RESEARCH ARTICLE

Astragaloside IV protects RGC-5 cells against oxidative stress

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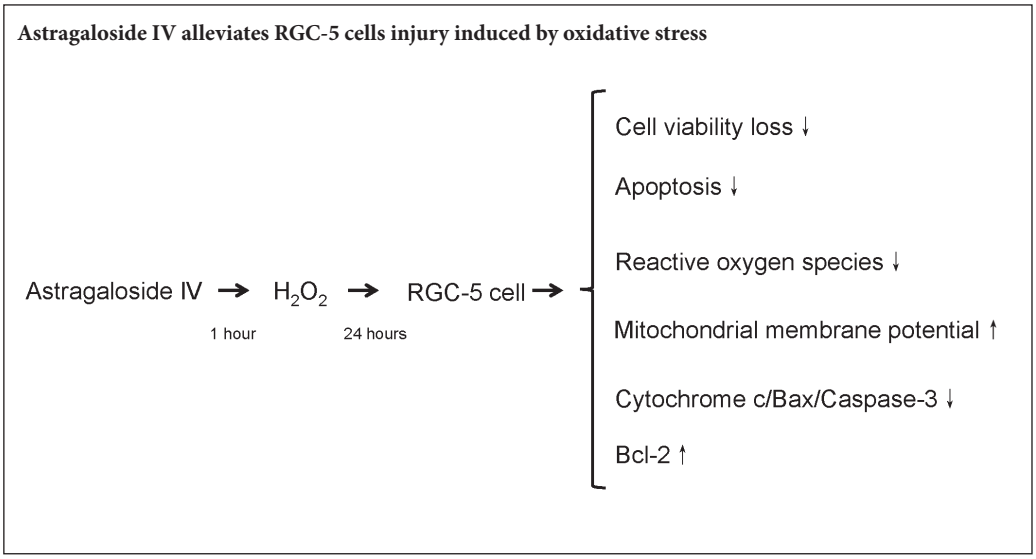
Graphical Abstract



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Abstract

Astragaloside IV is the main active compound of *Astragalus membranaceus*. Astragaloside IV has strong anti-oxidative activities and protective effects against progression of peripheral neuropathy. In this study, we determined whether astragaloside IV protects retinal ganglion cells (RGC) from oxidative stress injury using the rat RGC-5 cell line. Hydrogen peroxide (H_2O_2) was used to induce oxidative stress injury, with the protective effect of astragaloside IV examined. Cell Counting Kit-8 and 4′,6-diamidino-2-phenylindole staining showed that astragaloside IV increased cell survival rate and decreased apoptotic cell number. Flow cytometry showed that astragaloside IV decreased H_2O_2-induced reactive oxygen species levels. While laser confocal microscopy showed that astragaloside IV inhibited the H_2O_2-induced decrease of mitochondrial membrane potential. Western blot assay showed that astragaloside IV reduced cytochrome c release induced by H_2O_2, inhibited Bax and caspase-3 expression, and increased Bcl-2 expression. Altogether, these results indicate that astragaloside IV has potential protective effects against H_2O_2-induced oxidative stress in retinal ganglion cells.

Key Words: *nerve regeneration; Astragalus membranaceus; hydrogen peroxide; H_2O_2; retinopathy; neuroprotective effects; retinal ganglion cells; apoptosis; reactive oxygen species; mitochondrial membrane potential; mitochondrial pathway; neural regeneration*

Introduction

Astragalus membranaceus is a traditional herb and food used in China and India that is also used as a food additive. *Astragalus membranaceus* can treat the common cold, fatigue, diarrhea, and cardiac disease (Sun et al., 2008; Wang et al., 2008). *Astragalus membranaceus* is an immunomodulating agent that alleviates immunodeficiency disease (Ma et al., 2002). Recently, an increasing number of reports have shown *Astragalus membranaceus*-induced protection of the nervous system. *Astragalus membranaceus* can enhance recovery of stroke patients by reducing the cerebral infarction area, and also has anti-oxidative qualities (Xu et al., 2008; Chen et al., 2012). Studies have shown that *Astragalus membranaceus* markedly inhibits oxidative stress and improves oxygen-free radical-scavenging abilities (Zhang et al., 2009). Astragaloside IV (As-IV), or 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosylcycloastragenol (chemical structure shown in **Figure 1**), is the main active compound of *Astragalus membranaceus*. Previous studies have shown that As-IV has strong anti-inflammatory, anti-cerebral edema, anti-cardiac hypertrophy, anti-diabetic, and anti-oxidative activities, with protective effects against progression of peripheral neuropathy (Li et al., 2014; Lu et al., 2014; Sun et al., 2016; Qiao et al., 2017).

