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Original Article

BuyangHuanwu decoction (BYHWD) protects against retinal ischemic injury

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Abstract: Severe ipsilateral or bilateral carotid artery stenosis or occlusion is the most common cause of ocular ischemic syndrome (OIS). This study aimed to evaluate the protective effects of BuyangHuanwu decoction (BYHWD) against the retinal ischemia induced by ligation of carotid arteries in rats. The effects of BYHWD (0.64 g/kg, i.g.) on ischemia-induced retinal damage were examined with histological staining, RT-PCR and western blot. The protective effects of BYHWD on the ischemia retinal ganglion cells (RGCs) model induced by hypoxia were examined in vitro. The results indicated that BYHWD significantly prevented the decrease in density of cells in ganglion cell layer (GCL) and in the thickness of total retinal, and the increase in the numbers of TUNEL-positive cells. In addition, BYHWD decreased the expression of Caspase-3, Caspase-8 and Bax, and increased the expression of Bcl-2 in retina after ischemia. Similar to the results in vivo, BYHWD significantly suppressed the hypoxia-induced changes of apoptosis related gene and protein expression. These findings suggest that BYHWD may prevent ischemia-induced retinal ischemia through its inhibition of apoptosis.

Keywords: Retinal ischemia, ocular ischemic syndrome, BuyangHuanwu decoction, apoptosis

Introduction

Retinal ischemia, which leads to irreversible retinal cell death, is involved in many ocular diseases, such as retinal vein occlusion, diabetic retinopathy, ischemic optic neuropathy, and ocular ischemic syndrome [1-3]. OIS is a variable spectrum of aggregated ocular signs and symptoms that result from ocular hypoperfusion due to stenosis or occlusion of the common or internal carotid arteries [4]. OIS is manifested as amaurosis fugax, loss of visual acuity, orbital pain, changes of the visual field, and various anterior and posterior segment signs. Bilateral common carotid artery ligation in rats (also called common carotid artery occlusion, BCCAO) is a model to simulate the clinical ocular ischemic lesions caused by carotid stenosis [Slakter [5]]. Study showed the inner retina in BCCAO model is the most vulnerable, manifested as RGCs apoptosis and thinning of inner plexiform layer [6, 7]. Our previous study also found out the thinning of inner plexiform layer along with the ganglion cells significantly reduced in BCCAO model [8].

The treatments for OIS include systemic like carotid stenosis or conservative surgery, and local like eye drug, laser or surgery [4]. Effective drugs relevant to treatment of retinal ischemia have been less. Several studies reported the exploring of drug treatments for the disease, and animal experiments had confirmed the efficacy of the drugs, however, they have not been used in clinical application [9-17].

BuyangHuanwu decoction (BYHWD) was originally recorded in the traditional herbal literature of Yi-Lin-Gai-Guo written by Qing-Ren Wang in 1830 during the Qing Dynasty. According to the Chinese Pharmacopoeia [5], the decoction is comprised of seven commonly used Chinese herbal drugs: (1) Radix Astragali (*huangqi*), the dried roots of *Astragalus membranaceus* (Fisch.) Bge. var., mongholicus (Bge.) Hsiao; (2) The carda part of Radix Angelicae Sinensis root (*guiwei*), the dried lateral roots of *Angelica sinensis* (Oliv.) Diels; (3) Radix Paeoniae Rubra (*chishao*), the dried roots of *Paeonia lactiflora* Pall.; (4) Rhizoma Chuanxiong (*chuanxiong*), the dried rhizomes of *Ligusticum chuanxiong* Hort;

(5) FlosCarthami (*honghua*), the dried flowers of *Carthamus tinctorius* L.; (6) Semen Persicae (*taoren*), the dried seeds of *Amygdalu spersica* L.; and (7) Pheretima (*dilong*), the dried bodies of *Pheretima aspergillum* (E. Perrier), in the ratio of 120:6:4.5:3:3:3:3 on a dry weight basis, respectively. According to the traditional Chinese medical literature, it is used to enhance blood circulation and activate energy (qi) flow through energy meridians. This formula has been shown to provide neuroprotective effects for conditions such as brain ischemia [6, 7], stroke-induced disability [8, 9] and act against cerebral ischemia/reperfusion (CI/R) injury [10-12]. A previous study has found that BYHWT have the potential improvement for ischemic stroke and extended lifespan, primarily by regulating inflammation, apoptosis, angiogenesis and blood coagulation, as well as by mediating neurogenesis and nervous system development [13]. However, the nothing is known about effects of BYHWD in the OIS model, in which retinal ischemia is induced in rats by ligation of common carotid arteries.

In the present study, we used the BCCAO model to examine the protective effects of BYHWD intragastrically administered against structural retinal damage. To that end, we performed retinal cell apoptosis analyses, and we also explored the underlying mechanism.

Materials and methods

Animals

Male Brown Norway (BN) rats (10-12 weeks old, weight 250-300 g) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All rats were maintained in a light-controlled room at temperature of 22 ± 2°C and a relative humidity of 55 ± 5%. All animal uses were in accordance with the Guide for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China. Animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of Peking University (Beijing, China).

Preparation and quality control of Buyang-Huanwu decoction (BYHWD)

The crushed herbs of Radix Astragali, the carda part of Radix Angelicae Sinensis root, Radix Paeoniae Rubra, Rhizoma Chuanxiong, FlosCarthami, Semen Persicae, and Pheretima were mixed with a ratio of 120:6:4.5:3:3:3:3 (dry weight). The decoction was made by boiling the mixture in distilled water at 100°C for 30 min twice. The drug solution was then cooled and dried to give the drug powder, which was dissolved in distilled water at a final concentration of 2.0 g/ml (equivalent to dry weight of raw materials). All materials were purchased from local suppliers and were identified by the traditional Chinese medicine (TCM) professionals at National Research Institute of Chinese Medicine (NRICM) and with HPLC fingerprinting. The voucher specimens were deposited at the Herbarium of NRICM, Taiwan.

The High Performance Liquid Chromatography (HPLC) fingerprint of the BYHWD and of each individual component of the BYHWD were used as quality controls for the BYHWD preparation and for the materials used. The chemical profiling of each component of the BYHWD was verified by comparing it with the standard HPLC profile of the authentic materiamedica in the botanical drug database that has been build up at the NRICM. Each botanical drug was consistent in properties with respect to the crude drug, decoction pieces, microscopic morphology, DNA sequence of the internal transcribed spacer (ITS) region, and HPLC fingerprint when compared to the database of the NRICM (see supporting data for an example). Briefly, the HPLC profiling was performed using an Agilent 1100 series equipped with a quaternary solvent delivery system, auto-sampler, and a photodiode array (PDA) detector. The separation was performed on a Cosmosil ARII (250 mm × 4.6 mm, 5 μm) column. The mobile phase consisted of (A) 0.1% phosphoric acid in purified water and (B) acetonitrile. The linear gradient elution used had been optimized for BYHWD and was as follows: 2-2% B (0-5 min), 2-30% B (5-50 min), 30-60% B (50-70 min), with the re-equilibration time of the gradient elution being 15 min. The flow rate was 1 ml/min. The monitoring UV wavelengths were set at 203, 230 and 280 nm, and the scan range for the PDA was 190-400 nm.

