·Basic Research·

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

Lian Liu, Wei Lao, Qing-Shan Ji, Zhi-Hao Yang, Guo-Cheng Yu, Jing-Xiang Zhong

Department of Ophthalmology, the First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong Province, China

Co-first authors: Lian Liu and Wei Lao

Correspondence to: Jing-Xiang Zhong. Department of Ophthalmology, the First Affiliated Hospital of Jinan University, 613 Huangpu Road, Guangzhou 510630, Guangdong Province, China. zjx85221206@163.com

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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 lines, were exposed to different concentrations of H_2O_2 for 24h, then cell viability was measured by Cell Counting Kit-8 (CCK-8) assay to get the properly concentration of H_2O_2 which can induce half apoptosis of APRE-19. With different concentrations of LBP pretreatment, the ARPE-19 cells were then exposed to appropriate concentration of H_2O_2, 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.**

• **RSULTS: LBP significantly reduced the H_2O_2-induced ARPE-19 cells´ apoptosis. LBP inhibited the H_2O_2-induced down-regulation of Bcl-2 and up-regulation of Bax.**

• **CONCLUSION: LBP could protect ARPE-19 cells from H_2O_2-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 chorioid 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 H_2O_2 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% CO_2 at 37℃. When grown to 80%-85% confluence, the cells were collected for different assays.

H_2O_2-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 H_2O_2 (0, 300,

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℃ 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 [18]. 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, 5000 μg/mL, respectively) for 24h. Then the cells were exposed to 500 μmol/L H_2O_2 and incubated for 24h. After that, the cells were incubated with 10 μL of CCK-8 solution for 2h at 37℃ 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 500 μg/mL LBP for 24h before 500 μmol/L H_2O_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℃, 10min at 95℃ for polymerase activation, 45 cycles of 15s at 95℃ (denaturation), 30s at 60℃, 15s at 72℃ and 15s at 95℃ (annealing and extension). Finally, melting was curried out at 60℃ -95℃ (0.5℃ 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.

Table 1 RT- PCR primers used (Fermentas, Canada)

Gene	Primer
Bcl-2 (F)	5' GATAACGGAGGCTGGGATGC 3'
Bcl-2 (R)	5' CAGGCATGTTGACTTCACTTGTG 3'
Bax (F)	5' TTGCTTCAGGGGATGATTG 3'
Bax (R)	5' CAAAGTAGAAAAGGGCGACA 3'
β-actin (F)	5' ACAGAGCCTCGCCTTTGCCGAT 3'
β-actin (R)	5' CTTGCACATGCCGGAGCCGTT 3'

F: Forward; R: Reverse.

RESULTS

Lycium Barbarum Polysaccharides Reduced H_2O_2 – induced Cell Apoptosis in Retinal Pigment Epithelial Cells ARPE-19 cells were incubated with different concentrations of H_2O_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_2O_2-induced apoptosis model in order to analyze the protection of LBP.

ARPE-19 cells were incubated with different concentrations of H_2O_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_2O_2-induced cell damage, we detected the viability of ARPE-19 cells after H_2O_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_2O_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.

ARPE-19 cells were cultured with 0-5000 μg/mL LBP for 24h before incubated with 500 μmol/L H_2O_2 for 24h. Cell viability was measured by CCK-8 assay. Data are represented as (means±SD) of five individual experiments. $^aP<0.05$, $^bP<0.01$ 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_2O_2 (500 μmol/L) for 24h. Under the inverted microscope, we found a high portion of cells appeared apoptosis-like changes in H_2O_2 treatment group, such as detachment and cytoplasmic-condensation leading to rounding.

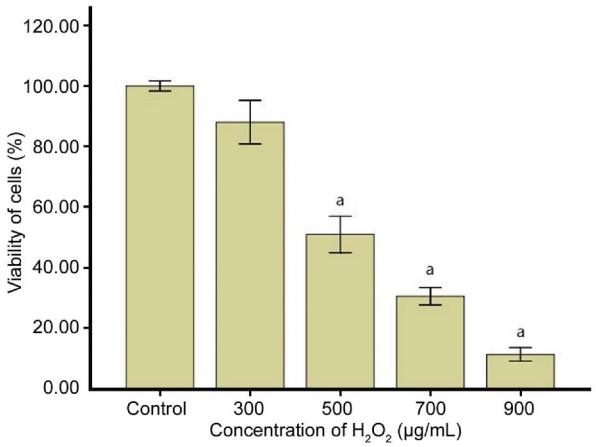

Figure 1 Effect of different concentrations of H_2O_2 to ARPE-19 cells [a]P<0.05, compared with control.

