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Astaxanthin protects retinal ganglion cells from acute glaucoma via the Nrf2/HO-1 pathway

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ABSTRACT

The death of retinal ganglion cells (RGCs) during acute glaucoma causes progressive degeneration of the retinal nerve and irreversible blindness. Astaxanthin (AST) is a type of xanthophyll carotenoids and naturally synthesized by multiple halobios. It has been reported to protect the retina from acute glaucoma due to its anti-oxidative and anti-neuroinflammatory properties. However, the mechanism underlying this process remains unclear. We designed a mouse model with acute glaucoma and AST was administered by oral gavage. Hematoxylin and eosin staining was utilized to evaluate the condition of retina and the number of ganglion cells was counted. QRT-PCR was performed to evaluate the mRNA levels of *Bax* and *Bcl2* while Western blot assay was used to determine the protein levels of Bax, Bcl2, Nrf2 and HO-1. AST protected the retinal integrity of mice with acute glaucoma. The apoptosis of RGCs induced by ischemia and reperfusion was repressed by AST. The protective functions of AST on the retinal and ganglion cells decreased with the knock-down of *Nrf2*. AST promoted the activation of *Nrf2* and *Ho-1* in the RGCs of the model mice. AST protected the RGCs from apoptosis during acute glaucoma and alleviated the severe retinopathy symptoms through the *Nrf2/Ho-1* pathway.

1. Introduction

Glaucoma has become the second major cause of irreversible blindness and around 61 million people worldwide were suffering from the disease in 2010 (Cook and Foster, 2012; Mudumbai, 2013). As one of the most prevalent types of glaucoma, acute glaucoma is characterized by rapid increase in the intraocular pressure and neuro-retinal destruction induced by retinal ischemia and reperfusion (I/R) (Baden et al., 2016; Bonomi et al., 1999). Diabetes is one of the leading causes of acute glaucoma in clinic, which induces the apoptosis and necrosis of retinal ganglion cells (RGCs) (Kimura et al., 2017). Although the detailed mechanism of the diabetic retinopathy remains unclear, accumulating evidence has indicated the crucial role that oxidative stress and reactive oxygen species (ROS) play in the complications induced by diabetes (Ghirlanda et al., 1997). Due to the high oxygen consumption and active energy metabolism, the retina is rather sensitive to anoxemia and ROS attack (Cui et al., 2006; Madsen-Bouterse and Kowluru, 2008). Therefore, it is urgent for us to identify new strategies to relieve the oxidative stress on the retina and treat acute glaucoma more efficiently.

Astaxanthin (AST) is a type of xanthophyll carotenoids and a red-orange color pigment which can be naturally synthesized in algae, yeast, shrimp, lobster, quail retina and salmon (Ambati et al., 2014; Laslett et al., 2012; Visioli and Artaria, 2017). Accumulating evidence has revealed the anti-oxidize effect of AST. For instance, the anti-oxidative effect of AST on salivary gland in an irradiation-induced mouse model has been reported (Yamada et al., 2010). In addition, research has also demonstrated the effect of AST on the apoptosis and autophagy signaling pathways including AMPK, PI3K/Akt and JNK signaling. Thus, AST may alleviate the neuro-retinal degeneration induced by the apoptosis of RGCs in diabetic patients (Hong-Brown et al., 2017; Lee et al., 2010). Considering the anti-oxidative, anti-inflammatory and anti-diabetic effects of AST, its applications in the prevention and treatment of acute glaucoma are potentially valuable.

The transcription factor nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) or NF-E2 related factor 2 (*Nrf2*) responds to endogenous and exogenous stress such as anoxemia and chemical insults, which induces multiple signaling modulations for cell defense (Krajka-Kuzniak et al., 2017). *Nrf2* and its downstream transcription regulator heme oxygenase-1 (*Ho-1*) have been reported to participate in the maintenance of tissue homeostasis in the retina under stress or trauma (Cameron et al., 2018).

In this research, we aimed to identify the alleviating effect of AST on

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acute glaucoma and reveal the detailed mechanism. We hereby reported that AST protected the RGCs from apoptosis and alleviated the retinopathy symptoms in acute glaucoma mice through the *Nrf2/Ho-1* pathway.

2. Methods

2.1. Animals

All experiments and procedures performed in animals in this research were examined and approved by the Animal Use Committee in Shandong University. The adult *Nrf2*$^{-/-}$ C57BL/6 mice and corresponding wild type C57BL/6 mice were purchased from Biocytogen (Beijing, China). CRISPR/Cas9 technology was used to knock out the *Nrf2* gene in C57BL/6 mice. Briefly, sgRNA targeting *Nrf2* gene and Cas9 mRNA were co-injected into fertilized eggs. Cas9 nuclease combined with genomic target sequence guiding by sgRNAs and cleaved the double-stranded DNA. All mice (total n = 30 for each group) were housed in the facility with a virus/antigen-free ventilation system and an air conditioning system with constant temperature and humidity. Mice received water and food *ad libitum*. The anesthetic utilized in the surgery was 2% isoflurane with oxygen. The intraocular pressure of left eye of each mouse participating in the research was elevated by an instillation needle containing 0.9 % saline. The intraocular pressure was kept to 110 mmHg for 60 min during the procedure to squeeze the retinal artery to induce ischemia. The disappearance of the red reflex on the iris of treated mice was identified as the symbol of retinal ischemia, and the acquirement of the red reflex on the iris was considered as the retinal reperfusion. The mice in the sham group were treated with the same needle but no elevated pressure and the right eyes of these mice were left untreated as the negative control. Mice were sacrificed after 72 h of surgery for further experiments.

AST (HLPC content 98 %) was purchased from Shanghai Winherb Medical S & T Development Co. Ltd. (Shanghai, China). 50 mg/kg of AST was administered to mice by oral gavage. The administration started one week before the operation and continued until three days after the operation, once a day. The administration strategy of wild type and Nrf2$^{-/-}$ C57BL/6 mice was exactly the same. C57BL/6 mice was used as the control. The mice in the Sham group had no additional vehicle treatment in terms of feeding because AST was administered by oral gavage, not by injection.

2.2. Hematoxylin and eosin (H&E) staining

The eye tissues were fixed in 10 % formalin. Different concentrations of alcohol were used as dehydrating agent to gradually remove water from the tissue blocks. The alcohol in the tissue blocks was replaced with xylene and then the tissues were embedded with wax. Thin sections were obtained with a microtome. H&E were used for the stain of slices.