Oxidative stress is involved in vision-threatening diseases including diabetic retinopathy, age-related macular degen-

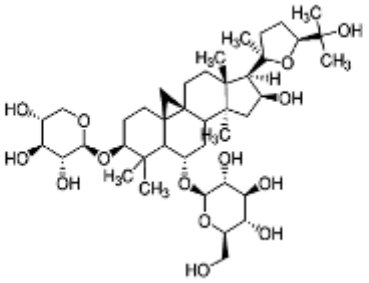

Figure 1 Chemical structure of astragaloside IV.

eration, glaucoma, uveoretinitis, and many other retinal diseases (Bearse et al., 2004; Klein et al., 2007; Meyer-Rüsenberg et al., 2007; Yau et al., 2012). Because of higher oxygen consumption in the retina, it is more easily damaged by oxidative damage than most other body tissues (Zhang et al., 2008b; Han et al., 2016). Increased reactive oxygen species (ROS) affect neuronal cells, causing neuronal cell apoptosis and visual impairment (Ozawa et al., 2011; Masuda et al., 2017). Although anti-oxidative stress is beneficial for the treatment of several ocular diseases, an effective treatment has not yet been developed.

In the present study, we used an oxidative stress model for retinal diseases. Specifically, we used hydrogen peroxide (H_2O_2) to induce cellular damage in the retinal ganglion cell (RGC)-5 line. The RGC-5 cell line has the advantages of generational stability and straightforward survival. Indeed, the molecular mechanism and maturation of cells are basically the same as for primary RGCs. Accordingly, this cell line has been widely used in ophthalmology research in recent years. In this study, we determined whether As-IV attenuates impairments in damaged RGC-5 cells and investigated the mitochondrial mechanism of As-IV-mediated protection.

Materials and Methods

RGC-5 cell culture and cell survival assay

Rat RGC-5 cells (PTA6600) were purchased from the cell collection of the American Type Culture Collection, which have been verified to be of the correct lineage. Cells cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Beijing, China), containing 10% fetal bovine serum with 100 U/mL of penicillin and 100 μg/mL of streptomycin, were incubated in 5% CO_2 at 37°C. Cells were passaged at a ratio of approximately 1:8 every 2 to 3 days. To passage, 5000 cells/well were dispensed into 96-well plates. To build an oxidative stress cell model, the plate was pre-incubated for 24 hours in a humidified incubator at 37°C, 5% CO_2 (HF90; Heal Force, Hong Kong, China). Cells were switched to DMEM containing 0 μM H_2O_2 group (control group) or 100, 200, 400, 600, or 800 μM H_2O_2 to determine the optimal concentration of H_2O_2 for establishing RGC-5 oxidative stress. Cells were incubated for 24 hours. Next, 10 μL of Cell Counting Kit-8 (CCK-8) solution (C0038; Beyotime, Hunan, China) was added to each well and incubated for 2 hours. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The appropriate concentration of H_2O_2 (600 μM) for the RGC-5 cell line was initially measured.

RGC-5 cells were plated into 96-well plates at a density of 5×10^3 or 10^5 cells per well of a 6-well plate. Cells were incubated for 24 hours in a humidified incubator at 37°C, 5% CO_2. Cells were then divided into six groups. The control group was incubated with normal culture medium without As-IV or H_2O_2. The intervention group included five subgroups, which were pre-treated with various concentrations (5, 10, 50, 100, or 200 mg/L) of As-IV for 1 hour. After removing As-IV, cells were incubated with medium containing H_2O_2 (600 μM) for an additional 24 hours. RGC-5 cells were incubated in 96-well plates. After H_2O_2 exposure, 10 μL of CCK-8 solution was added to each well and incubated for 2 hours. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad), followed by initial measurement of the appropriate concentration of As-IV (100 mg/L) for the RGC-5 cell line.

