Lycium barbarum polysaccharides protected human retinal pigment epithelial cells against oxidative stress–induced apoptosis

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Abstract

- **AIM:** To investigate the protective effect and its mechanism of lycium barbarum polysaccharides (LBP) against oxidative stress–induced apoptosis in human retinal pigment epithelial cells.

- **METHODS:** ARPE-19 cells, a human retinal pigment epithelial cell line, were exposed to different concentrations of H₂O₂ for 24h, then cell viability was measured by Cell Counting Kit–8 (CCK–8) assay to get the properly concentration of H₂O₂ which can induce half apoptosis of ARPE-19. With different concentrations of LBP pretreatment, the ARPE-19 cells were then exposed to appropriate concentration of H₂O₂ cell apoptosis was detected by flow cytometric analysis. Expression levels of Bcl-2 and Bax were measured by real time quantitative polymerase chain reaction (RT–PCR) technique.

- **RESULTS:** LBP significantly reduced the H₂O₂–induced ARPE–19 cells’ apoptosis. LBP inhibited the H₂O₂– induced down–regulation of Bcl-2 and up–regulation of Bax.

- **CONCLUSION:** LBP could protect ARPE–19 cells from H₂O₂–induced apoptosis. The Bcl –2 family had relationship with the protective effects of LBP.

- **KEYWORDS:** lycium barbarum polysaccharides; retinal pigment epithelial cell; apoptosis; age-related macular degeneration

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INTRODUCTION

Age-related macular degeneration (AMD), a kind of macular retinal disease with degenerative changes, can be divided into atrophy and exudative. It is characterized by the progressive atrophy of retinal pigment epithelial (RPE) cells and the formation of choroidal neovascularization (CNV). RPE cells are located between the choroid and the photoreceptor cell layer and provide nutrition to the latter. If the oxidative damage was happened to RPE cells, the breakdown of photoreceptor cells will happen soon and the visual acuity will be damaged obviously.

Lycium barbarum is a well-known traditional Chinese herbal medicine which has multiple pharmacological and biological functions including neuroprotection [1-4], antioxidant [5-7], anti-aging [8-9], cytoprotection [10-11] and immuno-modulating [6,12]. Lycium barbarum polysaccharides (LBP), extracted from lycium barbarum fruit, is thought to be the main component responsible for these biological activities [13]. Based on the antioxidant activity of LBP, many studies have demonstrated that LBP has protective effect against oxidative injury in various cells and tissues [14-17]. However, whether LBP can protect retinal pigment epithelial cells from oxidative stress and its pathogenesis is still unclear.

In the present study, we assessed the ability of LBP to protect human retinal pigment epithelial (ARPE-19) cells from H₂O₂ damage, analyzed its effects on cell viability, apoptosis and the mechanism in vitro.

**MATERIALS AND METHODS**

**Cell Culture and Treatment** The human retinal pigment epithelial cell line, ARPE-19, was obtained from the Cancer Institute of the Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified 5% CO₂ at 37°C . When grown to 80%-85% confluence, the cells were collected for different assays.

**H₂O₂–induced Oxidative Damage Model** The ARPE-19 cells were seeded into 96-well plates with six replicates for each group. When grown to 80%-85% confluence, cells were treated with different concentrations of H₂O₂ (0, 300,
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500, 700, 900 µmol/L, respectively). After 24h, the cells were incubated with 10 µL of Cell Counting Kit-8 (CCK-8) solution for 2h at 37°C and measured by absorbance at an optical density of 450 nm.

Concentration Series with Lycium Barbarum Polysaccharides to Retinal Pigment Epithelial Cells The preparation for LBP extracts was the same as reported previously [10]. To determine the optimal and safe concentration of LBP, ARPE-19 cells were seeded into 96-well plates with six replicates for each group. When grown to 80% -85% confluence, cells were treated with different concentrations of LBP (0, 10, 50, 100, 500, 1000, 500 µg/mL, respectively) for 24h. Then the cells were exposed to 500 µmol/L H$_2$O$_2$ and incubated for 24h. After that, the cells were incubated with 10 µL of CCK-8 solution for 2h at 37°C and measured by absorbance at an optical density of 450 nm.

