Lycium Barbarum Polysaccharide-Based Protection to Combat H₂O₂-Induced Oxidative Stress via the Nrf2/HO-1 Pathway in ARPE-19 Cells

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Abstract

Background

Age-related macular degeneration (AMD) has been closely correlated to visual impairment in the elderly, in particular to the oxidative stress (OxS) and apoptosis of retinal pigment epithelial (RPE) cells. Lycium barbarum polysaccharide (LBP), has been ascertained to promote people's immune system, as well as to reduce neuronal damage and blood retinal barrier disruption. Nevertheless, the protective function of LBP on AMD has not been investigated. In current study, H_2O_2 was utilized to stimulate the occurrence of OxS in RPE cells, aiming to investigate the protective function of LBP pretreatment and the underlying principle.

Results:

The experimental results indicated that LBP pretreatment had a significant efficacy to reduce oxidative damage, in combination with the increased cell viability and inhibited cell apoptosis. Besides, LBP was ascertained to modulate the expression of apoptotic proteins and to activate the nuclear-related factor 2 (Nrf2) signaling pathway to protect cells.

Conclusion:

These results demonstrated that LBP could activate the Nrf2/HO-1 pathway, hence protecting ARPE-19 cells from H_2O_2 -induced cell damage.

Introduction

To our best knowledge, patients with AMD are suffering from progressive visional degeneration due to the affected macular area. It is reported that the worldwide population with AMD is approximately 200 million by 2020, the number will further increase to 300 million by 2040(1). There are two prominent pathological features of AMD, the first feature is the formation and accumulation of drusen, and another one is the damage of RPE cells(2). Therefore, protecting RPE from injury is essential in decelerating the pathological development of AMD.

As a critical part of the blood-retinal barrier (BRB), RPE plays essential and irreplaceable roles in supporting the neural retina and visual cycle, by protecting fundus tissue from oxidation(3). As compared with other tissue cells, the RPE cell layer is more easily damaged by reactive oxygen species (ROS) resulting from the markedly high oxygen consumption of the retina(4, 5). Moreover, the ROS-induced damage to RPE cells was demonstrated as an irreversible process, which was an early sign in AMD(6). Hence, therapies against OxS should be efficacious to protect normal RPE and impede the development of AMD.

The antioxidant Nrf2 is of essential importance in the immune defense system. For instance, Nrf2 in the cytoplasm is combined with the inhibitor epichlorohydrin-related protein 1 (Keap1) like Kelch(7) under

physiological conditions. When the cell is damaged, Nrf2 leaks out from Keap1 and accumulates in the nucleus, triggering the downstream gene expression, such as heme oxygenase-1 (HO-1)(8). Recent research has revealed that Nrf2 and HO-1 were participated in the origin of AMD(9) by dynamically balancing retinal tissue under stress or trauma(10). Therefore, the therapeutic activation of Nrf2 could be potentially useful for AMD treatments.

LBP has exhibited various crucial biological functions, including immunomodulation, neuroprotection, anti-aging and antioxidative capacities(11). For example, LBP was reported to render a reduced level of ROS and apoptosis in human lens epithelial cells(12). In addition, ischemia-induced retinal damage on diabetic rats(13) was avoided by LBP via activating antioxidant pathway(14). Nevertheless, the protective function of LBP on AMD has not been investigated. Therefore, we aimed to evaluate the inhibitory action of LBP onwards H₂O₂-induced OxS and apoptosis in RPE cells, as well as to investigate its effects on the Nrf2/HO-1 pathway. And the AMD model was employed by exposing human retinal epithelial cell lines (ARPE-19) to H₂O₂, to provide an alternative neoteric strategy for AMD therapy.