2.3. Retrograde labeling of RGCs with fluorogold

1% hydroxystilbamidine (Fluorogold, FG, Sigma-Aldrich) diluted in 0.9 % NaCl was used in this study. 10 mg/kg of xylazine was used to anesthetize the mice by intraperitoneal injection. FG was injected into both superior colliculi of mice 4 days before the I/R injury operation. All mice were allowed 7 days in this study for retrograde transport of FG before being sacrificed, while the administration of AST started 7 days before the operation and continued until three days after the operation, once a day. After 72 h of reperfusion, the mice were euthanized and their retinas were carefully and completely removed. Images were obtained at 1.2–2.0 mm from the optic disk in four quadrants (superior and inferior, nasal and temporal). Four non-overlapping images were captured from the median line of each quadrant, and the number of cells in 16 microscope fields per retina was counted. ImageJ software (NIH, Bethesda, MD, United States) was used for manual counting. Microglial cells, which displayed a small, irregular rod-shaped or ameboid morphology, were excluded.

2.4. Terminal-deoxy-transferase-mediated dutp nick end-labeling (TUNEL) Assay

Sections of retina tissues were fixed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 20 min and washed by PBS for three times afterwards. 0.3 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) was applied to permeabilize the cell membrane and 1% BSA buffer with 5% anti-goat serum (Life technologies, Carlsbad, CA, USA) was used to decrease background at RT for 1 h. The sections were then treated with the TUNEL assay kit following the instructions and 4′,6′-diamidino-2-phenylindole (DAPI) was used for the detection of apoptotic cells.

2.5. Oxidative stress analysis

A dihydroethidium (DHE) Assay Kit - Reactive Oxygen Species (ab236206, Abcam, Shanghai, China) was used to evaluate the superoxide and ROS levels in the retinal tissues of mice following instruction. Antimycin A, an inhibitor of complex III of the mitochondrial electron transport chain, was included as a positive control for ROS generation. N-acetyl Cysteine was included as an anti-oxidant control. ROS generation was represented as total DHE fluorescence. Malondialdehyde (MDA) level in the retinal tissues was detected by the Lipid Peroxidation (MDA) Assay Kit (ab118970, Abcam, Shanghai, China) following the instructions. The MDA in the sample reacted with thiobarbituric acid (TBA) to generate an MDA-TBA adduct which was quantified by a microplate reader (OD =532 nm). A SOD Activity Assay Kit (K335-100, Biovision, San Francisco, America) was used to test the MnSOD activity in the retinal tissues of mice following the instructions. An 8-hydroxy 2 deoxyguanosine (8-OHdG) ELISA Kit (ab201734, Abcam, Shanghai, China) was used to evaluate the accumulation of 8-OHdG in the retinal tissues of mice following the instructions.

2.6. Quantitative real-time PCR

TRIzol regent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract the RNA from the tissues of mouse retina following the instructions. The FastKing Transcription Kits (KR116-02, TIANGEN Biotech, Beijing, China) was then used for the reverse transcription with 2 μg RNA of each tissue sample to produce cDNA. Briefly, 2 μg of total RNA was used as a template. 5 × gDNA Buffer (for removing genomic DNA), FQ-RT Primer Mix, 10 × King RT Buffer and RNase-free double distilled water were used to configure a 20 μl reverse transcription system with the template on ice. Quantitative analysis of various mRNA levels mentioned in this study was then examined by SuperReal PreMix Plus (TIANGEN Biotech, Beijing, China) kit and S1000 PCR Thermal cycler (Thermo Fisher Labsystems, Waltham, USA). 200 ng cDNA per sample were used for PCR reaction. β-actin was used as the endogenous control. The data was transformed and analyzed by the $2^{-\Delta\Delta CT}$ method. The amplification efficiency of the reaction in our study was 93 %.

The primers verified in a previous study were used in this assay as follows (Huang et al., 2018):

Bax: Forward Primer CCGGCGAATTGGAGATGAACT, Reverse Primer CCAGCCCATGATGGTTCTGAT;

Bcl-2: Forward Primer GAGAGCGTCAACAGGGAGATG, Reverse Primer CCAGCCTCCGTTATCCTGGA;

β-actin: Forward Primer AGAAAATCTGGCACCACACC, Reverse Primer GGGGTGTTGAAGGTCTCAAA.

2.7. Western blot assay

For analysis of protein expression, retinal tissues were harvested. For analysis of Nrf2 nuclear translocation, nuclear extracts from retinal

tissues were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) under the instruction. RIPA buffer (ATCC, Manassas, VA, USA) was used to collect the protein samples. A Bicinchoninic Acid protein assay kit (Thermo Fisher Labsystems, Waltham, USA) was then used to quantify the concentrations of proteins for all samples. 12 % SDS-PAGE was used to separate different proteins in these samples with a voltage of 140 V, and the proteins were then transferred onto a polyvinylidene difluoride membrane (Thermo Fisher Labsystems, Waltham, USA) with an electric current of 300 mA. The membrane was co-incubated with rabbit anti-GAPDH (2118 L, 1:1000 dilution, Cell Signaling Technology), rabbit anti-Bcl-2 (3498S, 1:1000 dilution, Cell Signaling Technology), rabbit anti-Bax (2772S, 1:1000 dilution, Cell Signaling Technology), rabbit anti-Nrf2 (#ab89443, 1:1000 dilution, Abcam), rabbit anti-Histone H3 (4499S, 1:2000 dilution, Cell Signaling Technology) and rabbit anti-HO-1 (82206S, 1:1000 dilution, Cell Signaling Technology) at 37℃ for 1 h and washed by PBS buffer for 3 times. Then, goat anti-rabbit IgG (1:5000, Abcam, Shanghai, China) was incubated with the membrane at 37℃ for 1 h and an ECL Western Blotting Substrate kit (Abcam, Shanghai, China) was used for chemiluminescence imaging.

2.8. Statistical analysis

All the experiment had been repeated at least three times and the data were shown as the mean ± standard deviation (SD). One-way ANOVA analysis with a Bonferroni post hoc test was applied to analyze the distinction of all the repeats. Statistical significance was accepted at $P<0.05$.

