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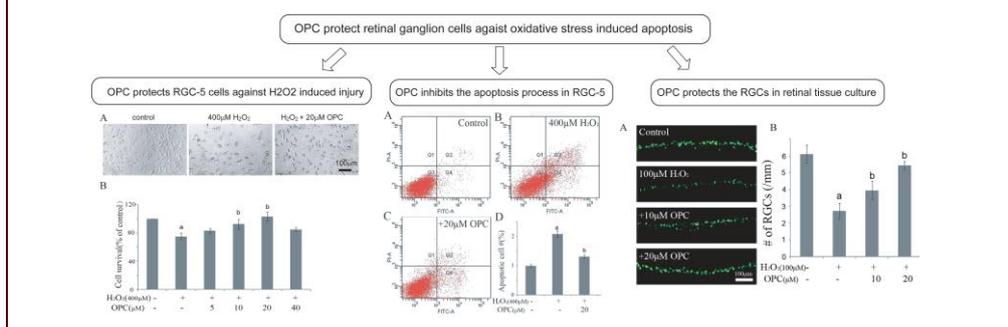
# Oligomeric proanthocyanidin protects retinal ganglion cells against oxidative stress-induced apoptosis

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## Graphical abstract



## Abstract

The death of retinal ganglion cells is a hallmark of many optic neurodegenerative diseases such as glaucoma and retinopathy. Oxidative stress is one of the major reasons to cause the cell death. Oligomeric proanthocyanidin has many health beneficial effects including antioxidative and neuro-protective actions. Here we tested whether oligomeric proanthocyanidin may protect retinal ganglion cells against oxidative stress induced-apoptosis *in vitro*. Retinal ganglion cells were treated with hydrogen peroxide with or without oligomeric proanthocyanidin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that treating retinal ganglion cell line RGC-5 cells with 20 μmol/L oligomeric proanthocyanidin significantly decreased the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced death. Results of flow cytometry and Hoechst staining demonstrated that the death of RGC-5 cells was mainly caused by cell apoptosis. We further found that expression of pro-apoptotic Bax and caspase-3 were significantly decreased while anti-apoptotic Bcl-2 was greatly increased in H<sub>2</sub>O<sub>2</sub> damaged RGC-5 cells with oligomeric proanthocyanidin by western blot assay. Furthermore, in retinal explant culture, the number of surviving retinal ganglion cells in H<sub>2</sub>O<sub>2</sub>-damaged retinal ganglion cells with oligomeric proanthocyanidin was significantly increased. Our studies thus demonstrate that oligomeric proanthocyanidin can protect oxidative stress-injured retinal ganglion cells by inhibiting apoptotic process.

## Key Words

neural regeneration; traditional Chinese medicine; retinal ganglion cell; oligomeric proanthocyanidin; oxidative stress; hydrogen peroxide; retinal explants; apoptosis; grants-supported paper; neuroregeneration

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**Supplementary information:** Supplementary data associated with this article can be found, in the online version, by visiting [www.nrnonline.org](http://www.nrnonline.org).

## INTRODUCTION

Retinal ganglion cells are critical neurons in the retina that transmit visual signals from the retina to the brain. Diabetic retinopathy and many optic nerve diseases including glaucoma, optic neuritis, and ischemic optic neuropathy are some of the vision impairments afflicting the retinal ganglion cells of the eye<sup>[1]</sup>. These diseases occur *via* a variety of mechanisms involving, for example, oxidative stress<sup>[2]</sup>, excitatory amino acid (glutamate)<sup>[3]</sup>, nitric oxide<sup>[4]</sup>, or reduced retinal perfusion<sup>[5]</sup>. Among these mechanisms, oxidative stress, leading to the formation of free radicals, has been implicated as part of the final common pathway for neurotoxicity in a variety of acute and chronic neurodegenerative diseases<sup>[6-7]</sup>. Therefore, it is important to reduce the death of retinal ganglion cells and develop neuroprotective strategies for retinal ganglion cells against oxidative stress.

Proanthocyanidin, a group of antioxidants subclass of polyphenols, is present abundantly in fruits such as apple, pear, grapes, and also in daily diet like chocolate, wine and tea<sup>[8]</sup>. Due to potent antioxidative activity, proanthocyanidins have been the subject of recent research, demonstrating antibacterial<sup>[9]</sup>, antiviral<sup>[10-11]</sup>, anticarcinogenic<sup>[12]</sup>, anti-inflammatory<sup>[13-14]</sup>, antiallergicvasodilatory<sup>[15-16]</sup>, antismelling<sup>[17]</sup> and neuroprotective activities<sup>[18-21]</sup>. More recently, Yang *et al*<sup>[22]</sup> verified that the grape seed extract was able to protect oxidative stressed-induced retinal ganglion cell damage by using differentiated retinal ganglion cell line RGC-5 cells. Based on these reports, we hypothesized that oligomeric proanthocyanidins, the most abundant phenolic compounds in grape seeds<sup>[23]</sup>, may protect retinal ganglion cells against oxidative stress. Thus we tested the protective effect of oligomeric proanthocyanidin on retinal ganglion cells against H<sub>2</sub>O<sub>2</sub> induced damage by using both RGC-5 cell line and retinal explant culture.