Nuclear staining for assessment of apoptosis

RGC-5 cells were plated in 6-well plates, pre-treated with As-IV (100 mg/L), and then incubated with H_2O_2 for 24 hours. Cells were washed with phosphate-buffered saline (PBS) and stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL) at 37°C for 10 minutes. Cells were washed with PBS. A fluorescence microscope (TE2000-U; Nikon, Tokyo, Japan) was used to capture fluorescence images.

Intracellular ROS production

Intracellular ROS was examined using the dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Intracellular radical species (H_2O_2, OH^-, and O^{2-}) can oxidize nonfluorescent dichlorofluorescein (DCFH) to fluorescent dichlorofluorescein (DCF). RGC-5 cells were pre-treated with As-IV (100 mg/L) and then incubated with H_2O_2 for 24 hours. DCFH-DA dye (10 μM) was added and incubated for 30 minutes at 37°C. Cells were washed twice with PBS and fluorescence analyzed by flow cytometry (FACS Aria™ Cell Sorter; BD, Franklin Lakes, NJ, USA) through the FL1 channel.

Mitochondrial membrane potential

Mitochondrial membrane potential (Δψm) was measured using the fluorescent probe, JC-1 (Beyotime). JC-1 is capable of selectively entering mitochondria where it forms monomers and emits green fluorescence when Δψm is relatively low. At high Δψm, JC-1 aggregates and emits a red fluorescence. After drug treatment, cells were incubated in 6-well plates and 0.5 mL JC-1 working solution added for 20 minutes at 37°C. JC-1 staining buffer was used to wash cells twice. Cells were scanned and imaged on a confocal microscope (Zeiss 510; Zeiss, Oberkochen, Germany).

Western blot assay

After the designated treatment, cells were collected and washed with PBS. Cellular proteins were extracted with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentration was determined using the BCA Pro-

tein Assay Kit (P0011; Beyotime). Protein samples (40 μg) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat dried milk at room temperature for 1 hour, and then incubated with primary antibodies overnight at 4°C. Membranes were washed with Tris-buffered saline–Tween 20 and incubated with the appropriate secondary antibodies at room temperature for 1 hour. The primary antibodies used were rabbit anti-cytochrome c polyclonal antibody (1:1000; sc-7159; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Bax polyclonal antibody (1:1000; sc-526; Santa Cruz), rabbit anti-Bcl-2 polyclonal antibody (1:1000; sc-492; Santa Cruz Biotechnology), rabbit anti-caspase-3 polyclonal antibody (1:1000; 9662; Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-β-actin polyclonal antibody (1:1000; TA-09; ZSGB-BIO, Beijing, China). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (1:50,000; ZSGB-BIO). Afterwards, membranes were washed again with Tris-buffered saline–Tween 20. Membranes were incubated with enhanced chemiluminescence (P0018A; Beyotime) and detected using the Chemi-Doc XRS gel documentation system (Bio-Rad). Protein bands were quantified by Image Lab (Bio-Rad), with β-actin used as an internal control.

Statistical analysis

Values are presented as the mean ± SD, and were processed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by Student-Newman-Keuls test was used to determine the significance of differences between means. All experiments were performed at least three times. $P < 0.05$ was considered statistically significant.

Results

As-IV protected RGC-5 cells against H_2O_2-induced toxicity

To investigate H_2O_2-induced cytotoxicity, RGC-5 cells were incubated with increasing concentrations of H_2O_2, and cell survival rate determined using CCK-8 at 24 hours after incubation. As shown in **Figure 2A**, H_2O_2 at 100 to 800 μM significantly induced cytotoxicity ($P < 0.05$). Compared with the control group, cell survival rate in the 600 μM H_2O_2 group was approximately 50%. Therefore, 600 μM H_2O_2 was used in the following experiments.

To confirm the protective effect of As-IV on RGC-5 cells exposed to H_2O_2, serial concentrations of As-IV (200, 100, 50, 10, and 5 mg/L) were incubated with RGC-5 cells prior to induction of H_2O_2. Cell survival rates of H_2O_2-induced RGC-5 cells incubated with As-IV improved (**Figure 2B**). Moreover, as the dose of As-IV increased, cell survival rate visibly increased. The optimum concentration of As-IV was 100 mg/L, which was used in the following study.