3. Results

3.1. AST protected the retinal tissues and RGCs

To verify the effects of AST on the retina of mice treated with I/R, H&E staining was performed. As shown in Fig. 1A, the retinal ganglion cell layer (GCL) and inner nuclear layer (INL) disappeared after the treatment of I/R, and the thickness of outer nuclear layer (ONL) also decreased dramatically. The oral administration of AST alleviated the damage caused by I/R treatment compared to the second group. Interestingly, knock-down of *Nrf2* repressed the protective function of AST on the damaged retina. Similarly, the number of RGCs decreased with the I/R treatment and AST prevented the RGCs from reduction. The knock-down of *Nrf2* inhibited the protection of AST on the survival of RGCs (Fig. 1B). The thickness of retina decreased with the treatment of I/R, whereas the retina became much thicker in the I/R + AST group than the I/R + AST (*Nrf2* KD) group (Fig. 1C). Together, the protective function of AST on the retina and RGCs in model mice was rather significant and it was strictly restricted by the expression of *Nrf2*.

3.2. Protective effect of AST on FG-labeled RGCs after the I/R treatment in mice

To further identify the protective function of AST on the retina of the I/R-treated mice, retrograde labeling with FG was performed. As shown in Fig. 2A, the number of FG-labeled RGCs decreased after the procedure and AST increased the number of surviving RGCs only in the presence of *Nrf2*. Similarly, as shown in Fig. 2B, the density of RGCs declined with I/R. In the IR + AST group, the density of RGC was restored, whereas in the IR + AST (*Nrf2* KD) group the density of RGC decreased again.

3.3. AST inhibited the apoptosis of RGCs to protect the retina

To investigate the mechanism underlying AST protection on the retina and RGCs, TUNEL assay was performed. The apoptosis rate of RGCs increased with I/R. Oral gavage administration of AST effectively inhibited the apoptosis of RGCs in the presence of *Nrf2* (Fig. 3). This result indicated that AST protected the retina of I/R-treated mice through inhibiting the apoptosis of RGCs.

Pro-apoptosis protein Bax and anti-apoptosis protein Bcl-2 were two crucial markers of cell apoptosis. To further demonstrate that AST inhibited the apoptosis of cells in the retinal tissues, we examined the expression of *Bax* and *Bcl-2* in retinal tissues of mice. As shown in Fig. 4A and B, the mRNA level of *Bax* increased while *Bcl-2* decreased after the I/R treatment, indicating that I/R induced the progressive apoptosis of cells in the retinal tissues. AST reversed the apoptosis and changed the mRNA levels of *Bax* and *Bcl-2*. Similarly, the protein levels of Bax and Bcl-2 showed the same trends among these group (Fig. 4 C-E), indicating that AST inhibited the apoptosis of cells in the retinal tissues through the *Nrf2* pathway.

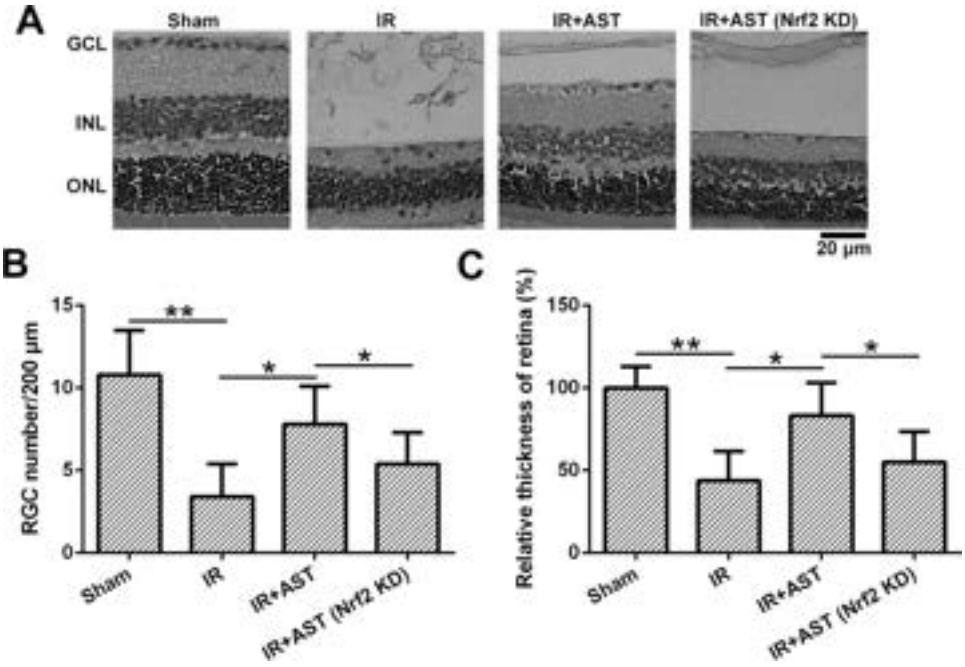

Fig. 1. The effects of Astaxanthin on the number of retinal ganglion cells (RGCs) and the thickness of the retinal tissue in ischemia/reperfusion (IR) mice. Hematoxylin and eosin staining of retinal cross-sections (A), the number of RGCs every 200 μm (B) and the relative retinal tissue thickness (C) of different groups were analyzed. Data are presented as mean ± SD, n = 6 for each group, *p < 0.05, **p < 0.01 between the indicated groups. GCL ganglion cell layer, INL inner nuclear layer, ONL outer nuclear layer. Sham vs IR + AST: p = 0.017 in B and 0.042 in C.

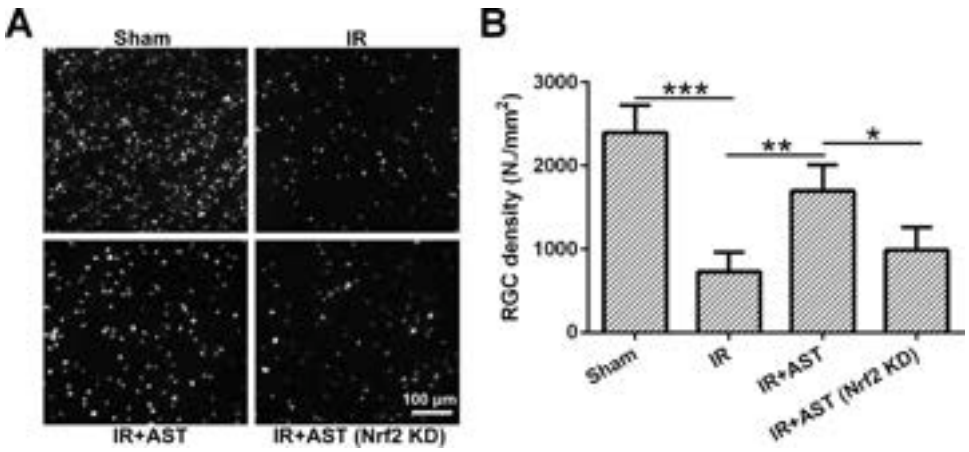

Fig. 2. Protective effect of Astaxanthin on FG-labeled RGCs after the ischemia/reperfusion (IR) in mice. A) Representative images of FG-labeled surviving RGCs in the retinal flatmounts from different groups captured 72 h after IR. B) Quantitative analysis of FG-labeled RGC densities in different groups. Data are presented as mean ± SD, n = 6 for each group, *p < 0.05, **p < 0.01 and ***p < 0.001 between the indicated groups. Sham vs IR + AST: p = 0.023.

