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Published in:
Experimental Eye Research

DOI:
[10.1016/j.exer.2020.108291](https://doi.org/10.1016/j.exer.2020.108291)

Publication date:
2020

Document Version
Author accepted manuscript

[Link to publication in ResearchOnline](#)

Citation for published version (Harvard):

Alhasani, RH, Zhou, X, Biswas, L, Li, X, Reilly, J, Zeng, Z & Shu, X 2020, 'Gypenosides attenuate retinal degeneration in a zebrafish retinitis pigmentosa model', *Experimental Eye Research*, vol. 201, 108291. <https://doi.org/10.1016/j.exer.2020.108291>

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Gyenosides attenuate retinal degeneration in a zebrafish retinitis pigmentosa model

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Abstract

Retinitis pigmentosa (RP) is a collection of heterogeneous genetic retinal disorders resulting in cumulative retinal deterioration involving progressive loss of photoreceptors and eventually in total blindness. Oxidative stress plays a central role in this photoreceptor loss. Gynenosides (Gyp) are the main functional component isolated from the climbing vine *Gynostemma pentaphyllum* and have been shown to defend cells against the effects of oxidative stress and inflammation, providing protection in experimentally-induced optic neuritis. The zebrafish model has been used to investigate a range of human diseases. Previously we reported early retinal degeneration in a mutant zebrafish line carrying a point-nonsense mutation in the retinitis pigmentosa GTPase regulator interacting protein 1 (*rpgrip1*) gene that is mutated in RP patients. The current study investigated the potential protective effects of Gyp against photoreceptor degeneration in the *Rpgrip1* deleted zebrafish. *Rpgrip1* mutant zebrafish were treated with 5 µg/ml of Gyp in E3 medium from 6 hours post fertilization (hpf) till 1 month post fertilization (mpf). *Rpgrip1* mutant zebrafish treated with 5 µg/mL of Gyp showed a significant decrease by 68.41% ($p=0.0002$) in photoreceptor cell death compared to that of untreated mutant zebrafish. Expression of antioxidant genes *catalase*, *sod1*, *sod2*, *gpx1*, *gclm*, *nqo-1* and *nrf-2* was significantly decreased in *rpgrip1* mutant zebrafish eyes by 61.51%, 77.40%, 60.11%, 81.17%, 72.07%, 78.95% and 85.42% (all $p<0.0001$), respectively, when compared to that of wildtype zebrafish; superoxide dismutase and catalase activities, and glutathione levels in *rpgrip1* mutant zebrafish eyes were significantly decreased by 87.21%, 21.55% and 96.51% (all $p<0.0001$), respectively. There were marked increases in the production of reactive oxygen species (ROS) and malondialdehyde (MDA) by

2738.73% and 510.69% (all $p < 0.0001$), respectively, in *rpgr1* mutant zebrafish eyes; expression of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α was also significantly increased by 150.11%, 267.79% and 190.72% (all $p < 0.0001$), respectively, in *rpgr1* mutant zebrafish eyes, compared to that of wildtype zebrafish. Treatment with Gyp significantly counteracted these effects. This study indicates that Gyp has a potential role in the treatment of RP.

Keywords: Retinitis pigmentosa; RPGRIP1; photoreceptor death; zebrafish; Gypenosides

1. Introduction

Retinitis pigmentosa (RP, MIM# 268000) is the term applied to a diverse range of inherited retinal disorders characterised by initial death of rod photoreceptors followed by the death of cone photoreceptors. RP is one of the most common retinal dystrophies, affecting 1 in 3000-4000 people worldwide (Petra-Silva and Linden, 2014; Phelan and Bok, 2000; Raghupathy et al., 2013). Leber congenital amaurosis (LCA), the congenital version of RP, was first recognised and described by Leber (1869). LCA is perhaps the severest form of RP, affecting 1 in 40,000-80,000 worldwide and causing significant visual impairment within one year of birth (Sherwin et al., 2008; Stone, 2007). Characterisation of LCA is based on its clinical features: photophobia, sensory nystagmus, early severe visual loss, absent or reduced electroretinogram (ERG) waveform, and amaurotic pupils (Stone, 2007).

