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Original article

Neuroprotective effect of *Erigeron Breviscapus (vant) Hand-mazz* extract on retinal ganglion cells in rabbits with chronic elevated intraocular pressure

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Background: Retinal ganglion cells (RGCs) are protected in rats with acute elevated intraocular pressure (IOP) by Erigeron breviscapus (vant.) hand-mazz (EBHM). However, it is unclear whether EBHM has neuroprotective effect on RGCs in animal with chronic elevated IOP.
Objective: Investigate the protective effect of EBHM extract on RGCs in rabbits with chronic elevated IOP.
Methods: Unilateral chronic elevated IOP was produced in rabbits by repeated injection of 2% methylcellulose into the anterior chamber. Secondary degeneration was measured with and without EBHM extract treatment for 60 days. At 60 days, the cells density of the RGCs layer, the thickness of retinal nerve fiber layer (RNFL), and the optic nerve axons were observed and analyzed using an image analysis system. The ultrastructural changes of RGCs and optic nerve axons were observed using transmission electron microscopy.
Results: Compared with their contralateral control eyes with normal IOP, in the retinas of 3-4 mm from the optic disc, the cells density of the RGCs layer in the eyes with chronic elevated IOP was 23.2±6.5 cells (n = 6) and 36.0±8.9 cells (n = 10) per three 400x fields at 60 days in untreated and EBHM-treated group, respectively. The RNFL thickness in eyes with chronic elevated IOP was 3.4±0.4 μm (n = 6) and 5.0±1.0 μm (n = 10) at 60 days in untreated and EBHM-treated group, respectively. The axons number per 15057.8 μm^2 in eyes with chronic elevated IOP was 370.4±41.0 (n = 6) and 439.0±50.8 (n = 10) at 60 days in untreated and EBHM-treated group, respectively. The number of the organelles in RGCs plasm appeared decreased and mitochondrion vacuolated in the elevated IOP eyes of EBHM-treated group, while some dispersive mitochondrion and rough surfaced endoplasmic reticulum and ribosome still existed in the RGCs plasm. The myelin sheath plates condensed and degenerated, and the microfilaments and microtubules decreased or disappeared in the elevated IOP eyes, but the axons degeneration in the chronic elevated IOP with EBHM treatment was less than that in the chronic elevated IOP without treatment.
Conclusion: EBHM extract provided a neuroprotective effect on retinal ganglion cells in rabbits with chronic elevated IOP.

Keywords: Chronic elevated IOP, Erigeron Breviscapus (Vant.) Hand-Mazz extract, neuroprotective effect, rabbit, retinal ganglion cells

Glaucoma is a progressive optic neuropathy involving characteristic structural pathological changes in the optic nerve head [1-3]. Reducing intraocular pressure (IOP) is the most practiced therapeutical approach of glaucoma patients [4-6]. However, progressive loss of visual field or blindness may still occur in some glaucoma patients with controlled IOP [7]. This disease has its major detrimental effect upon the eye by killing retinal ganglion cells (RGCs). Therefore, neuroprotection-based therapies to protect

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neurons are the most important for clinical management.

Erigeron breviscapus (vant.) hand-mazz (EBHM) (Deng-zhan-hua in Chinese) is a well-known traditional Chinese medicinal plant for heart disease [8]. Previous pharmacological studies demonstrated that EBHM could stimulate the fibrinolysis and anticoagulation of endothelial cells, dilate the blood vessels, reduce vascular resistance, increase blood flow, improve microcirculation, and inhibit thrombosis [9, 10]. According to a multi-center clinical trial by Wang et al. [11] and a retrospective study by Jiang [12], EBHM could improve the visual field in glaucoma patients with controlled IOP. In previous experimental studies using rats [13, 14], we showed that EBHM could improve the activity of cytochrome oxidase in RGCs and optic nerve axoplasmic transport of rat models with acute elevated IOP.

Although those experimental studies were based on the acute elevated IOP models of rats, most glaucoma patients in clinics show chronic progressive optic neuropathy process. Thus, it is most important to conduct further study using chronic elevated IOP model to confirm the neuroprotective effect of EBHM. In this study, we investigated the neuroprotective effect of EBHM on RGCs in rabbits with chronic elevated IOP.

