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Short Communication

Neuroprotective effects of *Lycium barbarum* Lynn on protecting retinal ganglion cells in an ocular hypertension model of glaucoma

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Abstract

Glaucoma is one of the major neurological disorders in eye leading to irreversible blindness in elderly. Increase in intraocular pressure (IOP) has been considered to be the major risk factor for the progressive loss of retinal ganglion cells (RGCs) in retina. While attenuation of IOP has been a major pharmaceutical target, reduction of IOP cannot prevent progressive loss of RGCs. In this regard, urgent need for alternative treatment has to be investigated. Anti-aging medicinal herb *Lycium barbarum* L. has been used for centuries in Eastern World to protect the eyes and maintain good health. Using an ocular hypertension (OH) model in rat by laser photocoagulation of episcleral and limbal veins, we attempted to investigate whether *L. barbarum* can promote RGCs survival against elevated IOP. Oral administration of *L. barbarum* in Sprague-Dawley rats (250–280 g) significantly reduced the loss of RGCs, although elevated IOP was not significantly altered. Rats fed with the 1 mg/kg extract could nearly totally escape from pressure-induced loss of RGCs. In conclusion, this is the first in vivo report showing the therapeutic function of *L. barbarum* against neurodegeneration in the retina of rat OH model. The results demonstrate that this extract may be a potential candidate for the development of neuroprotective drug against the loss of RGCs in glaucoma.
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Glaucoma is widely recognized as a neurodegenerative disease, characterized by optic nerve atrophy and progressive loss of vision from the periphery towards the central vision field (Quigley et al., 1981; Laquis et al., 1998; Gazzard et al., 2002; Foster et al., 2002). Death of retinal ganglion cells (RGCs) can attribute to visual loss in glaucoma patients (Flammer et al., 2002; Harwerth et al., 2002). Elevation of the intraocular pressure (IOP) has been considered to be a major pathological factor leading to the death of RGCs in glaucoma (Goldblum and Mittag, 2002; Wax and Tezel, 2002; Wein and levin, 2002). As a general practice, treatments of glaucoma usually focus on reducing the IOP. However, inhibition on the degeneration of RGCs can be accomplished to a certain extent only. Therefore, effort has been made to find out other neurotoxic factors in glaucoma. Glutamate released from the injured or dead neurons and nitric oxide (NO) (Neufeld et al., 1999; Haefliger et al., 1999) triggered by glutamate excitotoxicity threatening the survival of neighboring neurons have been reported to induce progressive degeneration of RGCs. Knowing that the extracts of anti-aging herbs, *Lycium barbarum*, exhibit cytoprotective effects on many cell types including neurons (Wang et al., 2002; Luo et al., 2004; Yu et al., 2005, 2006), we aim to investigate whether this kind of extract can protect RGCs in an animal experimental model of ocular hypertension.

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L. barbarum has long been known to improve eyesight in Chinese pharmacopoeia for centuries (Lam and But, 1999; Sommerburg et al., 1999; Leung et al., 2001). The lutein and zeaxanthin components of *L. barbarum* have been shown to restore visual functions in experimental light-induced phototoxicity and macular degeneration, probably by protecting RGCs from glutamate- and nitric oxide (NO)-induced neuronal apoptosis in the retina (Lam and But, 1999; Sommerburg et al., 1999; Leung et al., 2001). However, the polysaccharide from *L. barbarum* on the nervous system has not been systematically characterized although the content of polysaccharide in *L. barbarum* is more than 40%. While neuroprotective effects of the polysaccharide in neuronal cultures have been demonstrated (Yu et al., 2005, 2006), it is doubtful whether the polysaccharide can cross through blood–brain barrier or blood–retina barrier to elicit neuroprotection. Therefore, experiments in animal model of glaucoma were performed to investigate its neuroprotective effects.

Adult female Sprague-Dawley rats (10–12 weeks with weights 250–280 g) were housed in temperature-controlled animal room subjected to a 12-h light/12-h dark cycle and provided with sufficient food and water supply. Before surgery, animals were anesthetized with intraperitoneal injection of 80 mg/kg ketamine and 8 mg/kg xylazine. Proparacaine hydrochloride (Alcon 0.5% Alcaine®) was applied topic anaesthetics. After the surgery, ophthalmic ointment (Alcon Tobrex® tobramycin) was pasted onto the cornea to prevent inflammation. Each group had five to seven rats for the experiments. All the experimental protocols and procedures for animals have followed the tenets of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Aqueous extract of dried fruits of *L. barbarum* Lynn (purchased from Ningxia, China) was prepared by decoloration and de-lipidation in alcohol and boiling in distilled water. The extracts were then freeze-dried as powder for storage. The polysaccharide concentration of the *L. barbarum* extract was found to be 72% (Yu et al., 2005). For experiments, the extract was dissolved in phosphate-buffered saline (PBS). Animals were fed daily with 1 ml of either PBS or different dosages of crude extract of *L. barbarum* (LBP), including 0.01, 0.1, 1, 10, 100 and 1000 mg/kg, for a total of 21 days or 35 days. We arbitrary chose 1 week for the experiment as Chinese usually drinks the soup containing *L. barbarum* for several days. Nevertheless, we have also investigated a long-term effect of *L. barbarum*.