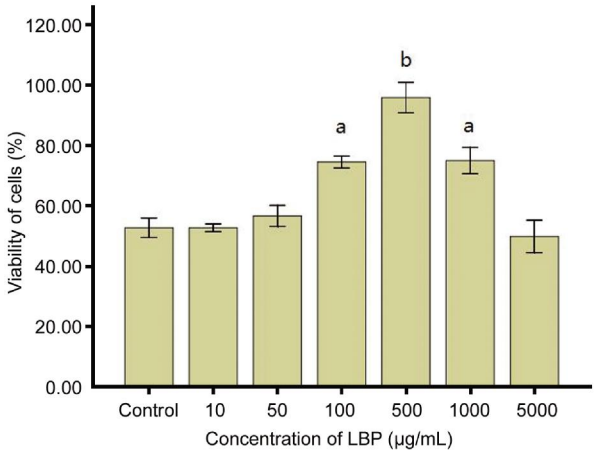

Figure 2 LBP increased the viability of H_2O_2 treated cells in appropriate concentrations [a]P <0.05, [b]P<0.01 compared with control.

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+H_2O_2 group, much lower than (40.1±1.46)% in the H_2O_2 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. H_2O_2 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. [a]P<0.05, [b]P<0.01 compared with control; [d]P<0.01 compared with H_2O_2 group.

Lycium Barbarum Polysaccharides Suppressed Bax and Motivated Bcl-2 in Retinal Pigment Epithelial Cells As shown in Figure 4, RT-PCR was used to measure the level of Bax and Bcl-2 mRNA in the control group, H_2O_2 group and the

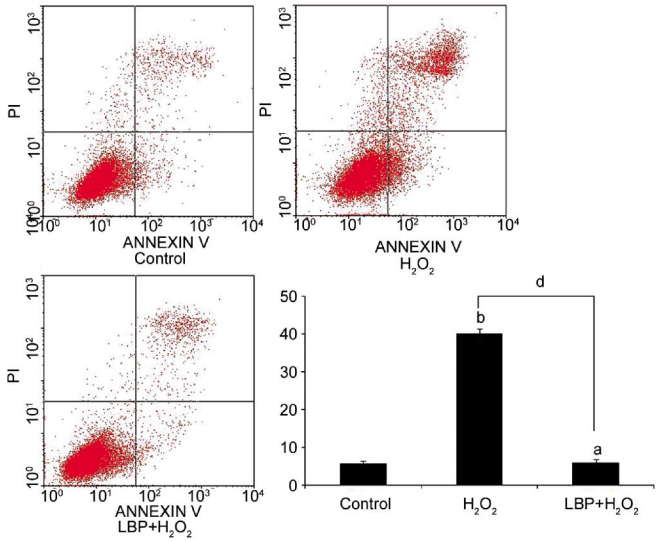

Figure 3 LBP reduced H_2O_2-induced ARPE-19cell apoptosis [a]P<0.05, [b]P<0.01 compared with control; [d]P<0.01 compared with H_2O_2 treatment alone.

(LBP+H_2O_2) group. The computerized tomographynumber (CT) of Bax were (1±0.06), (1.53±0.04) and (1.27±0.07) respectively，while the Bcl-2 were (1±0.04), (0.54±0.09) and (0.87 ±0.03). The CT of Bax and Bcl-2 had statistic difference between the (LBP+H_2O_2) group and the H_2O_2 group. Compared with the control group, Bcl-2/Bax ratio decreased in the (LBP+H_2O_2) group and the H_2O_2 group. However, compared with the H_2O_2 group, Bcl-2/Bax ratio increased significantly in the (LBP+H_2O_2) group. The results implied that pretreated with 500 μg/mL LBP distinctly modulated the levels of Bax and Bcl-2 gene in H_2O_2-exposed cells.