Materials and methods

Rabbit model of chronic elevated IOP

Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Three months old male New Zealand albino rabbits, weighing approximately 2.5 kg - 3.0 kg, were obtained from the Experimental Animal Center of Medical College, Shanghai Jiaotong University. Animals were fed and maintained in temperature-controlled rooms. Animals were weighed weekly to monitor their general health.

Unilateral chronic moderately elevated IOP was produced in 24 rabbits by injection of 2% methylcellulose into the anterior chamber according to the method by Manni et al. [15] and Zhu et al. [16]. Briefly, we measured the IOP using Goldmann applanation tonometer (Berne, Swiss) with one drop of 0.5% proparacaine hydrochloride applied to each eye. The baseline IOP of each rabbit was measured three times within a week before surgery. The eye was topically anesthetized three times using 0.5% proparacaine hydrochloride with five minutes interval. The anterior chamber puncture was performed and 2% methylcellulose (Sigma, St. Louis, MO, USA) (dissolved in saline) was injected. Thirty minutes later, the IOP was measured. If the IOP <25mmHg, the additional 2% methylcellulose was injected until the IOP ≥25mmHg. In the contralateral eye, the same anterior chamber puncture and aqueous humor aspiration were performed, and the same dose of saline was injected into anterior chamber.

The eyes were treated with antibiotic ointment and levofloxacin for a few days after surgery to avoid infection. The IOPs of both eyes were measured for each two days. If a repeated injection of methylcellulose was needed in the model eye, a similar repeat injection of saline was performed in the control eye.

EBHM extract treatment

EBHM extract was provided by Hunan Xiangya Pharmaceutical Co. Changsha, China). After the first 2% methylcellulose anterior chamber injection, the rabbits were divided randomly into two groups. One group of 14 rabbits was treated with EBHM extract, while the other group of 10 rabbits was untreated. In EBHM-treated group, 200 mg/kg per day EBHM extract (mixed with saline) was irrigated into the stomachs of each rabbit, and the treatment period last 60 days. The control group was not treated, but received the same dose saline stomach irrigation every day, for 60 days.

Rabbit tissue collection

All animals were anesthetized with 2.5% pentobarbital sodium (30mg/kg body weight) before tissue collection. Both eyes were quickly enucleated and chilled in iced phosphate-buffered saline. Optic nerve segments 1 mm from the back of the globe were dissected, and eyes were dissected as eyecups without cornea and lens. For histological analysis, optic nerve segments and eye cups were immediately fixed for 48 hours in the mixed fixation solution (100 mL fixation solution contained 40% formaldehyde 10 mL, acetic acid 5 mL, 95% alcohol 50 mL, 25% glutaraldehyde 5 mL and double distilled H_2O 30 mL) at 4°C. For ultrastructural analysis, optic nerve segments and eyecups were immediately fixed for six hours in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C.

Retinal histological analysis

Eyes for histological analysis were processed by paraffin embedding, and longitudinal sections (5 μm) were cut through the globe along the anterior-posterior axis. Sections were deparaffinized and rehydrated, stained with hematoxylin-eosin and mounted for microscopy and photography. The RGCs layer cells count and the retinal nerve fiber layer (RNFL) thickness measurement were conducted by three investigators in a masked fashion. The cells density of the RGCs layer was counted using an image analysis system (KS-400, Zeiss, Jena, Germany) in three 400 × fields at a distance of 3-4 mm from the optic disc. The distance between inner limiting membrane and retinal ganglion cell layer was defined as the RNFL thickness and measured by the image analysis system in three 400 × fields at a distance of 3-4 mm from the optic disc, and the average value was obtained.