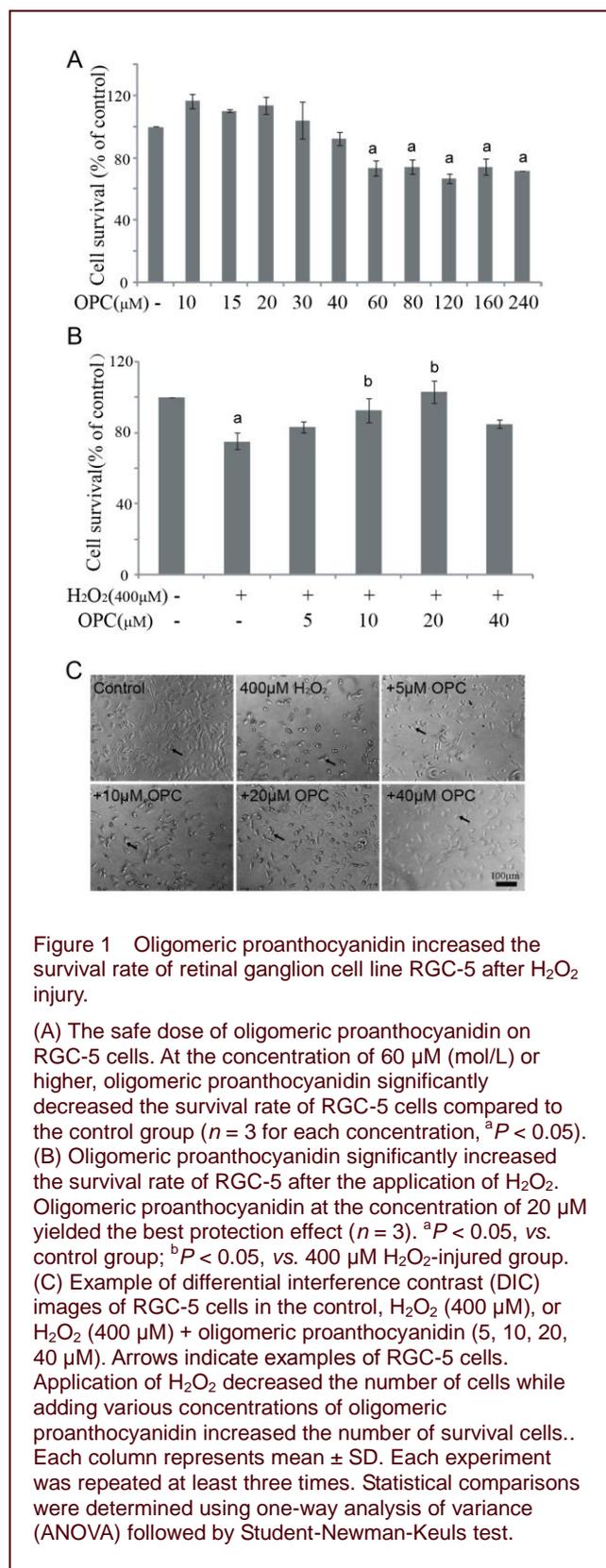
There are two major pathways of oxidative stress-induced apoptosis, including mitochondrial-mediated or receptor-mediated pathways. Both pathways lead to caspase-3 activation and cell death. In the mitochondrial pathway, the Bcl-2 family of pro- and anti-apoptotic proteins is the key regulator. For example, Bcl-2 inhibits apoptosis by inhibiting cytochrome c release and caspase activation, while Bax promotes apoptosis by inducing the release of cytochrome c, which then triggers the downstream apoptosis event<sup>[24]</sup>. Thus, in the current experiment, we tested whether oligomeric proanthocyanidin could reduce the cytochrome c-Bcl-2-Bax-caspase-3 mitochondrial-mediated apoptosis by measuring the expression change of Bax, Bcl-2 and caspase-3.

## RESULTS

### Oligomeric proanthocyanidin protected RGC-5 cells against H<sub>2</sub>O<sub>2</sub> induced injury

Before the continued experiment, we first identified the retinal ganglion cell line RGC-5 by immunochemical staining for Thy1 and Brn3 (retinal ganglion cells specific antibodies)<sup>[25]</sup> and found all the cells in culture were stained for Thy1 and Brn3 (data not shown). We further tested with glial fibrillary acidic protein (GFAP) antibody (antibody against glial cell) and there were no cells stained for GFAP, which confirmed that RGC-5 cells we used were transformed neurons.

We then screened for the safe dose range of oligomeric proanthocyanidin using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Among the series of concentrations that we tested, 20 and 40 μmol/L oligomeric proanthocyanidin did not cause obvious death in RGC-5 ( $P > 0.05$ ). But when we increased the concentration to 60 μmol/L and even higher at 240 μmol/L, all the survival rates of RGC-5 were significantly lower than the control group ( $P < 0.05$ ; Figure 1A). Thus for future test, we used the concentration of oligomeric proanthocyanidin lower than 40 μmol/L.



We next tested the protective effect of oligomeric proanthocyanidin on RGC-5 cells against the injury by H<sub>2</sub>O<sub>2</sub>. With 400 μmol/L H<sub>2</sub>O<sub>2</sub> alone in the medium for 7 hours, the survival rate of RGC-5 dropped to 0.75 times

( $n = 3$ ) of the control group ( $P < 0.05$ ). Treating cells with oligomeric proanthocyanidin significantly increased the survival rate to higher than 0.83 times ( $P < 0.05$ ; Figure 1B, C) of the control group. Oligomeric proanthocyanidin at the concentration of 20 μmol/L showed the highest survival rate at 1.03 times of control group, indicating that oligomeric proanthocyanidin was able to protect RGC-5 against H<sub>2</sub>O<sub>2</sub> injury.