Mutations in the Retinitis Pigmentosa GTPase Regulator Interacting Protein 1 (*RPGRIP1*) gene cause LCA (LCA6) (Dryja et al., 2001; Gerber et al., 2001), juvenile RP (Booij et al., 2005), late onset cone-rod dystrophy (CORD13) in humans (Hameed et al., 2003; Huang et al., 2013; Khan et al., 2013) and cone-rod dystrophy in dogs (Mellersh et al., 2013). Around 5% of all LCA cases is thought to be caused by mutations in the *RPGRIP1* gene (Kumaran et al., 2017). Depletion of *RPGRIP1* in human retinal pigment epithelial (RPE) cells causes cilia defects and abnormal cytoskeleton (Patnaik et al., 2018). Loss of *RPGRIP1* in mice results in developmental defects in photoreceptor outer segments, mislocalization of rhodopsin, and early retinal degeneration (Won et al. 2009; Zhao et al., 2003). Recently we have characterized a mutant zebrafish line that carries a nonsense homozygous mutation in the *rpgrip1* gene. The mutant zebrafish demonstrate defects

in the development of the rod outer segment and in the trafficking of ciliary protein, early retinal degeneration and impaired visual function (Raghupathy et al., 2017).

Gynostemma pentaphyllum (*G. pentaphyllum*) Makino (Jiaogulan in Chinese) is a herbaceous vine plant – more accurately a kind of cucurbitaceous liana (gourd family with woody vines to support its growth) – that is widely used in traditional medicine throughout Asia, including the mountainous regions of Vietnam, Japan, Korea and China. *G. pentaphyllum* Makino contains a variety of compounds including saponins, carotenoids, chlorophylls, flavonoids, polysaccharides, sterols, amino acids, vitamins and minerals (Razmovski-Naumovski et al., 2005). Gypenosides (Gyp), the dammarane-type saponins extract derived from the *G. pentaphyllum* (Thunb) Makino, are yellow in colour and bitter tasting, and have anti-diabetic, anti-hyperlipidemic, anti-tumour, anti-oxidative and anti-inflammatory properties (Li et al., 2019). We recently demonstrated that Gyp protected RPE cells from H₂O₂-induced oxidative damage and inflammation (Alhasani et al., 2018).

Current therapeutic strategies for RP include gene therapy, cell therapy, retinal prostheses and pharmacological approaches (Dias et al., 2018). High genetic heterogeneity of RP and the complexity of photoreceptor degeneration pathways make RP treatment extraordinarily challenging. Oxidative stress has been implicated in the progression of RP. Treatment with antioxidants has been shown to reduce oxidative damage and preserve photoreceptor function in RP animal models (Komeima et al., 2006; Lee et al., 2011; Shen et al., 2005). There is a close link between oxidative stress and inflammation. Mouse RP models exhibit increased proinflammatory cytokines and chemokines preceding photoreceptor degeneration (Karlstetter et al., 2015; Yoshida

et al., 2013a; Xun et al., 2019). RP patients also have significantly higher levels of proinflammatory cytokines and chemokines in the vitreous fluid (Yoshida et al., 2013b). Microglial activation has been observed in the retinas of RP patients and RP rodent models and is associated with rod cell death (Gupta et al., 2003; Karlstetter et al., 2015; Zhao et al., 2015). Activated microglia phagocytose pre-apoptotic rod cells and secrete proinflammatory cytokines, which exacerbates rod death in RP mouse models; by contrast, inhibition of microglial activation attenuates rod cell death (Zhao et al., 2015). Antioxidants and anti-inflammatory agents offer a therapeutic option (Guadagni et al., 2015), and so there is a pressing need to identify novel anti-oxidative and anti-inflammation agents for the treatment of RP. In the current study we appraised the therapeutic potential of Gyp for RP by using it to treat *rpgrip1* mutant zebrafish and investigating subsequent biochemical and histological changes. We found that Gyp treatment delayed photoreceptor death, increased antioxidant capacity and inhibited endoplasmic reticulum (ER) stress and inflammation.

2. Materials and methods

2.1. Toxicity assay

All animal work received approval from the UK Home Office with Project licence PPL 70/8697. Gypenosides (Gyp), containing several gypenoside molecules, were purchased from Xi'an Jiatian Biotech Co. Ltd, China with 98% purity and dissolved in 100% Dimethyl sulfoxide (DMSO) as a stock (10 mg/mL). To determine the toxicity of Gyp in zebrafish embryonic stages, toxicity assay was carried out using previously described methods (Scholz et al., 2008). Zebrafish embryos at 6 hours post fertilization (hpf) were treated at 28 °C in twenty-four-well plates (10 embryos/well) containing 500 µl/well of Gyp at concentrations of 0, 2.5, 5.0, 15.0, 25.0 and 50.0 µg/ml in E3

medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄). Mortality, heartbeat and hatching were recorded at 48, 72, 96 and 120 hpf. Heartbeat measurement was performed by putting individual embryos into E3 medium on a glass slide and visually counting ventricle beating (beats per minutes, BPM) under Evos microscope.