Cell Apoptosis Assay To quantify apoptosis cells, Annexin V and propidium iodide staining were used. Briefly, ARPE-19 cells were grown on a six-well plate at 2×10$^5$ cells per plate and incubated with or without 50 µg/mL LBP for 24h before 500 µmol/L H$_2$O$_2$ treatment. Thereafter, cells were collected and stained with Annexin V-FITC and PI in binding buffer for 20min. The stained cells were analyzed by flow cytometry (BD FACS Aria™).

Real Time Polymerase Chain Reaction Real time polymerase chain reaction (RT-PCR) was used to confirm Bcl-2 and Bax gene expression. ARPE-19 cells were grouped and treated just as above steps. The quantification of the selected genes by RT-PCR was performed using Rotor Gene 6000 (Corbett Life Science, USA) using the primers shown in Table 1. Every reaction consisted of 0.8 µL cDNA, 0.3 µL of each primer (10 µmol/L), 10 µL of 2×Master Mix and 8.1 µL reaction buffers (Water, nuclease-free) (total reaction volume 20 µL) (Fermentas). RT-PCR cycles consisted of: 2min at 50°C, 10min at 95°C for polymerase activation, 45 cycles of 15s at 95°C (denaturation), 30s at 60°C, 15s at 72°C and 15s at 95°C (annealing and extension). Finally, melting was carried out at 60°C -95°C (0.5°C increments) for 15s for each step. β-actin of each sample served as intrinsic control. The threshold cycle (CT) of each sample was normalized to β-actin. Relative quantification analysis was carried out with the Rotor Gene 6000 series software version 1.7. The analysis uses the sample's crossing point, the efficiency of the reaction, the number of cycles completed and other values to compare the samples and generate the ratios.

Statistical Analysis Data were analyzed using the SPSS 16.0 (SPSS Inc., Chicago, USA). Values were presented as mean±SD. For comparison of the different groups, analysis of variance (ANOVA) with the least significant difference (LSD) test was used. $P<0.05$ was considered statistically significant.

RESULTS Lycium Barbarum Polysaccharides Reduced H$_2$O$_2$-induced Cell Apoptosis in Retinal Pigment Epithelial Cells ARPE-19 cells were incubated with different concentrations of H$_2$O$_2$ (0, 300, 500, 700, 900 µmol/L, respectively) for 24h and the cells viability were ($99±0.68$)%, (88.04 ±2.89)%, (50.94 ±2.42)%, (30.50 ±1.17)%, and (11.28±0.89)% (Figure 1). We chose 500 µmol/L to construct the H$_2$O$_2$-induced apoptosis model in order to analyze the protection of LBP.

ARPE-19 cells were incubated with different concentrations of H$_2$O$_2$ (0-900 µmol/L) for 24h. Cell viability was measured by CCK-8 assay. Data represented the (means ±SD) and obtained from five independent experiments. $^aP<0.05$, compared with control.

In order to confirm whether LBP protect ARPE-19 cells against H$_2$O$_2$-induced cell damage, we detected the viability of ARPE-19 cells after H$_2$O$_2$ (500 µmol/L) incubation for 24h, with or without LBP pretreatment in different concentrations. As shown in Figure 2, cell viability was down to (52.14±1.83)% after exposed to H$_2$O$_2$ (500 µmol/L) for 24h, LBP pretreatment with a variety of concentrations from 10 µg/mL to 5000 µg/mL for 24h prevented the loss of cell viability. LBP exerted the maximal protective effect at 500 µg/mL.

| Table 1 RT-PCR primers used (Fermentas, Canada) |
|-----------------|------------------|
| Gene            | Primer           |
| Bcl-2 (F)       | 5’ GATAACGAGGGCTGGGATGC 3’ |
| Bcl-2 (R)       | 5’ CAGGCACTGGTACTTCACTGG 3’ |
| Bax (F)         | 5’ TTGCTTCAGGGATGTTG 3’ |
| Bax (R)         | 5’ CAAATGAGAAAAGCCGAC 3’ |
| β-actin (F)     | 5’ ACAGAGCTGCCTTTGCGCAT 3’ |
| β-actin (R)     | 5’ CTTGCACTAGCGGAGCCGTT 3’ |

F: Forward; R: Reverse.

