

Original Article

Lycium barbarum polysaccharides can reduce the oxidative damage of the retinal nerve cells in diabetic rats

Yuanyuan Wang^{1*}, Li Ding^{2*}, Yuanmei Li¹, Changrong Guan², Jian Guo³

¹Department of Endocrinology, Xixi Hospital of Hangzhou, Hangzhou 315000, Zhejiang Province, PR China; ²Department of Endocrinology, The People's Hospital of Lishui, Lishui 323000, Zhejiang Province, PR China; ³Ophthalmology Institute of Fujian Province, College of Pharmacy, Fujian Medical University, Fujian, PR China. *Equal contributors.

Received August 8, 2016; Accepted January 26, 2017; Epub March 15, 2017; Published March 30, 2017

Abstract: Objective: To evaluate the protection effects of lycium barbarum polysaccharides (LBP) on oxidative damage of retinal nerve cells in diabetic rats. Methods: Twenty SD rats were selected in this study and divided into blank control group with ($n=5$) and diabetes mellitus (DM) group with ($n=15$). 13 of the 15 rats in DM group were successfully made the diabetes mellitus rats. The 13 DM rats were randomly divided in to LBP ($n=7$) and DM group ($n=6$). The rats in the LBP group were given 6% LBP 0.5 ml intragastric administration *qd.* and rats in the DM group were treated with normal saline. After 24 weeks, the VEGF mRNA and protein, the retinal tissue ultrastructure changes of the three groups were compared. Results: The weight in DM and LBP group were significant lower than the blank control group ($P<0.05$); The blood glucose in the DM and LBP group were statistical higher than the control group ($P<0.05$) and no statistical difference of DM and LBP group ($P>0.05$); Compared with DM group, the SOD activity was decreased and MDA level were elevated in the LBP group ($P<0.05$); The VEGF mRNA and protein expression was not expressed in control group and decreased in LBP group compared with DM group ($P<0.05$). Conclusion: LBP can significantly reduce pathological changes of the mitochondria, prevent nerve cell apoptosis, blocking the vascular lesion change development through its antioxidant effect.

Keywords: Lycium barbarum polysaccharides, diabetes mellitus, rats, diabetic retinopathy, oxidative damage

Introduction

Diabetic retinopathy (DR), formerly called "diabetic microangiopathy", is the most common cause of irreversible blindness in diabetic patients [1-3]. Almost all patients with type 1 diabetes mellitus (DM) and more than 60% of patients with type 2 DM will develop some degree of retinopathy after a 20-year history of diabetes [4]. Hypertension, obesity, sex, hyperlipidaemia, chronic kidney disease, smoking and myopia are all systemic risk factors of diabetic retinopathy. However, its pathogenesis remains unknown [5]. Recent studies have shown that oxidative damage to retinal nerve cell mitochondria [6] may be a factor inducing the occurrence and development of DR.

Lycium barbarum, small red berry, has been used as a traditional Chinese herbal medicine to treat diabetes, cancer, abdominal pain, infer-

tility, dry cough, headache and fatigue, and also to increase longevity [7, 8]. Lycium barbarum polysaccharides (LBP), the dietary active ingredient extracted from *L. barbarum.*, has been discovered in bioactivities such as enhancing immune function, regulating blood sugar and protecting the nervous system [9, 10]. But whether it has effects on DR is largely unknown.

Many research showed that growth factors such as vascular endothelial growth factor (VEGF), have a close relation with the breaking of blood-retinal barrier [11-13]. VEGF is a soluble protein that may promote vascular endothelial cell proliferation and division. Besides, it can improve vasopermeability by regulating other retinal cells proliferation and function by synthesis growth factors. In addition, VEGF plays important roles in processes of pathological and physiological neovascularization.

Lycium barbarum polysaccharides reduce oxidative damage

In the present study, LBP is used to intervene streptozocin (STZ) in rats with diabetes mellitus (DM), after which oxidative damage to retinas is observed. This study provides an animal experimental basis for the clinical application of LBP in treating DR.