2.2. Zebrafish treatment

To investigate whether Gyp can inhibit or delay photoreceptor degeneration, *rpgr1* mutant embryos at 6 hpf were treated with Gyp in E3 medium till one month post fertilization (mpf). There were 3 groups: wildtype in E3 medium (0.05 % DMSO), *rpgr1* mutants in E3 medium (0.05 % DMSO), and *rpgr1* mutants treated with Gyp (5 µg /mL in 0.05% DMSO). The treatment was carried out in 60mm×15mm petri dishes in which the sibling and *rpgr1* mutant embryos (6 hpf) were incubated at 28°C in 4 mL E3 medium with or without Gyp (15 embryos/petri dish in triplicates). After 5 days, the embryos were transferred to 1-litre water tanks (15 fish/tank in 100 ml E3 medium) and kept at 24°C in the zebrafish facility for 25 days. During the treatment, fish were fed with ZM-100 fry food and brine shrimp (Zebrafish Management Ltd, UK). E3 medium (0.05 % DMSO) with or without Gyp (5µg /mL) was changed every two days. After the treatment, zebrafish were killed using Schedule one method and eyes were dissected for further examination.

2.3. Measurement of reactive oxygen species (ROS)

Eyes from wildtype, untreated and Gyp-treated *rpgr1* mutant zebrafish at 1mpf were collected and homogenized using the FastPrep®-24 homogenizer (MP Biomedicals, UK) on ice in lysis buffer containing 1 mM MgCl₂, 20 mM HEPES, 0.32 mM sucrose, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF, pH 7.4). The lysates were centrifuged at 10,000Xg for 5 minutes at 4°C and the supernatants were treated with Dichloro-dihydro-fluorescein Diacetate (DCFH-DA) solution (20

µg/ml, 8.3 µl/well) in 96-well plates and incubated in darkness at 37°C for 30 min. The fluorescent signal was measured using the Fluostar Optima microplate reader (BMG-labtech) at 485 nm (excitation) and 525 nm (emission). The ROS level was calculated as previously described (Alhasasni et al., 2018).

2.4. Histology and immunohistochemistry (IHC)

After one month of treatment, zebrafish eyes were dissected, fixed at 4°C for 24 hours in 4% paraformaldehyde (PFA) solution, then washed with PBS twice, dehydrated with ethanol and embedded in paraffin to make tissue wax blocks for histology; alternatively, the eyes were incubated with 5%, 15% or 30% sucrose for 2 hours and then embedded in Optimal Cutting Temperature (OCT) compound for cryosections. Eye samples of 8 µm thickness were sectioned by microtome or cryotome. For histology, sections were dewaxed and stained with haematoxylin-eosin. For measurement of the photoreceptor layer, five eyes from five individual fish from each group were used, two retinal sections of each eye were selected and images were taken from inferior sides. Thickness of the photoreceptor layer was measured using Image J software at the location 0.4 mm from the optic nerve head. For IHC, cryosections were blocked for 30 minutes in 2% bovine serum albumin (BSA)/PBS and incubated with primary antibody (anti-rhodopsin, Cat. ab98887, Abcam, 1:500 dilution) in 2% BSA/PBS at room temperature for 2 hours. The sections were washed three times (5 minutes/each) with PBS and then incubated with secondary antibody (goat anti-mouse IgG FITC-conjugated, Cat. F0257, Sigma, UK (1:500 dilution) in 2% BSA/PBS at room temperature for 1 hour. After washing with PBS five times (5 minutes/each), the sections were mounted with Vectashield 4',6-diamidino-2-phenylindole (DAPI)

(D9542-Sigma, UK). Images were taken with ZEISS LSM 800 confocal microscopy. To quantify the rhodopsin fluorescence signal, 10 retinal sections from five eyes (two sections/each eye) were collected from the control (wildtype zebrafish), *rpgr1* mutants, or *rpgr1* mutants treated with Gyp. In each section, one region (10 $\mu\text{m} \times 10 \mu\text{m}$, under $\times 400$ magnification) in the superior side (0.4 mm from the optic nerve head) and one region (10 $\mu\text{m} \times 10 \mu\text{m}$, under $\times 400$ magnification) in the inferior side (0.4 mm from the optic nerve head) were chosen for quantification of fluorescent signals with ImageJ software. The signals from both the superior region and the inferior region were averaged for the final calculation.

2.5. Gene expression

Total RNA was extracted from 20 eyes of wildtype, *rpgr1* mutant, or Gyp-treated *rpgr1* mutant zebrafish at 1 mpf by using the TRIzol reagent (Sigma, UK) according to the manufacturer's guidance. cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using Platinum[®] SYBR[®] Green QPCR SuperMix-UDG w/ROX kit (ThermoFisher Scientific, UK) following the manufacture's protocol. The primers for qRT-PCR are listed in Table S1. β -actin was used to normalize mRNA levels of candidate genes.