### Oligomeric proanthocyanidin inhibited the apoptosis of RGC-5 cell line

Based on the data of cell viability, we used Hoechst 33324 staining method to evaluate the extent of apoptosis on RGC-5 cells induced by H<sub>2</sub>O<sub>2</sub>. The cells of control group displayed low-intensity blue fluorescence from the Hoechst dye and normal nuclear morphology (Figure 2A). In contrast, in the 400 μmol/L H<sub>2</sub>O<sub>2</sub> group, the number of cells with high-intensity nuclei, indicators of apoptosis, was significantly increased (Figure 2B). The percentage of apoptotic cells increased to 4.78 times of the control group ( $P < 0.05$ ). But the changes were attenuated by 20 μmol/L oligomeric proanthocyanidin (Figure 2C): the apoptotic cell numbers dropped from 4.78 to 1.43 times of the control group ( $P < 0.05$ ; Figure 2D). This result suggested a protective effect of oligomeric proanthocyanidin against apoptosis.

To verify whether oligomeric proanthocyanidin had anti-apoptotic effect in H<sub>2</sub>O<sub>2</sub>-induced RGC-5 cells, Annexin V-FITC/PI assay was applied. Flow cytometry results showed that 400 μmol/L H<sub>2</sub>O<sub>2</sub> induced 20–25% apoptosis in RGC-5 cells, which was almost twice as high as the control group ( $2.08 \pm 0.13$ ). After treatment with 20 μmol/L oligomeric proanthocyanidin, the ratio of the apoptotic cells was decreased to 10% (data for one experiment are shown in Figure 2E–G), which was 1.31 times of the control group and significantly lower than the injury group ( $P < 0.05$ ) (Figure 2H). This result showed that the oligomeric proanthocyanidin effectively suppressed H<sub>2</sub>O<sub>2</sub>-induced apoptosis in RGC-5 cells.

Then western blots were applied to test the expression of several apoptosis related proteins, including Bcl-2 (an inhibitor of apoptosis), Bax (a pro-apoptotic Bcl-2 family) and caspase-3<sup>[26-27]</sup>. After 400 μmol/L H<sub>2</sub>O<sub>2</sub>-induced injury, the expression of Bcl-2 significantly decreased to 0.58 times of the control group ( $P < 0.05$ ; Figure 3A, B), while the expression of Bax ( $P < 0.05$ ; Figure 3A, B) and caspase-3 significantly increased to 1.85 and 1.68 times of the control respectively ( $P < 0.05$ ; Figure 3C, D). After 10 and 20 μmol/L oligomeric proanthocyanidin treatment, those changes were reversed.

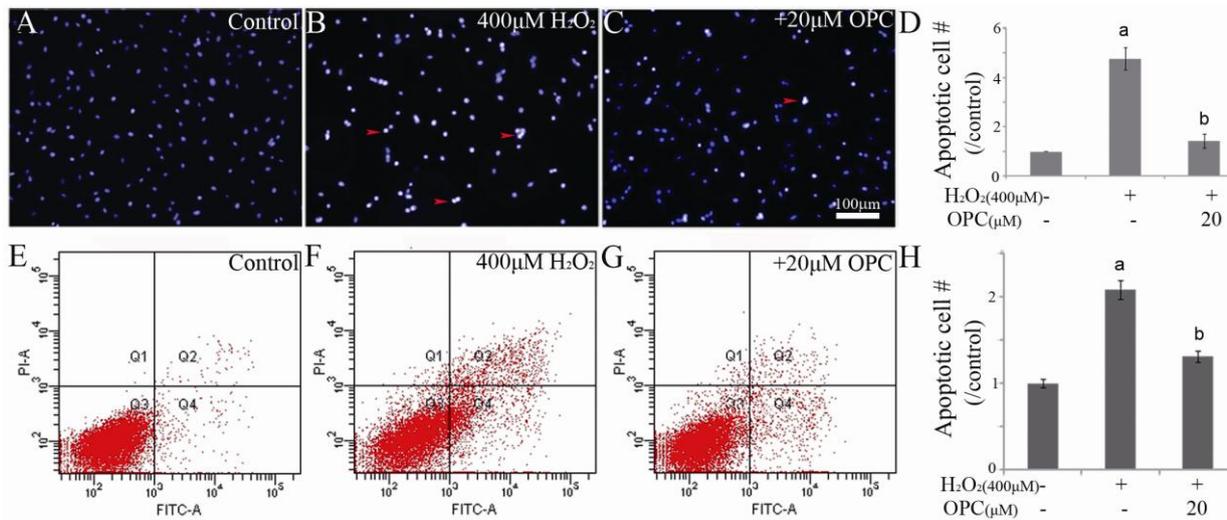


Figure 2 Oligomeric proanthocyanidin reduced H<sub>2</sub>O<sub>2</sub>-induced apoptosis in retinal ganglion cell line RGC-5.