Materials and methods

Rats and drugs

Twenty SPF male Sprague-Dawley (SD) rats were purchased for tail venous blood sampling from Shanghai SLAC Laboratory Animal Co., Ltd. The rats had body weights of 250 ± 25 g and ≤ 6.75 mmol/L [14] blood sugar, measured with a OneTouch II blood glucose meter purchased from Johnson & Johnson. No lesions were revealed in the extraocular or fundus examinations, and the rats were housed in the SPF Laboratory for Rodents of the Experimental Animal Center of Fujian Medical University [14]. STZ (Sigma) was dissolved in 0.1 mol/L sterile citrate-citrate buffer (pH 4.4) and maintained at 1% solution before use. LBP was provided by Professor Huang Liping of the Department of Pharmacy Practice, Fujian Medical University. Before use, 1.05 g LBP was weighed and dissolved in 17.5 ml normal saline (NS).

Modeling of STZ in rats with DM

The rats with SD were divided into two groups: 15 rats with DM and a control group of 5 normal rats, all of which were adaptively fed for 1 week and prepared for modeling after 10 h fasting. Afterward, 1% STZ solution (65 mg/kg) was introduced to the lower left abdominal cavity of rats in the DM group. The same dose of citrate-citrate buffer was injected in rats of the control group [15]. Following a 72 h period, tail venous blood samples were taken to measure blood sugar by a glucometer (BAYER, Germany) three times at each period of time. Rats with blood sugar equal to or greater than 16.7 mmol/L were tested again following 1 week. If the measured blood sugar remained equal to or greater than 16.7 mmol/L, the modeling was considered a success.

Grouping and observations

Thirteen successful rats were randomized into two groups: the group with DM and the group

treated with LBP. The rats of the LBP group were intragastrically administered 0.5 ml 6% LBP every morning, whereas those of the DM group were similarly administered 0.5 ml NS. Body weight and blood sugar were measured every 4 weeks.

MDA expression and SOD activity

Following 24 weeks of LBP and NS administration, each rat was weighed and their blood sugar was measured. After pentobarbitalum natrium ($60 \text{ mg} \cdot \text{kg}^{-1}$) was intraperitoneally given for anesthesia, the eyeballs were extirpated and the retinas were dissected under an ophthalmic operating microscope. The retinal tissues were placed in 4°C pre-cooled NS, rinsed twice with volume ratio of 1:9, and centrifuged for 10 min at 2000 rpm. Exactly 40 μl of supernatant was then taken as per the instructions given on the kits (MDA ELISA Kit from Abcore-inc and SOD Activity Assay Kit from Sigma).

VEGF mRNA expression

The expressions of VEGF mRNA in the retinas of each group of rats were detected by RT-PCR. The eyeballs of each group of rats were extirpated, and the retinas were dissected under an ophthalmic operating microscope. Total RNA was extracted using TRIzol. cDNA was prepared by reverse transcription (RT) and amplified by polymerase chain reaction (PCR). Reaction parameters are given in **Table 1**. The PCR products received agarose gel electrophoresis. The absorbance (A) of VEGF and β -actin was recorded using Bio-Rad GEL imaging system for gray-scale scanning, and the ratio of VEGF to β -actin was calculated. The *vegf* gene primers were as follows: F: 5'-GAAGGAGGAGGGCAGAAT-3', R: 5'-CGATTGGATGGCAGTAGC-3'; The β -actin primers were F: 5'-CTGAATGGCCCAGGTCTGAG-3' and R: 5'-AAGTCAGTGTACAGGCCAGC-3'.

Western blot

Total protein of the eyeball retinas were extracted using RIPA buffer (Beyotime). The protein concentration was measured using the BCA method. A total of 80 μg of protein was used for the analysis of VEGF expression and β -actin was used as an internal control. Anti-VEGF (1:1000) and anti- β -actin (1:5000) (Cell Sign-

Lycium barbarum polysaccharides reduce oxidative damage

Table 1. The difference of body weight during observation (g)

Group	Before treatment (g)	After treatment (g)	ΔBody weight (g)
Blank control (n=5)	281.0±11.81	564.9±38.61	283.9±33.31
DM model (n=6)	283.7±10.14	254.3±31.16*	-29.4±29.60*
LBP treatment (n=7)	283.0±13.46	253.3±41.32*	-29.7±35.49*

*P<0.05, compared with blank control group.