Optic nerve axon analysis

Optic nerve cross sections from 2% methylcellulose eyes and contralateral eyes were masked and assessed by light microscopy for counting axons density. Optic nerve segments were dehydrated and embedded. Sections (3 μm) were deparaffinized and rehydrated, stained with 1% toluidine blue, and mounted for microscopy and photography. For each nerve, a cross section from approximately 2 mm behind the globe was chosen at random. The axons were identified by the color difference between the axons zone and the connective tissue zone, and analyzed by three investigators in a masked fashion. The axons quantity was counted by the image analysis system in a 1000 × field. For each sample, three different fields (a total area of 15057.8 μm^2) were chosen at random.

Ultrastructural analysis of RGCs and axons

Six rabbits (three: EBHM-treated, three: untreated group) were randomly chosen for the ultrastructural analysis. Six hours after fixation in 2.5% glutaraldehyde, optic nerves and superior and inferior retinas were rinsed with PBS, and post-fixed in 1% OsO_4 for two hours at room temperature. Optic nerves and retinas were dehydrated in graded alcohols to 100% ethanol, washed with 100% propylene oxide, and embedded in Spurr resin (Electron Microscopy Sciences, Fort Washington, USA). Thin sections were cut, collected on 200 mesh grids, and stained with uranyl acetate and lead citrate. The sections were examined and photographed using a transmission electron microscope (Hitachi H-500, Tokyo, Japan).

Statistical analysis

Data are expressed as means ± SD. Statistical analyses were performed using the SAS Version 6.12 software package. Independent Student's *t* test was used to compare data between two groups. Newman Keuls test was used to compare data among three or more group. A p-value of less than 0.05 was considered to be statistically significant.

Results

Rabbit model with chronic elevated IOP

Rabbit weight was 3.0±0.1 kg and 2.9±0.1 kg in the untreated group (n=9), and EBHM-treated group (n = 13), respectively, at the end of experiment. All rabbits required 5-7 times injections of methylcellulose to maintain an elevated IOP, and the injection interval was 8-12 days. Two rabbits developed cornea infection because of repeated puncture into the anterior chamber, and were excluded from the samples. **Figure 1** shows that IOP was elevated in all model eyes at seven measurement time-points compared with the contralateral eye. Interestingly, the elevated IOPs in methylcellulose-injected eyes were similar between untreated and MBHM-treated group. The success rate was 22 out of 24.

Histological study

Figure 2 showed the cells density of the RGCs layer by comparing the eyes with elevated IOP to the contralateral, control eyes in untreated and EBHM-treated group. The cells density in eyes with chronic elevated IOP was 23.2±6.5 cells (n = 6) and 36.0±8.9 cells (n = 10) per three 400 × fields at 60 days in untreated and EBHM-treated group. The cells density of the RGCs layer in control eyes of animals treated with EBHM extract was not significantly different to that in control eyes of animals without EBHM extract treatment (p = 0.9174).

Figure 3 shows the thickness of RNFL by comparing the eyes with elevated IOP to the contralateral, control eyes in untreated and EBHM-treated group. The RNFL thickness in eyes with chronic elevated IOP was 3.4±0.4 μm (n = 6) and 5.0±1.0 μm (n = 10) at 60 days in untreated and

EBHM-treated group. The RNFL thickness in control eyes of animals treated with EBHM extract was not significantly different to that in control eyes of animals without EBHM extract treatment (p =0.729).

Figure 4 shows light microscopic analysis of optic nerves revealed axons damage in the elevated IOP eyes. Interestingly, the well-distributed axons were observed in **A, B**. some axons swellings (white arrow) and some gliosis (black arrow) appeared in **C, D**. The axons damage in the chronic elevated IOP with EBHM extract treatment group (**D**) was less than that in the chronic elevated IOP without treatment group (**C**).