OIS model in rat

Rats were randomly assigned to either OIS or sham OIS groups. OIS animals underwent a

BOCCA procedure [18], under anesthesia induced by intraperitoneal injection of a 7% solution of chloral hydrate at a dose of 0.42 mg/g. A ventral midline incision was made, and the common carotid arteries were bilaterally separated from the carotid sheath and vagus nerve. Each artery was rigidly occluded with 3-0 silk sutures. Sham OIS animals were submitted to the same procedure without occlusion of the common carotid arteries. After OIS or sham OIS surgery, the wound was closed and disinfected, and animals recovered in their home cage. Experimental animals were followed for a 3-month period.

Drug administration and animal grouping

The rats were randomly divided into five groups (n = 10 of each), named control (Sham OIS) plus 30 ml PBS (i.g. twice daily), OIS plus 30 ml PBS (i.g. twice daily), OIS plus BYHWD (0.64 g/kg, i.g., twice daily), OIS plus Ginaton (200 mg/kg, i.g., twice daily), and OIS plus Aspirin (45 mg/kg, i.g., twice daily). All animals were allowed to move and take food freely.

Histology

After 3 months, animals were sacrificed by overdose 10% chloral hydrate, and one eye was used for paraffin embedded and the other for biochemistry. The eyes were enucleated and immersed in 10% formalin for at least 24 h, and were then embedded in paraffin. Sections were cut at a thickness of 7 μm along the horizontal/vertical meridian through the optic disc. After staining with hematoxylin and eosin, the number of cells in the retinal ganglion cell layer (RGCL) and the total retinal thickness (TRT) were measured. All measures were made at 400 × magnification, approximately 200 μm from the optic disc using a Leica DM6 B (400 ×) microscope with a charge-coupled device camera and image Pro Plus software 4.1 (Media Cybernetics, Silver Spring, Md., USA).

The number of cells in the RGCL was counted as the liner cell density (cells/mm). In each retina, the numbers of cells and the thickness (μm) of total retina were obtained as the mean value of five different measurements.

TUNEL assay

Terminal dexy nucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) of paraffin-embedded tissue sections were performed using the In Situ Cell Death Detection Kit (Promega, Madison, WI, USA). After deparaffinisation and rehydration, retinal sections were digested with proteinase K solution for 25 min, rinsed with 0.1 mol/L PBS for 15 min at room temperature, incubated with the TUNEL reaction mixture for 1 h at 37°C in a humid atmosphere and then incubated with horseradish peroxidase (HRP)-conjugated streptavidin at room temperature for 5 min. Finally, DAB was used as a chromogen, and the sections were further counterstained with haematoxylin, dehydrated and mounted. Negative controls were performed by incubating sections without the recombinant TdT enzyme. Apoptotic cell nuclei appeared as dark brown/black under an E600 light microscope (Nikon, Tokyo, Japan). For the quantification of TUNEL-positive (apoptotic) cells, a minimum of 200 cells were counted at five random fields in each eye with three adjacent areas on both sides of the optic nerve head (1 mm from the optic nerve head), and the percentage of the positively-labeled cells was calculated.

Western blot

Rats were euthanized, their eye balls were rapidly removed, and the retinas were carefully separated and quickly frozen in dry ice. For protein extraction, the retina tissue or RGCs was homogenized on ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, pH 8.0 and 100 mg/L phenylmethylsulfonyl fluoride; Sigma), followed by centrifugation at 12,000 × g for 20 min at 4°C. Supernatants were collected. The protein concentrations were measured using a BCA kit (Pierce Chemical, Rockford, IL, USA). A mixture of equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 10% SDS-PAGE. The separated protein was then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). After being blocked with 5% non-fat dry milk in Tris-buffered saline/Tween-20 (TBST) buffer for 1 h, the membranes were incubated overnight at 4°C with the primary antibody. For immunoblotting, the following primary antibodies were used: Caspase-3 rabbit polyclonal antibody (1:500), Caspase-8 rabbit polyclonal antibody (1:500), Bax goat polyclonal antibody (1:100), Bcl-2 goat polyclonal antibody (1:100), and β-actin rabbit polyclonal antibody (1:1000) (Cell Signaling). Subsequently, the membranes

Table 1. PCR primer sequences

Gene	Primer sequences
Caspase-3	F: 5'-ATGCTTACTCTACCGCACCCG-3'
	R: 5'-GGTTAACACGAGTGAGGATGTGC-3'
Caspase-8	F: 5'-ATCCCAGATGAGGCAGACTTTC-3'
	R: 5'-GCTCTGGCAAAGTGACTGGAT-3'
Bax	F: 5'-TGAACTGGACAACAACATGGAG-3'
	R: 5'-AGCAAAGTAGAAAAGGGCAACC-3'
Bcl-2	F: 5'-GGAGGAACTCTTCAGGGATGG-3'
	R: 5'-GACAGCCAGGAGAAATCAAACAG-3'
β-actin	F: 5'-ACGTTGACATCCGTAAAGAC-3'
	R: 5'-GGACTCATCGTACTCCTGCT-3'

were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Amersham Biosciences, Piscataway, NJ, USA) at 37°C for 1 h. After TBST washing, enhanced chemiluminescence (ECL) reagent (Tiangen Biotech Co., Ltd., Beijing, China) was added to the membranes. Western blot bands were read for integrated optical density (IOD) and quantified using Quontity One software.

RT-PCR

Total RNA was extracted from retina tissue and RGCs in each group using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). RNA purity was estimated by calculating the 260/280 nm absorbance and reverse transcribed into cDNA using Transcript First-strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) in accordance to the manufacturer's protocol. RT-PCR was performed using a Super Real PreMix Plus (SYBR Green) kit (Tiangen, Beijing, China) with candidate gene sets (detailed in **Table 1**) or β-actin primers. The reactions were performed three times using an ABI Prism 7500 Sequence Detection System (ABI, Foster City, CA, USA). All data from each sample were assessed by $2^{-\Delta\Delta Ct}$ analysis. The measured genes that displayed a significant difference ($p< 0.01$) were considered differentially expressed conggenes.

Hypoxic injury to RGC-5 cells

The transformed retinal ganglion cell line, RGC-5 (PTA6600, ATCC) was grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA), supplemented with 10% fetal bovine serum. Cells were cultured in a standard growth environment. RGC-5 cells of passages 10-20 were used in our experiments, and cells were used at 80% confluence for all experiments.

$CoCl_2$ (Sigma-Aldrich, St. Louis, MO) was used to induce hypoxia-related cell injury. $CoCl_2$ were dissolved in distilled H_2O and sterilized through a 0.2 μm filter and added in the medium at a series of concentrations. RGC-5 cells were incubated with different concentrations of $CoCl_2$ (0, 0.1, 0.5, 1.0 and 2.0 mM) for 24 h.

The effect of $CoCl_2$ on cell viability following hypoxic insult was evaluated using the conventional MTT reduction assay. Briefly, 96-multiwell plates were seeded at 5×10^3 cells/well. After treatment, cells were incubated with 20 μL solution of 5 mg/ml MTT for 4 h at 37°C. Then the medium was removed. The formazan crystals were dissolved in 150 μL of DMSO and the absorbance was measured at 490 nm in a plate reader. Results were normalized against controls and presented as percentage of cell viability, compared with controls. Three independent experiments were performed.

Cell viability

BYHWD or Ginaton was dissolved in normal saline and Aspirin was dissolved in water. To determine the effect of the dugs on RGC-5 cell viability, cells were cultured with or without 1.0 mM $CoCl_2$, and vehicle, BYHWD, Ginaton, Aspirin for 24 h and results were analyzed under microscope.