Table 2. Blood glucose comparison of the two groups (mmol/L)

Group	Before treatment	After treatment
Blank control (n=5)	4.97±0.75	4.84±0.60
DM model (n=6)	23.01±2.44*	27.49±2.79*
LBP treatment (n=7)	22.54±2.19*	26.66±3.64*

*P<0.05, compared with blank control group.

Table 3. The effect of LBP on the SOD and MDA of DM rats retina ($\bar{x} \pm s$)

Groups	SOD (U·mgprot ⁻¹)	MDA (mol·mgprot ⁻¹)
Blank control (n=5)	171.51±4.79	4.12±0.43
DM model (n=6)	129.70±6.97*	8.23±0.43*
LBP treatment (n=7)	179.82±4.02* [#]	4.58±0.25 [#]

*P<0.05, compared with blank control group; [#]P<0.05, compared with DM model group.

aling Technology) were used as the primary antibodies. Goat anti-mouse IgG conjugated to horseradish peroxidase (1:1000) was used as the secondary antibody (Beyotime). The bound antibodies were detected using the ECL Plus Western Blotting Detection System (Pierce, Thermo) and the chemiluminescent signals were detected by using high-performance chemiluminescence film (KODAK).

Transmission electron microscope (TEM)

Twenty four weeks after administration, tail venous blood samples were taken from the rats to measure blood sugar, and pelltobarbitalum natricum (60 mg/kg) was injected intraperitoneally. The rats' hearts were perfused with 30 g/L glutaraldehyde. Their eyeballs were extirpated immediately. Retinal tissues were taken and cut into 1.5 mm × 3.0 mm rectangular pieces; fixed in 3% glutaraldehyde and 1.5% paraformaldehyde overnight and in 1% osmium tetroxide-1.5% potassium ferrocyanide for 1.5 h; rinsed with PBS; stained with 70% alcohol saturated with uranyl acetate; gradiently dehydrated with alcohol-acetone; and embedded in

epoxy resin 618. The tissues were sliced into 80 nm pieces, separately stained with uranyl acetate and lead citrate for 5 min, and observed and photographed under Philips EM 208.

Statistical analysis

SPSS13.0 was used for statistical analysis. The measured value of blood sugar was expressed by $\bar{x} \pm s$. The differences between the groups were analyzed by one-way ANOVA, P<0.05 was considered statistically significant.

Results

Changes in the body weight before and after treatment

Significant differences in body weight were observed between the group with DM (or the group treated with LBP) and the control group (P<0.01). Insignificant differences were found between the group with DM and the group treated with LBP (P>0.05) (**Table 1**).

Changes in the blood sugar before and after treatment

Significant differences were found in the blood sugar of the group with DM (or the group treated with LBP) and the control group (P<0.01). Insignificant differences were found between the group with DM and the group treated with LBP (P>0.05) (**Table 2**).

Effects of LBP on the total SOD activity and MDA expression level in the retina of rats with DM

The SOD activity in the retina of rats with DM was reduced, whereas the MDA expression level was elevated. SOD activity in the group treated with LBP was higher than in the group with DM, but the MDA expression level was greatly reduced, having significant differences (P<0.05) (**Table 3**).

Effects of LBP on the expression of VEGF mRNA in the retina of the rat with DM

As revealed by RT-PCR, VEGF mRNA was expressed at a low level in the control group. VEGF mRNA and protein level were most highly expressed in the group with DM and was visibly reduced in the group treated with LBP (P<0.05) (**Figure 1**).