2.6. Measurement of activities of superoxide dismutase (SOD) and catalase (CAT) and quantification of glutathione (GSH) and malondialdehyde (MDA) levels

Activities of SOD and CAT and levels of GSH and MDA were measured in zebrafish eye samples using individual commercial kits (STA-340 for SOD, STA-341 for CAT, STA-312 for GSH and STA-330 for MDA from Cell Biolabs) following the manufacturer's instructions.

2.7. Western blotting

199 Twenty eyes from the groups of wildtype, *rpgr1* mutant and Gyp-treated *rpgr1* mutant
200 zebrafish were homogenized in protein lysis buffer (T-PER, Thermo Fisher Scientific UK) using a
201 FastPrep®-24 homogenizer (MP Biomedicals) on ice. Concentration of protein samples was
202 measured by a Bradford assay. 100µg of individual protein samples was loaded in the pre-cast
203 protein gel (Cat. No. 4569034, Bio-Rad) then electrophoresed for 45-60mins at 200V in the running
204 buffer. The gel was removed from the electrophoresis apparatus and assembled into a sandwich
205 containing three sponges, filter paper, pre-cast protein gel, nitrocellulose membrane, filter paper
206 and three more sponges. The sponge-gel-membrane sandwich was then placed into a transfer
207 cassette in the Wet Tank Transfer System (Cat. No. EI9051, Thermo Fisher Scientific) containing the
208 transfer buffer to transfer the protein from the pre-cast protein gel onto the nitrocellulose
209 membrane under 175mA for 90 minutes. The nitrocellulose membrane was blocked with Tris
210 buffered saline with 0.1% Tween-20 (TBST) and 5% (w/v) non-fat dry milk for 2 hours at room
211 temperature. The membrane was incubated with primary antibody (anti-acetylated tubulin
212 antibody, Cat. T7451, Sigma, UK, 1:1000 dilution, anti-Caspase-3 antibody, Cat. ABIN1883652,
213 Antibodies-online, UK, 1:1000 or anti-TNF-α antibody, Cat. ABIN1884227, Antibodies-online, UK,
214 1:1000 dilution) in TBST with 5% (w/v) non-fat dry milk for two hours. After washing three times
215 with TBST, the membrane was incubated with secondary antibody (goat anti-rabbit antibody, Cat.
216 926–32211 or donkey anti-mouse antibody, Cat. 926–68072, 1:10,000 diluted in TBST with 5%
217 non-fat dry milk) at room temperature for 2 hours. The membrane was washed five times (5
218 minutes/each). The band intensity of targeted proteins was measured using the LI-COR Odyssey FC
219 Imaging System.

2.8. Cell death detection

Cell death in zebrafish eye sections was detected using DeadEnd™ fluorometric TUNEL assay (Promega, UK) according to the manufacturer's instructions. Briefly, following dewaxation zebrafish eye sections were treated with proteinase K solution (20 µg/ml), then incubated with rTDT reaction at 37°C for one hour. The reaction was terminated with 2×SSC. The sections were mounted with Vectashield DAPI (D9542-Sigma, UK) and the images were taken under ZEISS LSM 800 confocal microscopy. For quantification of cell death, six sections were chosen from six eyes (one section/each eye) of control, *rpgr1* mutant or Gyp-treated *rpgr1* mutant group; in each section, a region (50 µM×20 µM, long × wide) in the superior side (0.4 mm from the optic nerve head) was chosen; TUNEL-positive photoreceptor cells in each region were counted and presented as a percentage of the total number of photoreceptor cells in the same region.

2.9. Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 6 software via one-way ANOVA (analysis of variance) followed by Bonferroni post hoc test. The data were calculated from at least three independent experiments and shown as mean ± SEM (standard error of the mean). A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Toxic effect of Gyp on zebrafish embryos and larvae

Potential toxicity of Gyp was assessed to allow utilization of a safe dose for subsequent Gyp treatment. Zebrafish embryos at 6 hpf were exposed to Gyp at different concentrations (2.5, 5, 15 and 25 µg/ml) for 5 days. Treatment with Gyp at 2.5 and 5 µg/ml resulted in mortality over this period that was no different to that of the control group. Higher concentrations, however, caused

