Protective effects of CY-09 and astaxanthin on NaIO₃-induced photoreceptor inflammation via the NLRP3/autophagy pathway

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Abstract

● AIM: To study the effect of the NLRP3/autophagy pathway on the photoreceptor inflammatory response and the protective mechanism of CY-09 and astaxanthin (AST).

● METHODS: ICR mice were intraperitoneally injected NaIO₃, CY-09, AST successively and divided into 5 groups, including the control, NaIO₃, NaIO₃+CY-09, NaIO₃+AST, and NaIO₃+CY-09+AST groups. Spectral domain optical coherence tomography and flash electroretinogram were examined and the retina tissues were harvested for immunohistochemistry, enzyme linked immunosorbent assay (ELISA), and Western blotting. Retinal pigment epithelium cell line (ARPE-19 cells) and mouse photoreceptor cells line (661W cells) were also treated with NaIO₃, CY-09, and AST successively. Cell proliferation was assessed by cell counting kit-8 (CCK-8) assay. Apoptosis was analyzed by flow cytometry. Changes in autophagosome morphology were observed by transmission electron microscopy. Quantitative polymerase chain reaction (qPCR) was used to detect NLRP3 and caspase-1. NLRP3, caspase-1, cleaved caspase-1, p62, Beclin-1, and LC3 protein levels were measured by Western blotting. IL-1β and IL-18 were measured by ELISA.

● RESULTS: Compared with the control group, the activity of NaIO₃-treated 661W cells decreased within 24 and 48h, apoptosis increased, NLRP3, caspase-1, p62, Beclin-1, and LC3 protein levels were measured by Western blotting. IL-1β and IL-18 were measured by ELISA.

● CONCLUSION: CY-09 and AST inhibit NaIO₃-induced inflammatory damage through the NLRP3/autophagy pathway in vitro and in vivo. CY-09 and AST may protect retina from inflammatory injury.

● KEYWORDS: CY-09; astaxanthin; retinal degeneration; photoreceptor cells; inflammation; NLRP3

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INTRODUCTION

Retinal degenerative diseases are a group of chronic diseases that are characterized by the apoptosis of retinal photoreceptor cells, which leads to vision loss. Chronic inflammation in the retina may be an important mechanism that initiates photoreceptor cell death in age-related macular degeneration (AMD). Intravitreal injection of vascular endothelial growth factor (VEGF) inhibitors is the first-line treatment for wet AMD. But dry AMD that cannot be effectively controlled. When exposed to harmful stimuli, this retinal tissue activity may contribute to the establishment of a chronic inflammatory state in the local microenvironment and abnormal immune activation may occur. Photoreceptor cell death involves many molecular mechanisms, including NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome-dependent pyroptosis. Some studies have shown that the NLRP3/autophagy signaling pathway plays a key role in the inflammatory response of photoreceptors. When stimulated, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) play an important role in inflammasome activation. When activated, NLRP3 cleaves pro-caspase-1 to its activated form, which further promotes the maturation of inflammatory precursor proteins to the activated forms of interleukin (IL)-1β and IL-18.
IL-1β\(^{[13-14]}\). Activated caspase-1 can also play a proinflammatory role\(^{[15-16]}\). There are three main mechanisms by which NLRP3 were activated: K\(^{+}\) outflow\(^{[17-18]}\), the uptake of crystalline and particulate substances by phagocytes\(^{[19]}\), the production of reactive oxygen species (ROS)\(^{[20]}\). NaIO\(_3\) can induce retinal pigment epithelium (RPE) cell death through mechanisms\(^{[21]}\). In adult retinal pigment epithelial cell line-19 (ARPE-19) cells, NLRP3 inhibitor can prevent NaIO\(_3\)-induced lipid peroxidation, mitochondrial damage, DNA damage and cell death. In mouse models, NLRP3 inhibitor pretreatment can inhibit NaIO\(_3\)-induced cell death\(^{[22]}\). In this study, the NLRP3 inhibitor CY-09 and astaxanthin (AST) were administered to study whether the combined treatment exerts anti-inflammatory and protective effects on photoreceptor cells and to elucidate the underlying mechanism.