As-IV reduced H_2O_2-induced apoptosis of RGC-5 cells

Apoptotic morphology is typified by physiological cell death and includes chromatin condensation and DNA fragmentation. DAPI was used to measure apoptosis. The control group (**Figure 3A**) showed homogeneous and round nuclei, while nuclei in the H_2O_2 group showed chromatin condensation and DNA fragmentation (**Figure 3B**). In **Figure 3C**, pre-treatment with As-IV at 100 mg/L induced a significant reduction of apoptosis in RGC-5 cells.

As-IV prevented intracellular ROS accumulation in RGC-5 cells in H_2O_2 medium

Accumulation of ROS is a critical indicator of H_2O_2-induced oxidative stress. As illustrated in **Figure 4**, average fluorescence intensity of DCFH increased after hydrogen peroxide was added (peak value to right deviation). Furthermore, when AS-IV was added, average fluorescence intensity decreased (peak value to left deviation). These results indicate that H_2O_2 visibly increases intracellular ROS levels, whereas pre-treatment with As-IV induced a marked decrease in ROS production.

As-IV inhibited loss of mitochondrial membrane potential in H_2O_2 medium

Loss of mitochondrial membrane potential is a marker of primary cell apoptosis. After 24 hours of H_2O_2 exposure, mitochondrial membrane potential in the H_2O_2 group was reduced (**Figure 5B**), reflected by increased green fluorescence. However, As-IV-treated cells mitigated this loss of mitochondrial membrane potential, indicating a protective effect of As-IV (**Figure 5C**).

As-IV supported cytochrome c release, suppressed intracellular Bax and caspase-3 expression levels, and increased Bcl-2 expression

Expression of apoptosis-related proteins was examined by western blot assay. **Figure 6** shows higher Bax expression in the H_2O_2 group compared with the control group ($P < 0.05$). However, pre-treatment of RGC-5 cells with 100 mg/L As-IV, followed by 600 μM H_2O_2 for 24 hours, significantly suppressed H_2O_2-induced expression of Bax. Compared with the H_2O_2 group, As-IV prevented the decrease in Bcl-2 expression ($P < 0.05$). Release of cytochrome c from mitochondria to the cytoplasm is an indicator of mitochondrial dysfunction, which ultimately triggers apoptosis *via* a caspase-3 pathway.

Representative immunoblots (**Figure 7A**) show cytochrome c and caspase-3 expression in the control, H_2O_2, and As-IV groups. Compared with the H_2O_2 group ($P < 0.05$), substantial decrease of cytosolic cytochrome c was found in cells pre-treated with As-IV (**Figure 7B**), while caspase-3 was partially suppressed (**Figure 7C**). Thus, our results indicate that As-IV has an anti-apoptotic effect.

Discussion

Oxidative stress participates in over 50 common diseases and is defined by an imbalance between the oxidant and antioxidant systems (Kowluru et al., 2007; Zhang et al., 2008a; Zhao et al., 2010; Zhu et al., 2012). However, intracellular signaling pathways of H_2O_2-induced cell death have not extensively investigated (Cai., 2013). Compared with other

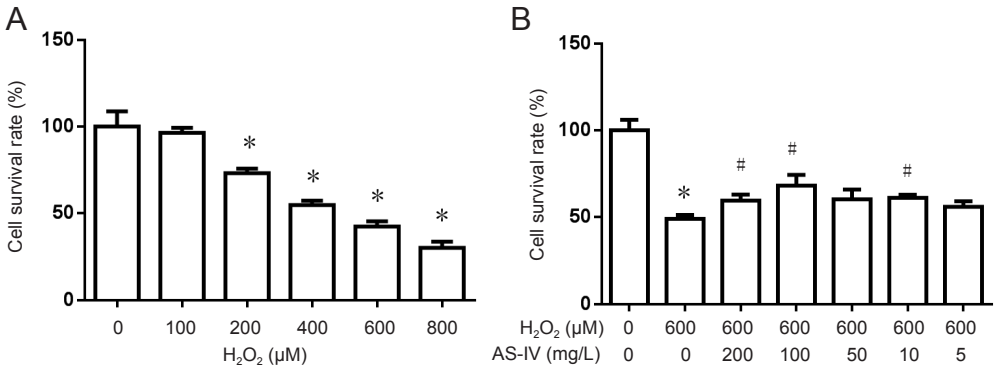

Figure 2 Astragaloside IV improved survival rate of RGC-5 cells following exposure to hydrogen peroxide (CCK-8 assay).