*AP<0.05, *bP<0.01 compared with control.

Based on the results of CCK-8 assay, ARPE-19 cells were incubated with or without 500 µg/mL LBP for 24h and then subjected to H$_2$O$_2$ (500 µmol/L) for 24h. Under the inverted microscope, we found a high portion of cells appeared apoptosis-like changes in H$_2$O$_2$ treatment group, such as detachment and cytoplasmic-condensation leading to rounding.
However, the proportion of apoptosis-like cells decreased in the group which was pretreated with 500 μg/mL LBP. Flow cytometric analysis showed the apoptosis rate was (6.1±0.77)% (n=3) in the LBP+H2O2 group, much lower than (40.1±1.46)% in the H2O2 group (n=3) (Figure 3), which demonstrated that LBP pretreatment (500 μg/mL) led to a significant decrease of apoptotic cells.

The cells were stained with enhanced green fluorescence protein (EGFP)-conjugated Annexin V and propidium iodide (PI). The EGFP and PI fluorescence was measured using flow cytometer with FITC-A and PI-A filter. H2O2 treatment significantly increased ARPE-19 cell apoptosis, while LBP pretreatment (500 μg/mL) led to a significant decrease of apoptotic cells. Data are represented as (means±SD) of three individual experiments. *P<0.05, †P<0.01 compared with control; ‡P<0.01 compared with H2O2 treatment alone.

**DISCUSSION**

RPE cells play crucial roles in protecting the outer retina from photooxidative stress, in the digestion of shed photoreceptor outer segments which contain oxidized lipids, and in inhibition of retinal edema and neovascularization[9]. Dysfunction and degeneration of RPE cells are crucially involved in the pathogenesis of AMD[20,21]. AMD is a degenerative disease of the retina and the leading cause of blindness in the elderly[22]. Although the etiology of dry AMD remains unclear, oxidative stress and chronic inflammation are believed to be triggered by genetic and environmental risk factors to drive dry AMD pathogenesis. The apoptotic death of RPE cells, followed by photoreceptor cell death, is a major factor contributing to the pathogenesis.
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of the dry form of AMD\textsuperscript{[21]}. Consistent with a critical role for oxidative stress in AMD, clinical studies have shown that AMD disease progression can be slowed with antioxidant vitamins and zinc supplements\textsuperscript{[23,24]}. The retina is particularly vulnerable to oxidative damage, which increases its reactive oxygen species production\textsuperscript{[25,26]}. Most in vitro data also attribute apoptosis as a major mechanism of RPE cell death in response to prooxidants, including hydrogen peroxide (H$_2$O$_2$) and its stable form tert-butyl hydroperoxide (tBHP)\textsuperscript{[27-34]}. Here, we systematically investigated the antioxidant activities of LBP and evaluated their role in H$_2$O$_2$–induced ARPE-19 cells damage. The results showed that pretreated with LBP could effectively modulate expression of Bcl-2 and Bax gene and protect ARPE-19 cells from H$_2$O$_2$–induced oxidative stress injury. Lycium barbarum (Wolfberry) has been used as a traditional anti-aging herb in Chinese pharmacopoeia for a long history\textsuperscript{[9,35]}. LBP, the main effective ingredient of lycium barbarum, isolated from the aqueous extracts of lycium barbarum, has a complicated role in the life and death of cells. Many studies had reported LBP could exhibit cytoprotective effects on neurons in the eye as well as on hepatocytes\textsuperscript{[36-39]}. Ho et al.\textsuperscript{[40]} investigated whether LBP could protect neurons against Homocysteine (Hcy) excitotoxicity. The results showed that LBP significantly reduced Hcy-induced neuronal cell death and apoptosis in rat primary culture cortical neurons as detected by lactate dehydrogenase assay and caspase-3-like activity assay.