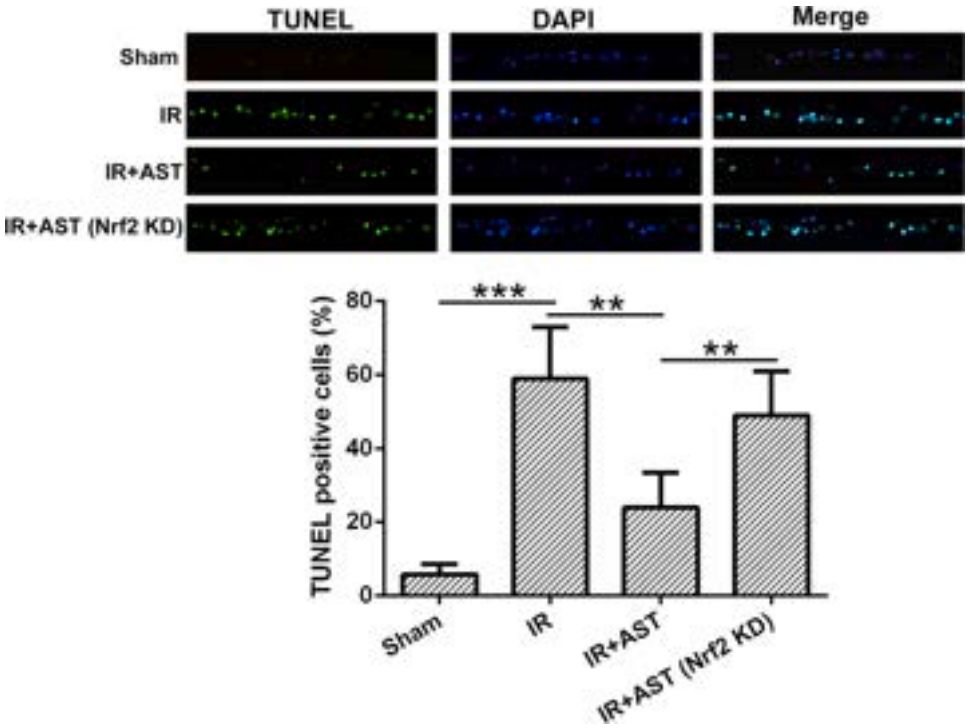

Fig. 3. Protective effect of Astaxanthin on the apoptosis of RGCs after the ischemia/reperfusion (IR) in mice. A) Representative images of TUNEL staining in the GCL of retinal sections from different groups. B) Quantitative analysis of TUNEL staining among different groups. Data are presented as mean ± SD, n = 6 for each group, **p < 0.01 and ***p < 0.001 between the indicated groups. Sham vs IR + AST: p = 0.028.

3.4. AST protected mouse retina from oxidative stress

DHE, MDA, 8-OHdG and MnSOD activity were closely related to the oxidative stress and ROS damage. The trend showed in Fig. 5A, B, C and D indicated that the I/R treatment induced the oxidative stress and oxidative DNA damage in RGCs. However, the oral administration of AST functioned as an anti-oxidant and protected the retina from oxidative stress and ROS. In addition, *Nrf2* played a crucial role in the modulation induced by AST.

3.5. AST promoted the upregulation of Nrf2 and Ho-1

To demonstrate the mechanism responsible for AST protection on the retinal tissues from oxidative stress and apoptosis, we performed Western blot assay to evaluate the protein levels of Nrf2 and HO-1 among all the groups. As shown in Fig. 6A to E, the protein levels of Nrf2 and HO-1 increased with the treatment of I/R. The total and nuclear Nrf2 protein levels and the HO-1 protein level increased with the oral administration of AST, indicating that AST protected the retina in model mice through the *Nrf2* and *HO-1* pathway.

4. Discussion

Acute glaucoma includes a series of heterogenous diseases with diversiform symptoms and unoptimistic prognosis (Ban et al., 2017). Although the detailed mechanism and pathogenesis of acute glaucoma remain unclear, accumulating evidence has demonstrated that high intraocular pressure, increased oxidative stress, neuro-inflammation