242 significantly increased mortality: 15 and 25 $\mu\text{g/ml}$ Gyp resulted in, respectively, $\sim 30\%$ and $\sim 60\%$
243 mortality at 120hpf (Figure S1A). The toxic effect of Gyp on cardiovascular development was
244 studied by monitoring the heart rate in control and treated groups at 72hpf. Only 25 $\mu\text{g/ml}$ Gyp
245 treatment had a significant effect on heart rate compared to untreated controls. Heart rate in the
246 control group was typically $\sim 100\text{-}120$ beats/min; a similar rate was observed in zebrafish larvae
247 exposed to Gyp at 2.5, 5 or 15 $\mu\text{g/ml}$, whereas the 25 $\mu\text{g/ml}$ Gyp-treated group showed a
248 significantly reduced heart rate of ~ 75 beats/min (Figure S1B). The hatching rate of zebrafish
249 embryos incubated with Gyp at different concentrations was also monitored at 72 hpf. The
250 hatching rate of 2.5 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ Gyp-treated embryos was 100%, identical to that of the
251 controls. However, zebrafish embryos treated with 15 or 25 $\mu\text{g/ml}$ Gyp had a marked decrease in
252 hatching rate compared to the untreated control group (Figure S1C). Based on the above toxic
253 effects of Gyp on zebrafish embryos, we chose Gyp at 5 $\mu\text{g/mL}$ for use in subsequent treatments.

254 3.2. Gypenoside treatment rescued photoreceptor degeneration in *rpgr1* mutant zebrafish

255 We have shown previously that outer / inner segments of *rpgr1* mutant zebrafish retina were
256 significantly shorter compared to the wildtype retina and that most rod cells had degenerated at
257 1mpf (Raghupathy et al., 2017). In the current study we performed retinal histology of wildtype,
258 Gyp-treated and untreated *rpgr1* mutant zebrafish at 1 mpf and confirmed that the
259 photoreceptor layer of *rpgr1* mutants was significantly shorter compared to that of wildtype
260 zebrafish. The thickness of the wildtype photoreceptor layer was $\sim 56\ \mu\text{m}$, while that of *rpgr1*
261 mutant zebrafish was $\sim 36\ \mu\text{m}$. *Rpgr1* mutant zebrafish treated with 5 $\mu\text{g/ml}$ Gyp had a
262 significantly thickened photoreceptor layer of $\sim 41\ \mu\text{m}$, though the thickness of photoreceptor layer

in Gyp-treated *rpgr1* mutant zebrafish was significantly less than that of wildtype zebrafish (Figure 1).

Rhodopsin has been shown to be mislocalized to the outer nuclear layer (ONL) of mouse *Rpgr1* mutant retinas ((Won et al., 2009). Immunofluorescence staining of retinal sections with 4D2 antibody (detection of rhodopsin) revealed that only a weak signal of mislocalized rhodopsin was present in ONL of zebrafish *rpgr1* mutant retinas, demonstrating early rod degeneration. Treatment of *rpgr1* mutant zebrafish with Gyp resulted in significantly increased signals of 4D2 staining (Figure 2A,B); when compared to wildtype, Gyp-treated zebrafish had significantly reduced 4D2 signals. *Rpgr1* mutant zebrafish also had markedly lower mRNA level of *rhodopsin*, while Gyp treatment significantly increased *rhodopsin* mRNA level although still not as high as that seen in wildtype zebrafish (Figure 2C).

3.3. Gyp reduced photoreceptor cell death in *rpgr1* mutant zebrafish

Rpgr1 mutant zebrafish have been shown to display significant cell death at examined time points: 14 days post fertilization (dpf), 1 mpf and 3 mpf (Raghupathy et al., 2017). In order to examine the protective role of Gyp, we carried out TUNEL assay to assess photoreceptor cell death. We observed significantly increased cell death in the ONL of the mutant zebrafish retinas, around 5-fold more compared to the control group. Gyp-exposed *rpgr1* mutant embryos showed a marked decrease in photoreceptor cell death compared to that seen in untreated *rpgr1* mutant zebrafish, while no difference was found between Gyp-treated and wildtype zebrafish (Figure 3).

We further examined whether the photoreceptor death was caspase-dependent by measuring the expression levels of caspase proteases, the key executioners of the apoptotic

process. Relative gene expression analysis using qRT-PCR showed significantly increased expression of *caspase3* in *rpgr1* mutant zebrafish eyes compared to those of wildtype zebrafish. Gyp treatment resulted in significantly decreased expression of *caspase 3* gene in the mutant zebrafish (Figure S2A). Furthermore, we examined the protein level of Caspase 3 by Western blotting and found that Caspase 3 was significantly increased in *rpgr1* mutant eyes compared to that of wildtype zebrafish, while Gyp treatment significantly counteracted these changes (Figure S2B). When compared to wildtype zebrafish, Gyp-treated mutant zebrafish still had higher expression of Caspase 3 at mRNA and protein levels (Figure S2).