**MATERIALS AND METHODS**

**Ethical Approval** All the animal experiments were approved by the Ethics Committee of Animal Center, West China Hospital, Sichuan University (Ethics number: 20230406006) and were conducted in accordance with the Guiding Opinions on the Treatment of Laboratory Animals issued by the Ministry of Science and Technology of China.

**Cells and Animals** The cell lines included mouse photoreceptor cells (661W cells) and ARPE-19 cells were purchased from Guangzhou Ginio Biotechnology Co., Ltd., China. The ICR mice were 4-6 weeks old, weighed 25±3 g, and purchased from Beijing Huafukang Company, China.

**Cell Culture** 661W cells were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hangzhou Tianhang Biotechnology Co., Ltd., China), 100 U/mL penicillin (HyClone, Logan, UT, USA), and 100 μg/mL streptomycin (HyClone). The ARPE-19 cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (Hangzhou Tianhang Biotechnology Co., Ltd., China), 100 U/mL penicillin (HyClone), and 100 μg/mL streptomycin (HyClone). The cells were cultured in an incubator in an environment with 5% CO\(_2\), saturated humidity and 37℃. When cell passage was performed, the cells were washed with phosphate buffer saline (PBS), and 2 mL of 0.25% pancreatic enzyme containing EDTA (Geno Biopharmaceutical Technology Co., Ltd., China) was added to digest the cells for 2-5min; then, the cells were centrifuged at 1000 rpm/min for 4min and resuspended for further experiments.

**Cell Counting Kit-8 Assay** The cells were cultured overnight in SHEL LAB and subjected to concentration gradients of NaIO\(_3\) (0, 100, 300, 600, 900, 1200, 1500, 1800, and 2400 μg/mL). Then, three NaIO\(_3\) concentrations were established and cell proliferation was analyzed after incubation with NaIO\(_3\). A gradient design was carried out to expose cells to different concentrations of AST. Cells were cocultured with three concentrations of CY-09 and NaIO\(_3\), 10 μL cell counting kit-8 (CCK-8) solution was added and incubated at 37℃ for 1-4h, and the absorbance was measured at 450 nm by a microplate system (Diatek Company., USA).

**Flow Cytometry** After cells were cultured overnight, NaIO\(_3\), CY-09, AST or a mixed solution containing the appropriate concentration was added to the cells. After incubation for 24h, the cells were transferred to 2 mL EP tubes and centrifuged. The cells were precipitated. Then 1 mL precooled PBS was added to resuspend the cells and centrifuged again. The cells were suspended in diluted binding buffer, and the concentration was adjusted to (1-5)×10\(^4\) cells/mL. The cell suspensions were mixed with 5 μL of Annexin V/Alexa Fluor, and incubated at room temperature for 5min in the dark. Then, 10 μL and 20 μg/mL propidium iodide solution (Apoptosis kit: 4A BIOTECH Company., China) was added, 400 μL PBS was added, and the cells were immediately analyzed by flow cytometry.

**Quantitative Real-Time Polymerase Chain Reaction** A spectrophotometer was used to determine the concentration of the total RNA that was extracted. The total RNA samples were diluted in proportion, and subsequent quantitative real-time polymerase chain reaction (qPCR) experiments were immediately conducted. cDNA was then transcribed using a designed primer (Beijing Tsingke Biotech Co., Ltd., China) and gDNA Eraser (Vazyme Biotech Co., Ltd., China). qPCR was performed using the SYBR Green Master kit (Vazyme Biotech Co., Ltd., China) and fluorescent quantitative PCR apparatus (Life Technologies, USA). GAPDH was used as an endogenous control. The data were analyzed by the 2\(^{-}\Delta\Delta CT\) method (Table 1).