The mRNA of Bcl-2 and Bax in ARPE-19 cells were detected by RT-PCR. LBP significantly inhibited the Bcl-2 down-regulation and Bax up-regulation in ARPE-19 cells. [a]P<0.05, [b]P<0.01 compared with control; [c]P<0.05, compared with H_2O_2 group; [d]P<0.01, compared with H_2O_2 group.

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[19]. 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

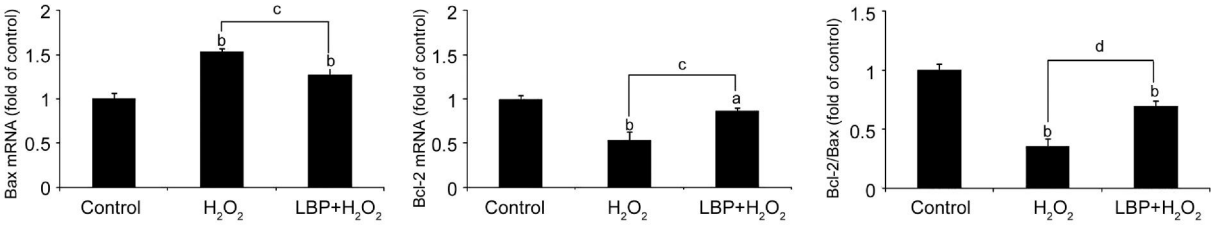

Figure 4 LBP inhibited the H_2O_2-induced down-regulation of Bcl-2 and up-regulation of Bax in ARPE-19cells [a]$P<0.05$, [b]$P<0.01$ compared with control; [c]$P<0.05$, [d]$P<0.01$, compared with H_2O_2 treatment alone.

of the dry form of AMD[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[23,24].

The retina is particularly vulnerable to oxidative damage, which increases its reactive oxygen species production [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_2O_2) and its stable form tert-butyl hydroperoxide (tBHP) [27-34]. Here, we systematically investigated the antioxidant activities of LBP and evaluated their role in H_2O_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_2O_2-induced oxidative stress injury.

Lycium barbarum (Wolfberry) has been used as a traditional anti-aging herb in Chinese pharmacopoeia for a long history [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[36-39].

Ho *et al* [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 [39]. Furthermore, LBP obviously increase the survival rate and promote the growth of mixed cultured rat retinal ganglion cells[41].

In the present study, we demonstrated that LBP increased ARPE-19 cells survival under acute (500 μmol/L H_2O_2) oxidative stress conditions. Under the inverted microscope, ARPE-19 cells exposed to H_2O_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_2O_2-treated cells. These indicated that LBP exerted a protective effect by inhibiting H_2O_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 [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 [43,44]. In the present study, incubated with H_2O_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_2O_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_2O_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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REFERENCES

1 Wang T, Li Y, Wang Y, Zhou R, Ma L, Hao Y, Jin S, Du J, Zhao C, Sun T, Yu J. Lycium barbarum polysaccharide prevents focal cerebral ischemic injury by inhibiting neuronal apoptosis in mice. *PLoS One* 2014;9 (3): e90780

2 Mi XS, Feng Q, Lo AC, Chang RC, Lin B, Chung SK, So KF. Protection of retinal ganglion cells and retinal vasculature by Lycium barbarum polysaccharides in a mouse model of acute ocular hypertension. *PLoS One* 2012;7(10):e45469

3 Yang D, Li SY, Yeung CM, Chang RC, So KF, Wong D, Lo AC. Lycium barbarum extracts protect the brain from blood-brain barrier disruption and cerebral edema in experimental stroke. *PLoS One* 2012;7(3):e33596

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 Onc* 2013;8(12):e81339

5 Xiao J, Liong EC, Ching YP, Chang RC, Fung ML, Xu AM, So KF, Tipoe GL. Lycium barbarum polysaccharides protect rat liver from non-alcoholic steatohepatitis-induced injury. *Nutr Dia Betes* 2013;3:e81