3.4. Gyp treatment can enhance the antioxidant capacity in *rpgr1* mutant eyes

Excessive reactive oxygen species (ROS) production causes cell death through the oxidation and damage of macromolecules. We have shown previously that Gyp can suppress H₂O₂-induced ROS production in retinal epithelial cells (Alhasani et al., 2018). Therefore, we conjectured that excessive ROS production underlies increased photoreceptor cell death in *rpgr1* mutant zebrafish retinas and that Gyp might reverse this effect. In fact, ROS production in *rpgr1* mutant zebrafish eyes was 2766% higher than that in wildtype zebrafish. Treatment of mutant zebrafish with Gyp resulted in a significant decrease in ROS production by 63% compared to that of untreated *rpgr1* mutant zebrafish eyes; however, even when treated with Gyp, mutant zebrafish had a higher production of ROS than did wildtype zebrafish (Figure 4A).

We then assessed the effect of Gyp on antioxidant gene expression using qRT-PCR. We observed that expression of *catalase*, *sod1*, *sod2*, *glutathione peroxidase 1 (gpx1)*, *glutamate-cysteine ligase modifier subunit (gclm)*, *NAD(P)H quinone oxidoreductase-1 (nqo1)* and

305 NF-E2-related factor-2 (*nrf2*) genes were markedly decreased in the mutant zebrafish eyes when
306 compared to that of wildtype zebrafish. Gyp exposure significantly increased the expression of the
307 examined antioxidant genes compared to that of untreated *rpgr1* mutant zebrafish. When
308 compared to wildtype zebrafish, Gyp-treated zebrafish had, with the exception of *sod2*, a lower
309 expression of the examined genes (Figure 4B-H).

310 We also measured SOD and catalase activities and the levels of glutathione (GSH) and
311 malondialdehyde (MDA) in the eyes of wildtype, untreated and Gyp-treated *rpgr1* mutant
312 zebrafish. SOD and catalase activities in *rpgr1* mutant zebrafish eyes were significantly decreased
313 when compared to that of wildtype zebrafish; Gyp treatment significantly increased SOD and
314 catalase activities compared to that of untreated *rpgr1* mutant zebrafish, though activities of
315 both enzymes were still lower than that in wildtype zebrafish (Figure 5A, B). GSH level was
316 significantly lower in *rpgr1* mutant zebrafish eyes compared to that of wildtype zebrafish;
317 treatment with Gyp significantly increased GSH level compared to that of untreated *rpgr1*
318 mutant zebrafish; when compared to wildtype zebrafish, Gyp-treated zebrafish eyes had a lower
319 level of GSH (Figure 5C). The level of MDA was higher in *rpgr1* mutant zebrafish eyes compared
320 to wildtype zebrafish, while Gyp treatment resulted in a significantly decreased MDA level
321 compared to that of untreated *rpgr1* mutant zebrafish; compared to wildtype zebrafish, MDA
322 level in Gyp-treated mutant zebrafish eyes was higher (Figure 5D).

323 3.5. Protective effect of Gypenoside against ER stress

324 ROS production and ER stress are closely related events. ER stress leads to increased transcription
325 and translational responses, thereby causing an increased expression of ER stress inducers (Hetz et

al., 2011). Accordingly, we examined expression of *xbp1t* (total) and its spliced variant *xbp1s*, *atf4*, *atf6* and *bip*, all of which are induced in ER stress. Expression of all these genes was significantly higher in *rpgr1* mutant zebrafish eyes compared to wildtype zebrafish, while expression was significantly lower in *rpgr1* mutant zebrafish eyes that were treated with Gyp compared to those that were untreated. Expression of *atf6*, *xbp1s* and *xbp1t* (but not *atf4* or *bip*) remained significantly higher in mutant zebrafish treated with Gyp compared to that of wildtype zebrafish (Figure 6).

3.6. Gyp inhibited inflammation in *rpgr1* mutant zebrafish

We also examined expression of proinflammatory cytokines (*il-1 β* , *il-6* and *tnf- α*) using qRT-PCR and found that expression of these genes was significantly higher in *rpgr1* mutant zebrafish eyes compared to that of wildtype zebrafish; Gyp treatment significantly reduced expression of the three cytokines (Figure 7A). The change of *Tnf- α* protein level in Gyp-treated *rpgr1* mutant was also confirmed by Western blotting. Expression of the proinflammatory cytokines was higher in Gyp-treated zebrafish eyes compared with that of wildtype zebrafish eyes (Figure 7B).

4. Discussion

The morphological and functional retinal cell impairment observed in RP is often a result of molecular changes triggered by the retinal damage (Dias et al. 2018). This retinal degeneration might be prompted by several factors including genetic predispositions, higher intraocular pressure, increased blood glucose levels, oxidative/nitrosative stress, or even aging. Irrespective of the trigger factor, a subsequent cascade of cell signals leads to well-established morphological and functional modifications such as apoptosis and retinal remodelling (Dias et al. 2018). Moreover,

oxidative stress, inflammatory response and induction of apoptotic pathways are characteristic traits in RP (Guadagni et al. 2015).