(A–C) Hoechst 33342 staining of RGC-5 cells in the control, H<sub>2</sub>O<sub>2</sub> (400 μM) and H<sub>2</sub>O<sub>2</sub> (400 μM) + oligomeric proanthocyanidin (20 μM) groups respectively under fluorescence microscopy. The arrowheads pointed to apoptotic cells marked by high intensity of blue fluorescence and nuclear condensation. (D) Normalized number of the apoptotic cells identified by Hoechst staining. (E–G) Flow cytometry assay of RGC-5 cells in the control (E), H<sub>2</sub>O<sub>2</sub> (400 μM) (F) and H<sub>2</sub>O<sub>2</sub> (400 μM) + oligomeric proanthocyanidin (20 μM) groups (G). Early apoptotic cells are shown in PI(-)/FITC(+) quarter (Q3) and late apoptotic cells are shown in PI(+)/FITC(+) (Q4) quarter. (H) Normalized percentage of apoptotic cells in Q2 and Q4 regions. <sup>a</sup>*P* < 0.05, vs. control group; <sup>b</sup>*P* < 0.05, vs. H<sub>2</sub>O<sub>2</sub> group. Each column represents mean ± SD (*n* = 3). Each experiment was repeated at least three times. Statistical comparisons were determined using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. μM: μmol/L.

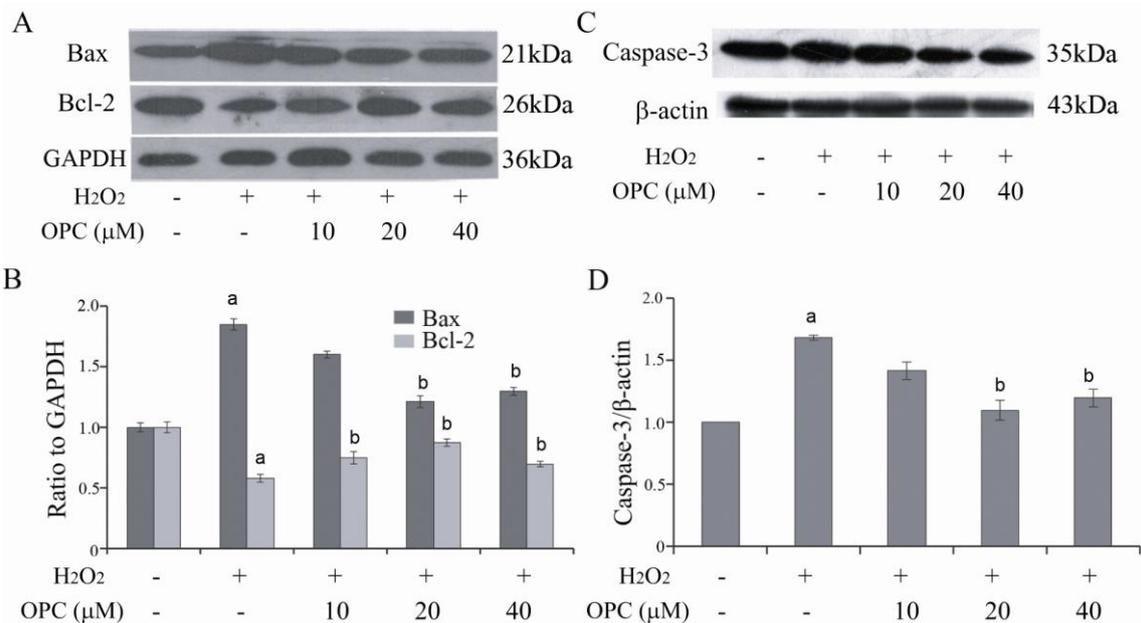


Figure 3 Oligomeric proanthocyanidin prevented H<sub>2</sub>O<sub>2</sub>-induced changes of apoptotic proteins in retinal ganglion cell line RGC-5.

(A) Representative immunoblots showing the expression of Bax and Bcl-2 under different conditions. GAPDH was used as the reference protein. (B) Normalized ratio of Bcl-2 and Bax to GAPDH in the control, H<sub>2</sub>O<sub>2</sub> (400 μM) and H<sub>2</sub>O<sub>2</sub> (400 μM) + oligomeric proanthocyanidin (10, 20, 40 μM) groups. (C) Representative immunoblots showing the expression of caspase-3 under different conditions. β-actin was used as the reference protein. (D) Normalized ratio of caspase-3 to β-actin. <sup>a</sup>*P* < 0.05, vs. the control group; <sup>b</sup>*P* < 0.05, vs. H<sub>2</sub>O<sub>2</sub> group. Each column represents mean ± SD (*n* = 3). Each experiment was repeated at least three times. Statistical comparisons were determined using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test.

After 20  $\mu\text{mol/L}$  oligomeric proanthocyanidin treatment, the expression of Bcl-2 increased from 0.58 to 0.87 times of the control group while Bax, caspase-3 dropped to 1.21 and 1.10 times of the control group respectively ( $P < 0.05$ ). The results indicated that oligomeric proanthocyanidin protected RGC-5 against  $\text{H}_2\text{O}_2$ -induced apoptosis by increasing the ratio of Bcl-2/Bax and inhibiting caspase-3 activity.