6 Su CX, Duan XG, Liang LJ, Feng W, Zheng J, Fu XY, Yan YM, Ling-Huang, Wang NP. Lycium barbarum polysaccharides as an adjuvant for recombinant vaccine through enhancement of humoral immunity by activating Tfh cells. *Vet Immunol Immunopathol* 2014;158(1-2):98-104

7 Chen W, Cheng X, Chen J, Yi X, Nie D, Sun X, Qin J, Tian M, Jin G, Zhang X. Lycium barbarum polysaccharides prevent memory and neurogenesis impairments in scopolamine-treated rats. *PLoS One* 2014;9 (2):e88076

8 Yi R, Liu XM, Dong Q. A study of Lycium barbarum polysaccharides (LBP) extraction technology and its anti-aging effect. *Afr J Tradit Complement Altern Med* 2013;10(4):171-174

9 Chang RC, So KF. Use of anti-aging herbal medicine, Lycium barbarum, against aging-associated diseases. What do we know so far? *Cell Mol Neurobiol* 2008;28(5):643-652

10 Jin M, Huang Q, Zhao K, Shang P. Biological activities and potential health benefit effects of polysaccharides isolated from Lycium barbarum L. *Int J Biol Macromol* 2013;54:16-23

11 Liu Q, Li Y, Hu L, Wang D. Lycium barbarum polysaccharides attenuate cisplatin-induced hair cell loss in rat cochlear organotypic cultures. *Int J Mol Sci* 2011;12(12):8982-8992

12 Zhu J, Zhang Y, Shen Y, Zhou H, Yu X. Lycium barbarum polysaccharides induce Toll-like receptor 2- and 4-mediated phenotypic and functional maturation of murine dendritic cells via activation of NF-kappaB. *Mol Med Rep* 2013;8(4):1216-1220

13 Amagase H, Sun B, Borek C. Lycium barbarum (goji) juice improves *in vivo* antioxidant biomarkers in serum of healthy adults. *Nutr Res* 2009;29 (1):19-25

14 Niu AJ, Wu JM, Yu DH, Wang R. Protective effect of Lycium barbarum polysaccharides on oxidative damage in skeletal muscle of exhaustive exercise rats. *Int J Biol Macromol* 2008;42(5):447-449

15 Zhao Z, Luo Y, Li G, Zhu L, Wang Y, Zhang X. Thoracic aorta vasoreactivity in rats under exhaustive exercise: effects of Lycium barbarum polysaccharides supplementation. *J Int Soc Sports Nutr* 2013;10:47

16 Luo Q, Li Z, Huang X, Yan J, Zhang S, Cai YZ. Lycium barbarum polysaccharides: protective effects against heat-induced damage of rat testes and H2O2-induced DNA damage in mouse testicular cells and beneficial effect on sexual behavior and reproductive function of hemicastrated rats. *Life Sci* 2006;79(7):613-621

17 Li XM. Protective effect of Lycium barbarum polysaccharides on streptozotocin-induced oxidative stress in rats. *Int J Biol Macromol* 2007; 40:461-465

18 Yu MS, Leung SK, Lai SW, Che CM, Zee SY, So KF, Yuen WH, Chang RC .Neuroprotective effects of anti-aging oriental medicine Lycium barbarum against beta-amyloid peptide neurotoxicity. *Exp Geronto l* 2005; 40(8-9):716-727

19 Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev* 2005;85(3):845-881

20 Roth F, Bindewald A, Holz FG. Keypathophysiologic pathways in agerelated macular disease. *Graefes Arch Clin Exp Ophthalmol* 2004;242 (8):710-716

21 Nowak JZ. Age-related macular degeneration (AMD): pathogenesis and therapy. *Pharmacol Rep* 2006;58(3):353-363

22 Friedman DS, O'Colmain BJ, MuÑoz B, Tomany SC, McCarty C, de Jong PT, Nemesure B, Mitchell P, Kempen J; Eye Diseases Prevalence Research Group. Prevalence of age-related macular degeneration in the United States. *Arch Ophthalmol* 2004;122(4):564-572

23 Age-Related Eye Disease Study 2 Research Group. Lutein+zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. *JAMA* 2013;309(19):2005-2015

24 Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol* 2001; 119(10):1417-1436