As a result of the high metabolic activity of photoreceptors, which have the greatest oxygen consumption rate per gram of any body tissue (Ames III, 1992), oxidative stress contributes significantly to the progression of RP (Komeima et al., 2006; Shen et al., 2005; Usui et al., 2009). Numerous animal models of RP suggest that ROS is a stimulus that triggers apoptotic photoreceptor cell death in RP and is aggravated by oxidative stress (Cuenca et al., 2014). Several studies have suggested that the secondary loss of cone cells that is characteristic of RP is a result of damage arising from the higher oxygen consumption and subsequent increased ROS levels when rod cells die (Carmody and Cotter, 2000; Shen et al., 2005). Moreover, ROS production due to light exposure has been demonstrated to exacerbate retinal degeneration in hereditary and age-linked retinopathies including RP (Paskowitz et al., 2006; Sui et al., 2013).

Previous studies have demonstrated that Gyp enhance antioxidant capacity, inhibit inflammation and suppress apoptotic cell death in *in vivo* animal models, such as ischemia/reperfusion injury rodent models (Ye et al., 2016; Yu et al., 2016a; Yu et al. 2016b; Yu et al., 2016c; Zhao et al., 2014). More recently, Gyp have been shown to exhibit protective effects in rodent optic neuritis models by inhibiting lipopolysaccharide (LPS) or myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide-induced inflammation (Wang et al., 2018; Zhang et al., 2017). There are few studies, however, on its role in retinal degeneration. Our previous *in vitro* study demonstrated that Gyp suppress ROS generation in the ARPE-19 cell line via its effects on gene expression and protein level of several antioxidant scavengers and apoptosis-related genes

368 (Alhasani et al., 2018). Our current research has shown that *rpgr1* mutant zebrafish had
369 significantly higher production of ROS and MDA, lower expression of antioxidant genes, less GSH
370 and high level of rod cell death; Gyp treatment significantly reduced generation of ROS and MDA
371 and increased antioxidant gene expression and GSH level, arresting rod cell degeneration.

372 The NRF2 pathway plays an important role in cellular defence against oxidative damages
373 (Lim et al., 2014). NRF2 is involved in the regulation of several cellular defence mechanisms that
374 counteract the potentially lethal consequences of ROS (Lim et al., 2014). In stress-free conditions
375 NRF2 is localized to the cytoplasm but in the presence of oxidative stress is phosphorylated and
376 translocated to the nucleus, in turn activating several cytoprotective genes (Lim et al., 2014).
377 Activation of the NRF2 functional pathway has been reported to protect against photoreceptor
378 degeneration in RP animal models. Nakagami et al. (2016) demonstrated that NRF2 activation and
379 upregulation of its effector proteins slowed photoreceptor cell death in a RP rabbit model.
380 Adeno-associated virus (AAV)-mediated overexpression of NRF2 in the retina alleviated
381 photoreceptor degeneration in three RP mouse models (Xiong et al., 2015). We too found that
382 *rpgr1* mutant zebrafish eyes had lower expression of NRF2 and its targeting antioxidant genes
383 and that Gyp treatment increased expression of these genes, suggesting that Gyp protection is
384 possibly mediated by the NRF2 signalling pathway.

385 Impairment of protein folding and maturation in ER causes ER stress, leading to the unfolded
386 protein response (UPR). UPR is mediated by protein kinase RNA-like endoplasmic reticulum kinase
387 (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) signalling
388 pathways. ER stress has been implicated in the pathogenesis of RP, while pharmacological

389 inhibition of ER stress has been shown to slow retinal degeneration in RP animal models (Dias et
390 al., 2018; Zhang et al., 2014). Previous studies demonstrated that Gyp protected cardiomyocytes
391 from ischemia-reperfusion injury-induced apoptosis via inactivation of C/EBP homologous
392 protein-10 (CHOP) (Yu et al., 2016c). Here we showed that *rpgr1* mutant eyes had higher
393 expression of *atf4*, *atf6*, *xbp1* and *bip* genes, which encode proteins involved in the UPR. Gyp
394 treatment led to significantly decreased expression of these genes, suggesting that Gyp inhibited
395 ER stress that may contribute to decreased rod apoptosis in Gyp-treated *rpgr1* mutant zebrafish.

396 Inflammatory cytokines are overproduced upon exposure to ROS/oxidative stress. Several
397 studies have reported the secretion of various proinflammatory cytokines and chemokines into
398 the vitreous cavity in RP and other ocular diseases, with these cytokines and chemokines being
399 partly responsible for the initiation and development of the condition (Funatsu et al., 2005;
400 Yoshida et al., 2013a; Yoshida et al., 2013b; Yoshimura et al., 2009). Our previous study
401 demonstrated that deletion of RPGR, an RPGRIP1 interaction protein, caused microglial activation
402 and higher IL-1 β expression in mouse retinas (Zhang et al., 2019). In the current study, we also
403 found higher expression of proinflammatory cytokines (il-1 β , il-6 and tnf- α) in *rpgr1* mutant eyes
404 and showed that Gyp significantly decreased expression of these genes.