#### Oligomeric proanthocyanidin protected retinal ganglion cells in retinal tissue culture against $\text{H}_2\text{O}_2$ injury

In order to investigate if oligomeric proanthocyanidin could actually protect retinal ganglion cells in retina from  $\text{H}_2\text{O}_2$  injury, we checked the survival rate of retinal gan-

glion cells in cultured retinal tissue (Figure 4A). In the control group, the mean density of retinal ganglion cells was  $6.1 \pm 0.5/\text{mm}$ . In the 100  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  group, the mean density of retinal ganglion cells was  $2.7 \pm 0.5/\text{mm}$ , which was 56.5% lower than the control group ( $P < 0.05$ ; Figure 4B, C). In the oligomeric proanthocyanidin group, the retinal ganglion cells density was restored. In the 10  $\mu\text{mol/L}$  oligomeric proanthocyanidin group, the retinal ganglion cell density was recovered to  $3.9 \pm 0.7/\text{mm}$  (64.5% of the control group). In the 20  $\mu\text{mol/L}$  oligomeric proanthocyanidin group, the retinal ganglion cell density was restored to  $5.4 \pm 0.9/\text{mm}$  (88.8% of the control), which was significantly higher than the  $\text{H}_2\text{O}_2$  group ( $P < 0.05$ ). Our data thus proved that oligomeric proanthocyanidin was able to protect retinal ganglion cells *in vitro*.

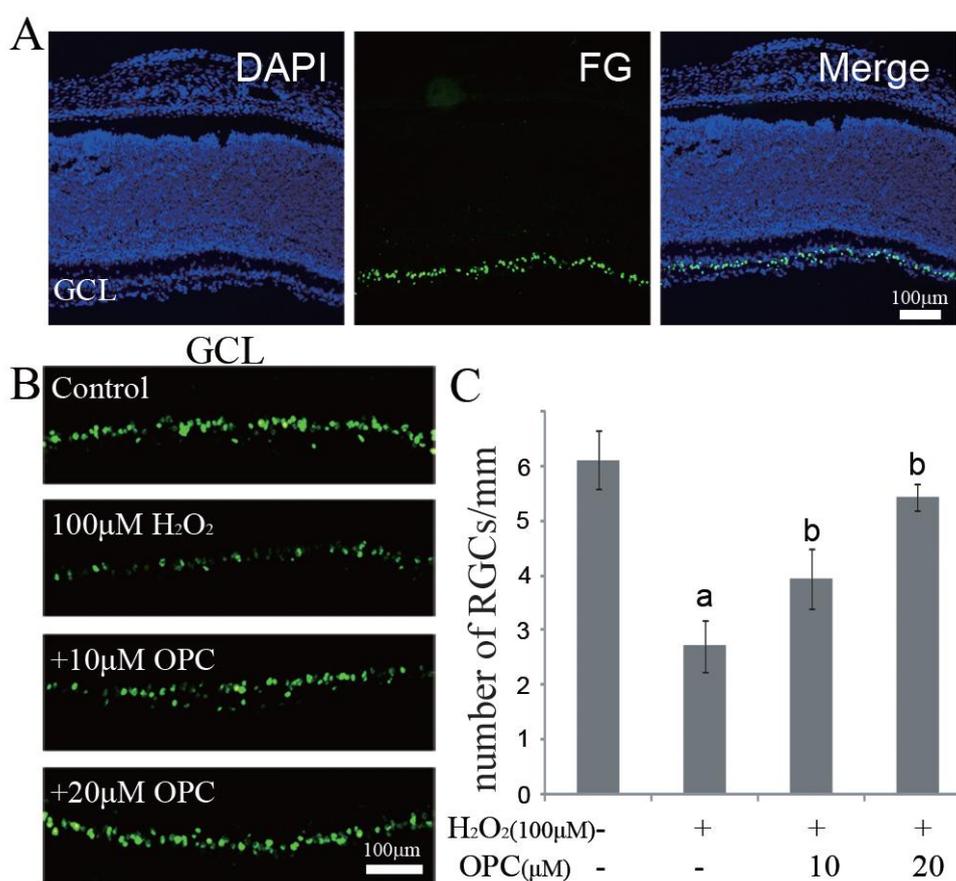


Figure 4 Oligomeric proanthocyanidin increased the survival rate of retinal ganglion cells in retinal explant culture after  $\text{H}_2\text{O}_2$  injury.

(A) DAPI staining of cultured retinal slices showed that all green fluorescence labeled cells were located in retinal ganglion cell layer (GCL), confirming that retinal ganglion cells (green cells) were back-labeled by flurogold (FG) injection in the superior colliculus. (B) Images of FG marked green retinal ganglion cells in the GCL layer of retinal slices under Leica microscope in the control,  $\text{H}_2\text{O}_2$  (400  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (400  $\mu\text{M}$ ) + oligomeric proanthocyanidin (10, 20  $\mu\text{M}$ ) groups. (C) Quantification of the number of retinal ganglion cells. The number of retinal ganglion cells was significantly decreased in the  $\text{H}_2\text{O}_2$  group compared to the control group ( $^aP < 0.05$ ;  $n = 3$ ). However, when we added oligomeric proanthocyanidin, the number of retinal ganglion cells was significantly increased from that in the injury group ( $^bP < 0.05$ ;  $n = 3$ ). Each column represents mean  $\pm$  SD. Each experiment was repeated at least three times. Statistical comparisons were determined using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test.  $\mu\text{M}$ :  $\mu\text{mol/L}$ .

## DISCUSSION

Our results demonstrate that oligomeric proanthocyanidin has a protective effect on retinal ganglion cells against H<sub>2</sub>O<sub>2</sub>-induced injury, as confirmed by using both RGC-5 cell lines and retinal explant culture. We further show that oligomeric proanthocyanidin inhibits the apoptosis induced by oxidative stress, possibly by inhibiting mitochondrial apoptosis pathway.

