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Neuroprotective effect of *Lycium barbarum* on retina of Royal College of Surgeons (RCS) rats

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Abstract

Hereditary retinal dystrophy usually leads to blindness. Using Royal College of Surgeons (RCS) rats as a hereditary retinal dystrophy model, we investigated the possible neuroprotective effects of the aqueous extract of dried Lycium barbarum (LBA). Sixty postnatal RCS rats were selected and randomly divided into a control group (CG, thirty rats) and an experimental group (EG). Ten days after birth, EG rats were treated by 1 mg/kg of LBA per day, and CG rats were normally fed. These rats were killed at postnatal day (P) 25, P35 and P50, and retinal tissue was prepared for analysis. Photoreceptor cells were assessed by hematoxylin and eosin (HE) staining, TUNEL detection and Caspase-2 protein expression. We found that in rats at P25, the outer nuclear layer (ONL) of EG was thicker and more photoreceptor cells survived. Meanwhile, the TUNEL expression in EG was obviously reduced compared with CG. The Caspase-2 positive cells were found in the ganglion cell layer and inner nuclear layer in both CG and EG at 25-50 postnatal days, but the expression in EG rats was significantly lower than in CG at P25. The results demonstrated that LBA might have a neuroprotective role on the retinal tissue of RCS rats at the early stage by protecting photoreceptors and inhibiting apoptosis involving Caspase-2 protein.

Key words: *retinal degeneration, Lycium barbarum, apoptosis, RCS rat.*

Introduction

Hereditary retinal dystrophy is a major cause of inherited blindness worldwide, with an estimated prevalence of 1 : 3,000 [6] ~ 1 : 1,490 [15]. It mostly results from the degenerative deficiency of photoreceptors and/or the retinal pigment epithelium [3,8,14]. The Royal College of Surgeons (RCS) rats were first reported by Bourne *et al.* [4], and have been widely adopted as the animal model for studying the hereditary retinal dystrophy. In the RCS rats, the retinal pigment epithelium cannot phagocytose the outer segment discs that are continually shed from photoreceptors. The resulting accumulation of debris in the subretinal space leads to a progressive loss of photoreceptors [16,21].

Previous studies indicated that apoptosis is the dominant mechanism of photoreceptor degeneration in the RCS rats [18,19]. A possible mechanism for the apoptosis in these animals is oxygen toxicity. Yu *et al.* [22] reported that the intraretinal oxygen distribution in dystrophic RCS rats was clearly affected after P30, reflect-

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ing a loss of photoreceptor oxygen consumption. Similarly, another study found that during the critical period (P16-20) hyperoxia slowed photoreceptor death, whereas hypoxia accelerated death [20].

It has been suggested that lutein and zeaxanthin, which are two common carotenoids found in plants and are constituents of the yellow macular pigment in the human retina, might decrease the risk of age-related macular degeneration [13]. *In vitro* and *in vivo* studies have shown that these carotenoids are also potent antioxidants [10,11,13]. Additionally, Thomson *et al.* [17] also reported that long-term dietary supplementation with zeaxanthin reduced photoreceptor death. This effect is probably achieved by protecting retinal ganglion cells from glutamate-and nitric oxide (NO)-induced neuronal apoptosis in the retina [12].

Barbary wolfberry fruit (Goji berry), which is the dried fruit of *Lycium barbarum* (LBA), has long been used to nourish different organs and maintain the healthy state of body as an anti-aging herbal medicine in the Eastern world. Considering this, some experiments have investigated the therapeutic effect of the LBA on the visual defect. As LBA is one of the richest natural sources of zeaxanthin [2], previous investigations found that daily dietary supplementation with LBA could increase plasma zeaxanthin and antioxidant levels as well as protects from hypopigmentation in the macula of elderly subjects [5]. In addition, oral administration of LBA could significantly reduce the loss of retinal ganglion cells in rats with glaucoma by decreasing the ocular hypertension [7].

In the present experiment, we treated RCS rats with LBA to investigate its possible therapeutic role on the apoptosis of photoreceptor cells. To our knowledge, this is the first preclinical research about the treatment of hereditary retinal dystrophy by means of LBA. The results bring new insights into the application of Chinese medicinal herbs. However, further studies are needed to elucidate the exact constituents of LBA and their interaction with photoreceptor apoptosis mechanisms.