**Western Blotting** After cells were washed, RIP A lysis buffer (Beyotime Biotechnology Co., Ltd., China) containing

### Table 1 List of real-time polymerase chain reaction primer sequences

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Nucleotide sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-NLRP3-F</td>
<td>TCTCAAGTCTAAGCACCACCGG</td>
</tr>
<tr>
<td>M-NLRP3-R</td>
<td>CGAAGCGACATTGATGAC</td>
</tr>
<tr>
<td>M-caspase-1-F</td>
<td>AGGGATAATTGGGGACCACATAC</td>
</tr>
<tr>
<td>M-caspase-1-R</td>
<td>CTGAACTTTTAAACACACACTCC</td>
</tr>
<tr>
<td>M-GAPDH-F</td>
<td>TGAAGGGTGAGGGCAAAAG</td>
</tr>
<tr>
<td>M-GAPDH-R</td>
<td>AGTCTCTCTGGTGGCAGGTAT</td>
</tr>
<tr>
<td>M-IL-1β-F</td>
<td>GAAATGGCACAATGGGCTAGT</td>
</tr>
<tr>
<td>M-IL-1β-R</td>
<td>TTCTCACAGCCCAATGGTAT</td>
</tr>
<tr>
<td>M-IL-18-F</td>
<td>AGTAAAGTGGGGAGGGTTTG</td>
</tr>
<tr>
<td>M-IL-18-R</td>
<td>ATCATGACAGCCTGGGATT</td>
</tr>
<tr>
<td>M-Bax-F</td>
<td>CACTAAAGTGGCCGCAGCTGA</td>
</tr>
<tr>
<td>M-Bax-R</td>
<td>GGTCGCGAAATGGGAGAGGA</td>
</tr>
</tbody>
</table>
phosphatase inhibitor (AS-PEN Biotechnology Co., Ltd., China) and protease inhibitor (Beyotime Biotechnology Co., Ltd., China) was added, and the cells were lysed on ice for 3-5 min. The lysates were centrifuged at 12 000 g and 4°C for 5 min. The protein concentrations were determined with a BCA kit (Beyotime Biotechnology Co., Ltd., China). Before the protein loading buffer (Bio-Rad Laboratories Co., Ltd., USA) was used, β-mercaptoethanol was added and mixed. Then, 4× loading buffer was added, mixed, and incubated in a 100°C water bath for 10 min. The SDS-PAGE gel (BIOSHARP Company, China) was prepared. The appropriate SDS-PAGE separation gel was selected according to the molecular weights of the target proteins. The sample proteins and the protein prestained marker (Beyotime Biotechnology Co., Ltd., China) were added to the sample wells of an SDS-PAGE gel, and electrophoresis was carried out at 80 V for 30 min. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Merck, USA) and marker position to make the sandwich for protein transfer. After transfer, the PVDF membranes were incubated in 50 mL of 5% skim milk (BD Company, USA) on a shaker for 1-2 h. Then, primary antibodies were diluted in PBS with Tween-20 (PBST), added to the membranes, and incubated at 4°C overnight. The primary antibodies included the following: rabbit anti-NLRP3 antibody (US CST Corporation, USA, #15101), rabbit anti-caspase-1 antibody (AbBiotech, AUS #AF5418), rabbit anti-cleaved caspase-1 antibody (AbBiotech, #AF4095), rabbit anti-P62 antibody (CST Corporation, #39749), rabbit anti-Beclin-1 antibody (CST Corporation, #3495), rabbit anti-LC-3 antibody (CST Corporation, #4108), and rabbit anti-GAPDH antibody (Abcam Corporation, UK, # ab181602). Then, the primary antibodies were removed, and were washed with PBST 3 times for 5 min each. The corresponding diluted fluorescent secondary antibodies (LI-COR Corporation, USA) were added and incubated at room temperature for 1 h in the dark. Then, the PVDF membranes were washed with PBST in the dark and immediately placed in a Western blotting image analysis system to collect and save images.

**Immunofluorescence Staining** Cellular immunofluorescence was performed according to standard procedures, which included cell preparation, fixation, permeabilization, blocking, primary antibody incubation, secondary antibody incubation, washing, mounting, observation and imaging.