### Oligomeric proanthocyanidin protect retinal ganglion cells against oxidative stress induced apoptosis

In glaucoma, elevated intraocular pressure is generally taken as the most significant risk factor for accelerated retinal ganglion cells death. It is widely accepted that oxidative damage in response to pressure elevation is an important underlying mechanism of elevated intraocular pressure induced cellular damage and neuronal death<sup>[2, 28]</sup>. Thus finding an effective way to protect retinal ganglion cells against oxidative damage would provide a potential clinical application in the treatment of glaucoma.

In the present experiment, we used the established RGC-5 as a model cell type for retinal ganglion cells<sup>[29]</sup>. As a model of oxidative stress, we treated RGC-5 cells with hydrogen peroxide, which at 300 μmol/L, was sufficient to induce cell damage, including apoptosis<sup>[30]</sup>. By using RGC-5 cell line, we showed that oligomeric proanthocyanidin at 20 μmol/L almost completely blocked H<sub>2</sub>O<sub>2</sub>-induced RGC-5 cell apoptosis, as evaluated by the MTT cell viability assay, nuclear condensation, flow cytometry, increase in Bax and caspase-3 expression and decrease in Bcl-2 expression. Oligomeric proanthocyanidin at 20 μmol/L also rescued the retinal ganglion cells in primary retinal explant culture. Our result was consistent with Yang's report that the grape seed extract (rich in oligomeric proanthocyanidin) was able to protect oxidative induced retinal ganglion cell damage<sup>[22]</sup> using differentiated RGC-5 cell line. As grape seed extract has multiple components other than oligomeric proanthocyanidin, it is hard to exclude the contribution of other components in the protection of retinal ganglion cells. Here in our experiment, we showed for the first time that oligomeric proanthocyanidin (proanthocyanidine B5), found in grape seed extract and many other plants, protected retinal ganglion cells *in vitro*.

While RGC-5 cells have got some criticism for their derivation and purity as a cell line, they are believed to be retinal neuronal precursor cells<sup>[25]</sup>. Expressing markers of

retinal ganglion cells (Thy-1) in RGC-5 cells indicates that they are retinal ganglion cell-like neurons and can be used as a guide to retinal ganglion cells behavior. A greater significance is that our findings from the RGC-5 cells were able to be replicated with retinal ganglion cells directly from rat retinal explant. The protective effect of oligomeric proanthocyanidin should be investigated further in an animal model of retinal ganglion cell damage to determine whether oligomeric proanthocyanidin is a potential neuroprotective agent of retinal ganglion cells.

### Possible mechanism for the neuroprotective actions of oligomeric proanthocyanidin toward retinal ganglion cell apoptosis

The oxidative insult induced by H<sub>2</sub>O<sub>2</sub> is known as an exogenous source for reactive oxygen species production and to cause obviously molecular damage within cells. Excessive activation of reactive oxygen species caused by H<sub>2</sub>O<sub>2</sub> can induce cytotoxicity through the mitochondrial pathway or extracellular pathway<sup>[31]</sup>. In the current study, we tested the possible pathway by checking the expression of Bcl-2, Bax and caspase-3.

Our results show that H<sub>2</sub>O<sub>2</sub>-induced apoptotic cell death is mediated by the downregulation of Bcl-2 and up-regulation of Bax and caspase-3. Oligomeric proanthocyanidin, on the other hand, effectively prevents the change of those proteins in a concentration-dependent manner. These data suggest that oligomeric proanthocyanidin may protect retinal ganglion cells from apoptotic death by inhibiting the mitochondrial apoptosis pathway. However, as intracellular signaling pathways are very complex, we cannot exclude other pathways that are involved in these processes.

### Neuroprotection of oligomeric proanthocyanidin

Our current finding regarding the protective effect of oligomeric proanthocyanidin on retinal ganglion cells is consistent with previous reports on its neuronal protective properties.

Neuroprotection of proanthocyanidin was probably firstly indicated in the cases of applying grape seed extract (rich in oligomeric proanthocyanidin) to protect neuron from amyloid-induced death<sup>[18]</sup> and to reduce neural damage in ischemia animal model<sup>[21, 32]</sup>. Proanthocyanidin was later reported to be capable of attenuating cognitive deterioration and reducing brain neuropathology in animal models of Alzheimer's disease<sup>[33]</sup>. Report of inhibiting tau aggregation associated with Alzheimer's disease by oligomeric proanthocyanidin extracted from cinnamon<sup>[20]</sup> also suggested the neuroprotective effect of oligomeric proanthocyanidins. Mechanism underlying the

neuroprotective effect of oligomeric proanthocyanidin and its metabolism in the brain were also explored<sup>[19, 34]</sup>. Here we further demonstrated that oligomeric proanthocyanidin was able to protect retinal ganglion cells from death induced by H<sub>2</sub>O<sub>2</sub>, by possibly reducing the apoptotic process through mitochondrial pathway.

The present results suggest that oligomeric proanthocyanidin plays an important role in neuroprotection. As oligomeric proanthocyanidins can cross the blood-brain barrier and affect neuronal cells<sup>[35]</sup>, our results imply a potential application of oligomeric proanthocyanidin in the clinical treatment of many neural diseases, from glaucoma, ischemia to neurodegenerative disease.

## MATERIALS AND METHODS

### Design

An *in vitro* experiment.

### Time and setting

This study was performed at GHM Institute of CNS Regeneration, Jinan University in China between May 2011 to March 2013.