AO/EB staining

Cell apoptosis was assessed by Dual AO/EB fluorescent staining. Cells were resuspended in 100-300 mL PBS and stained with 5 mL cocktail of AO (100 mg/mL) and EB (100 mg/mL) in PBS (AO/EB, Sigma, St. Louis, MO). 10 mL suspension was applied onto a microscope slide and then covered with a coverslip. The morphology of apoptotic cells was examined and 500 cells were counted within 20 min using a fluorescent microscope (OLYMPUS, Japan). Live cells are permeable to AO but not to EB and stained green, and dead cells permeable to both AO and EB, and EB stains the DNA red. This red staining is distinctive from AO staining of autolysosomes (red) in the cytoplasm. Dual acridine orange/ethidium bromide (AO/EB) staining method was repeated 3 times at least.

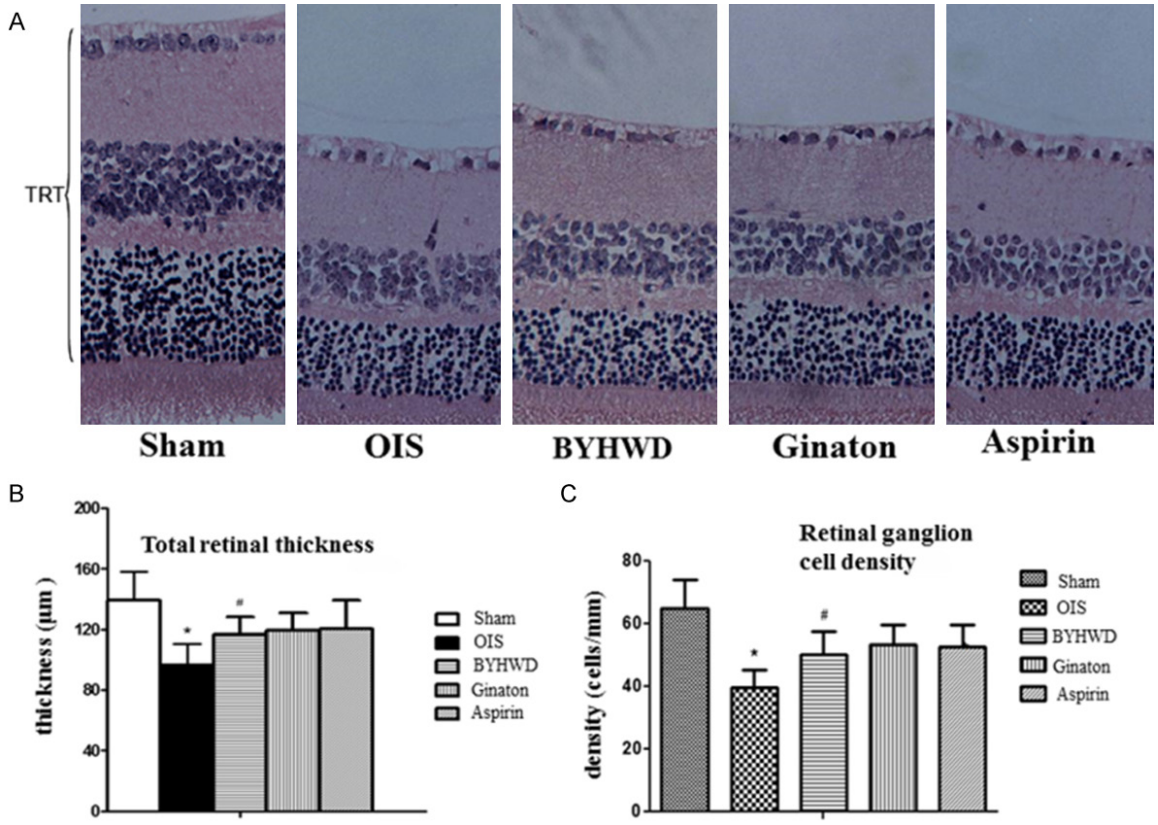

Figure 1. Effect of BYHWD on ischemia-induced retinal morphological damage. (A) Representative photomicrographs of hematoxylin and eosin-stained retinal sections in control, OIS, OIS plus BYHWD, OIS plus Ginaton, and OIS plus Aspirin groups. Quantitative analysis of total retinal thickness (B), and the numbers of cells in GCL (C). Layer thickness and retinal ganglion cell number are expressed as mean ± SD of layer thickness (μm) and cells per millimeter (cells/mm), respectively (n = 10). *P<0.05 vs. control group; #P<0.05 vs. OIS group.

DNA fragmentation assay

In brief, cells were incubated for 5 min in the lysis buffer containing 50 mM Tris base, 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 5 units RNase at 37°C (the pH of the buffer was adjusted to the 7.4). Then total proteins were denaturized with 500 μL of chloroform/isoamialcohol (24:1) and total DNA was separated by centrifugation at 12,000 rpm. Total DNA was precipitated with isopropranol and was quantified by UV-vis spectroscopy and 10 μg of DNA was electrophoresed in a 1.2% agarose gel containing ethidium bromide in a mini gel tank containing Tris-Boric acid-EDTA buffer for 2 hours under 90 V. Then the gel was visualized under UV transilluminator.

Statistical analysis

Statistical analysis was performed with SPSS software version 13.0. Results were expressed as the mean ± standard deviation (SD). Significance of differences between two groups was tested by Student's t test or ANOVA. A value of $P<0.05$ was considered statistically significant.

Results

BYHWD protected against the structural retinal damage induced by ischemia

Figure 1A compares representative retinal sections obtained from an animal in each experimental group. The overall retinal thickness of the OIS retina was significantly thinner (**Figure 1B**), along with a significant reduction in the number of cells within the GCL layer (**Figure 1C**). Oral administration of BYHWD significantly prevented such reductions in the GCL cell number and total retinal thickness (versus the OIS group) (**Figure 1A-C**).

