• Basic Research •

Autophagy serves as a protective effect against inflammatory injury of oxidative stress in ARPE-19 cell

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Abstract

● **AIM:** To test the effect of autophagy on inflammatory damage resulting from oxidative stress in adult retinal pigment epithelial cell line (ARPE-19).

● **METHODS:** ARPE-19 cells were pretreated with 200 and 600 μmol/L hydrogen peroxide (H_2O_2) at various time intervals. The changes of cell morphology, cell viability, reactive oxygen species (ROS) level, autophagic activity, and the inflammatory cytokines (TNFα, IL-6, and TGFβ) were measured at baseline and after treatment with autophagy inducer rapamycin (Rapa) and suppressor wortmannin (Wort) or shATG5.

● **RESULTS:** The levels of ROS, cytokines (TNFα, IL-6, and TGFβ), and autophagic activity were significantly increased in ARPE-19 cells after pretreated with H_2O_2 (all P<0.05) and IL-10 was significantly decreased (P<0.05). By upregulating autophagy, Rapa significantly reduced oxidative stress-induced secretion of pro-inflammatory factors (TNFα and IL-6) and ROS (all P<0.05), yet elevated the production of TGFβ (P<0.05). In contrast, suppression of autophagy through Wort or ATG5 knockdown reduced cell viability, increased cell apoptotic rate, and exacerbated the generation of ROS and inflammatory cytokines (TNFα, IL-6, and TGFβ; all P<0.05).

● **CONCLUSION:** Autophagy demonstrates a protective effect on ARPE-19 cell through mitigating oxidative damage and oxidative stress-induced inflammatory response. Regulation of autophagy may be a potential way for age-related macular degeneration.

● **KEYWORDS:** retinal pigment epithelium; autophagy; reactive oxygen species; inflammation

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INTRODUCTION

Autophagy is present in various eukaryotic cells, facilitating the preservation of intracellular homeostasis by removing the abnormal accumulation of proteins and other harmful substances. Besides, autophagy is also a vital self-protective mechanism for cells[1], exerting a crucial regulatory function in cellular growth, development, and various physiological and pathological processes[2-3]. Numerous investigations have revealed that autophagy plays a critical role in the pathogenesis of various diseases[4]. Insufficient autophagy can cause excessive accumulation of intracellular metabolic waste, contributing to the incidence and development of age-related macular degeneration (AMD) to a certain extent[5].

AMD is a kind of retinal disorder mainly manifested by progressive central visual function impairment, which is also the predominant factor contributing to severe eye damage and blindness in individuals aged 50 and above[6]. More and more studies believed that AMD was a degenerative fundus disease involving many factors, such as genes, age, metabolic disorders, oxidative stress, immune inflammation, and others[7]. Among these factors, senescence, oxidative stress, and inflammatory response have been confirmed as key contributors.

Retinal pigment epithelium (RPE) is comprised of a monolayer of epithelial cell[7], acting as an important nutrient, maintenance, and metabolic tissue for the retina, and performs a variety of functions[8]. They play key roles in retinal and choroidal homeostasis, phagocytosing photoreceptor

outer segments, transporting nutrients and metabolites to photoreceptors, and so on. Studies have confirmed that RPE cell impairment is the core event in AMD. As individuals age, the scavenging ability of RPE cells decreased, causing a continual accumulation of waste products. The gradual and irreversible destruction of photoreceptor cells following RPE degeneration eventually leads to blindness[9].

RPE cells exhibit a strong susceptibility to oxidative stress due to their abundant oxygen levels, vigorous metabolism, and direct contact with light sources. Oxidative stress occurs when the body's antioxidant system fails to clear excessive reactive oxygen species (ROS), causing damage to tissue and cells[10]. However, the ability of RPE cells to respond to increased oxidative stress declines with age, resulting in a variety of age-related changes that eventually result in a loss in RPE function and cell death[11]. Damage to the RPE layer and subsequent cell death causes a disruption in the retina's internal environment, which in turn harms the photoreceptors in the surrounding area and leads to AMD[9].