### Materials

RGC-5 cell was a generous gift from Dr. Mengfei Chen (Zhongshan Ophthalmic Center, Guangzhou, China). Oligomeric proanthocyanidin was purchased as proanthocyanidine B5 (Aladin Inc, Shanghai, China; CAS: 4852-22-6). Rats were purchased from the Animal Center of Southern Medical University in China (certification No. #0114926).

### Methods

#### RGC-5 cell culture

RGC-5 cell is a transformed retinal ganglion cell line that has been previously shown to express ganglion cell markers and exhibit ganglion cell-like behavior in culture<sup>[36]</sup>. The RGC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific Hyclone, Logan, Utah, USA) containing 4 500 mg/L glucose and 10% fetal bovine serum (Thermo Scientific Hyclone) at 37°C in a humidified atmosphere of 95% air and 5%CO<sub>2</sub> for 1–4 days till experiment. Cells were monitored with Zeiss HAL100 microscope (Carl Zeiss Microscopy, Jena, Germany) for the morphological changes during growth.

#### Immunocytochemistry

After being incubated for 48 hours, the cells were washed twice with phosphate buffer and fixed in 4% pa-

raformaldehyde for 20 minutes. The cells were soaked in blocking buffer containing 0.5% Triton X-100, 5% bovine serum in phosphate buffer for 25 minutes at room temperature. Then cells were incubated with primary antibodies at 4°C overnight, washed, incubated for 2 hours at 37°C with secondary antibodies conjugated to a fluorescence marker, rinsed, mounted, and then observed under Leica 6100M fluorescent microscope (Leica, Wetzlar, Germany). Primary antibodies included anti-Thy1.1 (1:300; Abcam Inc., Cambridge, MA, USA), Brn3 (1:100; Cell Signaling Technology, Boston, MA, USA), and GFAP (1:1000, Cell Signaling Technology).

#### MTT assay for cell viability

MTT assay was applied to test the viability of the cells. Cells ( $3 \times 10^3$ /well) were grown in 96-well plates. After various treatments, the culture medium was gently disposed and the cells were washed with new culture medium. Next, 150  $\mu$ L of culture medium containing 15  $\mu$ L of MTT (5 mg/mL; Sigma, St. Louis, MO, USA) was added to each well, and the wells were incubated for additional 4 hours at 37°C in a 5% CO<sub>2</sub> incubator. The purple-blue MTT formazan precipitate was dissolved in 150  $\mu$ L of dimethylsulfoxide. Absorbance of each well was measured on a plate reader (Tecan, Männedorf, Switzerland) at 570 nm. The values from each well were then compared to estimate the viability of cells.

#### Treatment of H<sub>2</sub>O<sub>2</sub> and oligomeric proanthocyanidin

Cells ( $3 \times 10^3$ /well) were grown for 24 hours in 96-well plates, then treated with different concentrations of oligomeric proanthocyanidin from 5, 10, 15, 20, 30, 40, 60, 80, 120, 160 to 240  $\mu$ mol/L to screen for the safe dosage range. As the half life of oligomeric proanthocyanidin was 7 hours, we chose 7 hours as the exposure time. In separate experiment, cells were pretreated with different concentrations of H<sub>2</sub>O<sub>2</sub> (200, 300, 400, 600 and 800  $\mu$ mol/L) for 7 hours. After 7-hour exposure to 400  $\mu$ mol/L H<sub>2</sub>O<sub>2</sub>, we found that the cell survival rate was about 70% of the control group (supplementary Figure 1 online). So 400  $\mu$ mol/L H<sub>2</sub>O<sub>2</sub> was chosen as the damage dose in order to see a clear protective effect of oligomeric proanthocyanidin. In separate experiment testing the protective effect of oligomeric proanthocyanidin, oligomeric proanthocyanidin and H<sub>2</sub>O<sub>2</sub> were added together into the culture medium and incubated for 7 hours.

#### Hoechst 33342 staining for cell apoptosis

After the aforementioned H<sub>2</sub>O<sub>2</sub> and oligomeric proanthocyanidin treatments for 7 hours, the cells were washed twice with PBS and fixed in 4% paraformaldehyde for 20 minutes. The cells were then stained with

10  $\mu\text{mol/L}$  Hoechst 33342 (Sigma, Shanghai, China) for 20 minutes and imaged using Zeiss fluorescence microscopy. High intensity cells were counted in five fields of view ( $872 \mu\text{m} \times 653 \mu\text{m}$ /field of view) in each group with Image pro plus software (Media Cybernetics, Inc., Rockville, MD, USA). Counted numbers were averaged and normalized to the control group.