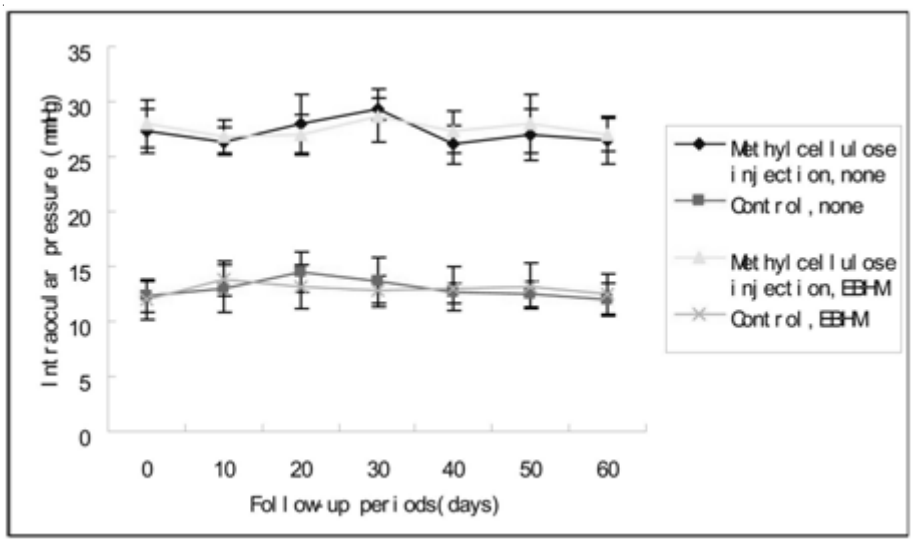

Figure 1. Elevated IOP in rabbit eyes with 2% methylcellulose injected into the anterior chamber. In one group (n = 13), EBHM extract was irrigated into the stomachs of the rabbits. In the control group (n = 9), saline was irrigated into the stomachs of the rabbits. Throughout the 60-day follow-up, IOP was elevated in all 2% methylcellulose injection eyes compared with control eyes ($p < 0.05$). Elevated IOP was not significantly different in untreated and EBHM-treated group.

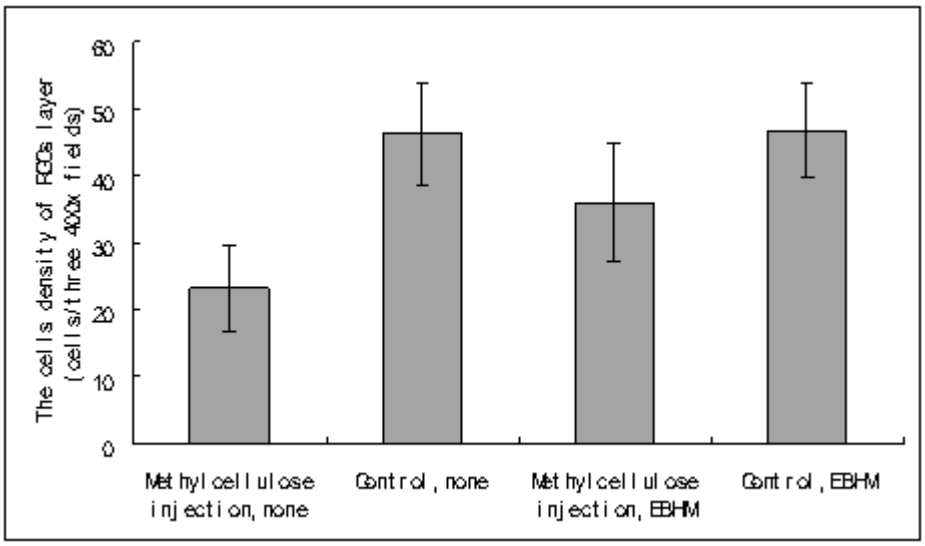

Figure 2. The cells density of RGCs layer in the rabbit retinas of the chronic elevated IOP eyes and control eyes. The graph depicted the mean ±SD of 10 rabbits treated with EBHM extract and six rabbits treated with vehicle. A significant difference was evident in RGCs density in eyes with chronic elevated IOP between EBHM-treated and control (vehicle) group (p =0.013).