**Enzyme-Linked Immunosorbent Assay** Enzyme-linked immunosorbent assay (ELISA) was performed following the steps in the kit instructions (ELK Biotechnology, China). These steps included coating, blocking, sample addition, primary antibody binding, washing, secondary antibody binding, substrate addition, reaction termination, detection and analysis.

**Establishment of the Animal Model** The experimental animals were raised in the standard specific pathogen free (SPF) environment. NaIO3 powder was dissolved at a concentration of 12.5 mg/mL (4 mL). The mice were divided into different group (25, 50, and 100 mg/kg), and NaIO3 was delivered by intraperitoneal injection. Experimental mice were randomly divided into 5 groups, included the control group, NaIO3 group, NaIO3+CY-09 group, NaIO3+AST group and NaIO3+CY-09+AST group. CY-09 powder was dissolved in a solution containing 10% DMSO+90% corn oil. The AST was dissolved in PBS by ultrasonication for 15 min. In the treatment groups, NaIO3 solution was intraperitoneally administered only once. CY-09 was intraperitoneally starting on the second day after the establishment of the model and continued for 1 wk. AST was continuously administered for 1 wk starting on the second day after the establishment of the model.

**Hematoxylin and Eosin Staining** Tissues were fixed with formalin. Then, the tissues were washed with PBS to remove the fixative. Then, the tissues were dehydrated with a series of graded alcohol solutions and were stained with hematoxylin to stain the cell nuclei, followed by eosin to stain the cytoplasm. Then were treated with a clearing agent such as xylene to render them transparent. The slides then were mounted with neutral balsam, and were observed under a microscope.

**Spectral Domain Optical Coherence Tomography** Fundus imaging was performed with spectral domain optical coherence tomography (SD-OCT) to evaluate changes in retinal layers from model mice. All the examinations were performed by the intraperitoneal injection of tribromoethanol solution under general anesthesia. The examinations were carried out on the 3rd and 7th day after treatment.

**Flash Electoretinogram** On the 3rd and 8th day, flash electoretinogram (FERG) was performed under the conditions of light adaptation (LA) and dark adaptation (DA). All the examinations were performed under general anesthesia. First, DA examination was performed, in which the mice were exposed to stimulation ranging from low to high, and then, LA examination was performed. After the test, the latent value and amplitude value of the FERG a and b waves were measured and evaluated by the same experienced ophthalmologist.

**Statistical Analysis** SPSS and GraphPad Prism 9 were used for statistical description and analysis. Measurement data with normal distribution were presented as the mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparisons of more than two groups and then multiple comparison of LSD, and the difference between two groups was analyzed by Student’s t-test. P<0.05 was considered statistical significance.
RESULTS

Effects of NaIO3, CY-09, and AST on 661W and ARPE-19 Cells Proliferation and Activity

In the cell proliferation experiment, and concentrations of 0, 100, 300, 900, 1200, 1500, 1800, 2100, and 2400 µg/mL NaIO3 was tested. Then, three different concentrations of NaIO3 within the appropriate range, 250, 500, and 750 µg/mL NaIO3, were tested at time points of 24, 48, and 72h (Figure 1A). The results suggested that the medium concentration and high concentration of NaIO3 caused cell damage after 24h and 72h of treatment, and cell proliferation was inhibited at the lower concentration (Figure 1B). The AST concentrations included 0, 10, 20, 40, and 80 μmol/L, and the cells were cocultured with 500 µg/mL NaIO3 solution for 24h (Figure 1C). Then, low, medium and high concentrations of AST (5, 10, and 20 μmol/L) were chosen and combined with 500 µg/mL NaIO3 to treat 661W cells and ARPE-19 cells. It was found that with increasing AST concentration, cell activity increased (Figure 1D).