Results

LBP remitted H₂O₂-induced cell viability damage

The toxicity of LBP against ARPE-19 cells was firstly assessed. After 24 hours of pretreatment with LBP (0, 0.25, 0.5, 1 or 2 mg/ml), the cell viability was subsequently measured utilizing CCK 8 assay (Fig. 1A). Experimental results presented that the cell viability was retained before and after 24 hours of LBP pretreatment, indicating that the tested concentration of LBP was safe for the cells. To evaluate the potential impact of H₂O₂, ARPE-19 cells were incubated with 0–1,000 µM H₂O₂ for 2 hours, and the resulting data of cell toxicity were assessed (Fig. 1B). Notably, as compared to the control group, the cell viability was markedly reduced after the H₂O₂ treatment. For instance, the cell viability was decreased by 53.6% (P < 0.01) at the concentration of 500 µM H₂O₂, and 500 µM was chosen for the following analysis. The antioxidant effect of LBP pretreatment was further evaluated. ARPE-19 cells were first progressively treated with LBP (0.5, 1 or 2 mg/ml) and 500 µM H₂O₂, the final cell viability was determined via CCK 8 kit. In Fig. 1C, ARPE-19 cell viability was resumed up to 90.33% after pretreatment with 2 mg/ml LBP. These results suggested that 24 hours of pretreatment with LBP (0.5-2mg/ml) effectively avoided H₂O₂-induced damage in ARPE-19 cells.

LBP ameliorated H₂O₂-triggered OxS

To explore the protection mechanism of LBP, the indicators of intracellular OxS and the levels of antioxidant enzymes were evaluated. In the current work, DCFH-DA assay was employed to measure ROS levels, as well as to the ability of LBP to scavenge H₂O₂-induced ROS. As illustrated in Fig. 2A and B, in comparison with the control, both ROS and MDA levels in APRE-19 cells were evidently increased upon exposure to H₂O₂ (P < 0.001). However, the LBP pretreatment significantly reduced the ROS level and MDA

level in response to the LBP dose. These results demonstrated the critical influence of LBP on inhibiting the H₂O₂-treated OxS in ARPE-19 cells. Additionally, the antioxidant stress indicators (SOD, CAT and GSH-Px) were monitored with or without LBP and H₂O₂. As illustrated in Fig. 2C and Fig. 2D, H₂O₂ was capable to reduce the activities of antioxidant enzymes, while LBP pretreatment effectively restored the activities of SOD and CAT. Similar phenomenon was obtained in Fig. 2E, where the level of GSH-Px ratio was enhanced by LBP pretreatment as compared to that with H₂O₂ treatment. Therefore, LBP pretreatment was ascertained to be useful for ameliorating OxS.

LBP prevented H₂O₂-induced apoptosis

Flow cytometry was employed to determine the extent of apoptosis occurring in ARPE-19 cells under different experimental conditions. As illustrated in Fig. 3A and Fig. 3B, the extent of apoptosis in the H₂O₂ group was markedly larger than the control group. Notably, the H₂O₂-caused apoptosis in ARPE-19 cells was inhibited (Fig. 3C, D and E) after incubation with varying doses of LBP. On the other hand, to identify the anti-apoptotic effect of LBP at the protein expression level, apoptosis-related proteins including Bax and caspase-3 and the anti-apoptotic protein Bcl-2 were detected by Western blotting. In Fig. 3G, as a comparison with the control, cells exposed to 500 µM H₂O₂ exhibited higher levels of Bax and caspase-3 but lower level of Bcl-2, which were consistent with the results obtained from flow cytometry. However, the higher level of Bcl-2 but lower levels of Bax and caspase-3 were observed after 24 hours of LBP pretreatment, indicating the dose-dependent capability of LBP on reversing H₂O₂-caused apoptosis with a statistically significant difference (Fig. 3G). In addition, the Bcl-2/Bax ratio was markedly increased in the LBP pretreatment groups while it was declined evidently in the H₂O₂ group, demonstrating its effectively protection of H₂O₂-induced apoptosis in ARPE-19 cells.