Lycium barbarum polysaccharides reduce oxidative damage

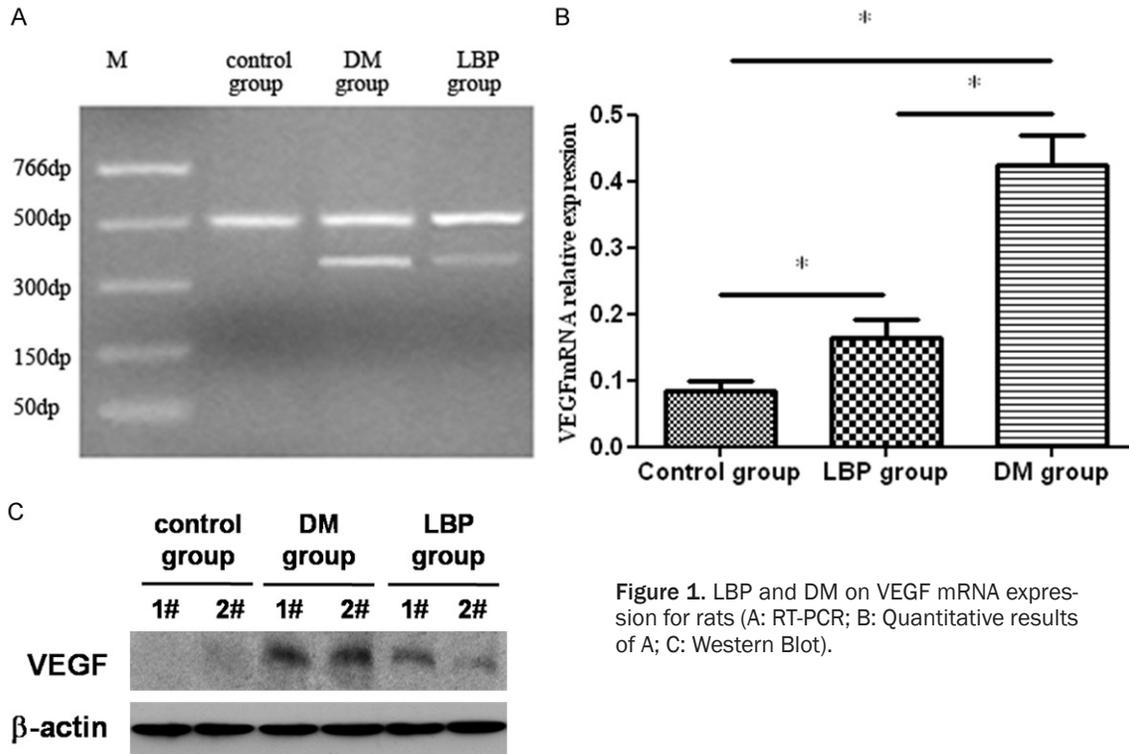


Figure 1. LBP and DM on VEGF mRNA expression for rats (A: RT-PCR; B: Quantitative results of A; C: Western Blot).