(A) Survival comparison of control and hydrogen peroxide (H_2O_2) groups: RGC-5 cells were maintained *in vitro* and H_2O_2 added at concentrations of 100, 200, 400, 600, and 800 μM. In the control group, H_2O_2 was not added. Histograms of the results were plotted. The objective was to obtain a concentration 50% lower than the control group. (B) Survival rate at which astragaloside IV (As-IV) protects RGC-5 cells from H_2O_2: cells were pre-treated with various concentrations (5, 10, 50, 100, and 200 mg/L) of As-IV for 1 hour. The H_2O_2 group was incubated in the same H_2O_2 medium but without As-IV. ∗$P < 0.05$, *vs.* control group (control group was incubated in the same medium but without As-IV or H_2O_2); #$P < 0.05$, *vs.* H_2O_2 group (mean ± SD, one-way analysis of variance followed by the Student-Newman-Keuls test). Experiments were performed at least three times.

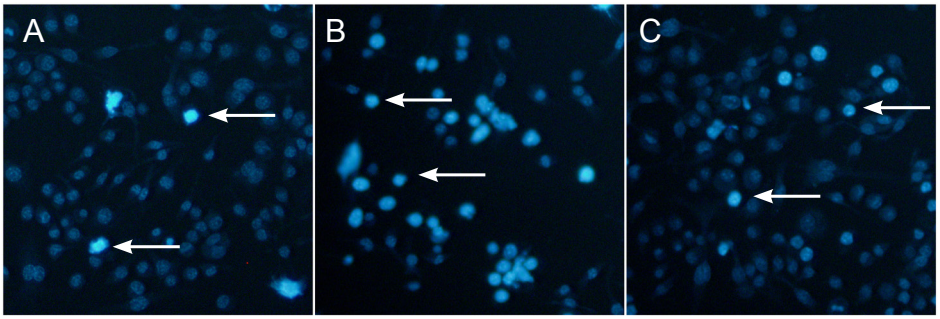

Figure 3 Astragaloside IV suppressed RGC-5 cell apoptosis induced by hydrogen peroxide DAPI staining, fluorescence microscopy).
RGC-5 DAPI staining was observed by immunofluorescence microscopy (× 200). Arrows show cell nuclear condensation (bright blue) representing apoptosis. (A) Control group; (B) hydrogen peroxide group; and (C) astragaloside IV group. DAPI: 4′,6-Diamidino-2-phenylindole

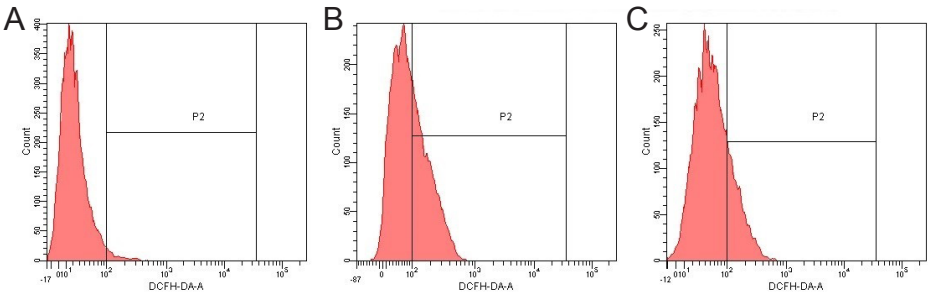

Figure 4 Astragaloside IV prevented intracellular reactive oxygen species accumulation in RGC-5 cells.
Representative plots show the positive status of intracellular reactive oxygen species in RGC-5 cells in the control group (A), hydrogen peroxide group (B), and astragaloside IV group (C).