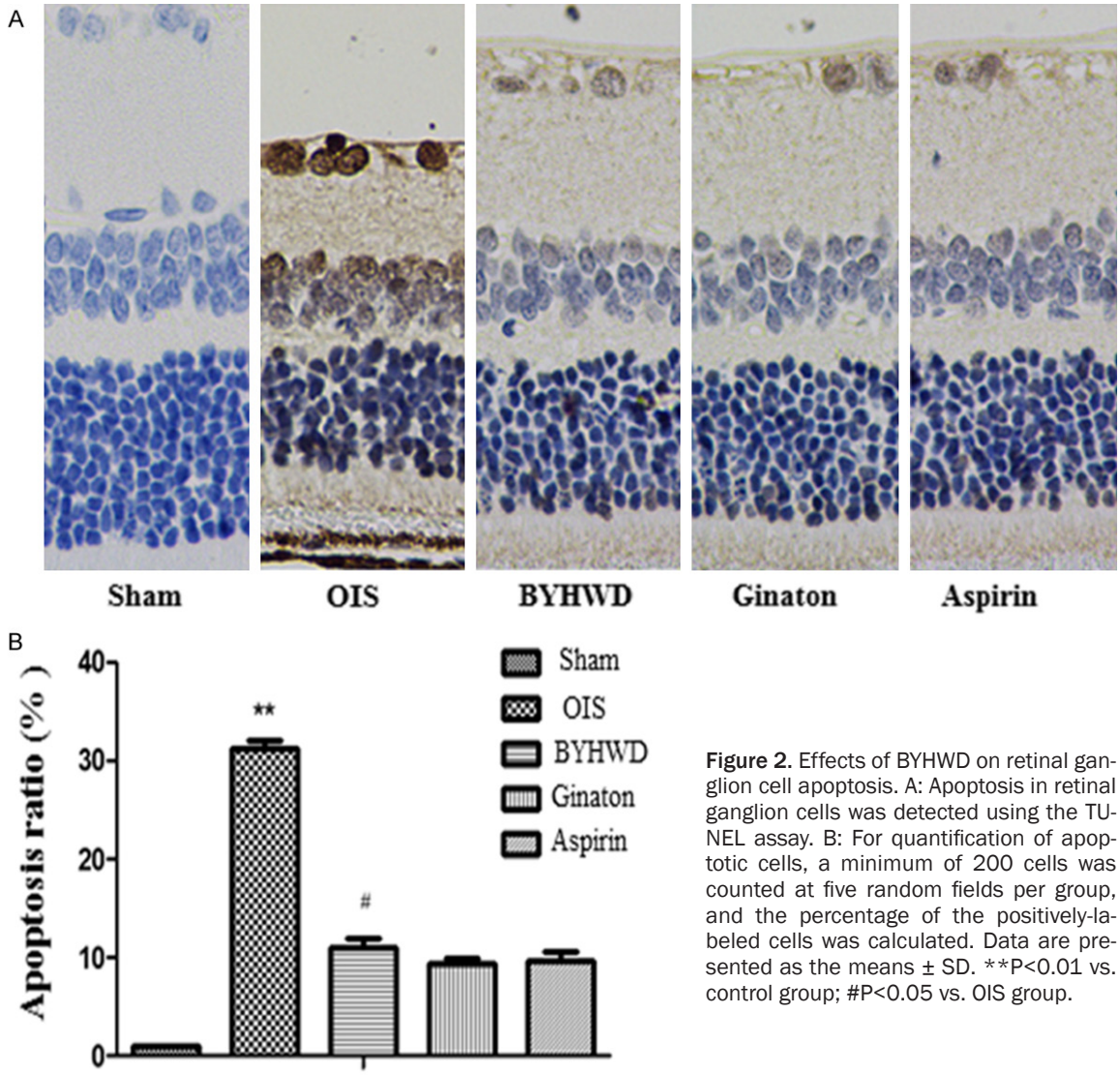

Figure 2. Effects of BYHWD on retinal ganglion cell apoptosis. A: Apoptosis in retinal ganglion cells was detected using the TUNEL assay. B: For quantification of apoptotic cells, a minimum of 200 cells was counted at five random fields per group, and the percentage of the positively-labeled cells was calculated. Data are presented as the means ± SD. **P<0.01 vs. control group; #P<0.05 vs. OIS group.

BYHWD suppressed the increase in cell apoptosis induced by ischemia

To evaluate apoptosis, TUNEL staining (**Figure 2A**) was performed. Higher levels of apoptotic bodies were observed in the retinal ganglion cell layer (RGCL) of all OIS group as compared to the Sham group (**Figure 2A** and **2B**). Oral administration of BYHWD significantly reduced the number of TUNEL-positive cells in GCL (versus the vehicle-treated retina) (**Figure 2A** and **2B**).

BYHWD stimulated the expression of Bcl-2 and prevented the expression of Caspase-3, Caspase-8 and Bax in rat retina following ischemia

This experiment investigated the mechanisms responsible for OIS-induced cell death in the retina and the effects of BYHWD. As can be seen in **Figure 3A**, Western blot analysis revealed that the levels of Caspase-3, Caspase-8 and Bax were markedly increased in the OIS group compared with the Sham group (**Figure 3A-D**), while that of Bcl-2 was significantly decreased (**Figure 3A** and **3E**). Furthermore, RT-PCR analysis revealed the same trend in Caspase-3, Caspase-8, Bax and Bcl-2 mRNA levels (**Figure 4A-D**). BYHWD significantly reduced the OIS-induced expression of Caspase-3, Caspase-8 and Bax (**Figures 3A-D** and **4A-C**), and elevated the expression of Bcl-2 (**Figures 3E** and **4D**).

$CoCl_2$-induced injury of RGC-5 cells

$CoCl_2$ treatment decreased the RGC viability (**Figure 5**). This reduction at the 0.1 mM concentration was not significantly different than control cells. However, the 0.5 and 1.0 mM