LBP alleviated H₂O₂-induced cell damage via the Nrf2/HO-1 pathway

To explore the protective molecular mechanism, the signaling role of Nrf2/HO-1 throughout LBP-based protection was studied by respective investigation of H₂O₂-induced oxidative damage and apoptosis. From Western blotting assays, H₂O₂ treatment improved the nuclear transcription expression of Nrf2 protein, the main regulators of cellular antioxidant response. In comparison with the H₂O₂ group, LBP pretreatment also improved the nuclear transcription expression of Nrf2 protein, with a dose-dependent effectiveness (Fig. 4A). Additionally, the downstream gene HO-1 was expressed in a similar trend to that of Nrf2 (Fig. 4B). To further validate the mechanism of LBP on H₂O₂-induced ARPE-19 cells, individual LBP treatment showed a negligible influence on the expression of nuclear Nrf2 and HO-1. Conversely, a statistically significant increase was observed in nuclear Nrf2 and HO-1 upon induction of H₂O₂ (Fig. 4C). As such, the synergistic combination of LBP and OxS contributed to increasing the nuclear transport of Nrf2 protein.

As for the molecular mechanisms, Nrf2 gene knockout experiments were carried out by the siRNA mixed with lipofectamine 2000. After the intervention of Nrf2 siRNA, the expression of Nrf2 was markedly dropped in ARPE-19 cells (Fig. 4D) and the LBP-mediated expression of HO-1 was almost eliminated (Fig. 4D). Additionally, the intervention of Nrf2-siRNA aggravated the H₂O₂-caused cell death, offsetting the protection of LBP on cells (Fig. 4E). In conclusion, LBP could activate the Nrf2/HO-1 pathway, hence protecting ARPE-19 cells from H₂O₂-induced cell damage.

Discussion

As an essential part of BRB, RPE cells are of critical importance to maintain the structural integrity of the retina(15, 16). And RPE cells, susceptible to the negative effects of OxS, are generally exposed to high levels of ROS(17), resulting from the higher oxygen consumption of the retina. Previous studies have correlated the cumulative ROS-induced damage in RPE cells with the early stage of AMD(18). Therefore, early intervention measurements are of essential importance to prevent OxS-induced damage in RPE cells. The anti-oxidative and anti-apoptotic functions of LBP have so far been addressed on various eye diseases, including retinitis pigmentosa(19), glaucoma(20), retinal ischemia-reperfusion injury(21) and diabetic retinopathy(22). The chemical composition analysis of LBP showed that glycopeptides in LBP could alleviate lipid peroxidation(23–25). Given the above, our study aims to explore how LBP prevent OxS and apoptosis in ARPE-19 cells and its potential mechanism.

In our experiment, a classic model(26) was utilized to explore the influence of LBP on H₂O₂-triggered OxS(27–29). H₂O₂ was employed to imitate the pathogenesis of AMD. As demonstrated in CCK-8 assays, exposure to 500 μ M H₂O₂ distinctly reduced the viability of ARPE-19 cells, whereas LBP pretreatment reversed this phenomenon in a concentration-dependent manner, suggesting that LBP effectively prevented H₂O₂ from inducing cell damage.

It is reported that the H₂O₂-caused OxS is relative to the increase of ROS levels, the excessive ROS could be eliminated by strengthening antioxidant enzymes, thereby reducing the apoptotic state of ageing RPE cells(30, 31). Inspired by this, DCFH-DA staining was firstly used to detect ROS levels, and the subsequent flow cytometry data presented that the fluorescence intensity of ROS in the H₂O₂ group increased significantly. In contrast, LBP pretreatment reduced the H₂O₂-triggered ROS enhancement. The level of MDA was also consistent with the level of ROS. Besides, anti-oxidant levels in ARPE-19 cells, viz., SOD, CAT and GSH-Px, were maintained to a high extent in response to LBP pretreatment. These results indicated that LBP reduced H₂O₂-triggered OxS in ARPE-19 cells through improving endogenous antioxidant activity.

