.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Not applicable.

Contribution of authors

Xingli Zhou designed the study and supervised the data collection. Ximing Zhao analyzed and interpreted the data. Both the authors prepared the manuscript for publication, reviewed and approved the draft of the manuscript. All authors have read and approved the manuscript.

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