The protective effects of LBP against neuronal cell death were detected in retinal ischemia/reperfusion (I/R) injuries, pretreatment with LBP significantly attenuated neuronal cell apoptosis in the ganglion cell layer and the inner nuclear layer of I/R retina induced by surgical occlusion of the internal carotid artery\textsuperscript{[39]}. Furthermore, LBP obviously increase the survival rate and promote the growth of mixed cultured rat retinal ganglion cells\textsuperscript{[41]}. In the present study, we demonstrated that LBP increased ARPE-19 cells survival under acute (500 μmol/L H$_2$O$_2$) oxidative stress conditions. Under the inverted microscope, ARPE-19 cells exposed to H$_2$O$_2$ exhibited apoptotic-like signs. However, with the pretreatment of LBP, the proportions of apoptotic cells were significantly decreased. Moreover, flow cytometry analysis showed that LBP markedly reduced the apoptosis in H$_2$O$_2$–treated cells. These indicated that LBP exerted a protective effect by inhibiting H$_2$O$_2$–induced cell apoptosis.

Bcl-2 and Bax are discovered as a very close relationship's apoptotic gene in recent years, and apoptosis is regulated by maintaining the Bcl-2 and Bax balance\textsuperscript{[42]}. Bcl-2 is antiapoptotic gene and Bax is proapoptotic gene. When overexpressed Bax, forming homodimers of Bax/Bax, promotes cell death, and when overexpressed Bcl-2, forming heterodimers of Bcl-2/Bax, inhibits cell death. Therefore, the ratio of Bcl-2/Bax determines whether the cells accept signals to induce apoptosis\textsuperscript{[43,44]}. In the present study, incubated with H$_2$O$_2$ caused a significant reduction positive expression of Bcl-2 and an elevation positive expression of Bax in ARPE-19 cells, and the Bcl-2/Bax ratio decreased impressively, which suggested the cell apoptosis increased. However, treated with LBP before incubated with H$_2$O$_2$ could significantly increase the Bcl-2/Bax ratio by up-regulating the expression of Bcl-2 and down-regulating that of Bax in comparison.

In conclusion, LBP can prevent APRE-19 cell against the apoptosis induced by H$_2$O$_2$. The mechanism relates to up-regulate the expression of Bcl-2 and down-regulate the expression of Bax. Compared with chemically synthesised medicines, LBP, a natural complex isolated from a traditional Chinese herb, has few side effects and is more suitable for long-term clinical use, which may be proved to be a new potential anti-AMD drug.

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Figure 4 LBP inhibited the H$_2$O$_2$–induced down–regulation of Bcl–2 and up–regulation of Bax in ARPE–19 cells $^aP<0.05$, $^bP<0.01$ compared with control; $^cP<0.05$, $^dP<0.01$, compared with H$_2$O$_2$ treatment alone.
REFERENCES
4 Chu PH, Li HY, Chin MP, So KF, Chan HH. Effect of Lycium barbarum (wolfberry) polysaccharides on preserving retinal function after partial optic nerve transection. *PloS One* 2013;8(12):e81339
32 Rodrigues GA, Maurier–Mahe F, Shurland DL, McLaughlin A, Luks K,
LBP protected ARPE−19 cells against H$_2$O$_2$−induced apoptosis


34 Faghiri Z, Bazan NG. PI3K/Akt and mTOR/p70S6K pathways mediate neuroprotectin D1−induced retinal pigment epithelial cell survival during oxidative stress−induced apoptosis. *Exp Eye Res* 2010;90(6):718−725


40 Ho YS, Yu MS, Yang XF, So KF, Yuen WH, Chang RC. Neuroprotective effects of polysaccharides from wolfberry, the fruits of Lycium barbarum, against homocysteine−induced toxicity in rat cortical neurons. *J Alzheimers Dis* 2010;19(3):813−827


42 Takagi−Morishita Y, Yamada N, Sugihara A, Iwasaki T, Tsujimura T, Terada N. Mouse uterine epithelial apoptosis is associated with expression of mitochondrial voltage−dependent anion channels, release of cytochrome C from mitochondria, and the ratio of Bax to Bcl−2 or Bcl−X. *Bio Reprod* 2003;68(4):1178−1184


44 Tsukahara S, Yamamoto S, Tin−Tin−Win−Shwe, Ahmed S, Kunugita N, Arashidani K, Fujimaki H. Inhalation of low−level formaldehyde increases the Bcl−2/Bax expression ratio in the hippocampus of immunologically sensitized mice. *Neuromuscul Disord* 2006;16(2):63−68