#### **Flow cytometry analysis for cell apoptosis**

RGC-5 cells ( $1 \times 10^4$  cells/mL) were seeded in 6-well plates. Double staining with Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN, Nanjing, China) was performed for quantification of apoptosis. Cells attached to the bottom of plates and the cells in the supernatant were collected, washed twice with ice-cold PBS and re-suspended in 200  $\mu\text{L}$  of Annexin binding buffer. Then, 2  $\mu\text{L}$  of Annexin V-FITC and 2  $\mu\text{L}$  of PI were added and incubated for 5–15 minutes in the dark at room temperature. After incubation, the samples were analyzed by flow cytometry (FACSAria<sup>TM</sup>, BD, San Jose, CA, USA)

#### **Western blot analysis for apoptosis-related protein expression**

Cells were seeded in 6-well plates at a density of  $1 \times 10^4$  cells/mL. The cells were then subjected to 400  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  injury in the presence or absence of oligomeric proanthocyanidin. The control cells were left untreated. At 7 hours after treatment, the cells were lysed, and protein samples were obtained. The protein extracts were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-Bcl-2 antibody (1:1 000; Cell Signaling Technology), anti-Bax antibody (1:1 000; Cell Signaling Technology), anti-caspase-3 antibody (1:1 000; Cell Signaling Technology), anti- $\beta$ -actin antibody (1:1 000; Cell Signaling Technology) and GAPDH antibody (1:1 000; Cell Signaling Technology). The membrane was then incubated with a goat anti-rabbit IgG secondary antibody (1: 5 000; Sigma) for 2 hours at room temperature and detected with a chromogenic horseradish peroxidase western blot substrate (Pierce Biotechnology Inc, Rockford, IL, USA). The apoptosis-related protein expression levels were evaluated by measuring the ratios of Bax, Bcl-2 to GAPDH or the ratio of caspase-3 to  $\beta$ -actin, and then normalized to the control group. Each experiment was repeated three times and the values were averaged. Immunoblot pixel area for each protein was quantified using Image pro

plus software (Media Cybernetics) and then normalized to that of the internal reference

#### **Isolation of retina**

Pregnant female Sprague-Dawley rats were purchased from the animal center of Southern Medical University in China (certification No. #0114926) and kept in animal facility of Jinan University (Guangzhou, Guangdong Province, China) under specific pathogen free level till the pups were delivered and taken for experiment. The mother rats were then sacrificed by intraperitoneal injection of 13.5 mg/kg chloride hydrate (Sigma, Shanghai, China). All animal procedures were performed according to guidelines and approved by competent ethics committees of Jinan University, China.

#### **Retrograde labeling of retinal ganglion cells**

Retinal ganglion cells were retrograde labeled before isolating the retina from new born Sprague-Dawley rats. For identification of retinal ganglion cells, 4% of FluroGold (Fluorochrome, Inc., Englewood, CO, USA) was injected into the superior colliculus 2 days prior to experiment to retrogradely label retinal ganglion cells. In retinal tissue culture experiment, Sprague-Dawley rats at 3 days after birth were anesthetized with 3.5% hydrochloride. Then an incision was made to expose the skull and a hole was opened above the area of the superior colliculus. Using a Hamilton syringe affixed to a micromanipulator on the stereotaxic apparatus, a needle was inserted and a total of 2.5  $\mu\text{L}$  FluroGold was delivered to each point at a depth of 1.5 mm and 2 mm separately. The needle was left in place for 2 minutes after each injection to allow dye absorption. After the injection, the hole in the skull was sealed and skin above the skull was sutured. The operated pup was kept on heating pat till fully recovered and then returned to the cage with mother.

#### **Retinal explant culture and retinal ganglion cell counting**

Retinal explant culture was performed under the standard protocol<sup>[37-38]</sup>. Basically new born SD rats were sacrificed by overdose anesthesia 2–3 days after labeling. Then freshly enucleated eyes were immersed in cold Hank's Balanced Salt Solution under sterile conditions, with the inner retinal facing up. Then retina was moved to the DMEM/F12 media (Hyclone, Logan, Utah, USA) which contained 10% fetal bovine serum in incubators with 5%  $\text{CO}_2$  at 37°C. Twelve hours later, the solution was changed to 100  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  media, with or without 10 or 20  $\mu\text{mol/L}$  oligomeric proanthocyanidin. After 7 hours of treatment, the retina was fixed by 4% paraformaldehyde (PFA) overnight, then embedded in

optimum cutting temperature (OCT) compound. The retina was cut into 15 µm-thick slices. Number of retinal ganglion cells was counted with ImageJ (NIH). As the cells distributed unequally in different areas of retina, we only counted the retinal ganglion cells in the sections with optic disc, and within 150 µm distance away from the optic disc on each side of the optic disc in each slice. For each eye, retinal ganglion cells in about five slices were counted in every group and the numbers were averaged cross each slice.

### Statistical analysis

All experiments were repeated at least three times. Data were expressed as mean ± SD. Statistical comparisons were determined using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test using SPSS software 17.0 (SPSS, Chicago, IL, USA). Statistical significance was accepted at a value of  $P < 0.05$ .

**Research background:** In glaucoma and other many optic nerve diseases, retinal ganglion cells die and cause the loss of vision.

**Research frontiers:** In this study, we innovatively tested whether oligomeric proanthocyanidin, an antioxidative and neuroprotective agent and abundant in grape seed, may protect retinal ganglion cells against oxidative stress induced apoptosis.

**Clinical significance:** We are trying to investigate the clinical value of oligomeric proanthocyanidin in the treatment of glaucoma and other optic diseases.

**Academic terminology:** Oligomeric proanthocyanidin is the oligomer of proanthocyanidin, which presents abundantly in fruits such as apple, pear, grapes, and also in daily diet like chocolate, wine and tea and has proven to be antioxidant and neuroprotective.

**Peer review:** Our *in vitro* studies on both RGC-5 cell lines and retinal explants demonstrate that oligomeric proanthocyanidin can protect the oxidative stress injured retinal ganglion cells by inhibiting the apoptotic process, thus indicating a potential application of oligomeric proanthocyanidin in the clinical treatment of glaucoma and other optic diseases.

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