In the rat OH model, which simulates glaucoma, an increase in IOP was achieved by argon laser photocoagulation. This technique was adopted from the method developed by WoldeMussie et al. (2001) and have been adopted in our laboratory with track record of publications (Siu et al., 2002; Ji et al., 2004; Chan et al., 2005). Accordingly, the right eyes of the animals were photocoagulated while leaving the left eyes unoperated as a control. Argon laser photocoagulation (120–140 laser spots; power, 1 W; spot size, 50–100 μm; duration, 0.1 s) was applied to the three episcleral veins (two superior and one inferior at the temporal area) and the limbal veins in a 270° arc around perilimbal region (excluding the nasal quadrants) with a Streit® operation microscope. After 1 week, a second laser photocoagulation (80–120 laser spots with same power) was applied to block any neovascular reconnection. To monitor the condition of ocular hypertension, the animals were anesthetized for IOP measurement by using Tonopen XL tonometer (Mentor®, Norwell, USA). Using the argon laser photocoagulation model, we were able to keep the IOP elevation at about 1.5 times of the normal baseline of SD rats (Ji et al., 2004). There was no need to incise the conjunctiva and tenous capsule and the elevated IOP was comparable to the episcleral venous cautery model (Garcia-Valenzuela et al., 1995) which induced 1.5–1.8 times increase in IOP. In addition, the consistency of IOP elevation among ocular hypertensive eyes was as high as about 5 mm Hg difference. However, increase in IOP in hypertonic saline model was about 7 to 28 mm Hg (Morrison et al., 1997).

Four days before euthanasia, 2 μl of 6% Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) soaked in Gelfoam (Upjohn, Kalamazoo, MI, USA) was applied to the superior colliculus (SC) of each hemisphere, according to our published reports (Siu et al., 2002; Ji et al., 2004). After completion of labeling, the skins were sutured. Thus, dyes absorbed by RGCs via retrograde transport provided a marker for the viable cells.

Animals were kept alive for 2–4 weeks after the laser photocoagulation. The eyes were enucleated and fixed with 4% freshly made paraformaldehyde (in 0.1 M PBS, pH 7.4) for 60 min. The retina was then isolated and mounted onto gelatin-coated glass slide with Vectashield mounting media (Vector Laboratories, CA). FG-labeled RGCs were counted with a fluorescent microscope at 400× magnification. Labeled RGCs in seven areas ($200 \times 200\ \mu m^2$) with each 500-μm separation were counted (Fig. 1A). The total number of RGCs in these predefined seven areas represented approximately 3.0 to 3.8% of the total retinal area. Total number of survived RGCs in these seven areas in the OH eye was compared to that in the contralateral eye as control. This method had been reported in our previous publications (Siu et al., 2002; Ji et al., 2004). The results were expressed as percentage of RGC loss (%control, mean ± standard error of the mean (SEM)). The cell counting was done in a double-blinded manner.

The change of RGC density was expressed as percentage of RGC loss (percent as control, mean ± standard error of the mean (SEM)) by using one-way ANOVA for multiple comparison and Tukey test as post hoc test for determination of significance. To improve the confidence, $p < 0.001$ was regarded as significant different.

In the present study, an OH model in rat was utilized to induce ocular hypertension. Throughout this experiment, IOPs were measured before and after laser photocoagulation (3 days after each laser). The validity of the measurements was controlled by assessing the control eyes. Before the laser operation, the basal IOP of both eyes was 14.6 ± 0.4 mm Hg (Table 1). IOP was increased after laser photocoagulation, and the increase in IOP was retained for 2 weeks. Rats fed with the extracts from *L. barbarum* did not have a significant change in IOP and the pressure of the eyes was noted from 21.2 ± 0.2 mm Hg to

23.6 ± 0.6 mm Hg (Table 1), which was similar to that in the group of rats without feeding with extracts.