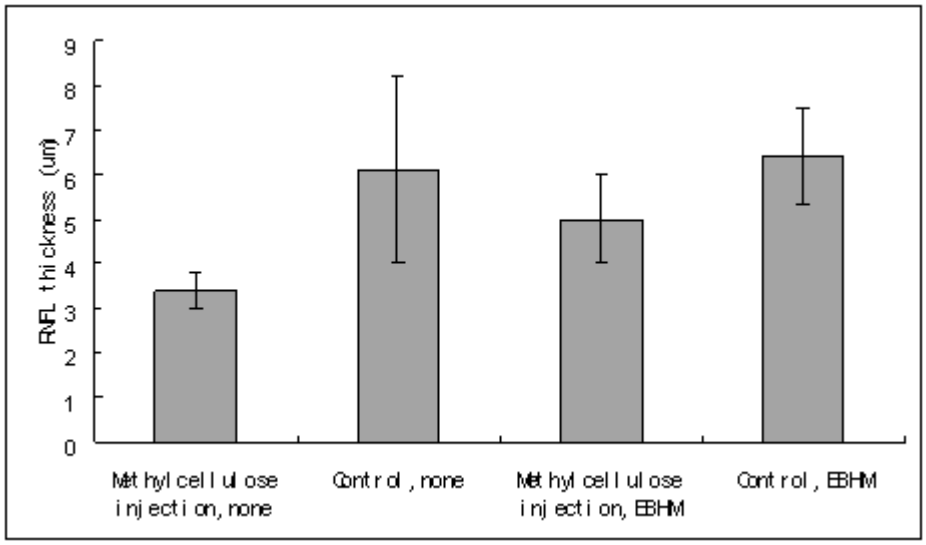

Figure 3. RNFL thickness in the rabbit retinas of the chronic elevated IOP eyes and control eyes. The graph depicted the mean±SD of 10 rabbits treated with EBHM extract and six rabbits treated with vehicle. A significant difference was evident in RNFL thickness in eyes with chronic elevated IOP between EBHM-treated and control (vehicle) group ($p = 0.0025$).

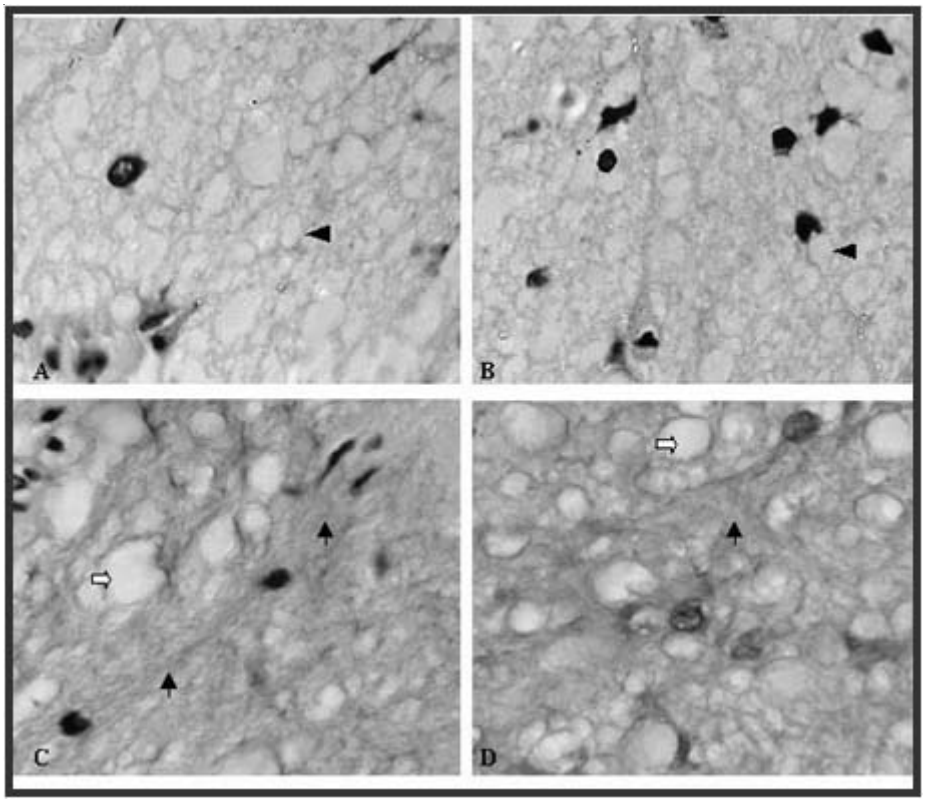

Figure 4. Light microscopic images of optic nerves revealed axons damage in the elevated IOP eyes. **A**: control, none; **B:** control, EBHM extract treatment; **C**: chronic elevated IOP, none; **D**: chronic elevated IOP, EBHM extract treatment. (Toluidine blue staining, 1000×).