Material and methods

Material

Sixty postnatal RCS rats were used in the current study. These animals were bred in our own animal facilities from colonies originally sourced from LaVail [14]. All procedures were conducted in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research. These RCS rats were randomly and equally divided into two groups, the control group (CG) and the experimental group (EG). Eexperimental group rats were given 1 mg/kg of LBA (from the Department of Anatomy, Hong Kong University) per os by a specially designed tube from the tenth day after birth, while CG rats were fed with the same amount of physical saline. Ten rats in each of CG and EG groups were killed at P25, 35 and 50, respectively.

Methods

The eyes were enucleated quickly after RCS rats were anesthetized by 10% chloral hydrate (400 mg/kg), and then fixed in a 4% buffered formaldehyde solution for 24 h, and dehydrated and embedded in paraffin. Finally, 4 μm sagittal sections through the posterior pole of retina were made. Three sections of each sample were stained by hematoxylin and eosin (HE) for observation by light microscopy. Adjacent sections were stained using the TdT-mediated DNA nick-end bio-dUTP labelling (TUNEL) and Caspase-2 immunohistochemistry as follows.

For TUNNEL staining the sections were deparaffinized and hydrated. Nuclei were stripped of protein by incubating with 20 μg/ml of proteinase K for 15 min. Terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP (Roche Co., USA) in TdT buffer were added on the sections incubating in humid atmosphere at 37°C for 2 h. The reaction was terminated by transferring the slides to PBS (PO_4^{2-} 0.01 M, NaCl 0.15 M, pH 7.4) with three 5 min washes. Finally, sections were incubated in fluorescence conjugated streptavidin (1 : 500) for 30 min at room temperature. The samples are analysed under a fluorescence microscope, with excitation wavelength in the range of 450~500 nm and detection in the range of 515~565 nm (green). Negative controls were processed as above except that incubation with biotinylated dUTP was omitted. Positive controls were digested by DNAase I before DNA nick-end labelling.

For Caspase-2 immunohistochemistry, the sections were deparaffinized and hydrated. Endogenous peroxidase was inactivated by covering the sections with 3% H_2O_2 for 10 min, then rinsed 3 times for 5 min in PBS, and incubated with 10% goat serum in humid atmosphere at 37°C for 20 min. Sections were incubated with primary antibody (1 : 500) overnight at 4°C, which was obtained from Zhong Shan Biotechnology. Sections were then washed with PBS with three 5 min washes, and incubated with biotin-conjugated secondary antibody (anti-rabbit IgG) at 37°C for 30 min. Sections were then washed with 0.01 M of PBS again,

and incubated with HRP-streptavidin for 20 min followed by DAB staining. Corresponding negative controls were prepared by substitution of the primary antibody with 10% normal goat serum in PBS.

The images were captured by an OLYMPUS BX51 (Japan) microscope. The number of photoreceptor cells per 30 μm length as well as number of Caspase2-labelled nuclei and TUNEL-labelled nuclei per 100 μm length was counted. The data were analysed statistically by *t*-test of the mean of two age-matched samples. Probability values of less than 0.05 were determined to be statistically significant.

Results

HE stain

The retina of CG had regular alignment at P25 (Fig. 1A'). Then, the outer nuclear layer (ONL) became thinner and some photoreceptor cells disappeared at P35. The ONL was the thinnest and the number of photoreceptors was the smallest at P50, but the inner nuclear layer (INL) and retinal ganglion cells were hardly altered. Compared with the age-matched CG, ONL of EG was thicker and more photoreceptors survived at P25 (Fig. 1A). However, each layer of the retina was similar to CG at P35 and 50 (Table I).

Caspase-2 immunohistochemistry

The Caspase-2 positive cells were observed in both the ganglion cell layer and inner nuclear layer in CG and EG from P25 to P50. The number of Caspase-2 positive cells was less at P25 in EG than CG ($P < 0.05$) (Table II, Figs. 1B, B').