Three different concentrations of CY-09 (25, 50, and 100 μmol/L) and 500 µg/mL NaIO3 solution were combined and incubated with 661W cells. Compared with the NaIO3 group, the cell activity increased with CY-09 concentration increasing (Figure 2A). In the control group, the apoptosis rate of 661W cells was 6.82%, and ARPE-19 cells was 7.15%. When NaIO3 was administered, the average apoptosis rate of 661W cells was 32.28%, and ARPE-19 cells was 23.25%. When NaIO3+CY-09 was administered, the mean apoptosis rate of 661W cells was 23.11%, and ARPE-19 cells was 20.90%. These results showed that with the addition of CY-09, the apoptosis rate of the two kinds of cells was decreased compared with that in NaIO3 group (P<0.01; Figure 2B).

The immunofluorescence staining results in 661W cells showed that a small amount of NLRP3 was uniformly expressed in the cytoplasm of the control group, and the cells were densely distributed. After NaIO3 treatment, cell death was increased and the green fluorescence in the cells was enhanced. The fluorescence of the NaIO3+CY-09 group was decreased (Figure 2C).

Effect of CY-09 Combined with Astaxanthin on Inflammatory Response and Apoptosis of 661W and ARPE-19 Cells

ELISA results showed that the levels of IL-1β and IL-18 in the NaIO3 group were significantly increased than in the control group (P<0.05) and decreased in NaIO3+CY-09 and NaIO3+AST group (P<0.05) especially in the NaIO3+CY-09+AST group (P<0.05) both in 661W and ARPE-19 cells (Figure 2D, 2E).

In the control group, the apoptosis rate of 661W cells was 5.05%, and ARPE-19 cells was 6.33%. In the NaIO3 group, the apoptosis rate of 661W cells was 31.55%, and ARPE-19 cells was 30.33%. In the NaIO3+CY-09 group, the apoptosis rate of
Figure 2 Effects of NaIO₃ and CY-09 on 661W and ARPE-19 cells

A: Compared with the NaIO₃ group, the cell activity increased with increasing CY-09 concentration in 661W and ARPE-19 cells; B: Apoptosis of 661W and ARPE-19 cells after treatment with NaIO₃ and CY-09; C: NLRP3 expression by immunofluorescence after CY-09 treatment in the 661W cell; D, E: Expression of IL-1β and IL-18 in 661W (D) and ARPE-19 (E) cells treated with CY-09 combined with AST using ELISA. *P<0.05, **P<0.01. 661W: Mouse photoreceptor cells line; ARPE-19: Adult retinal pigment epithelial cell line-19; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; CY-09: An inhibitor binds to NLRP3; IL: Interleukin; AST: Astaxanthin.
661W cells was 23.64%, and ARPE-19 cells was 24.00%. In the NaIO3+AST group, the apoptosis rate of 661W cells was 22.17%, and ARPE-19 cells was 18.54%. In the NaIO3+CY-09+AST group, the apoptosis rate of 661W cells was 14.79%, and ARPE-19 cells was 12.43%. These results showed that with the addition of CY-09 and AST, the apoptosis rates of these two kinds of cells were decreased compared with those that were treated with NaIO3 alone (*P<0.01; Figure 3).

The mRNA levels of NLRP3 and caspase-1 in NaIO3 group were higher than in the control group (*P<0.05) in 661W cell, and decreased in NaIO3+CY-09 and NaIO3+AST group, especially in NaIO3+CY-09+AST group (*P<0.05; Figure 4A). The protein levels of NLRP3 and cleaved caspase-1 also decreased in NaIO3+CY-09, NaIO3+AST, and NaIO3+CY-09+AST group (Figure 4B).

**Effect of CY-09 Combined with Astaxanthin on Autophagy in 661W and ARPE-19 Cells**

Compared with the NaIO3 group, the expression of p62 in NaIO3+CY-09 and NaIO3+AST groups were increased in both 661W and ARPE-19 cells (*P<0.05), while Beclin-1, LC3-I, LC3-II, and LC3-II/LC3-I were decreased (*P<0.05), especially in NaIO3+CY-09+AST group (*P<0.05; Figure 5).