Figure 5 shows the optic nerve axons number by comparing the eyes with elevated IOP to the contralateral, control eyes in animals not treated pharmacologically and in animals treated with EBHM extract. The axons number per 15057.8 μm^2 in eyes with chronic elevated IOP was 370.4±41.0 (n = 6) at 60 days in untreated animals and 439.0±50.8 (n = 10) at 60 days in treated animals with EBHM extract (p =0.020). The axons number in control eyes of animals treated with EBHM extract was not significantly different to that in control eyes of animals without EBHM extract treatment (p =0.793).

Ultrastructural study

Figure 6 shows electron microscopic analysis of RGCs of the control eyes and the elevated IOP eyes.

Figure 7 shows electron microscopic analysis of optic nerve axons of the control eyes and the elevated IOP eyes.

Discussion

A major goal of glaucoma research has been to develop analogous treatment approaches to prevent the death of ganglion cells of the retina. Risk factors such as elevated IOP [17-19], decreased neurotrophin support [20, 21], glutamate-associated excitotoxicity [22-24], hypoperfusion, and vasospasm [25-27] have been implicated in ganglion cell death in glaucoma. Neuroprotective strategies have focused on mitigating these risk factors associated with RGCs loss in glaucoma.

Manni et al. [15] reported that RGCs density significantly decreased in the rabbits 10 days after ocular hypertension. The present chronic elevated IOP model was consistent with the study by Manni et al. [15]. The repeated punctures and injections into the anterior chamber may result in an inflammatory response. Considering that the contralateral eyes received the same anterior chamber punctures and injections, the RGCs loss in the experiment eyes might be attributed to the chronic elevated IOP.

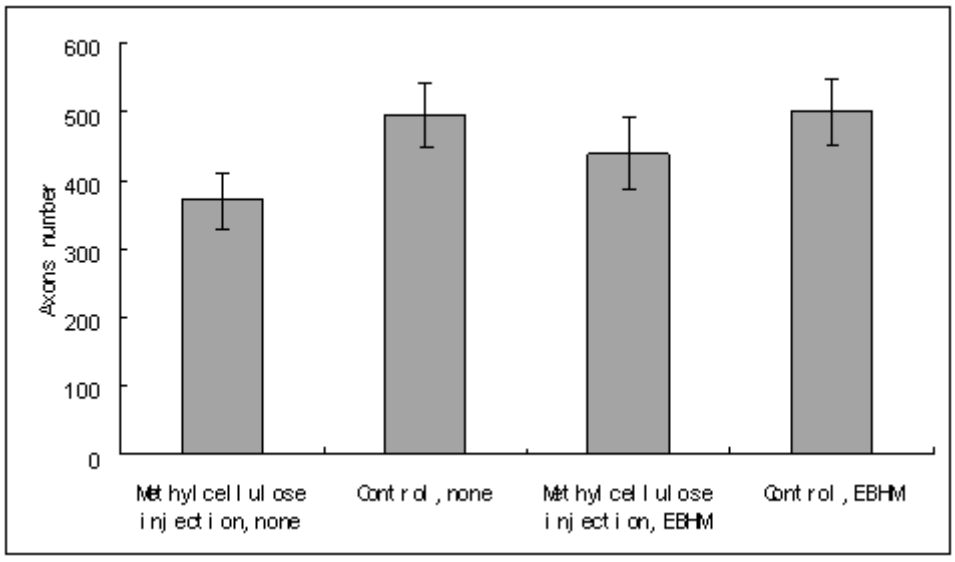

Figure 5. The optic nerve axons number in the rabbit retinas of the chronic elevated IOP eyes and control eyes. The axons number was randomly counted in three different fields (a total area of 15057.8 μm^2). The graph depicted the mean±SD of 10 rabbits treated with EBHM extract and six animals treated with vehicle. A significant difference was evident in the axons number in eyes with chronic elevated IOP between between EBHM-treated and control (vehicle) group (p =0.020).