TUNEL

In RCS rats, the positive labelled nuclei could be determined clearly only in the outer nuclear layer from P25 to P50. The number of the labelled nuclei reached the highest level at P35 and reduced dramatically at

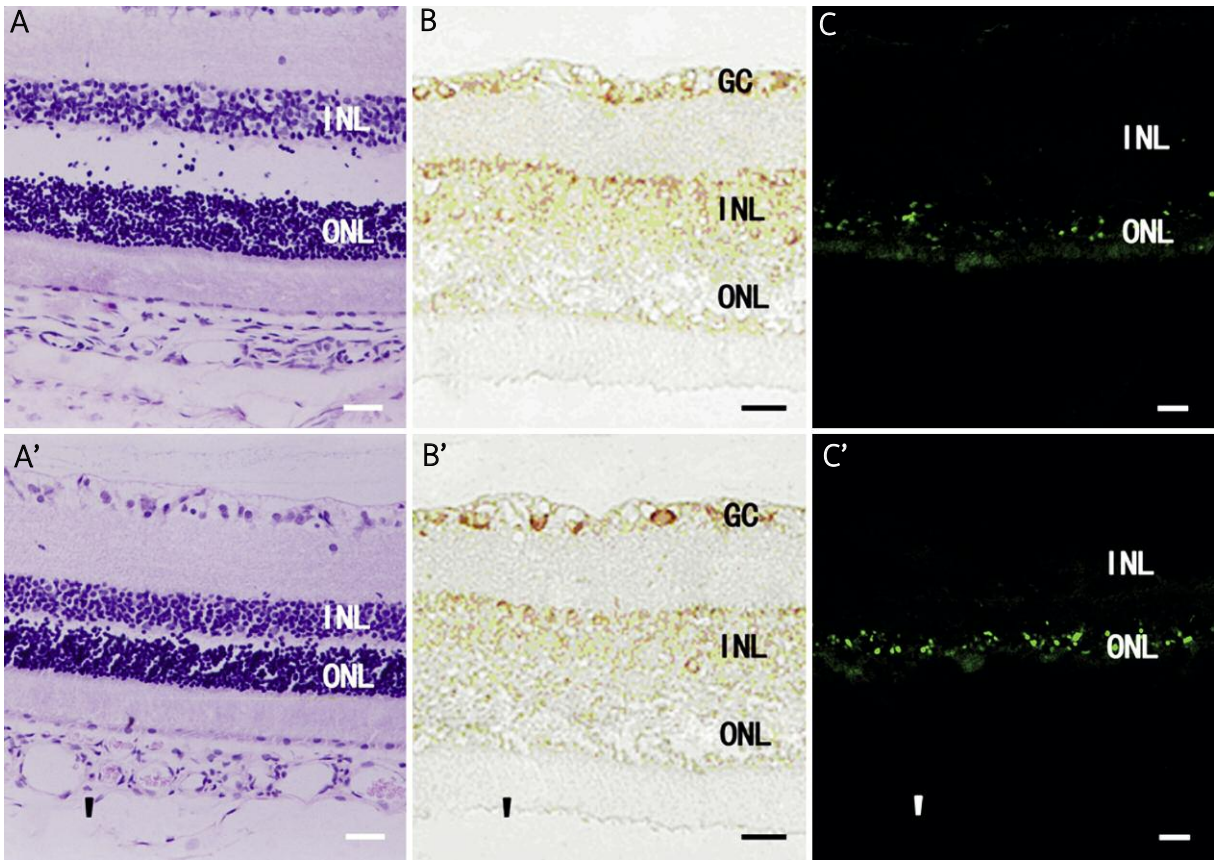

Fig. 1. Microscopic examination of the retina of RCS rats (× 200). **A**, **B**, **C)** HE, immunohistochemistry staining and TUNEL detection of EG at P25, respectively. **A', B', C')** HE, immunohistochemistry staining and TUNEL detection of CG at P25, respectively. GC – ganglion cell layer; INL – inner nuclear layer; ONL – outer nuclear layer

Table I. The number of photoreceptor cell changes per 30 μm length ($\bar{x} \pm s$)

Group	*N*	P25	P35	P50
CG	20	77.00 ± 4.12	54.00 ± 5.87	23.00 ± 6.36
EG	20	94.80 ± 0.84*	56.40 ± 4.56	23.60 ± 4.77

CG – control group, EG – experimental group, N – the number of eyes, P – postnatal day
**Compared with age-matched CG, P < 0.05*

Table II. Number of Caspase2-labelled nuclei per 100 μm length ($\bar{x} \pm s$)

Group	*N*	P25	P35	P50
CG	20	10.60 ± 1.87	30.70 ± 1.02	14.80 ± 1.61
EG	20	7.14 ± 1.86*	29.24 ± 1.72	15.31 ± 1.86

CG – control group, EG – experimental group, N – the number of eyes, P – postnatal day
**Compared with age-matched CG, P < 0.05*

Table III. Number of TUNEL-labelled nuclei per 100 μm length ($\bar{x} \pm s$)

Group	*N*	P25	P35	P50
CG	20	18.17 ± 2.14	57.17 ± 7.41	26.67 ± 4.97
EG	20	12.17 ± 1.72*	53.03 ± 4.62*	25.83 ± 3.72

CG – control group, EG – experimental group, N – the number of eyes, P – postnatal day
**Compared with age-matched CG, P < 0.05*

P50. Compared with age-matched CG, more apoptotic cells in EG were detected at P25 and P35 ($P < 0.05$) (Table III, Figs. 1C, C').