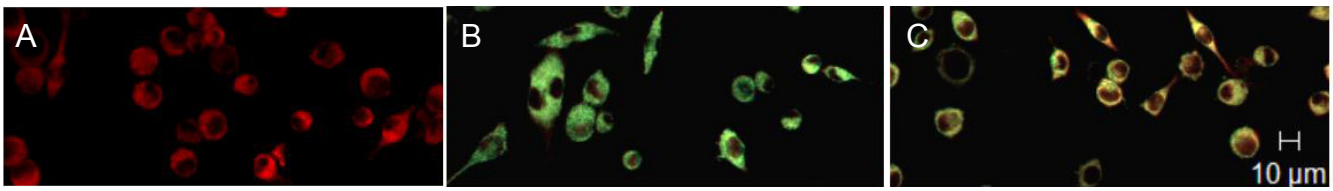

Figure 5 Astragaloside IV inhibited hydrogen peroxide-induced loss of mitochondrial membrane potential in RGC-5 cells.
Laser confocal microscopy shows changes in RGC-5 mitochondrial membrane potential, which was measured using the fluorescent probe JC-1. At high mitochondrial membrane potential, JC-1 aggregates emit a red fluorescence. At low mitochondrial membrane potential, JC-1 emits a green fluorescence. A mixture of red and green fluorescence is observed in (C). This represents the mitochondrial membrane potential in RGC-5 cells from the astragaloside IV group (C), which is higher compared with hydrogen peroxide group (B). (A) Control group.

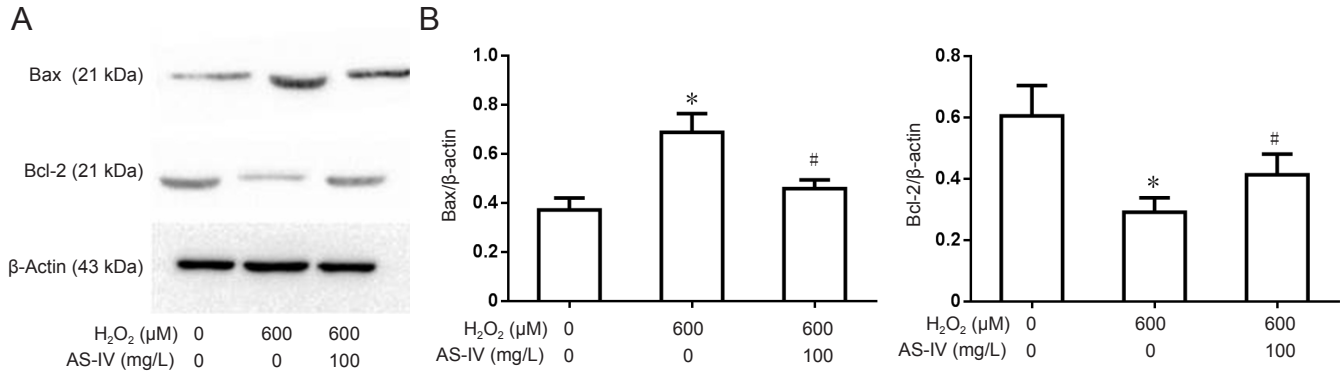

Figure 6 Effect of astragaloside IV on Bax and Bcl-2 expression.
(A) Expression of Bax and Bcl-2 in the control, hydrogen peroxide (H_2O_2), and astragaloside IV (As-IV) groups. (B) Quantitative analysis of the optical density of Bax and β-actin. (C) Quantitative analysis of the optical density of Bcl-2/β-actin. *P < 0.05, *vs.* control group; #P < 0.05, *vs.* H_2O_2 group (mean ± SD, one-way analysis of variance followed by the Student-Newman-Keuls test). Experiments were performed at least three times.