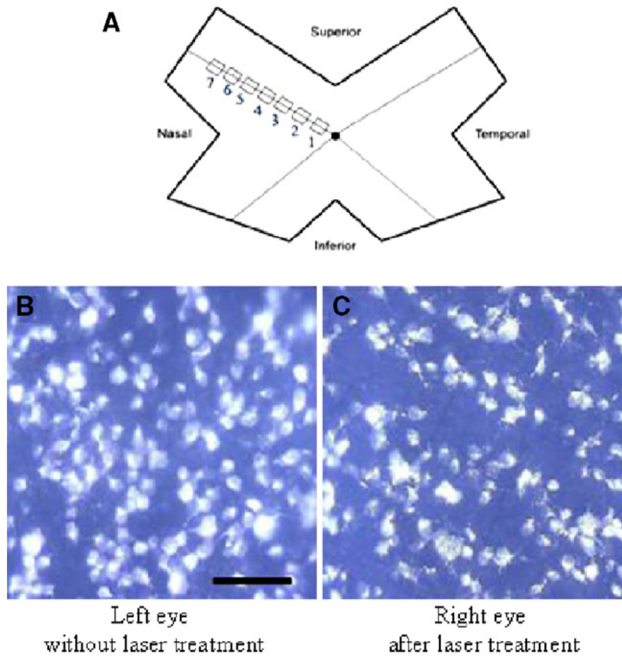
To assess the survival of RGCs after ocular hypertension, the total number of survived RGCs at the predefined areas in the eye with OH treatment was compared with that in control eye. After 2 weeks of laser photocoagulation, the percentage loss of RGCs was 17.0 ± 1.1% in the eye with OH treatment compared to the eye without OH.

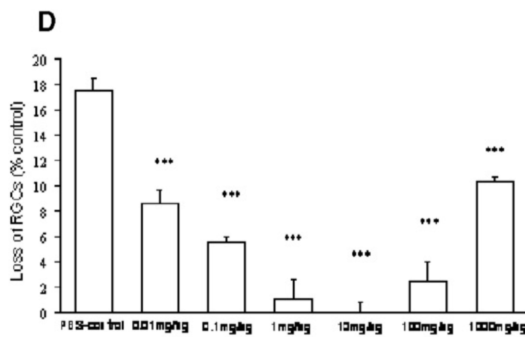
To determine whether *L. barbarum* displayed protective effects on RGCs against elevated IOP, we fed rats daily with different dosages of extract starting from 7 days prior to induction of ocular hypertension. The IOP level of the animals administrated with the extract was maintained at 21.2 ± 0.2 mm Hg to 23.6 ± 0.6 mm Hg after the laser operation, and this result was as high as that of the control. While there was no reduction of increased IOP, rats fed with the extract showed a significant reduction of RGC loss in the eye with OH treatment (Fig. 1D). The level of RGC loss was nearly undetectable in animals fed with 10 mg/kg extract (0.0 ± 0.9%, $n=7$, $p<0.001$). The dose–response curve for the neuroprotective effects of *L. barbarum* on RGC appeared as a U-shaped curve. There was no significant difference in RGC loss between the groups fed with 1 mg/kg (1.0 ± 1.6%, $n=5$, $p<0.001$) or 100 mg/kg (2.4 ± 1.7%, $n=6$, $p<0.001$). Rats fed with 0.01 mg/kg (8.6 ± 1.1%, $n=4$), 0.1 mg/kg (5.5 ± 0.6%, $n=4$) or with 1000 mg/kg (10.3 ± 0.5%, $n=4$) of the extracts from *L. barbarum* prevented nearly 50% RGC death (Fig. 1D).

Table 1
Increase in intraocular pressure (IOP) and percentage loss of retinal ganglion cell (RGC) loss in this laser photocoagulation model

	0 day	3 days	7 days	14 days	28 days
Right eye (IOP, mm Hg)					
PBS group ($n=7$)	14.6 ± 0.4	22.6 ± 1.1	24.6 ± 0.7	24.5 ± 0.7	22.1 ± 1.1
LBP group (1 mg/kg, $n=7$)	13.9 ± 0.6	18.2 ± 2.7	21.2 ± 0.2	23.6 ± 0.6	21.9 ± 0.9
Left eye (IOP, mm Hg)					
PBS group ($n=7$)	13.9 ± 1.7	13.0 ± 0.5	14.1 ± 0.3	12.2 ± 1.0	13.4 ± 0.7
LBP group (1 mg/kg, $n=7$)	13.7 ± 0.3	13.9 ± 0.8	12.7 ± 0.4	13.2 ± 0.2	14.1 ± 0.4
Percentage of RGC loss (%)					
PBS group ($n=6$)	–	4.1 ± 0.6	9.2 ± 0.4	14.5 ± 0.9	21.1 ± 1.6
LBP group (1 mg/kg, $n=5$)	–	0.5 ± 1.3	0.5 ± 1.1	1.0 ± 1.6	6.0 ± 1.9

The table describes an increase in IOP (mm Hg) in this OH model and loss of RGC (%). We use 1 mg/kg of LBP as an example of the treatment group for illustration.