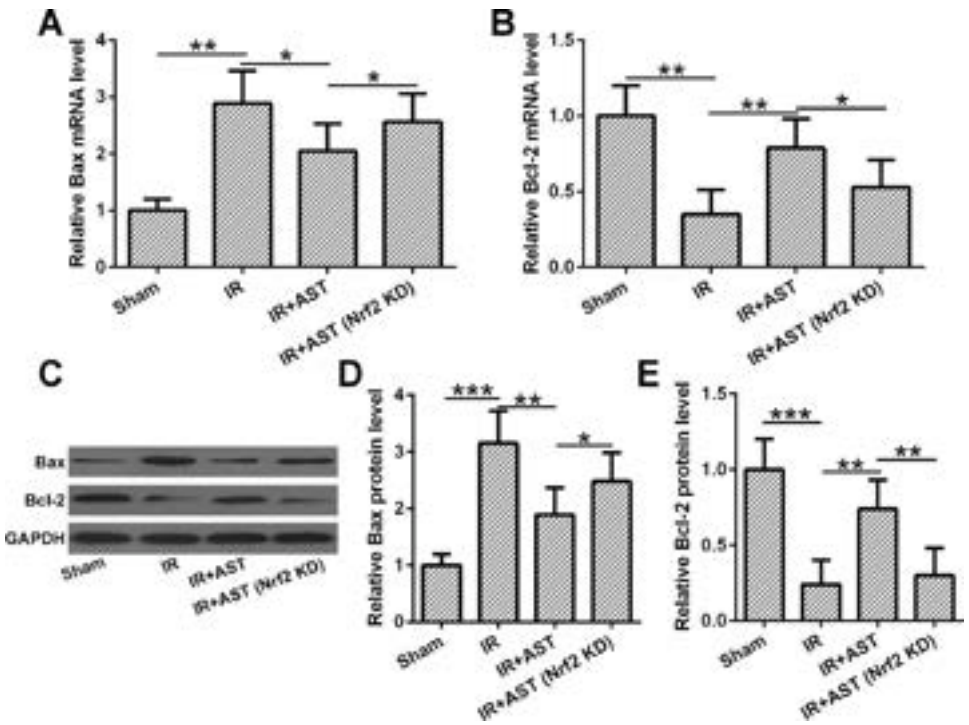

Fig. 4. Protective effect of Astaxanthin on the apoptosis of retina after the ischemia/reperfusion (IR) mice. A, B) qRT-PCR was used to determine the mRNA expressions of Bax (A) and Bcl-2 (B) in the whole retinas from different groups. C) Western blotting was used to determine the protein expressions of Bax and Bcl-2 in the whole retinas from different group and the relative expressions were normalized to Sham (D and E). Data are presented as mean ± SD, n = 8 for each group, *p < 0.05, **p < 0.01 and ***p < 0.001 between the indicated groups. Sham vs IR + AST: p = 0.008 in A, 0.036 in B, 0.029 in D and 0.033 in E.

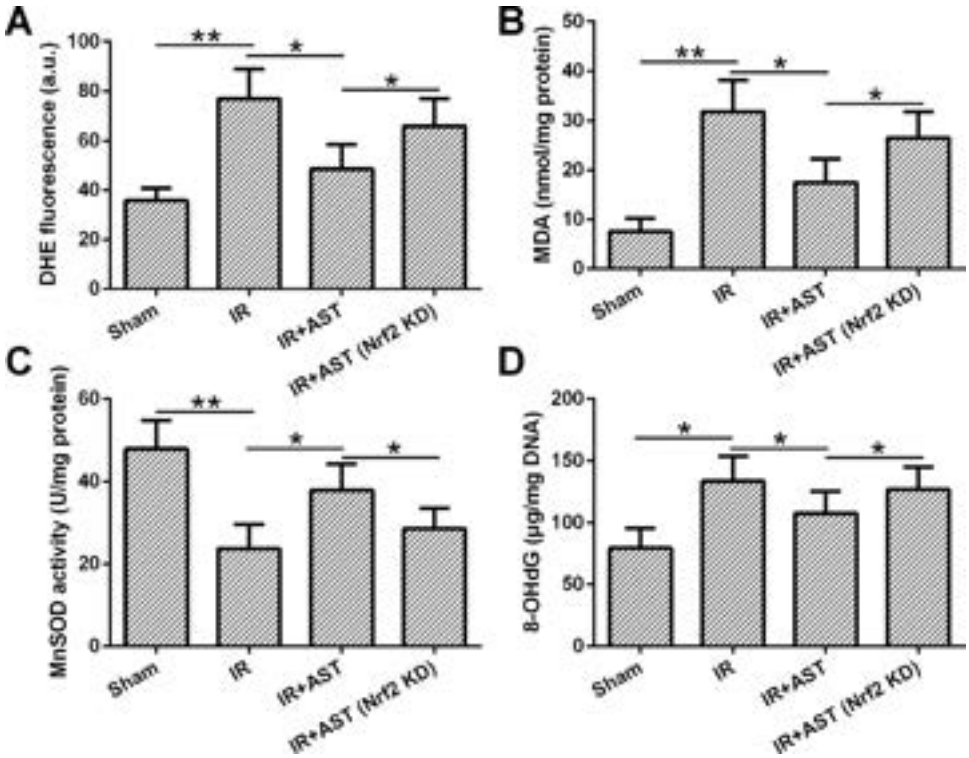

Fig. 5. Protective effects of Astaxanthin on the superoxide (DHE), MDA and 8−OHdG levels and MnSOD activity in the retinal tissues from ischemia/reperfusion (IR) mice. A) Superoxide level (DHE), B) MDA production, C) MnSOD activity and D) 8-OHdG level were measured according to the manufacturer's instructions for each assay. Data are presented as mean ± SD, n = 8 for each group, *p < 0.05, **p < 0.01 between the indicated groups. Sham vs IR + AST: p = 0.042 in A, 0.031 in B, 0.023 in C and 0.046 in D.

and irreversible apoptosis of RGCs promote the development of acute glaucoma and contribute to the final vision loss, thus seriously impacting the life quality of patients (Turalba and Chen, 2008). However, the treatment of acute glaucoma is rather limited and no effective treatment has been reported to restore the loss of vision (Plateroti et al., 2015). Therefore, it is urgent for us to identify an effective and credible treatment strategy to prevent the occurrence of retina damage and mitigate the progression of the symptoms. In our research, we firstly established an acute glaucoma mouse model. We used an intraocular pressure of 110 mmHg lasting for 60 min to induce the I/R injury of retinal tissues in mice. We wanted to use this model to simulate the rapid increase in intraocular pressure and the pathological changes of RGC in patients with acute glaucoma. And the severe acute glaucoma is exactly the prerequisite for AST to maximize its effectiveness. We reported that AST protected the retina and increased the amount of surviving RGCs in the I/R-treated mice. Mechanically, AST relieved the oxidative stress of