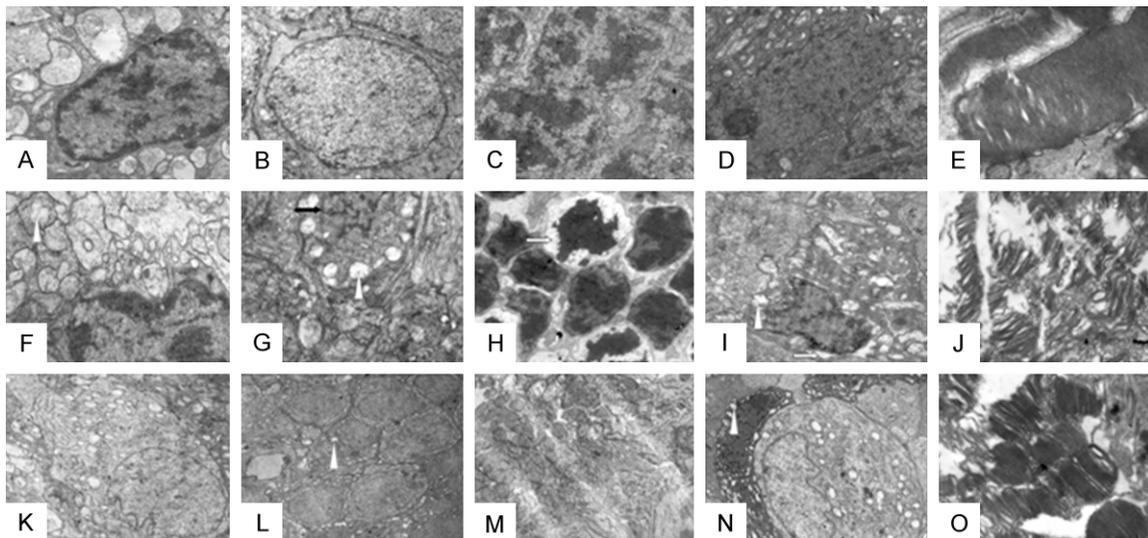


Figure 2. Ultrastructure of retina of experiment rats A-E control group, F-J DM group, and K-O LBP group (A: ganglion cell (TEM × 10⁵), B: bipolar cell (TEM × 8000), C: outer nuclear layer (TEM × 6000), D: müller cell (TEM × 8000), E: outer segment (TEM × 5000); F: ganglion cell (TEM × 8000), G: bipolar cell (TEM × 4800), H: outer nuclear layer (TEM × 10000), I: müller cell (TEM × 10000), J: outer segment (TEM × 5000); K: ganglion cell (TEM × 5000), L: bipolar cell (TEM × 3150), M: outer nuclear layer (TEM × 6000), N: müller cell (TEM × 3150), O: outer segment (TEM × 5000)).

Changes in the retinal ultrastructure of each group

The retinal ultrastructure of the control group was normal (**Figure 2A-E**). In the retina of the

group with DM, the neurons and glial cell mitochondria of different sizes were reduced. Numerous large mitochondria were visible in the inner nuclear layer, with less swelling and fractured mitochondrial cristae; whereas some

mitochondria were round vacuoles (see **Figure 2F, 2G, 2I**, marked in white triangles). In the LBP group, neither the retinal ganglion cell nor the photoreceptor exhibited obvious abnormality. However, the bipolar cells and Müller glial cells exhibited reduced and shorter mitochondrial cristae (see **Figure 2K, 2N**, marked in white triangles) and no abnormal changes in the nuclei. The cells were arranged regularly and closely in the inner nuclear layer.

Discussions

DR, as one of the most common ocular complications of DM, has long been regarded as microangiopathy [16, 17]. Animal studies have found that retinal neuropathy can appear early in the 1st week of the animal model of DM [18] as degeneration and apoptosis of ganglion, photoreceptor, and glial cells. Barber [19] believed that DR was an ocular neurodegenerative disease. The experimental results show that varying degrees of lesions occurred in almost all retinal nerve tissues in the 24th week of the DR animal model, manifesting as changes in the number and morphology of mitochondria of ganglion, photoreceptor, bipolar, and glial cells, with more heterochromatin in the nucleus, but fewer synaptic vesicles in the axon. Bipolar, photoreceptor, and Müller glial cells exhibited the most significant changes [20, 21]. The nuclei of some bipolar and photoreceptor cells exhibited early typical changes, such as chromatin condensed in the margin, shrunken nuclear membrane shrunken, and fragmented nucleus. Müller glial cells appeared with reactive hyperplasia. Müller glial cells are among the most important glial cells in retinal nerve tissues that provide nutrition and support [22]. Reactive hyperplasia may be caused by degeneration and apoptosis of peripheral neurons, especially bipolar cells. In the present experiment, ganglion cells exhibited no obvious pathological changes, but the number of the cells was reduced, probably due to the earlier changes in the ganglion cells. Apoptosis and necrosis of cells with more serious lesions occurred at an early stage. However, the remaining cells suffered mild diseases. Martin et al [18] reported that in modeling of mice with DM (C57BL/6 mice) induced by 75 mg/kg STZ, the blood sugar was between 320 and 460 mg/dl; they observed necrosis of ganglion cells in the 4th week and reported that ganglion

cells reduced by 20%-25% in the 14th week. Barber et al [19]. found that rats with DM SD induced by 65 mg/kg STZ had 242-334 mg/dl blood sugar, and ganglion cells reduced by 10% in the 30th week. Rats with SD in the present experiment received a 65 mg/kg dose of STZ and exhibited 400 mg/dl blood sugar. Hence, elevated blood sugar can cause considerable ganglion cell loss. The present study found that rats with extensive lesions in retinal nerve tissues manifested only individual microvascular pericytes with early pathological changes. However, neither the vascular endothelial cells nor basement membrane exhibited discernible lesions. The occurrence of DR likely started with pathological changes in nervous tissues.