Electron microscopy images of 3400×, 8500×, and 17500× of 661W cells showed that the control group had a few autophagosomes that were generally not large. In the NaIO3 group, there were obvious, large bulla-like autophagosomes and more fusion occurred. In the NaIO3+CY-09 and NaIO3+AST groups, the number of fused autophagosomes was significantly reduced compared with that in the NaIO3 group. The number of autophagosome vesicles in the NaIO3+CY-09+AST group was reduced compared with that in the other treatment groups (Figure 6).

**Establishment of an AMD Model by NaIO3 Injection**

On the 3rd day, retinal pigment disorder was observed in the high-dose group. In the medium-dose group, retinal

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Figure 3 661W and ARPE-19 cell apoptosis after treatment with CY-09 combined with AST *P<0.01. 661W: Mouse photoreceptor cells line; ARPE-19: Adult retinal pigment epithelial cell line-19; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; AST: Astaxanthin; CY-09: An inhibitor binds to NLRP3.

Figure 4 Effects of AST and CY-09 on NLRP3, caspase-1 expression in 661W and ARPE-19 cells A: The mRNA levels of NLRP3 and caspase-1 in 661W cells; B: The protein levels of NLRP3 and cleaved caspase-1 in 661W and ARPE-19 cells. *P<0.05, *P<0.01. 661W: Mouse photoreceptor cells line; ARPE-19: Adult retinal pigment epithelial cell line-19; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; AST: Astaxanthin; CY-09: An inhibitor binds to NLRP3.
pigment disorder and interlayer highly reflective spots in the corresponding area were observed. In the low-dose group, there were a few interlayer spots. On the 7th day, the retina of all groups exhibited retinal background pigment disruption and more interlayer dots (Figure 7).

Dark adaptation was mainly tested with different intensities of flash stimulation. The test conditions were as follows: DQQ DA 0.001, DQQ DA 0.01, DQQ DA 0.1, DQQ DA 1.0, DQQ DA 3.0, DQQ DA 3.0 OP, DQQ DA 10.0, and DQQ LA 3.0. The wave amplitudes and latency of the a- and b-waves in the control group were normal, and the wave amplitudes of the low-dose group, medium-dose group and high-dose group were decreased to different degrees (Figure 8).

Due to the influence of drug injection and anesthesia, the mortality rates were higher. Only the eyes of mice from the control group and low-dose group were harvested for hematoxylin and eosin (HE) and immunohistochemical staining. In the control group, the structure of retinal layers was clear, the retinal nerve layer were neatly arranged, cell morphology was good and the staining was uniform. In the NaIO₃ group, the retinal layer structure was obviously loose, the structure was not orderly, cell staining was not uniform, and some cells were irregular, swollen, displaced or absent (Figure 9).

**Expression of NLRP3 and Inflammatory Factors as well as Autophagy in Mouse** The retinal background image of the control group is clear, and the structure of each layer is clear and complete. In the NaIO₃ group, the retinal structure was thin, there were more high reflection points between layers, and the RPE layer changed unevenly. The NaIO₃+CY-09 group had uneven pigment disorder and a high interlayer dot reflex. The NaIO₃+AST group exhibited uneven, disordered changes in pigment (Figure 10).

The experimental mice were examined with FERG to evaluate the functional changes in the retina after different treatments (Figure 11).
In control group, the retinal tissue of mouse eyeballs showed complete structure, clear layers, close arrangement of cells, uniform and good staining, clear and good cell morphology. In treated groups, the structure of retinal layers was disordered and relaxed, the RPE pigment was disordered and displaced, and local photoreceptor cells were moved, resulting in unclear structure of retinal layers, irregular cell morphology, deformation and expansion. In the NaIO3+CY-09 group the retinal structure of each layer could be distinguished. In the NaIO3+AST group, retinal relaxation, RPE structure and pigment detachment were observed, and nuclear staining was obvious. The structure of each layer in the NaIO3+CY-09+AST group was improved (Figure 12).