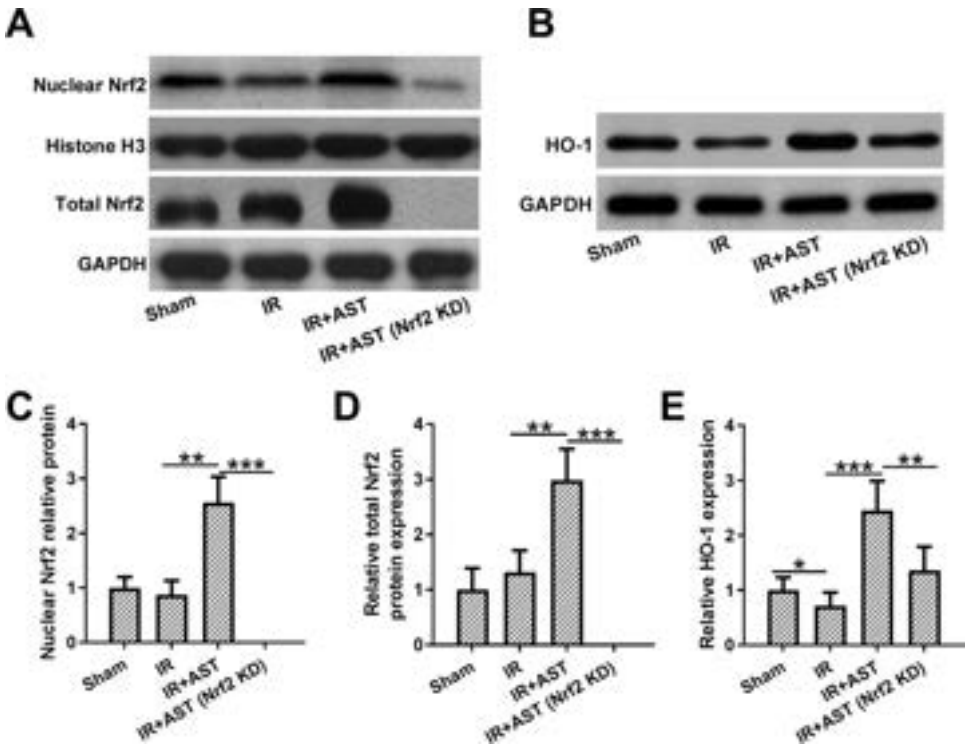

Fig. 6. Effects of Astaxanthin on Nrf2 activation and HO-1 expression in the retinal tissues from ischemia/reperfusion (IR) mice. The content of nuclear Nrf2, total Nrf2, Histone H3 and HO-1 were determined by Western blot analysis. C-E) The relative expressions were normalized to sham. Data are presented as mean ± SD, n = 8 for each group, **p < 0.01 and ***p < 0.001 between the indicated groups. Sham vs IR + AST: p = 0.006 in C, 0.004 in D and 0.002 in E.

mouse retina and thus suppressed the apoptosis of RGCs during acute glaucoma. We firstly demonstrated that *Nrf2* was an essential factor for AST to protect the retina from acute glaucoma. The treatment of AST induced the activation of Nrf2 and the upregulation of its downstream transcription factor HO-1, which mediated the cell defense against oxidative stress and ROS attack and suppressed the RGC apoptosis.

AST has drawn great attention of researchers due to its application in a variety of diseases. The chemical structure and its oxygenated β-ion-one-type ring systems have made AST one of the most efficient anti-oxidants among carotenes (Foss et al., 1987; Hussein et al., 2006). AST has been reported to protect important biomacromolecules, such as DNA, proteins and carbohydrates, from the oxidation and damage of ROS, singlet oxygen and other lipids produced during I/R (Yuan et al., 2011). For instance, AST functions as an inhibitor of the dysfunction of mitochondrial induced by oxidative stress (Kim and Kim, 2018). It has also been reported that AST plays an important role in the alleviation of the ethanol-caused liver injury by repressing oxidative stress through the STAT3 pathway (Han et al., 2018).

Diabetes and I/R cause the synapse dysfunction and irreversible damage of RGC axons, thus blocking the communication of the vision signal and causing irreversible apoptosis of RGCs (Agostinone and Di Polo, 2015; Anderson et al., 2002). Increasing evidence has shown that it is the oxidative stress that contributes to the damage of the retina, and the caspase-independent apoptosis of RGCs induced by ROS during acute glaucoma has drawn great research attention (Yeh et al., 2016). Finding a way to inhibit the apoptosis of RGC is significant for the treatment of acute glaucoma. AST has been reported to modulate the apoptosis activity in a variety of cells. For example, dietary AST has been suggested to inhibit the myonuclear apoptosis and thus attenuate the muscle atrophy in rats (Yoshihara et al., 2017). And it has been reported that AST repressed the glutamate-induced apoptosis by regulating the calcium influx and endoplasmic reticulum stress (Lin et al., 2017). Interestingly, the impact of AST on cell apoptosis can be a double-edged sword. It has also been reported that AST could induce the apoptosis of LS-180 cells (Hormozi et al., 2019). And AST promoted the radiation sensitivity of esophageal squamous cell carcinoma by inducing cell apoptosis (Qian et al., 2017). The dual function of AST in different types of cells requires us to identify the exact mechanism before using AST for the treatment of acute glaucoma in clinic. Interestingly, we have also investigated the toxicity of AST on the animals with the dose used in this research in the preliminary experiments, and no obvious side effects were observed. By comparing the retinal integrity and RGC numbers in mice of Sham + AST and Sham + Control groups, we identified that 50 mg/kg AST treatment had no obvious side effects on the retinal tissues of mice.

In addition, the anti-inflammation activity of AST has also been extensively studied. For example, the inflammation and reverse polarization of microglial cells can be repressed by AST through the LRP-1 pathway (Wen et al., 2017). AST also inhibited the inflammation and fibrosis of obesity-induced liver and adipose tissue in a mouse model (Kim et al., 2017). AST has also been used to treat neuropathy such as Alzheimer and Parkinson diseases due to its anti-neuroinflammation activity (Galasso et al., 2018).

Taken together, the anti-oxidative, anti-apoptosis and anti-inflammation properties of AST contribute to its valuable and potential applications in the protection of retinal tissues and treatment of acute glaucoma. Nakajima et al. reported that AST can protect retinal cells from oxidative stress both in vitro and in vivo in 2008, where they used a variety of factors including hydrogen peroxide, N-methyl-D-aspartate (NMDA), and starvation to increase the level of ROS in RGC-5 cells, and observed that AST treatment can reverse this process and inhibit the apoptosis of RGC-5 cells (Nakajima et al., 2008). Similarly, Otsuko et al. also reported AST protected against retinal damage, ameliorated the retinal dysfunction caused by I/R injury and maintained the structural integrity of the mouse retina, thereby suggesting that this process is achieved through the anti-oxidant effect of AST (Otsuka et al., 2016). In our research, we also identified that oral administration of AST protected the retina of model mice and increased surviving RGCs.

Similar to previous research, we also demonstrated that AST inhibited the oxidative stress induced by I/R treatment and thus suppressed the apoptosis of RGCs. However, we firstly revealed the detailed molecular mechanism of the protective effects of AST against retinal damage caused by I/R injury. We reported that AST treatment promoted the upregulation of both *Nrf2* and *Ho-1* in the retinal tissues of mice with acute glaucoma and thus protected the RGCs from oxidative stress.