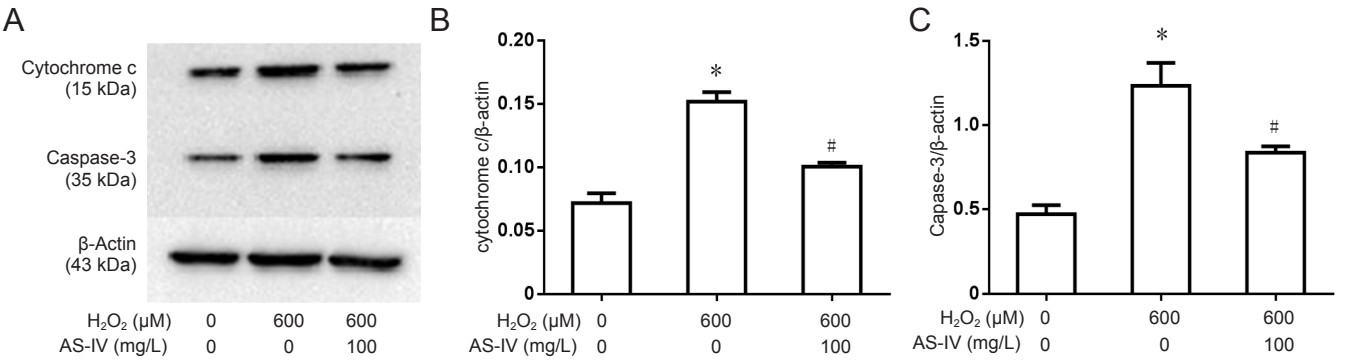

Figure 7 Effect of astragaloside IV on cytochrome c and caspase-3 expression in RGC-5 cells following exposure to H_2O_2 (western blot assay).
(A) Expression of cytochrome c and caspase-3 in the control, H_2O_2, and astragaloside IV (As-IV) groups. (B) Quantitative analysis of cytochrome c/β-actin band density. (C) Quantitative analysis of caspase-3 and β-actin band density. *P < 0.05, *vs.* control group; #P < 0.05, *vs.* H_2O_2 group (mean ± SD, one-way analysis of variance followed by the Student-Newman-Keuls test). Experiments were performed at least three times.

ROS, H_2O_2 plays a key role because it is relatively stable, generated from nearly all sources of oxidative stress (Zhao et al., 2011), and is freely diffusible within and between cells. Exogenous H_2O_2 can penetrate cells and induce high membrane permeability (Halliwell et al., 1992), which is predominantly generated during mitochondrial oxidative metabolism (Ray et al., 2012). Our study confirmed that RGC-5 cells incubated with H_2O_2 led to a dose-dependent loss in viability. *Astragalus membranaceus* can improve energy metabolism and inhibit apoptosis to alleviate nerve injury (Zhang et al., 2010; Huang et al., 2012). Many studies have shown that As-IV suppresses cell apoptosis (Chan et al., 2009, Sun et al., 2014). Furthermore, As-IV pre-treatment markedly and dose-dependently protects neurons (Chan et al., 2009), which is consistent with our study.

Pathogenesis of retinopathy not only relates to retinal vessels but is also strongly associated with neurons and glial cells of the retina. Appropriately, application of neuroprotective drugs can delay the occurrence of retinopathy (Zhang et al., 2011). In the retina, RGCs reflect the earliest differentiation of neurons and play a crucial role in visual signal processing of dark adaptation. Neurotrophic factors have been shown to protect neuronal and retinal cells, and delay the start of retinopathy (Hammes et al., 1995; Zhang et al., 2008b). Nevertheless, at present there is no study using As-IV for treatment for retinopathy. Here, using the RGC-5 cell line, we show a protective effect of As-IV that involves neuroprotective mechanisms through the anti-oxidative stress pathway. These results are coincident with previous studies showing that As-IV has diverse pharmacological properties such as anti-oxidative stress and neuroprotective effects (Chan et al., 2009). Thus, in RGCs, As-IV may have a neuroprotective effect that delays the start of retinopathy. This is a novel finding that has not been previously reported.

A major limitation of our current study is culture of the RGC-5 cell line *in vitro*. Consequently, we solely examined the effect of oxidative stress on cells and have not reproduced the in vivo environment. As-IV has been shown to have potential for prevention and treatment of retinal diseases including diabetic retinopathy, age-related macular degeneration, and glaucoma. In the future, we will further study the effect of As-IV *in vivo*.

In summary, our study shows for the first time that As-IV protects RGC-5 cells from H_2O_2-induced oxidative stress

and apoptosis. Our findings will aid understanding of the underlying mechanisms of retinal injury.

Author contributions: *HYK designed the study. MH performed experiments. YL and PC analyzed data. HJ and MH wrote the paper. All authors approved the final version of the paper.*
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Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
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