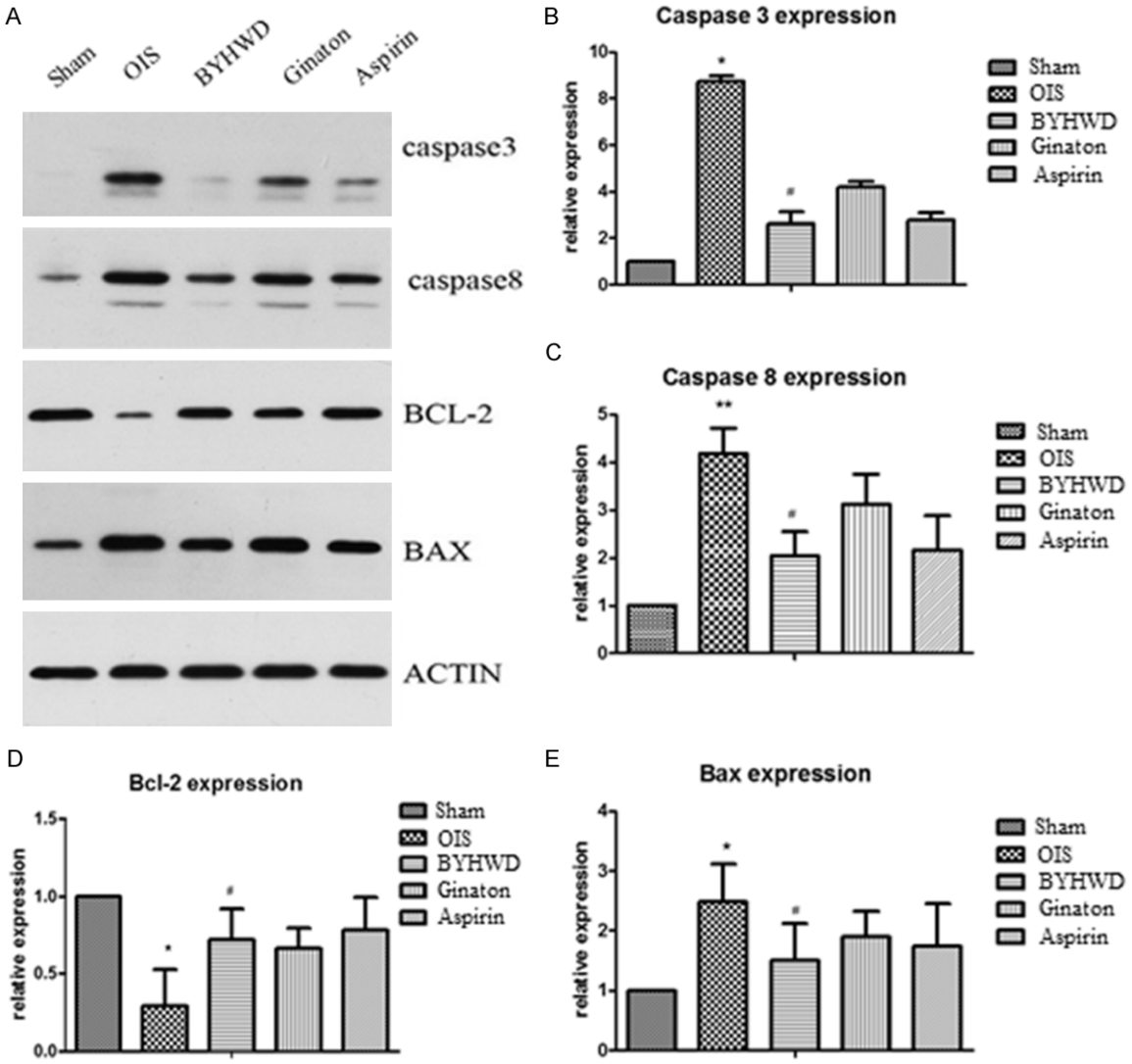

Figure 3. Effects of BYHWD on ischemia-induced protein expression of Caspase-3, Caspase-8, Bax and Bcl-2. (A) Representative band images show the expression of Caspase-3, Caspase-8, Bax and Bcl-2 in control, OIS, OIS plus BYHWD, OIS plus Ginaton and OIS plus Aspirin groups. (B-E) Quantitative analysis of band densities for (B) Caspase-3, (C) Caspase-8, (D) Bcl-2, and (E) Bax. Data are presented as the means ± SD. *P<0.05 vs. control group; **P<0.01 vs. control group; #P<0.05 vs. OIS group.

$CoCl_2$ concentrations conveyed significantly reduced cell viability in comparison with control cells (P<0.01). The 2.0 mM concentration, when contrasted with control, resulted in a reduction of cell viability, which was considered beyond the LD50. Since LD50 was surpassed at the 2.0 mM concentration, we performed subsequent hypoxia induction experiments within the 1.0 mM $CoCl_2$ concentration.

BYHWD prevented $CoCl_2$-induced RGC-5 apoptosis

The morphological characterization of RGC-5 cells showed that cells grew as a monolayer and exhibited axonal processes. Incubation with $CoCl_2$ (1.0 mM) for 24 h induced morphological changes of RGC-5 cells as shown by neurite retraction, cell body area reduction, condensation of the nucleus and cytoplasm and formation of cell fragments. However, these types of morphological changes were prevented by BYHWD treatment (**Figure 6A**).

In the case of apoptosis analysis, the effect of BYHWD on RGC-5 cells was further confirmed by dual AO/EB fluorescent staining and DNA fragmentation assay (**Figure 6B** and **6C**). No significant apoptosis was detected in BYHWD treatment group (**Figure 6B**). Early-stage apop-

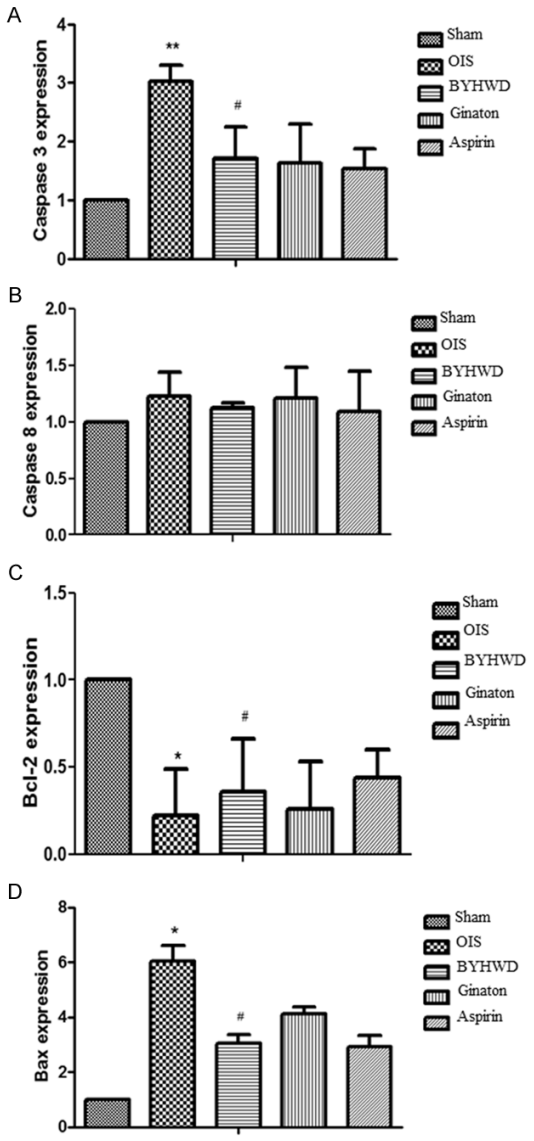

Figure 4. Effects of BYHWD on ischemia-induced mRNA expression of Caspase-3, Caspase-8, Bax and Bcl-2. (A-D) Quantitative analysis of the mRNA levels of (A) Caspase-3, (B) Caspase-8, (C) Bcl-2, and (D) Bax. Data are presented as the means ± SD. *P<0.05 vs. control group; **P<0.01 vs. control group; #P<0.05 vs. OIS group.

totic cells, marked by crescent-shaped or granular yellow-green AO nuclear staining, were detected in Aspirin treatment group (**Figure 6B**). Late-stage apoptotic cells, with concentrated and asymmetrically localized orange nuclear EB staining, were detected in the control group or Ginaton treatment group (**Figure 6B**). Necrotic cells increased in volume and showed uneven orange-red fluorescence at their periphery, and appeared to be in the process of disintegrating (**Figure 6B**), were also detected.

Gel electrophoresis showed the formation of the DNA ladder, which was induced by $CoCl_2$ (**Figure 6C**). **Figure 6C** obviously illustrates the formation of DNA-ladder (trailing) in the control group or Ginaton treatment group, which confirms occurrence of DNA breakage and fragmentation. This event can happen only due to cell apoptosis. Therefore, the protective effect of the BYHWD was exerted primarily against apoptosis.

BYHWD inhibited the hypoxia-induced apoptosis in RGC-5 by activating Bcl-2 and inhibiting Caspase-3 and Bax

Caspase-3, Bax and Bcl-2 were detected by western blot analysis. There was no statistical difference in the expression of Caspase-3 protein among the four groups (P>0.05). The expression of BAX was significantly increased in the $CoCl_2$-treated group compared with that of the control, while that of Bcl-2 was significantly decreased. BYHWD treatment significantly increased the expression of Bcl-2 (**Figure 7A**). RT-PCR analysis revealed that Caspase-3 and Bax mRNA levels were significantly increased, and Bcl-2 mRNA levels were significantly decreased (all P<0.05) (**Figure 7B-D**). BYHWD significantly reduced the level of Caspase-3 and Bax mRNA, and elevated that of Bcl-2, but without returning to the control levels.