NLRP3 was increased in the NaIO3 group, and the number of positive areas increased. The positive expression and region of positive expression in the NaIO3+CY-09 and NaIO3+AST groups were decreased compared with those in the NaIO3 group. The positive expression and region of positive expression in the NaIO3+CY-09+AST group were decreased compared with those in the NaIO3, NaIO3+CY-09 and NaIO3+AST groups. Caspase-1 staining revealed positive expression in the NaIO3 group, NaIO3+CY-09 group, NaIO3+AST group and NaIO3+CY-09+AST group with a large regions of positive staining (Figure 13).

IL-1β and IL-18 in the NaIO3+CY-09 group were lower than in the NaIO3 group (P<0.05). The NaIO3+CY-09 group had lower caspase-1 than the NaIO3 group (P<0.01). The NaIO3+CY-09+AST group had lower caspase-1 than the NaIO3+CY-09 group and NaIO3+AST group (P<0.05). Compared with that in the NaIO3 group, p62 in the other treatment groups was increased (P<0.05). The NaIO3+CY-09+AST group exhibited higher p62 than the NaIO3+CY-09 group and NaIO3+AST group (P<0.05). Compared with NaIO3 group, Beclin-1 in the other treatment groups was decreased (P<0.05). The NaIO3+CY-09+AST group exhibited lower LC3-II/LC3-I than the NaIO3+CY-09 group and NaIO3+AST group (P<0.05; Figure 15).

DISCUSSION

Retinal photoreceptor cells can directly convert external light into visual signals[23]. The damage and death of photoreceptor cells are important causes of visual loss in AMD[24]. Aging can induce retinal inflammation, which evolves into a chronic inflammatory state[25]. Photoreceptor cell death involves a variety of molecular mechanisms related to cell death, including inflammation and apoptosis; among these processes which includes the NLRP3 inflammasome as the core cell death pathway, photoreceptor autophagy and ferroptosis pathway[26]. Dry AMD is closely related to the NLRP3 inflammasome and autophagy[27].

There is currently no effective treatment for dry AMD[6]. The currently available animal models can simulate many pathological features of human AMD[29] and have their own diversity, advantages and limitations[29]. In this study, we chose NaIO3 induction to establish AMD models. This model can selectively cause RPE cell damage, making it a replicable[30]. NaIO3 induces retinal tissue senescence in vivo, significant age-related DNA damage in cells[31]. Therefore, in this study, 661W cells and ARPE-19 cells were treated with NaIO3, and NaIO3 was administered to mice via intraperitoneal injection.

Figure 7 SD-OCT images after NaIO3 administration  SD-OCT: Spectral domain optical coherence tomography.
Animal experiments were also performed with different doses. The study showed that the retinal thickness of the model group was reduced, the a-wave and b-wave of FERG were decreased, the cell level was disordered, the photosensitive cells in the outer plexiform layer were denatured, all the layers of the retina were affected, and visual function was affected. The results showed that NaIO₃-induced in vivo and in vitro AMD models were successfully and feasibly established, and these

Figure 8 Analysis of FERG images captured on the 8th day after NaIO₃ infusion  A: Waveform of each group after model establishment; B: Statistical analysis of the amplitudes of the wave. *P<0.05. FERG: Flash electroretinogram; DA: Dark adaptation; LA: Light adaptation.
models could be used for further experimental exploration.

Senescence, metabolism, inflammation and oxidative damage of photoreceptor cells and RPE may be related to AMD. Treatment of mice and cultured retinal cells, including 661W cells and ARPE-19 cells, with NaIO₃ significantly reduced photoreceptor cell function[32]. NaIO₃ reduced b-wave amplitude and retinal thickness, leading to a loss of normal RPE hexagonal shape[33]. After NLRP3 is activated, it can promote the recruitment of activate caspase-1 which can promote the secretion of IL-1β[34]. In vitro, NaIO₃ activated several cell death pathways in retinal cells[35]. This result was also observed in this study. The expression levels of IL-1β and IL-18 were significantly upregulated. Increased expression of NLRP3, caspase-1, and cleaved caspase-1 were observed. The levels of IL-1β and IL-18 were decreased, indicating that the combination of drugs had a certain inhibitory effect on inflammatory factor production. The transcription levels of NLRP3 and caspase-1 confirmed that CY-09 and AST exerted a certain inhibitory effect on the NaIO₃-induced inflammatory response, and the inhibitory effect of drug combination was stronger. Apoptosis experiments also confirmed that. In terms of protein expression, the same conclusion was obtained for the NLRP3, caspase-1, and cleaved caspase-1.