According to the previous studies, the activation of the apoptotic triggered by ROS presents an essential influence throughout AMD pathogenesis(32). In particular, the Bcl-2 family and caspase-3 proteins are two major regulators during cell apoptosis(33, 34). The impact of H₂O₂ exposure resulted in an increase in apoptotic proteins (Bax and caspase-3), but a decrease in the anti-apoptotic protein (Bcl-2). However,

24 hours of LBP pretreatment before H₂O₂ incubation reversed the previously observed phenomenon, as evidenced by the reduced expression of Bax and caspase-3 protein and the augment of Bcl-2 protein. These results indicated that LBP was capable to lower the apoptosis of ARPE-19 cells, hence preventing the internal oxidative damage.

Furthermore, Nrf2 has heavily participated in the process of cell redox homeostasis, which serves to resist OxS by promoting the expression of antioxidant enzymes(35, 36). However, few researches have focused on the relationship between LBP and the Nrf2 pathways in oxidative damage. Once the cells stimulated by OxS, Nrf2 would dissociate from Keap1 and transfer into the nucleus to activate HO-1(37, 38). Our results indicated LBP pretreatment alone haven't increased nuclear translocation of Nrf2, and similarly, the expression of HO-1 was not affected. However, under the stimulation of OxS, LBP heightened the nuclear translocation of Nrf2 and the expression of HO-1. Thus, H₂O₂ was essential for Nrf2 translocation and the expression of antioxidant protein, which was consistent with the results of Hao et al(39). Furthermore, our results indicated that Nrf2 siRNA partly reversed the preventing function of LBP on H₂O₂-caused cell death. Therefore, LBP-induced activation of the Nrf2/HO-1 pathway plays an effective role in preventing the H₂O₂-triggered OxS in ARPE-19 cells. However, the conclusion was limited as only one retinal cell line and in-vitro experiments were carried out in our study. Hence, other retinal cell lines and more in-vivo research are necessary to verify the effects of LBP and H₂O₂.

Conclusion

In conclusion, this work provided convincing evidence to prove the effect of LBP in ARPE-19 cells, especially its inhibition on H₂O₂-triggered OxS and apoptosis through enhancing the antioxidant enzyme system and activating the Nrf2/HO-1 pathway. This work suggests that LBP is potentially served as a nutritional supplement, which exhibits potential functions to reduce the risk of AMD and OxS-associated retinal disorders.

Materials And Methods

Materials and chemicals

LBP (purity > 90%) was purchased from Solarbio Company (Beijing, China). DMEM/F12 medium and foetal bovine serum (FBS) were obtained from the Procell Life Science & Technology (Wuhan, China). Primary antibodies against histone H3, Bcl2, Caspase-3, Bax and β actin were obtained from Abcam (Cambridge, MA, USA) and antibodies against Nrf2 and HO-1 were obtained from Cell Signaling Technology (Beverly, MA, USA). The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), Malondialdehyde (MDA), Superoxide dismutase (SOD), GSH-peroxidase (GSH-Px) and Catalase (CAT) commercial kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Secondary antibody terminally-conjugated with horseradish peroxidase, and enhanced

chemiluminescence (ECL) reagent were purchased from Beyotime Biotechnology (Shanghai, China) and all other chemicals were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture and treatment

DMEM/F-12 with FBS (10%), streptomycin (100 mg/ml) and penicillin (100 U/ml) were used to culture the ARPE-19 cells (Procell Life Science & Technology Co. Ltd., certified by STR) in a humidified incubator (5% CO₂, and 37°C). All these treatments were carried out when cells reached approximately 80% confluence.