Discussions

In the present study, after BOCCA, the ischemic retinas treated with the vehicle contained thinner RGC layer compared with the sham retinas, along with numerous apoptotic cells in the RGC layer. The ischemic retinas in rats administered with BYHWD contained significantly more RGCs and significantly fewer apoptotic cells in their RGC layer than the ischemic retinas treated with the vehicle. The protective effect of BYHWD on retinal cells may involve its ability to modulate the activity of the apoptotic pathway. This possibility is also supported by the observation that administering ischemic rats with BYHWD led to a significant decrease in Caspase-3, Caspase-8, Bax expression and increase Bcl-2 expression. To our knowledge, this study is the first reported account of BY-

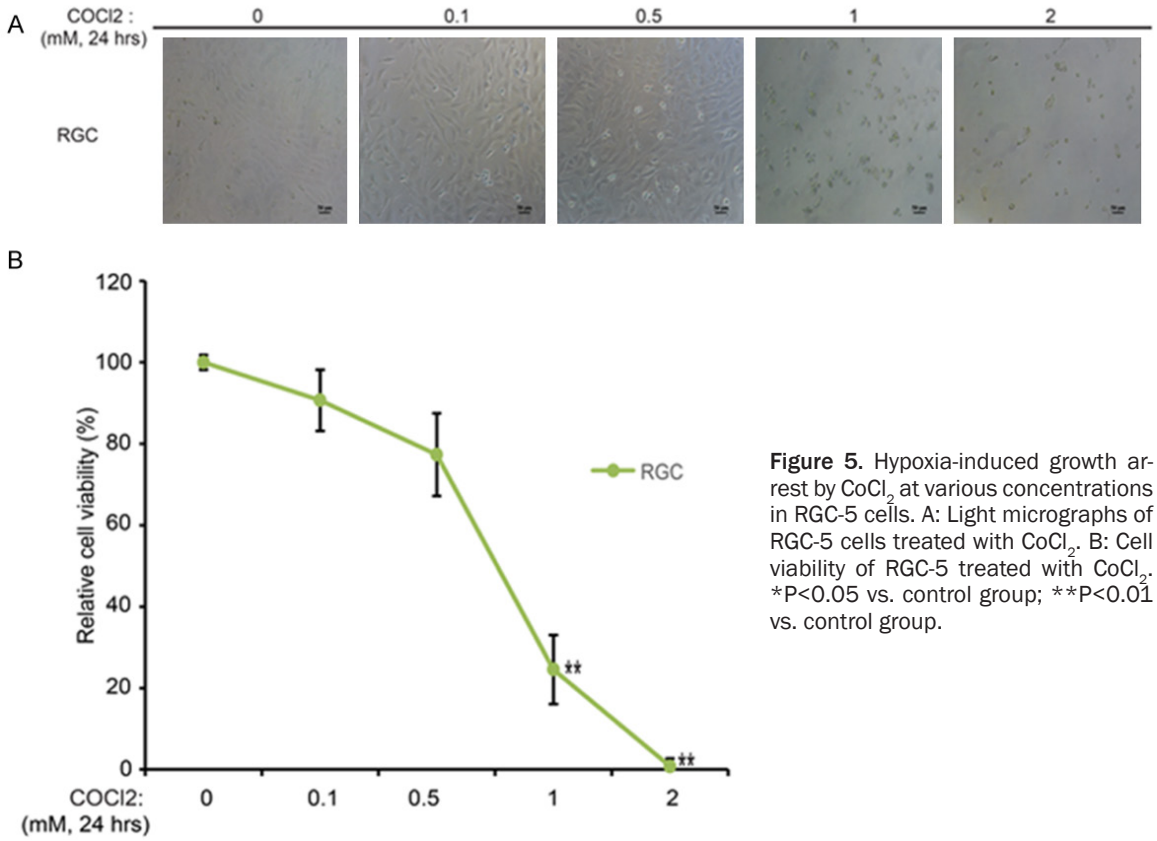

Figure 5. Hypoxia-induced growth arrest by $CoCl_2$ at various concentrations in RGC-5 cells. A: Light micrographs of RGC-5 cells treated with $CoCl_2$. B: Cell viability of RGC-5 treated with $CoCl_2$. *P<0.05 vs. control group; **P<0.01 vs. control group.

HWD as a protective agent against retinal ischemic injury. BYHWD could preserve the retinal structure, counteracting any reduction in the thickness of the total retina and avoiding RGC death. Furthermore, BYHWD inhibited $CoCl_2$-induced RGC death in vitro. BYHWD achieves these protective effects by suppressing apoptosis and thus providing cytoprotective effects after injury in neuronal organs.

The pathogenesis of retinal ischemia injury is complicated and is not completely understood. However, it is known that apoptosis plays an important role [14]. Neuronal cell death in the inner retina is induced by retinal ischemia, which can be caused by such maneuvers as central retinal artery occlusion [15], or increasing the intraocular pressure [16]. Furthermore, apoptosis is induced by retinal ischemia, and has been observed in the GCL, INL, and ONL following the retinal ischemia induced by occlusion of the central retinal artery [17]. One study revealed that cells in the inner retina especially RGCs are more vulnerable to ischemic damage compared to cells of the outer retina [19]. RGC loss caused by retinal ischemia injury occurs primarily by apoptosis, although it may also involve other cell death pathways [20]. In the present study, the number of cells in GCL and the thickness of total retinal were decreased after ligation of common carotid arteries, while TUNEL-positivity, which is displayed by apoptotic cells, was increased in the retina. Apoptosis of the GCL is progressive and irreversible [21]; therefore, anti-apoptosis therapy in retinal ischemic diseases maybe beneficial to improve retinal functions. In the present study, BYHWD (0.64 g/kg) inhibited not only the decrease in the number of cells in GCL and the decrease in total retinal thickness, but also the increases in TUNEL-positive cell numbers in GCL. This result indicates that BYHWD protects against the RGC apoptosis induced by retinal ischemia.

Apoptosis is a special kind of cell death and is regulated by multiple genes and proteins. The Bcl protein family plays an important regulatory role in the process of cell apoptosis, in which Bcl-2 is the most important anti-apoptotic protein, whereas Bax is known to promote apoptotic cell death [22, 23]. As the acknowledged