25 Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 2000;45(2):115-134

26 Khandhadia S, Lotery A. Oxidation and age-related macular degeneration: insights from molecular biology. *Expert Rev Mol Med* 2010; 12:e34

27 Ferrington DA, Tran TN, Lew KL, Van Remmen H, Gregerson DS. Different death stimuli evoke apoptosis *via* multiple pathways in retinal pigment epithelial cells. *Exp Eye Res* 2006;83(3):638-650

28 Chang JY, Bora PS, Bora NS. Prevention of oxidative stress-induced retinal pigment epithelial cell death by the PPARgamma agonists, 15-deoxy-delta 12, 14-prostaglandin J (2). *PPAR Res* 2008;2008:720163

29 Garg TK, Chang JY. Oxidative stress causes ERK phosphorylation and cell death in cultured retinal pigment epithelium: prevention of cell death by AG126 and 15-deoxy-delta 12, 14-PGJ2. *BMC Ophthalmol* 2003;3:5

30 Tsao YP, Ho TC, Chen SL, Cheng HC. Pigment epithelium-derived factor inhibits oxidative stress-induced cell death by activation of extracellular signal-regulated kinases in cultured retinal pigment epithelial cells. *Life Sci* 2006;79(6):545-550

31 Zou X, Feng Z, Li Y, Wang Y, Wertz K, Weber P, Fu Y, Liu J. Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: activation of Nrf2 and JNK-p62/SQSTM1 pathways. *J Nutr Biochem* 2012;23 (8): 994-1006

32 Rodrigues GA, Maurier-Mahe F, Shurland DL, McLaughlin A, Luhrs K,

Throo E, Delalonde-Delaunay L, Pallares D, Schweighoffer F, Donello J Differential effects of PPARgamma ligands on oxidative stress-induced death of retinal pigmented epithelial cells. *Invest Ophthalmol Vis Sci* 2011; 52(2):890-903

33 Kim JH, Kim JH, Jun HO, Yu YS, Min BH, Park KH, Kim KW. Protective effect of clusterin from oxidative stress-induced apoptosis in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2010;51 (1):561-566

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

35 Ho YS, So KF, Chang RC. Drug discovery from Chinese medicine against neurodegeneration in Alzheimer's and vascular dementia. *Chin Med* 2011;6:15

36 Chan HC, Chang RC, Koon-Ching Ip A, Chiu K, Yuen WH, Zee SY, So KF. Neuroprotective effects of Lycium barbarum Lynn on protecting retinal ganglion cells in an ocular hypertension model of glaucoma. *Exp Neurol* 2007;203(1):269-273

37 Chiu K, Chan HC, Yeung SC, Yuen WH, Zee SY, Chang RC, So KF. Modulation of microglia by Wolfberry on the survival of retinal ganglion cells in a rat ocular hypertension model. *J Ocul Biol Dis Infor* 2009;2(3): 27-136

38 Chiu K, Zhou Y, Yeung SC, Lok CK, Chan OO, Chang RC, So KF, Chiu JF. Up-regulation of crystallins is involved in the neuroprotective effect of wolfberry on survival of retinal ganglion cells in rat ocular hypertension model. *J Cell Biochem* 2010;110(2):311-320

39 Li SY, Yeung CM, Yu WY, Chang RC, So KF, Wong D, Lo AC. Lycium barbarum Polysaccharides reduce neuronal damage, blood-retinal barrier disruption and oxidative stress in retinal ischemia/reperfusion injury. *PLoS One* 2011;6(1):e16380

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

41 Yang M, Gao N, Zhao Y, Liu LX, Lu XJ. Protective effect of Lycium barbarum polysaccharide on retinal ganglion cells *in vitro*. *Int J Ophthalmol* 2011;4(4):377-379

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. *Biol Reprod* 2003;68(4):1178-1184

43 Yu X, Kubota H, Wang R, Saegusa J, Ogawa Y, Ichihara G, Takeuchi Y, Hisanaga N. Involvement of Bcl-2 family genes and Fas signaling system in primary and secondary male germ cell apoptosis induced by 2-bromopropane in rat. *Toxicol Appl Pharmacol* 2001;174(1):35-48

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. *Neuroimmunomodulation* 2006;13(2):63-68