Cell viability assay

ARPE-19 cells were seeded into 96-well plates (1 X 10⁴ cells per well) with six replicates for each group. After overnight cultured, the cells were incubated with varying concentrations of H₂O₂ (0, 125, 250, 500, 1000 µM, St. Louis, MO, USA) for 2 hours to identify the working concentration. On the other hand, ARPE-19 cells were pretreated with of LBP (0, 0.25, 0.5, 1, 2 mg/ml) for 24 hours to optimize the dose of LBP in our study. Besides, the 24 hours of LBP pretreatment were proceeded before co-incubation with H₂O₂ (500 µM) to assess the protective function of LBP on H₂O₂-triggered cell death. Subsequently, cell viability was detected according to the specifications of the CCK-8 kit. Briefly, the CCK-8 solution was added into each well and incubated for 2 hours at 37°C in a dark environment. Cell viability measurements were carried out using the microplate reader (BioTek, Winooski, VT USA). Cell viability (%) = [(absorbance of the test sample- absorbance of the control sample)/ mean absorbance of the control] ×100.

Measuring intracellular ROS

DCFH-DA method was utilized in this step. Briefly, ARPE-19 cells (1 X 10⁶ cells per well) were cultured with or without different amounts of LBP in six-well plates for 24 hours, before treatment with 500 µM H₂O₂. After that, these cells were cultured in the presence of DCFH-DA (10 mM) for 20 min within a dark environment. After thrice washed using cold PBS, the fluorescence intensity of harvested cells was determined using a FACSCalibur flow cytometer (Beckman Coulter, Brea, CA). All experimental results are shown as a percentage relative to that of the control sample.

Measuring levels of MDA, SOD, CAT and GSH-Px activities

In 1.5 mL Eppendorf tubes, ARPE-19 cells (1 X 10⁶ cells per well) after different treatments were co-incubated with 100 µL of RIPA lysis buffer and 10% protease inhibitor for 30 minutes. After lysis and 15 minutes of centrifugation (12000 g and 4°C), the protein in the resulting cell suspension was quantified using the BCA kit. Finally, the intracellular activities of MDA, SOD, and levels of CAT and GSH-Px were spectrophotometrically detected using the relevant commercial kits. Notably, SOD, CAT and GPX-Px activities were denoted as units/mg protein, while MDA levels were expressed as nmol/g protein. These experimental results are shown as percentages of the control value.

Quantitation of apoptotic cells

Similar to the previously described procedures, the centrifuged ARPE-19 cells were resuspended with 100 µL binding buffer with a cell density at 10⁶ cells/mL. Subsequently, 5 µL of Annexin V-FITC and 5 µL of PI

were added and gently mixed into the cell suspension. After 15 minutes of dark incubation at room temperature (RT), FACSCalibur flow cytometry and CellQuest software (BD Biosciences) were utilized for the quantitation of apoptotic cells. Notably, the early, normal, and late apoptotic cells were indicated by annexin V-FITC+/PI⁻, annexin⁻/PI⁻, and annexin+/PI⁺ cell populations, respectively.

Western blot analysis

Similarly, followed by the detection of protein concentration, SDS-PAGE with protein lysates (30 µg/lane) was transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After 2 hours of blocking treatment (5% skim milk) at RT, the membranes were immersed into the primary antibody at 4°C overnight. After washing, the secondary antibodies were subsequently modified at RT for 2 hours. The protein bands on the membranes were observed by incubation with ECL reagent and analyzed via Image Lab Software (BioRad). β-actin and histone H3 were chosen as internal controls.

siRNA Interference

ARPE-19 cells (1 X 10⁵ cells per well) were firstly transfected with 100 µM siRNA (control or Nrf2, GenePharma, China) using lipofectamine 2000 (Invitrogen) for 12 hours. After 24 hours of pretreatment of LBP and 2 hours of exposure to 500 µM H₂O₂, western blot and CCK 8 assay were finally used to evaluate the effect of LBP on protecting ARPE-19 cells and its underlying mechanism.

Statistical analysis

All data in the current study were depicted in the format of means ± SEM. GraphPad Prism software version 8.0, and one-way ANOVA and subsequent Tukey's multiple comparison test were utilized to determine the P value. A value of P < 0.05 was denoted as statistically significant and all assays were repeated at least in triplicate.