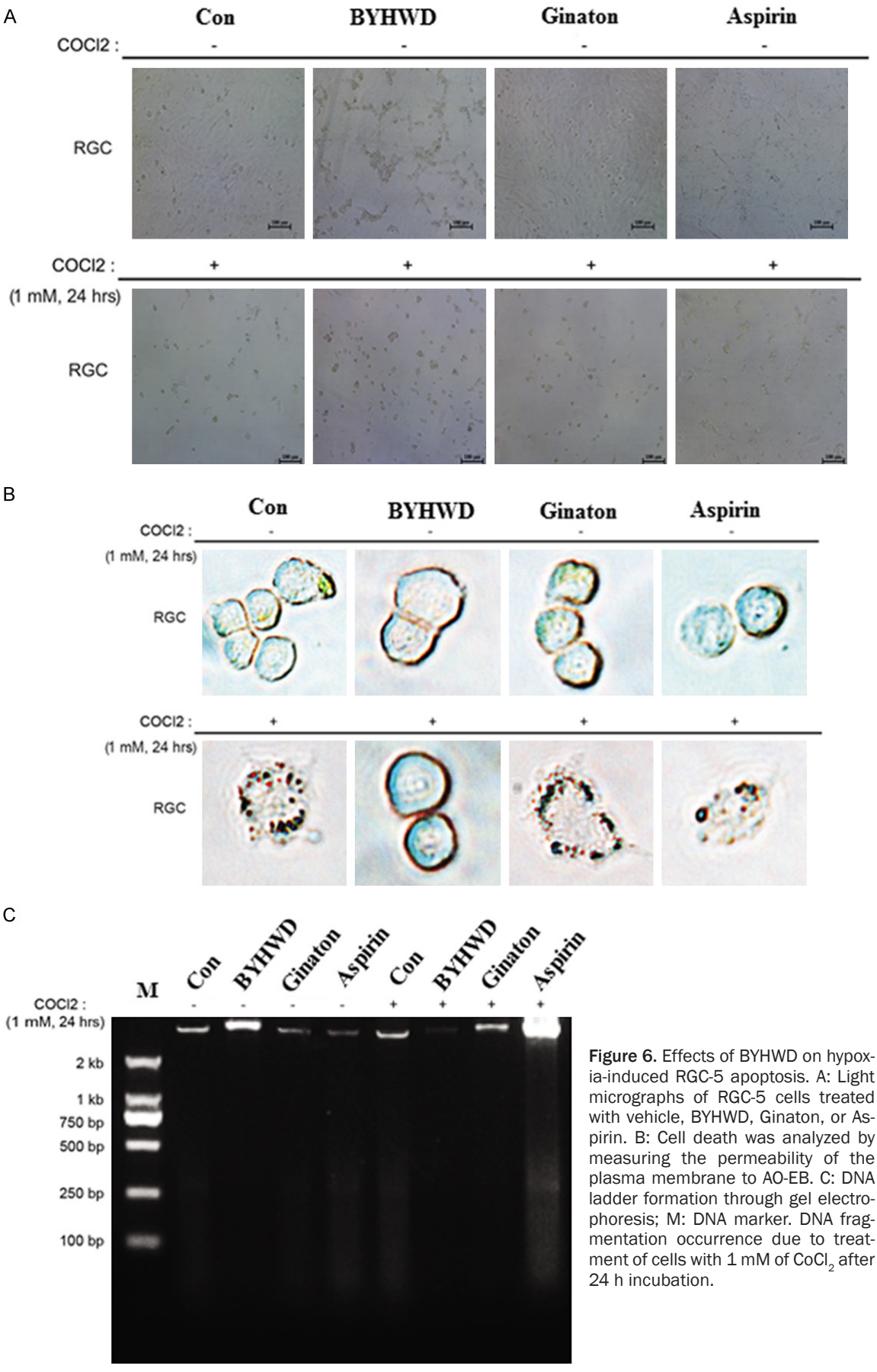

Figure 6. Effects of BYHWD on hypoxia-induced RGC-5 apoptosis. A: Light micrographs of RGC-5 cells treated with vehicle, BYHWD, Ginaton, or Aspirin. B: Cell death was analyzed by measuring the permeability of the plasma membrane to AO-EB. C: DNA ladder formation through gel electrophoresis; M: DNA marker. DNA fragmentation occurrence due to treatment of cells with 1 mM of $CoCl_2$ after 24 h incubation.

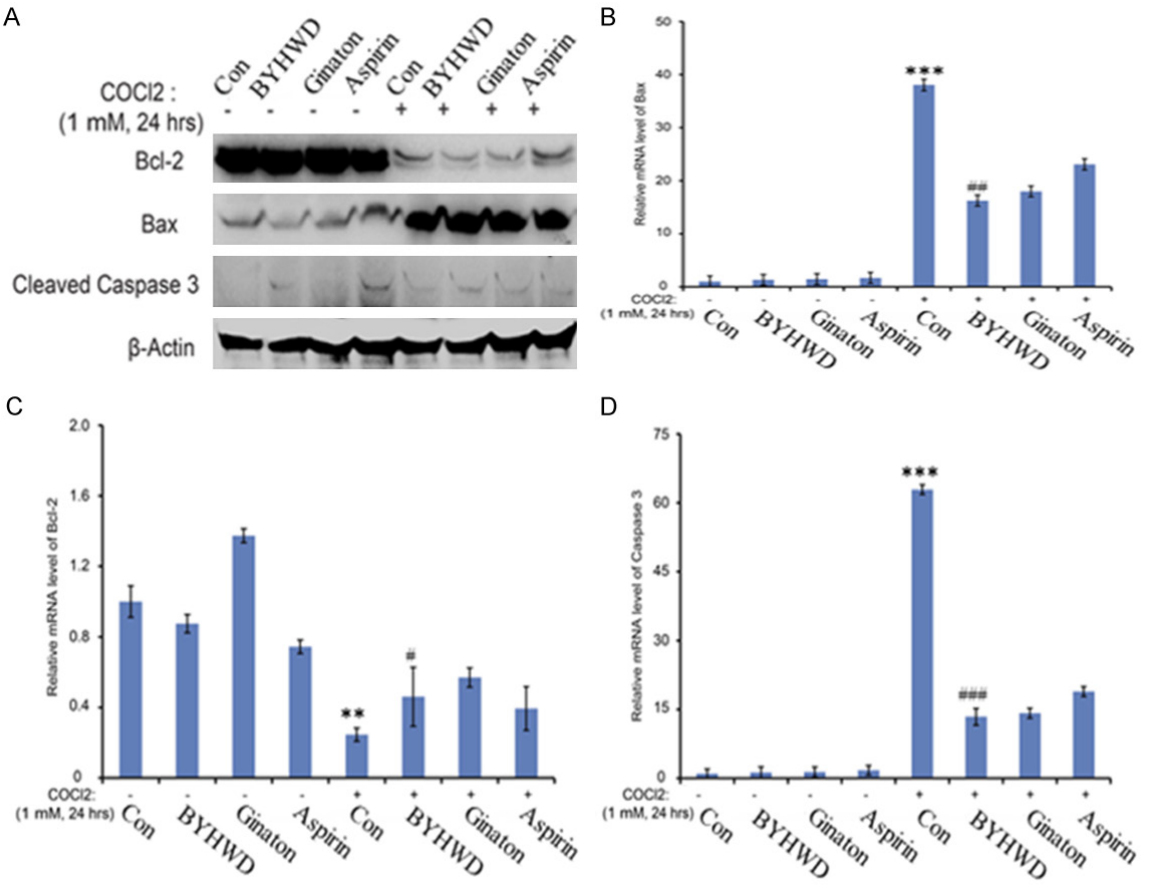

Figure 7. Effects of BYHWD on hypoxia-induced expression of Caspase-3, Bax and Bcl-2 in RGC-5 cells. (A) Representative band images show the expression of Bax, Bcl-2 and Caspase-3 in the Con, BYHWD, Ginaton, or Aspirin groups. (B-D) Quantitative analysis of the mRNA levels of (B) Bax, (C) Bcl-2, and (D) Caspase-3. Data are presented as the means ± SD. **P<0.01 vs. Con treated without $CoCl_2$ group; ***P<0.001 vs. Con treated without $CoCl_2$ group; #P<0.05 vs. CON treated with $CoCl_2$ group; ##P<0.01 vs. Con treated with $CoCl_2$ group; ###P<0.001 vs. Con treated with $CoCl_2$ group.

anti-apoptotic protein, Bcl-2 can prevent the formation of the Bax-induced cascade [24], the ratio of Bcl-2 to Bax plays an important role in cell fate (i.e., survival or death) following an apoptotic stimulus [9, 10]. Cleavage and activation of Caspase-3 and -8 are well-known hallmarks of RGC degeneration following optic nerve transection [25, 26]. Moreover, Caspase-3 and -8 levels negatively correlate with RGC survival [27]. Many studies of animal retinal ischemia found that the expression of Bax was remarkably increased in response to apoptosis signals, resulting in an up-regulation of Caspase-3 and other caspases downstream, thus implying activation of the mitochondrial apoptotic pathway [21, 28]. In this experiment, through the establishment of the retinal ischemia injury model, we observed the effects of BYHWD on the expression of Bcl-2/Bax, Caspase-3 and -8 in the retina. Retinal ischemia injury induced the up-regulations of pro-apoptotic factors including Bax, Caspase-3 and caspase-8, which were all reversed by BYHWD treatment. Surprisingly, BYHWD remarkably enhanced the up-regulation of Bcl-2 induced by retinal ischemia injury. Therefore, it appears that BYHWD administration contributes to protection against retinal ischemia injury by inhibiting the apoptosis of ganglion cells. This result is similar to findings in other organs. A growing body of evidence has suggested that BYHWD plays a protective role in ischemia/reperfusion injury by inhibiting apoptosis [6, 12]. To confirm that the protective effect of BYHWD associated apoptosis inhibition, we evaluated the effect of BYHWD against in vitro ischemia,

focusing on Bcl-2, Caspase-3 and Bax. It has been reported that Bcl-2, Caspase-3 and Bax are also expressed in RGC cultures [29]. In the present study, RGCs were destroyed by $CoCl_2$. BYHWD protects against $CoCl_2$-induced RGC apoptosis by activating Bcl-2 and inhibiting Caspase-3 and Bax.