Damaged RPE cells can not clear some metabolic waste and their own cell debris due to functional decline, which stimulates the production of local inflammatory signals and eventually causes a series of non-specific inflammatory reactions[9]. Hence, persistent inflammation is a mechanism that affects both AMD risk and disease development. NOD-like receptor protein 3 (NLRP3) is observable within the RPE of eyes donated by individuals with AMD. Additionally, significantly elevated levels of the interleukin (IL)-6, IL-8, interferon-γ (IFN-γ), and transforming growth factor β (TGFβ) were noticed in the peripheral blood and aqueous humor of individuals with AMD[12-14]. Moreover, a notable rise of IL-6, IL-18, IL-1β in ARPE-19 cells occurred following exposure to oxidative stress, indicating that oxidative stress could potentially trigger an inflammatory response[15]. Inflammation can contribute to choroidal neovascularization (CNV) and geographic atrophy (GA) in AMD, causing serious visual impairment or even blindness[14]. For patients with CNV, anti-vascular endothelial growth factor (VEGF) is currently the main treatment[16]. However, about one-third of the patients still have no obvious effect after anti-VEGF treatment. Currently, there is no successful therapy available for individuals with GA[17]. Therefore, the study of anti-inflammatory treatment for AMD appears to be necessary and reasonable.

As a protective mechanism, autophagy has been found to be enhanced by oxidative stress to eliminate oxidatively damaged proteins and organelles. Meanwhile, upregulating autophagy can also diminish ROS production induced by oxidative stress, confirming the regulatory role of autophagy in oxidative stress[18]. Previous studies have affirmed that numerous inflammatory cytokines, such as IL-6, IL-8, IL-1β, IL-4 and tumor necrosis factor α (TNFα), were released in an *in vitro* AMD model by exposing RPE cells to oxidative stress caused by H_2O_2 or lipopolysaccharides[19-21]. However, to the best of our knowledge, there were very few reports on whether autophagy plays a certain regulatory role in immune inflammation response in AMD, and the knowledge in this area was incomplete. Herein, the goal of the present study was to investigate the association between autophagy and inflammation further, aiming to gain a clearer understanding of autophagy's role in AMD.

MATERIALS AND METHODS

Materials Adult retinal pigment epithelial cell line (ARPE)-19 has been acquired from the China Center for Type Culture Collection (CCTCC, GNHu45, Shanghai, China) and authenticated by Shanghai iCell Bioscience Ltd. (Shanghai, China). Life Technologies supplied the subsequent items: fetal bovine serum (FBS), Dulbecco's modified Eagles medium/nutrient mixture F-12 (DMEM/F-12) cell culture medium, trypsin-EDTA solution (0.05% and 0.25%), TRIzol reagent, and Lipofectamine 2000 transfection reagent. We purchased and used the following items: hydrogen peroxide (Millipore, 88597-100ML-F), rapamycin (Rapa; MedChemExpress LLC, HY-10219), wortmannin (Wort; MedChemExpress LLC, HY-10197), the Bradford protein assay kit (Yeasen Biotechnology, 20202ES76), horseradish peroxidase (HRP)-conjugated goat anti-mouse (ABCAM, ab6789) and Alexa Fluor 680 conjugated goat anti-mouse (Invitrogen, A-21058) secondary antibodies, all based on the manufacturer's recommendation. ABCAM supplied the primary mouse monoclonal antibodies for this research, which consisted of anti-LC3 (ab243506), anti-p62 (ab280086), anti-ATG5 (ab238092), anti-BECN1 (ab114071) and anti-beta actin (ab8226).

Cultivation of Cell and Approaches of Treatment ARPE-19 cells underwent inoculation within DMEM/F-12 medium enriched with 10% FBS at a temperature of 37℃ under 5% CO_2, 95% air. Cells were subjected to 200 (low-dose exposure studies) or 600 μmol/L (high-dose exposure studies) hydrogen peroxide (H_2O_2) in basal medium. Selected studies were conducted following treatment with the autophagy inducer Rapa (10 nmol/L), the autophagy suppressor Wort (3 nmol/L), or knockdown of *ATG5* using shATG5. Cells that were untreated or treated with a vehicle were utilized as controls.