405 In conclusion, we observed that loss of Rpgrip1 in zebrafish resulted in a defective
406 antioxidant defense system and increased ER stress and inflammation. Gyp treatment inhibited
407 oxidative damage, ER stress and inflammation, resulting in slowdown of photoreceptor death in
408 *rpgr1* mutant zebrafish.

409 **Acknowledgments** This study was funded by a Saudi Arabia government PhD scholarship, the
410 Rosetrees Trust, National Eye Research Centre and the Lotus Scholarship Program of Hunan
411 Province, China. X.S. is a Visiting Professor to Shaoyang University.

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Figures and legends

Figure 1 (A) Representative image of hematoxylin and eosin stained transverse retinal sections of wildtype (WT), untreated (UT) *rpgr1p1* mutant and Gyp-treated *rpgr1p1* mutant zebrafish at 1 mph. (B) Quantification of the length of photoreceptor cell layer of the examined zebrafish. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelial cells. The data were analysed using one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean $\pm$ SEM. ** $p < 0.01$ and **** $p < 0.0001$.

Figure 2 (A) Immunostaining of transverse retinal sections of wildtype (WT), untreated (UT) and Gyp-treated *rpgr1* mutant zebrafish at 1mpf using 4D2 antibody. Rhodopsin localization in the rods was visualized by green fluorescent signals, while nuclei were

labelled in blue by staining with DAPI. (B) Quantification of the corrected total cell fluorescence (CTCF %) of rods in retinal sections of examined zebrafish. (C) Expression of *rhodopsin* was examined by qRT-PCR. INL, inner nuclear layer; ONL, outer nuclear layer. All data were analysed using one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3 (A) Photoreceptor cell death in wildtype (WT), untreated (UT) and Gyp-treated *rpgr1p1* mutant zebrafish at 1 mpf retinas was detected using TUNEL assay and photographed by ZEISS LSM 800. (B) The number of cells labelled with positive TUNEL signal was counted within a 50 μm x 20 μm (longxwide) area in the outer nuclear layer of the retinal section. The averaged percentage of cells with positive TUNEL signal was counted from six individual animals from each group. A negative control was set up using retinal sections from wildtype zebrafish at 1 mpf incubated with the buffer without rTDT. INL, inner nuclear layer; ONL, outer nuclear layer. Data of the fluorescent cells counting were analysed using one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean $\pm$ SEM. ns, no significance. *** $P < 0.001$ and **** $P < 0.0001$.

Figure 4. (A) ROS production in eye samples of wildtype (WT), untreated (UT) or Gyp-treated *rpgrip1* mutant zebrafish at 1 mpf was measured using DCFH-DA staining described in Materials and methods section. (B-H) Expression of antioxidant genes *catalase*, *sod1*, *sod2*, *gpx1*, *gclm*, *nqo-1* and *nrf2* in the eye samples of wildtype (WT), untreated (UT) or Gyp-treated *rpgrip1* mutant zebrafish at 1 mpf was measured by qRT-PCR. All data were analysed using one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 5. Gyp treatment significantly increased SOD (A) and catalase (B) activities and GSH level (C), decreased MDA level (D) in eye samples of wildtype (WT), untreated (UT) or Gyp-treated *rpgr1* mutant zebrafish at 1 mpf. All data were analysed by one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6. Expression of endoplasmic reticulum (ER) stress-related genes, *atf4* (A), *atf6* (B), *xbp1s* (C), *xbp1t* (D) and *bip* (E) in eye samples of wildtype (WT), untreated (UT) or Gyp-treated *rpgrip1* mutant zebrafish at 1 mpf was measured by qRT-PCR. All data were analysed using one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean $\pm$ SEM. ns, no significance. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 7. Gyp inhibited inflammation in *rpgr1* mutant zebrafish eyes. (A) Expression of *il-1β*, *il-6* and *tnf-α* in eye samples of wildtype, untreated (UT) or Gyp-treated *rpgr1* zebrafish at 1 mpf examined by qRT-PCR. (B) Protein level of Tnf-α in eye samples of wildtype, untreated (UT) or Gyp-treated *rpgr1* mutant zebrafish detected by Western blotting. Quantification of Tnf-α level by normalization of Tnf-α intensity to the intensity of acetylated Tubulin. All data were analysed using one-way ANOVA followed by appropriate post hoc test (Bonferroni) and presented as mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$.