Discussion

Dietary antioxidants, particularly lutein and zeaxanthin, are hypothesized to have the capacity to protect photoreceptor in the retina from oxidative damage and inflammation. However, it is still unknown whether such possibly protective effect is useful for the prevention of the apoptosis of photoreceptor cells in hereditary retinal dystrophy. By means of histological observation and measurement, we found that daily dietary supplementation with LBA could decrease the retinal degeneration of RCS rats at an early stage. However, such protective effect did not prevent the degenerative progress of photoreceptor at later stages of the disease.

It has been proven that the retinal pigment epithelium of RCS rats have defects in phagocytising the outer segment disks of photoreceptor cells, which is believed to be the primary cause of the retinal degeneration of RCS rats [21]. In addition, degeneration of retinal photoreceptor cells was the major pathologic factor in the retinal degeneration of RCS rats [14]. Consistent with these findings, our HE staining result showed that ONL was thinner and the number of photoreceptors was smaller in the retinas of the CG than in those of the age-matched EG at P25. Outer nuclear layer became the thinnest and the photoreceptors were hardly to survive by P50, but the INL and retinal ganglion cells were not altered significantly. Our findings verified that the accumulation of the outer segment disks might be the primary cause of the retinal photoreceptor cells degeneration. The outer segment disks accumulated continuously so that the width of ONL at P35 was smaller than that at P25, and almost all the retinal photoreceptor cells of the RCS rats disappeared by P50 in CG.

Caspases play critical roles in initiation and execution of apoptosis [1] and Caspase-2 is a direct effector of the mitochondrial apoptotic pathway [9]. In the current experiment, the Caspase-2 positive cells were seen in the ganglion cell layer and INL in the CG and EG from P25 to P50. The number of positive cells was significantly lower at P25 in the EG than in the CG, which was consistent with the TUNEL result, indicating that Caspase-2 protein may play an important role in the retinal degeneration and LBA might protect apoptotic neurons through inhibiting the expression of Caspase-2.

By TUNEL staining, the positive labelled nuclei could be determined clearly only in the outer nuclear layer from P25 to P50. The number of the labelled nuclei increased starting from P25, reached the highest level at P35 and reduced markedly at P50. In addition, the number of positive cells in the EG was obviously lower than in the CG at P25 and P35. Combined with the Caspase-2 immunohistochemistry staining, these results suggested that LBA might make more photoreceptors survive, and/or might preserve degenerated neurons at an early stage by inhibiting apoptosis. At the same time, we observed that LBA had almost no effect on the photoreceptors after P25; it might result from photoreceptor apoptosis at P25, and reached the peak of apoptosis so that the retina released the condition of hypoxia.

Since retinal dystrophy was first reported more than 150 years before, extensive investigations have been performed on this disease, but no effective treatment for the disease has been found. Currently, there are three primary methods to treat hereditary retinal dystrophy: gene therapy, photoreceptor cell transplantation and medicine intervention. The application of gene therapy and photoreceptor cell transplantation is limited because of technical issues, and the medicine intervention has great potential by influencing the process of the retinitis pigmentosa. This investigation demonstrated that LBA has a neuroprotective effect on the early stage of retinal degeneration of the RCS rats. Nevertheless, further investigation is still needed for practical clinical usage. Moreover, the mechanism by which LBA exerts its direct effect on the retina remains to be elucidated.

Conclusions

The results suggest that LBA has a neuroprotective effect on retinal degeneration of RCS rats at the early stage, resulting in preservation of more photoreceptors; it might preserve degenerated neurons in the early stage by inhibiting apoptosis because of the light absorbing effect of zeaxanthin and lutein present in LBA. Caspase-2 protein may contribute to the retinal degeneration and LBA might protect apoptotic neurons by inhibiting the expression of Caspase-2. However, the mechanism of action is unclear, given the lack of relationship between the complex constituents in LBA and degenerative mechanism for the photoreceptor cells in RCS rats.

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