LBP was extracted from Chinese wolfberry from Ningxia [17]. Pharmacological studies found that LBP can remove excess free radicals [23] and improve the activity of antioxidant enzymes [24, 25], increasing the antioxidant effect. The present experiment found insignificant differences in the blood sugars of the group treated with LBP and the group with DM. In addition, insignificant differences in body weight were found between the groups, indicating that LBP did not reduce blood sugar or control DM. The results were consistent with the findings of Liu Ping et al [26], who measured a blood sugar level of 13.43 ± 1.36 mmol/L in the successful model after administering low-dose 35 mg/kg STZ combined with high-fat and high-glucose diets. In the model with high-dose 65 mg/kg STZ alone, which was used in the present study, the blood sugar in successful animals was over 20 mmol/L, ruling out the possibility that LBP can directly cure DM by reducing blood sugar and controlling DM. In the present study, no pathological changes were found in ganglion cells or photoreceptor cells in the group treated with LBP, whereas very few and mild mitochondrial changes were visible in the cytoplasm of bipolar cells and Müller glial cells, such as shorter and reduced mitochondrial cristae. The SOD activity in the retina was remarkably increased, whereas MDA expression level decreased. This finding indicated that LBP and DFU can alleviate DM-induced retinal neuropathy by exerting an antioxidant effect on DM-induced oxidative damages to mitochondria in nervous tissues. The reduced VEGF demonstrated that we can inhibit the progress of retinal neuropathy and pathological changes to vascular tissue by antioxidant treatment.

In summary, the early retinopathy of DM mainly occurs in neurons and glial cells and manifests as changes in the number and morphology of mitochondria and cell apoptosis, as well as few mild vascular lesions. This result indicates that DM can trigger the occurrence of DR, as the mitochondrial pathway induces the apoptosis of nerve cells. LBP has an antioxidant effect, which can significantly reduce pathological changes in mitochondria, block the apoptosis of nerve cells, and prevent the progress of lesions toward vascular changes. LBP has potential for the prevention and treatment of retinal neuropathy in early DM.

Disclosure of conflict of interest

None.

Address correspondence to: Li Ding, Department of Endocrinology, The People's Hospital of Lishui, No. 15 Dazhong Road, Lishui 323000, Zhejiang Province, PR China. Tel: +86-0578-2780116; E-mail: dingli_lishui@163.com