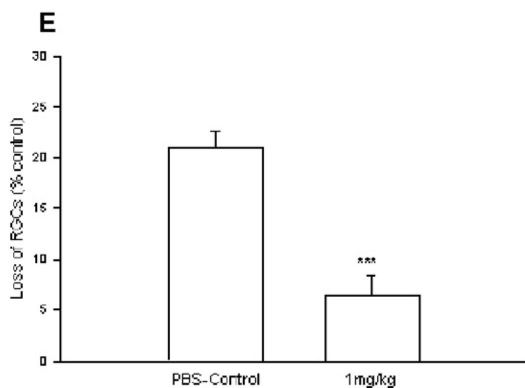


Fig. 1. Schematic diagram of counting Fluoro-gold labeled RGCs and percentage of RGC loss in animals daily fed with the extract of *Lycium barbarum*. (A) For quantification of RGCs, the FG-labeled RGCs were counted using a fluorescent microscope with UV-2 filter. Seven predefined areas (200 × 200 μm^2/microscope field) with a separation of 500 μm along each quadrant from central retina were studied in each retina (B and C). Photomicrographs of FG-labeled RGCs are shown in left eye without laser treatment (B) and right eye with laser treatment (C), scale bar, 50 μm. For the experiment using *L. barbarum*, all animals were fed daily with different treatments 7 days before first laser photocoagulation until sacrifice. Animals were allowed to survival for 2 weeks (D) and 4 weeks (E) after first laser photocoagulation. (D) Significant reduction of RGC loss was found in animals fed with a range of doses of *L. barbarum* from 0.01 to 1000 mg/kg (***$p<0.001$) 2 weeks after laser photocoagulation. With the dosage administrated with 1 mg/kg extract of *L. barbarum*, no RGC damaged was counted. (E) Significant reduction of cell loss was found in groups treated orally with 1 mg/kg for 4 weeks after laser photocoagulation (***$p<0.001$).

To investigate whether *L. barbarum* has long-term protective effects on RGCs, we examined the loss of RGCs on rats 4 weeks after laser treatment. Animals were fed in a similar way as mentioned above but were euthanized 4 weeks after first laser photocoagulation. Similar to the above findings, the increased IOP was not significantly altered throughout the experiment. After first laser photocoagulation, the percentage of cell loss in control group (21.1±1.6%) was significantly higher than that of 2 weeks. Rats fed with 1 mg/kg of extract had a significant reduction of RGC loss in eye with OH treatment (6.6±1.9%; $p<0.001$) (Fig. 1E). The results showed that neuroprotective effects of *L. barbarum* could be maintained to 4 weeks. Similar to what had been observed previously, neuroprotective effects were not achieved by reduction of elevated IOP (Table 1).

This is the first in vivo study demonstrating a possible neuroprotective effect of *L. barbarum* on RGC in retina against ocular hypertension. *L. barbarum*, as an anti-aging oriental herbal medicine, has long been used in Eastern world to nourish different organs and maintain the state of healthy body. However, its effects in the nervous system such as eyes or the brain are not clearly defined. Lutein and zeaxanthin in *L. barbarum* have been reported to protect photoreceptors (Lam and But, 1999; Sommerburg et al., 1999; Leung et al., 2001). These two ingredients are usually extracted using organic solvents. However, the dried fruits of *L. barbarum* used by us have been soaped with ethanol, and substances dissolved in the organic phase have subsequently been removed. Therefore, it is unlikely that our extract contains these two anti-oxidative small molecules. There is one report showing the possible interaction between warfarin and *L. barbarum* resulting in increased anti-coagulant effect (Lam et al., 2001). However, it is still unclear whether our extract exhibits this type of effects. While the beneficial effect of *L. barbarum* is noted in this report, we should still be caution of any possible unknown side effects.

Regarding the potential mechanisms, direct neuroprotective effects of *L. barbarum* on neurons cannot be neglected. We have recently reported that *L. barbarum* provides neuroprotection against β-amyloid peptide neurotoxicity (Yu et al., 2005, 2006). Inhibition of stress kinase such as c-Jun N-terminal kinase (JNK) by the extract has been demonstrated (Yu et al., 2005). Indeed, inhibition of JNK to elicit neuroprotection has also been reported in another herbal medicine (Lai et al., 2006). Therefore, it may be possible that the extract acts on RGC to exhibit neuroprotection by attenuating the activation of JNK. This may also explain why the dose-dependent curve appears in U-shaped as too much JNK-specific inhibitor also loss its protective effects (Yu et al., 2005). It has been questioned of whether neuroprotective effects of *L. barbarum* can be demonstrated in animal model of neurodegenerative diseases. The results show that polysaccharide from *L. barbarum* can elicit neuroprotection to eye, suggesting that neural tissue can be benefited by polysaccharide of *L. barbarum* regardless of the blood–brain barrier or blood–retina barrier.

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