Abbreviations

AMD

Age-related macular degeneration

LBP

Lycium barbarum polysaccharide

ROS

reactive oxygen species

OxS

Stress oxidative

MDA

malondialdehyde

SOD

superoxide dismutase

CAT

Catalase

GSH-Px

glutathione peroxidase

RPE

retinal pigment epithelium

RT

room temperature

Declarations

Authors' contributions

QZ and RL designed and performed the experiments. RL analyzed the data. QZ and MG wrote the manuscript. XH helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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None.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

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Figures

Figure 1

LBP remitted H₂O₂-induced cytotoxicity. (A) Effect of LBP on cell viability of ARPE-19 cells. ARPE-19 cells were treated with different concentrations of LBP (0–2 mg/ml) for 24h. (B) Effect of H₂O₂ on cell viability of ARPE-19 cells. ARPE-19 cells were treated with different concentrations of H₂O₂ (0–1000μM) for 2h. (C) Effect of LBP against H₂O₂-induced cytotoxicity in ARPE-19 cells. ARPE-19 cells were pretreated with different concentrations of LBP (0.5-2 mg/ml) for 24h followed by 500μM H₂O₂ for 2h. All results denote the means ± SEM (n = 3). *P<0.05 vs. control, **P<0.001 vs. control, ##P<0.001 vs. H₂O₂-induced cells without pretreatment with LBP.

Figure 2

LBP ameliorated H₂O₂ induced oxidative stress. (A) Effect of LBP on ROS level in H₂O₂-induced ARPE-19 cells. (B) Effect of LBP on MDA level in H₂O₂-induced ARPE-19 cells. (C) Effect of LBP on SOD activities in H₂O₂-induced ARPE-19 cells. (D) Effect of LBP on CAT activities in H₂O₂-induced ARPE-19 cells. (E) Effect of LBP on GSH-Px activities in H₂O₂-induced ARPE-19 cells. All results denote the means $\pm$ SEM (n = 3). ***P<0.001 vs. control, #P<0.01 vs. H₂O₂-induced cells without pretreatment with LBP. ##P<0.001 vs. H₂O₂-induced cells without pretreatment with LBP.

Figure 3

LBP protects H₂O₂-induced apoptosis. (A-E) Apoptosis was analyzed by being double stained with Annexin V-FITC and PI. Flow cytometry analysis of ARPE-19 cells in each group. (F) Quantification of apoptotic rate of each group. (G) Apoptosis related protein (Caspase 3, Bax, Bcl-2) was detected by Western blotting. All results denote the means ± SEM (n = 3). **P<0.01 vs. control, ***P<0.001 vs. control,

#P<0.01 vs. H2O2-induced cells without pretreatment with LBP. ##P<0.001 vs. H2O2-induced cells without pretreatment with LBP.

Figure 4

LBP alleviated H2O2-induced RPE cells oxidant damage though Nrf2/HO-1 pathway. ARPE-19 cells were incubated with LBP for 24h, then treated with or without 500μM H2O2 for 2h. (A) The level of relative protein expression of nuclear Nrf2 was determined by Western blotting. (B) The level of relative protein

expression of HO-1 was determined by Western blotting. (C) Protein expression level of nuclear Nrf2 and HO-1 was analyzed by Western blotting. (D) ARPE-19 cells were transfected by siRNA for 12h and then incubated with LBP for 24h and induced by H2O2 for 2h. Protein expression level of Nrf2 and HO-1 was analyzed by Western blotting. (E) ARPE-19 cells were transfected by siRNA for 12h and then incubated with or without LBP for 24h and induced by H2O2 for 2h. The cytoprotective effect of LBP was analyzed by CCK 8 method. + indicates with H2O2 or LBP, - indicates without H2O2 or LBP. *P<0.05 vs. control **P<0.01 vs. control, ***P<0.001 vs. control, #P<0.01 vs. H2O2-induced cells without pretreatment with LBP. ##P<0.001 vs. H2O2-induced cells without pretreatment with LBP.

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