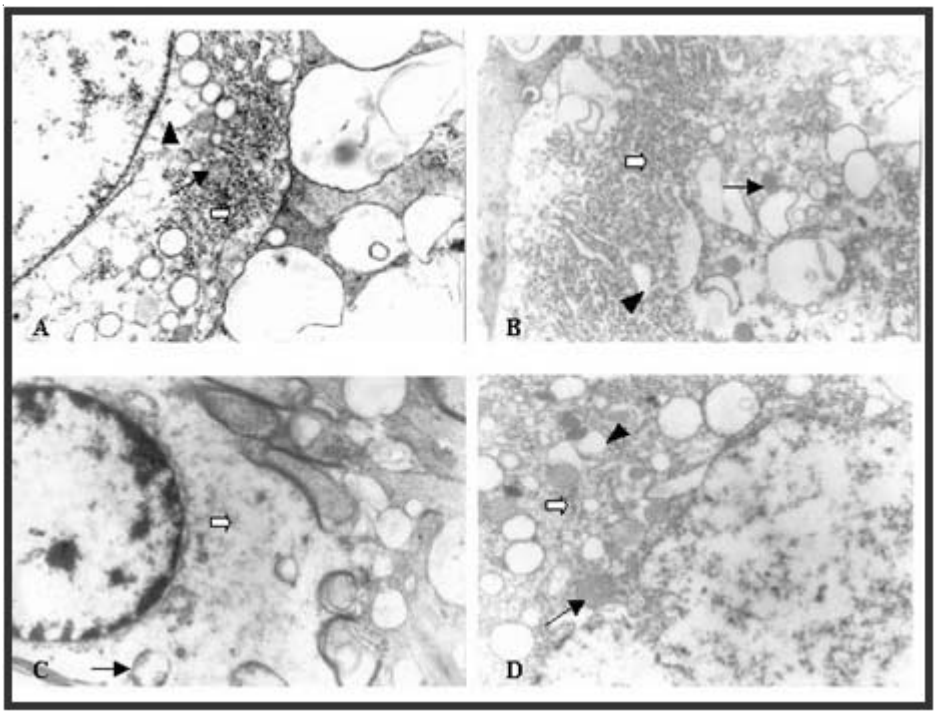

Figure 6. Electron microscopic images of RGCs of the control eyes (**A, B**) and the elevated IOP eyes (**C, D**). A: control, none; **B**: control, EBHM extract treatment; **C**: chronic elevated IOP, none; **D**: chronic elevated IOP, EBHM extract treatment. The abundant mitochondrion (black arrow), rough surfaced endoplasmic reticulum (block arrowhead) and ribosome (white arrow) appeared in RGCs plasm in control eyes of animals with or without EBHM extract treatment (**A, B**). The ribosome (white arrow) and the other organells in RGCs plasm appeared decreased, and mitochondrion vacuolated (black arrow) in the elevated IOP eyes of animals without EBHM extract treatment (**C**). Some dispersive mitochondrion (black arrow) and rough surfaced endoplasmic reticulum (block arrowhead) and ribosome (white arrow) still existed in the RGCs plasm in the elevated IOP eyes of animals with EBHM extract treatment (**D**). (Transmission electron microscopy, 14400×).