References

- [1] Pusparajah P, Lee LH and Abdul Kadir K. Molecular markers of diabetic retinopathy: potential screening tool of the future? *Front Physiol* 2016; 7: 200.
- [2] Lee JH and Song SJ. Current challenges in diabetic retinopathy: are we really doing better? *Endocrinol Metab (Seoul)* 2016; 31: 254-257.
- [3] Ting DS, Cheung GC and Wong TY. Diabetic retinopathy: global prevalence, major risk factors, screening practices and public health challenges: a review. *Clin Exp Ophthalmol* 2016; 44: 260-277.
- [4] Klein R, Klein BE, Moss SE and Cruickshanks KJ. The wisconsin epidemiologic study of diabetic retinopathy: XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in type 1 diabetes. *Ophthalmology* 1998; 105: 1801-1815.
- [5] Cai X and McGinnis JF. Diabetic retinopathy: animal models, therapies, and perspectives. *J Diabetes Res* 2016; 2016: 3789217.
- [6] Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I and Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; 404: 787-790.
- [7] Qi B, Ji Q, Wen Y, Liu L, Guo X, Hou G, Wang G and Zhong J. Lycium barbarum polysaccharides protect human lens epithelial cells against oxidative stress-induced apoptosis and senescence. *PLoS One* 2014; 9: e110275.
- [8] Yang M, Ding J, Zhou X, Zhang X, Tao H, Wang Y and Li G. Effects of lycium barbarum polysaccharides on neuropeptide Y and heat-shock protein 70 expression in rats exposed to heat. *Biomed Rep* 2014; 2: 687-692.
- [9] Huyan T, Li Q, Yang H, Jin ML, Zhang MJ, Ye LJ, Li J, Huang QS and Yin DC. Protective effect of polysaccharides on simulated microgravity-induced functional inhibition of human NK cells. *Carbohydr Polym* 2014; 101: 819-827.
- [10] Zhao R, Li QW, Li J and Zhang T. Protective effect of Lycium barbarum polysaccharide 4 on kidneys in streptozotocin-induced diabetic rats. *Can J Physiol Pharmacol* 2009; 87: 711-719.
- [11] Saint-Geniez M, Maharaj AS, Walshe TE, Tucker BA, Sekiyama E, Kurihara T, Darland DC, Young MJ and D'Amore PA. Endogenous VEGF is required for visual function: evidence for a survival role on muller cells and photoreceptors. *PLoS One* 2008; 3: e3554.
- [12] Matsumoto T, Ishida K, Nakayama N, Kobayashi T and Kamata K. Involvement of NO and MEK/ERK pathway in enhancement of endothelin-1-induced mesenteric artery contraction in later-stage type 2 diabetic Goto-Kakizaki rat. *Am J Physiol Heart Circ Physiol* 2009; 296: H1388-1397.
- [13] Wu Y, Feng B, Chen S, Zuo Y and Chakrabarti S. Glucose-induced endothelin-1 expression is regulated by ERK5 in the endothelial cells and retina of diabetic rats. *Can J Physiol Pharmacol* 2010; 88: 607-615.
- [14] Lin BQ, Zhou JY, Ma Y, Deng YJ, Zheng CJ and Lin JL. Preventive effect of danhong huayu koufuye on diabetic retinopathy in rats. *Int J Ophthalmol* 2011; 4: 599-604.
- [15] Agrawal SS, Naqvi S, Gupta SK and Srivastava S. Prevention and management of diabetic retinopathy in STZ diabetic rats by *Tinospora cordifolia* and its molecular mechanisms. *Food Chem Toxicol* 2012; 50: 3126-3132.
- [16] Lee R, Wong TY and Sabanayagam C. Epidemiology of diabetic retinopathy, diabetic macular edema and related vision loss. *Eye Vis (Lond)* 2015; 2: 17.
- [17] Jin M, Huang Q, Zhao K and Shang P. Biological activities and potential health benefit effects of polysaccharides isolated from *Lycium barbarum* L. *Int J Biol Macromol* 2013; 54: 16-23.
- [18] Martin PM, Roon P, Van Eils TK, Ganapathy V and Smith SB. Death of retinal neurons in streptozotocin-induced diabetic mice. *Invest Ophthalmol Vis Sci* 2004; 45: 3330-3336.
- [19] Barber AJ. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. *Prog Neuropsychopharmacol Biol Psychiatry* 2003; 27: 283-290.
- [20] Curtis TM, Hamilton R, Yong PH, McVicar CM, Berner A, Pringle R, Uchida K, Nagai R, Brock-

Lycium barbarum polysaccharides reduce oxidative damage

- bank S and Stitt AW. Muller glial dysfunction during diabetic retinopathy in rats is linked to accumulation of advanced glycation end-products and advanced lipoxidation end-products. *Diabetologia* 2011; 54: 690-698.
- [21] Mizutani M, Gerhardinger C and Lorenzi M. Muller cell changes in human diabetic retinopathy. *Diabetes* 1998; 47: 445-449.
- [22] Schellini SA, Gregorio EA, Spadella CT, Machado JL and de-Moraes-Silva MA. Muller cells and diabetic retinopathy. *Braz J Med Biol Res* 1995; 28: 977-980.
- [23] Liu Q, Li Y, Hu L and Wang D. Lycium barbarum polysaccharides attenuate cisplatin-induced hair cell loss in rat cochlear organotypic cultures. *Int J Mol Sci* 2011; 12: 8982-8992.
- [24] Shan X, Zhou J, Ma T and Chai Q. Lycium barbarum polysaccharides reduce exercise-induced oxidative stress. *Int J Mol Sci* 2011; 12: 1081-1088.
- [25] Cheng D and Kong H. The effect of Lycium barbarum polysaccharide on alcohol-induced oxidative stress in rats. *Molecules* 2011; 16: 2542-2550.
- [26] Liu Ping HL. Effect of Lycium barbarum polysaccharide on glucose and lipid metabolism in diabetic rats. *Journal of Ningxia Medical University* 2008; 30: 427-428.