Taken together, BYHWD efficiently improved retinal ischemia and would be helpful for OIS treatment.

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Disclosure of conflict of interest

None.

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References

[1] Bucolo C, Ward KW, Mazzon E, Cuzzocrea S, Drago F. Protective effects of a coumarin derivative in diabetic rats. Invest Ophthalmol Vis Sci 2009; 50: 3846-52.

[2] Bucolo C, Drago F. Carbon monoxide and the eye: implications for glaucoma therapy. Pharmacol Ther 2011; 130: 191-201.

[3] Bucolo C, Leggio GM, Drago F, Salomone S. Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin-induced diabetic rats. Biochem Pharmacol 2012; 84: 88-92.

[4] Terelak-Borys B, Skonieczna K, Grabska-Liberek I. Ocular ischemic syndrome-a systematic review. Med Sci Monit 2012; 18: RA138-144.

[5] Committee CP. China pharmacopoeia, 2005 ed. Chemical Industry Press 2005; Part One.

[6] Li XM, Bai XC, Qin LN, Huang H, Xiao ZJ, Gao TM. Neuroprotective effects of BuyangHuanwu decoction on neuronal injury in hippocampus after transient forebrain ischemia in rats. Neurosci Lett 2003; 346: 29-32.

[7] Cai G, Liu B, Liu W, Tan X, Rong J, Chen X, Tong L, Shen J. BuyangHuanwu decoction can improve recovery of neurological function, reduce infarction volume, stimulate neural proliferation and modulate VEGF and Flk1 expressions in transient focal cerebral ischaemic rat brains. J Ethnopharmacol 2007; 113: 292-9.

[8] Li T. Inhibitory effects of BYHWD on ROS generation in stroke rat brain tissues. Acta Biophysica Sinica 2003; 19: 441-447.

[9] Wang Q. Yilin Gaicuo (correction on errors in medical classics). People's Medical Publishing House; 2005.

[10] Zhao LD, Wang JH, Jin GR, Zhao Y, Zhang HJ. Neuroprotective effect of BuyangHuanwu decoction against focal cerebral ischemia/reperfusion injury in rats--time window and mechanism. J Ethnopharmacol 2012; 140: 339-44.

[11] Li TJ, Qiu Y, Rui YC, Li TZ, Zhang WD. [DNA microarray analysis of protective mechanism of Buyanghuanwu decoction on focal cerebral ischemia/reperfusion in rats]. Zhongguo Zhong Yao Za Zhi 2004; 29: 559-63.

[12] Tang YH, Li H, Chen BY. [Effect of active fraction of Buyanghuanwu decoction on caspase expression in rats after focal cerebral ischemic reperfusion]. Zhongguo Zhong Xi Yi Jie He Za Zhi 2006; 26: 533-7.

[13] Wang HW, Liou KT, Wang YH, Lu CK, Lin YL, Lee IJ, Huang ST, Tsai YH, Cheng YC, Lin HJ, Shen YC. Deciphering the neuroprotective mechanisms of Bu-yang Huan-wu decoction by an integrative neurofunctional and genomic approach in ischemic stroke mice. J Ethnopharmacol 2011; 138: 22-33.

[14] Nashine S, Liu Y, Kim BJ, Clark AF, Pang IH. Role of C/EBP homologous protein in retinal ganglion cell death after ischemia/reperfusion injury. Invest Ophthalmol Vis Sci 2014; 56: 221-31.

[15] Goldenberg-Cohen N, Dadon S, Avraham BC, Kramer M, Hasanreisoglu M, Eldar I, Weinberger D, Bahar I. Molecular and histological changes following central retinal artery occlusion in a mouse model. Exp Eye Res 2008; 87: 327-33.

[16] Li SY, Fu ZJ, Ma H, Jang WC, So KF, Wong D, Lo AC. Effect of lutein on retinal neurons and oxidative stress in a model of acute retinal ischemia/reperfusion. Invest Ophthalmol Vis Sci 2009; 50: 836-43.

[17] Daugeliene L, Niwa M, Hara A, Matsuno H, Yamamoto T, Kitazawa Y, Uematsu T. Transient ischemic injury in the rat retina caused by thrombotic occlusion-thrombolytic reperfusion. Invest Ophthalmol Vis Sci 2000; 41: 2743-7.

[18] Lavinsky D, Arterni NS, Achaval M, Netto CA. Chronic bilateral common carotid artery occlusion: a model for ocular ischemic syndrome in the rat. Graefes Arch Clin Exp Ophthalmol 2006; 244: 199-204.

[19] Schmid H, Renner M, Dick HB, Joachim SC. Loss of inner retinal neurons after retinal ischemia in ratsinner retinal neuron loss. Invest Ophthalmol Vis Sci 2014; 55: 2777-2787.
[20] Kuehn MH, Fingert JH, Kwon YH. Retinal ganglion cell death in glaucoma: mechanisms and neuroprotective strategies. Ophthalmol Clin North Am 2005; 18: 383-95, vi.
[21] Zhang Y, Cho CH, Atchaneeyasakul LO, McFarland T, Appukuttan B, Stout JT. Activation of the mitochondrial apoptotic pathway in a rat model of central retinal artery occlusion. Invest Ophthalmol Vis Sci 2005; 46: 2133-9.
[22] Lalier L, Cartron PF, Juin P, Nedelkina S, Manon S, Bechinger B, Vallette FM. Bax activation and mitochondrial insertion during apoptosis. Apoptosis 2007; 12: 887-96.
[23] Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407: 770-776.
[24] Antonsson B. Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. Mol Cell Biochem 2004; 256-257: 141-55.
[25] Cheung ZH, Chan YM, Siu FK, Yip HK, Wu W, Leung MC, So KF. Regulation of caspase activation in axotomized retinal ganglion cells. Mol Cell Neurosci 2004; 25: 383-93.
[26] Kermer P, Ankerhold R, Klocker N, Krajewski S, Reed JC, Bahr M. Caspase-9: involvement in secondary death of axotomized rat retinal ganglion cells in vivo. Brain Res Mol Brain Res 2000; 85: 144-50.
[27] Nikolaev A, McLaughlin T, O'Leary DD, Tessier-Lavigne M. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature 2009; 457: 981-9.
[28] Singh M, Savitz SI, Hoque R, Gupta G, Roth S, Rosenbaum PS, Rosenbaum DM. Cell-specific caspase expression by different neuronal phenotypes in transient retinal ischemia. J Neurochem 2001; 77: 466-75.
[29] Tchedre KT, Yorio T. Sigma-1 receptors protect RGC-5 cells from apoptosis by regulating intracellular calcium, Bax levels, and caspase-3 activation. Invest Ophthalmol Vis Sci 2008; 49: 2577-88.