The present study indicated that EBHM extract was able to protect RGCs in the chronic elevated IOP rabbit eyes. The main active constituents of EBHM extract are 4'-hydroscutellarein,4'-hydrobaicalein-7-β-D-plamyagin,4'-hydroscutellarein-7-β-D-glycuron methyl ester, pyromeconic acid, and several kinds of flavones and flavonoids [28-31]. According to Chu et al. [32], beneficial effects of EBHM extract come from the combined activity and a certain interdependency of several active constituents of the extract. Most likely, the protective and rescuing effects of EBHM extract may be attributable to its activity of microcirculation improvement. Bastianetto et al. [33] reported that the flavonoid fraction strongly inhibited both the toxicity and the free radical accumulation induced by sodium nitroprusside and/or 3-morpholinosydnonimine. Since EBHM extract contains several kinds of flavones and flavonoids, it also has the effect of inhibiting the toxicity and the free radical accumulation induced by elevated IOP.

In conclusion, EBHM extract is able to protect and rescue rabbit RGCs in a rabbit model of chronic glaucoma. Pharmacological neuroprotection, using EBHM extract, may be a viable approach to treat glaucoma. This approach may be effective with or without a pharmacological agent to lower IOP.

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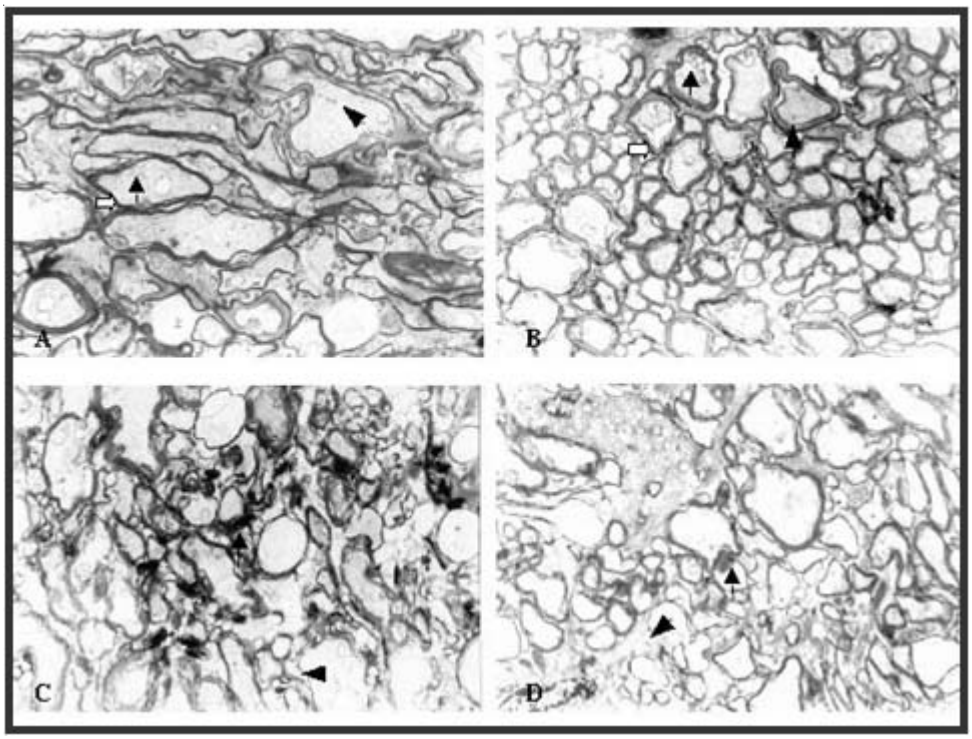

Figure 7. Electron microscopic images of optic nerve axons of the control eyes (**A, B**) and the elevated IOP eyes (**C, D**). **A**: control, none; **B:** control, EBHM extract treatment; **C**: chronic elevated IOP, none; **D**: chronic elevated IOP, EBHM extract treatment. The well-distributed myelin sheath plates (white arrow) and abundant microfilaments and microtubules (black arrow) and mitochondrion (black arrowhead) appeared in control eyes of animals with or without EBHM extract treatment (**A, B**). The myelin sheath plates condensed (black arrow) and degenerated (black arrowhead) and the microfilaments and microtubules decreased or disappeared in the elevated IOP eyes of animals with or without EBHM extract treatment (**C, D**) but the axons degeneration in the chronic elevated IOP with EBHM extract treatment (**D**) was less than that in the chronic elevated IOP without treatment (**C**). (Transmission electron microscopy, 5400×).

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