The expression of autophagy-related proteins and observe the autophagic flux with electron microscopy, were employed[36]. When the LC3 protein in the outer membrane of autophagic vesicles is fused with a fluorescent protein, it can be localized in the cell[37]. The change in autophagy can be measured
Figure 11 Visual electrophysiological evaluation of retinal function in mice after treatment

A: Retinal electrophysiological waveforms of mice from each group 10d after treatment; B: Statistical analysis of the amplitudes of waves in each group. *P<0.05. FERG: Flash electroretinogram; AST: Astaxanthin; CY-09: An inhibitor binds to NLRP3; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; DA: Dark adaptation; LA: Light adaptation.
Figure 12 HE staining of retinal tissues after different treatments

HE: Hematoxylin and eosin; AST: Astaxanthin; CY-09: An inhibitor binds to NLRP3; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3.

Figure 13 Immunofluorescence staining of NLRP3 (A) and caspase-1 (B) in retinal tissues after different treatments

by observing the number of substrate residues that are not degraded in phagolysosomes under an electron microscope [38]. By calculating the LC3-II to LC3-I ratio, the autophagic flux can be quantified [39]. In this study, through the detection of changes in LC3-II/LC3-I expression, found that compared with the NC group, the NaIO3-induced model indeed exhibited enhanced autophagy. However, upon the addition of the CY-09 and AST, autophagy was reduced, and inflammation was alleviated, and the effect of the drug combination was more obvious. Autophagosome formation was morphologically examined in 661W cells by transmission electron microscopy, indicating that the NaIO3-induced AMD model exhibits enhanced autophagy in photoreceptor cells and that autophagy may play an important role in photoreceptor cell death. In addition, the morphology of autophagosomes was significantly reduced, and autophagic vesicle numbers were decreased when the cells were treated with NLRP3 inhibitors and antioxidants.

The retinal thickness of NaIO3 group was reduced, the a-wave and b-wave of FERG were reduced and visual function was affected. CY-09 alleviated the degree of retinal damage caused by NaIO3, and with increasing dose. Reducing the level of NLRP3 can alleviate retinal immune inflammation, and reduce the damage effect. CY-09 alleviates inflammation by inhibiting NLRP3 inflammasome activation [40-41]. Activation and assembly of the NLRP3 inflammasome significantly affect the release of IL-1β [42]. Antioxidant drugs AST may have the potential to control dry AMD [43]. AST has powerful antioxidant, anti-inflammatory, antiapoptotic and immunomodulatory properties [44]. AST significantly inhibits the increase in TNF-α and IL-1β, thereby improving inflammation-related diseases [45]. After photoreceptor cells are stimulated, NLRP3, caspase-1, IL-1β and IL-18 are activated in the cells. CY-09 and AST inhibits the formation of NLRP3 and promotes autophagy, which can protect photoreceptor cells. These indicate that the NLRP3 and autophagy interact, and one factor can inhibit the other factor to a certain extent, but the overactivation of autophagy can also cause cell death.

In conclusion, in vitro treatment with NaIO3 promoted the expression of NLRP3 in 661W and ARPE-19 cells. The NLRP3 inflammasome may promote the inflammatory death of photoreceptor cells and CY-09 can inhibit NaIO3-induced photoreceptor cell damage in the AMD model. By inhibiting the NLRP3 with CY-09 and adding AST, the inflammatory response of photoreceptor cells and RPE cells could be inhibited, resulting in a protective effect. Autophagy and the NLRP3 interact. Autophagy can protect photoreceptor cells, and overactivation of autophagy can cause photoreceptor cell death. Inhibition of the NLRP3 can also inhibit autophagy to a certain extent.

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