Nrf2 is a transcription factor responding to the oxidative stress. Kelch-like ECH associated protein 1 (Keap1) is bound to Nrf2 and forms a heterodimer in the cytoplasm (Bellezza et al., 2018). The structure of Keap1 can be modulated by ROS or singlet oxygen and Nrf2 is then released and translocated into the nucleus to activate a series of downstream genes that regulate the cell defense under oxidative stress. Accumulating evidence has revealed the crucial role of Nrf2 in the progression of acute glaucoma. For instance, it has been reported that trimetazidine, a type of anti-oxidant, inhibited the apoptosis of RGCs in mice with acute glaucoma through the Nrf2 and HO-1 pathway (Wan et al., 2017). Another research has revealed that Nrf2 functions to protect the neurons in the retina after the treatment of I/R (Li et al., 2019; Ma, 2013). Interestingly, the activation of Nrf2 is also related to AST. AST has been indicated to promote the anti-inflammation and anti-oxidative activities of macrophages in Nrf2-dependent manners (Farruggia et al., 2018). Besides, the diabetes-induced renal fibronectin and collagen accumulation can be repressed by AST through the Nrf2 and ARE signaling pathways (Zhu et al., 2018). Similarly, we also demonstrated the close correlation between the protection of AST on the retina and RGCs and the activation of Nrf2. AST could not protect the retina or inhibit the apoptosis of RGCs in the absence of Nrf2. And we also discovered that dietary AST promoted the translocation of Nrf2 from the cytoplasm into the nucleus, indicating that AST might relieve the oxidative stress of RGCs through activating Nrf2 and its downstream gene *Ho-1*. In addition, as a crucial transcription factor, Nrf2 does trigger multiple downstream genes including catalase, SOD1/2, Thioredoxin and HSP-70, which can also play an important role in the cellular protection against oxidative stress. The activation and regulation of AST on these genes will be explored in our future research.

5. Conclusion

In conclusion, we hereby verify the protective function of AST on the retina and RGCs in a mouse model with acute glaucoma. AST inhibits the oxidative stress by activating *Nrf2* and *Ho-1* and thus suppresses the apoptosis of RGCs and protects the integrity of retina in acute glaucoma. Although AST alone cannot completely cure acute glaucoma, we believe that AST can be used as an auxiliary agent to participate in the treatment of acute glaucoma and significantly relieve the symptoms of patients.

Author statement

Yan Li, Cuiying Chu and Shu Liu conducted the experiments and analyzed the data. Qiang Wang conceived and supervised this study. Yan Li and Qiang Wang wrote the manuscript.

Research involving human participants and/or animals

All experiments and procedures performed in animals in this research were examined and approved by the Animal Use Committee in Shandong University.

Informed consent

Not applicable.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgment

None.

References

Agostinone, J., Di Polo, A., 2015. Retinal ganglion cell dendrite pathology and synapse loss: implications for glaucoma. Prog. Brain Res. 220, 199–216.
Ambati, R.R., Phang, S.M., Ravi, S., Aswathanarayana, R.G., 2014. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications–a review. Mar. Drugs 12, 128–152.
Anderson, M.G., Smith, R.S., Hawes, N.L., Zabaleta, A., Chang, B., Wiggs, J.L., John, S. W., 2002. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice. Nat. Genet. 30, 81–85.
Baden, T., Berens, P., Franke, K., Roman Roson, M., Bethge, M., Euler, T., 2016. The functional diversity of retinal ganglion cells in the mouse. Nature 529, 345–350.
Ban, N., Siegfried, C.J., Lin, J.B., Shui, Y.B., Sein, J., Pita-Thomas, W., Sene, A., Santeford, A., Gordon, M., Lamb, R., Dong, Z.Y., Kelly, S.C., Cavalli, V., Yoshino, J., Apte, R.S., 2017. GDF15 is elevated in mice following retinal ganglion cell death and in glaucoma patients. JCI Insight 2.
Bellezza, I., Giambanco, I., Minelli, A., Donato, R., 2018. Nrf2-Keap1 signaling in oxidative and reductive stress. Bba-Mol Cell Res 1865, 721–733.
Bonomi, L., Marraffa, M., Marchini, G., Canali, N., 1999. Perimetric defects after a single acute angle-closure glaucoma attack. Graefes Arch. Clin. Exp. Ophthalmol. 237, 908–914.
Cameron, B.D., Sekhar, K.R., Ofori, M., Freeman, M.L., 2018. The role of Nrf2 in the response to normal tissue radiation injury. Radiat. Res. 190, 99–106.
Cook, C., Foster, P., 2012. Epidemiology of glaucoma: what's new? Can. J. Ophthalmol. 47, 223–226.
Cui, Y., Xu, X., Bi, H., Zhu, Q., Wu, J., Xia, X., Qiushi, R., Ho, P.C., 2006. Expression modification of uncoupling proteins and MnSOD in retinal endothelial cells and pericytes induced by high glucose: the role of reactive oxygen species in diabetic retinopathy. Exp. Eye Res. 83, 807–816.
Farruggia, C., Kim, M.B., Bae, M., Lee, Y., Pham, T.X., Yang, Y., Han, M.J., Park, Y.K., Lee, J.Y., 2018. Astaxanthin exerts anti-inflammatory and antioxidant effects in macrophages in NRF2-dependent and independent manners. J. Nutr. Biochem. 62, 202–209.
Foss, P., Renstrom, B., Liaaenjensen, S., 1987. Natural occurrence of enantiomeric and meso astaxanthin 7-Star-Crustaceans including zooplankton. Comp Biochem Phys B 86, 313–314.
Galasso, C., Orefice, I., Pellone, P., Cirino, P., Miele, R., Ianora, A., Brunet, C., Sansone, C., 2018. On the Neuroprotective Role of Astaxanthin: New Perspectives? Mar. Drugs 16.
Ghirlanda, G., Di Leo, M.A., Caputo, S., Cercone, S., Greco, A.V., 1997. From functional to microvascular abnormalities in early diabetic retinopathy. Diabetes. Rev. 13, 15–35.
Han, J.H., Ju, J.H., Lee, Y.S., Park, J.H., Yeo, I.J., Park, M.H., Roh, Y.S., Han, S.B., Hong, J.T., 2018. Astaxanthin alleviated ethanol-induced liver injury by inhibition of oxidative stress and inflammatory responses via blocking of STAT3 activity. Sci. Rep. 8, 14090.
Hong-Brown, L.Q., Brown, C.R., Navaratnarajah, M., Lang, C.H., 2017. FoxO1-AMPK-ULK1 Regulates Ethanol-Induced Autophagy in Muscle by Enhanced ATG14 Association with the BECN1-PIK3C3 Complex. Alcohol. Clin. Exp. Res. 41, 895–910.
Hormozi, M., Ghoreishi, S., Baharvand, P., 2019. Astaxanthin induces apoptosis and increases activity of antioxidant enzymes in LS-180 cells. Artif Cell Nanomed B 47, 891–895.
Huang, W., Gao, F., Hu, F., Huang, J., Wang, M., Xu, P., Zhang, R., Chen, J., Sun, X., Zhang, S., Wu, J., 2018. Asiatic acid prevents retinal ganglion cell apoptosis in a rat model of Glaucoma. Front. Neurosci. 12, 489.
Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid with potential in human health and nutrition. J. Nat. Prod. 69, 443–449.
Kim, S.H., Kim, H., 2018. Inhibitory effect of astaxanthin on oxidative stress-induced mitochondrial Dysfunction-A mini-review. Nutrients 10.
Kim, B., Farruggia, C., Ku, C.S., Pham, T.X., Yang, Y., Bae, M., Wegner, C.J., Farrell, N.J., Harness, E., Park, Y.K., Koo, S.I., Lee, J.Y., 2017. Astaxanthin inhibits inflammation and fibrosis in the liver and adipose tissue of mouse models of diet-induced obesity and nonalcoholic steatohepatitis. J. Nutr. Biochem. 43, 27–35.
Kimura, A., Namekata, K., Guo, X., Noro, T., Harada, C., Harada, T., 2017. Targeting Oxidative Stress for Treatment of Glaucoma and Optic Neuritis. Oxid. Med. Cell. Longev. 2017, 2817252.
Krajka-Kuzniak, V., Paluszczak, J., Baer-Dubowska, W., 2017. The Nrf2-ARE signaling pathway: an update on its regulation and possible role in cancer prevention and treatment. Pharmacol. Rep. 69, 393–402.
Laslett, L.J., Alagona Jr, P., Clark 3rd, B.A., Drozda Jr, J.P., Saldivar, F., Wilson, S.R., Poe, C., Hart, M., 2012. The worldwide environment of cardiovascular disease: prevalence, diagnosis, therapy, and policy issues: a report from the American College of Cardiology. J. Am. Coll. Cardiol. 60, S1–49.
Lee, J.W., Park, S., Takahashi, Y., Wang, H.G., 2010. The association of AMPK with ULK1 regulates autophagy. PLoS One 5 e15394.
Li, H., Zhang, Q.Q., Li, W.F., Li, H.Y., Bao, J., Yang, C.P., Wang, A.P., Wei, J.F., Chen, S. Z., Jin, H.T., 2019. Role of Nrf2 in the antioxidation and oxidative stress induced developmental toxicity of honokiol in zebrafish. Toxicol Appl Pharm 373, 48–61.

Lin, X.T., Zhao, Y., Li, S.H., 2017. Astaxanthin attenuates glutamate-induced apoptosis via inhibition of calcium influx and endoplasmic reticulum stress. Eur. J. Pharmacol. 806, 43–51.
Ma, Q., 2013. Role of Nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. 53, 401-+.
Madsen-Bouterse, S.A., Kowluru, R.A., 2008. Oxidative stress and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives. Rev. Endocr. Metab. Disord. 9, 315–327.
Mudumbai, R.C., 2013. Clinical update on normal tension glaucoma. Semin. Ophthalmol. 28, 173–179.
Nakajima, Y., Inokuchi, Y., Shimazawa, M., Otsubo, K., Ishibashi, T., Hara, H., 2008. Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress in-vitro and in mice in-vivo. J. Pharm. Pharmacol. 60, 1365–1374.
Otsuka, T., Shimazawa, M., Inoue, Y., Nakano, Y., Ojino, K., Izawa, H., Tsuruma, K., Ishibashi, T., Hara, H., 2016. Astaxanthin protects against retinal damage: evidence from in vivo and in vitro retinal ischemia and reperfusion models. Curr. Eye Res. 41, 1465–1472.
Plateroti, P., Plateroti, A.M., Abdolrahimzadeh, S., Scuderi, G., 2015. Pseudoexfoliation syndrome and pseudoexfoliation Glaucoma: a review of the literature with updates on surgical management. J. Ophthalmol. 2015, 370371.
Qian, X., Tan, C., Yang, B., Wang, F., Ge, Y., Guan, Z., Cai, J., 2017. Astaxanthin increases radiosensitivity in esophageal squamous cell carcinoma through inducing apoptosis and G2/M arrest. Dis. Esophagus 30.
Turalba, A.V., Chen, T.C., 2008. Clinical and genetic characteristics of primary juvenile-onset open-angle glaucoma (JOAG). Semin. Ophthalmol. 23, 19–25.
Visioli, F., Artaria, C., 2017. Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps. Food Funct. 8, 39–63.
Wan, P.X., Su, W.R., Zhang, Y.Y., Li, Z.D., Deng, C.B., Zhuo, Y.H., 2017. Trimetazidine protects retinal ganglion cells from acute glaucoma via the Nrf2/Ho-1 pathway. Clin. Sci. 131, 2363–2375.
Wen, X.J., Xiao, L.J., Zhong, Z.Y., Wang, L.M., Li, Z., Pan, X.P., Liu, Z.L., 2017. Astaxanthin acts via LRP-1 to inhibit inflammation and reverse lipopolysaccharide-induced M1/M2 polarization of microglial cells. Oncotarget 8, 69370–69385.
Yamada, T., Ryo, K., Tai, Y., Tamaki, Y., Inoue, H., Mishima, K., Tsubota, K., Saito, I., 2010. Evaluation of therapeutic effects of astaxanthin on impairments in salivary secretion. J. Clin. Biochem. Nutr. 47, 130–137.
Yeh, P.T., Huang, H.W., Yang, C.M., Yang, W.S., Yang, C.H., 2016. Astaxanthin inhibits expression of retinal oxidative stress and inflammatory mediators in streptozotocin-induced diabetic rats. PLoS One 11.
Yoshihara, T., Yamamoto, Y., Shibaguchi, T., Miyaji, N., Kakigi, R., Naito, H., Goto, K., Ohmori, D., Yoshioka, T., Sugiura, T., 2017. Dietary astaxanthin supplementation attenuates disuse-induced muscle atrophy and myonuclear apoptosis in the rat soleus muscle. J. Physiol. Sci. 67, 181–190.
Yuan, J.P., Peng, J.A., Yin, K., Wang, J.H., 2011. Potential health-promoting effects of astaxanthin: a high-value carotenoid mostly from microalgae. Mol. Nutr. Food Res. 55, 150–165.
Zhu, X.Y., Chen, Y.J., Chen, Q., Yang, H.Y., Xie, X., 2018. Astaxanthin promotes Nrf2/ARE signaling to alleviate renal fibronectin and collagen IV accumulation in diabetic rats. J. Diabetes Res.