Cell Viability Test Cell proliferation was conducted utilizing the cell counting kit-8 (CCK-8) assay (MedChemExpress LLC, HY-K0301, USA). Each group contained 3×10^3 cells inoculated in 96-well plates for 24h. Then different concentrations of H_2O_2 (with/without Wort, or with/without transinfected with shATG5) were added for the drug toxicity experiments. After being cultured for the specific time points according to the experiment design, 10 μL CCK-8 reagent was added, followed

Table 1 Primer sequences in qRT-PCR analysis

Gene	Forward	Reverse
BECN1	5′-GGCCAATAAGATGGGTCTGA-3′	5′-GCTGCACACAGTCCAGAAAA-3′
ATG5	5′-AAGCAACTCTGGATGGGATT-3′	5′-GCAGCCACAGGACGA AAC-3′
ATG7	5′-CAGTCCGTTGAA GTCCTC-3′	5′-TCAGTGTCCTAGCCACATTAC-3′
ATG9	5′-CCAGAACTACATGGTGGCACT-3′	5′-GTCCCCAGAAGAGGATCAGC-3′
p62	5′-GACTACGACTTGTGTAGCGTC-3′	5′-AGTGTCCGTGTTTCACCTTCC-3′
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-GGGGTCGTTGATGGC AACA-3′

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

by re-incubation for 2h. The absorbance measurement was conducted at 450 nm, and growth curve was plotted.

Reactive Oxygen Species Assay Evaluation of alterations within intracellular levels of ROS was conducted utilizing a 2′,7′-dichlorofluorescein diacetate DCFH-DA kit (Beyotime Biotechnology, Shanghai, China, S0033S) based on the guidelines provided by the manufacturer. ARPE-19 cells were washed with D-Hank and incubated with 10 μmol/L DCFH-DA at 37°C in the absence of light for 20min. Subsequently, the distribution of DCF fluorescence was analyzed using a fluorescence spectrophotometer, employing an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Western Blot Analysis ARPE-19 cells were lysed using RIPA buffer (Beyotime Biotechnology, Shanghai, China, P0013C) enriched with protease inhibitor (Roche Applied Science, 5892970001). Protein levels were determined using a BCA assay. Each channel was loaded with equal amounts of cell lysate protein and separated using an SDS-polyacrylamide gel. The proteins were then moved onto nitrocellulose membranes (0.22 mm; Bio-Rad Laboratories, 162-0112), and to prevent nonspecific binding, a solution of 5% nonfat milk was applied as the blocking agent. The membranes were then maintained overnight in an incubator utilizing the following primary antibodies: LC3 (1:1000), p62 (1:1000), BECN1 (1:1000), ATG5 (1:1000) and beta actin (1:3000, loading control). After rinsing with 0.1% tris buffered saline Tween (TBST), the corresponding horse radish peroxidase (HRP) conjugated secondary antibody (1:5000) was added and left to incubate at room temperature for 1h. Protein bands were identified using an improved chemiluminescence technique (Millipore, USA) and then subjected to X-ray film for visualization. Protein bands were scanned using computer-aided software ImageJ (NIH, Bethesda, MD, USA) for further processing and analysis to quantify target protein expression.

Reverse Transcription Polymerase Chain Reaction Total RNA was isolated from ARPE-19 cells utilizing TRIzol reagent, following the instructions provided by the manufacturer. RNA was reverse transcribed to cDNA using the BeyoRT™ II cDNART kit (Beyotime Biotechnology, Shanghai, China), and then was amplified using primers for *BECN1*, *ATG5*, *ATG7*, *ATG9*, *p62* and *GAPDH* (Table 1), Universal SYBR Green I detection assays (Bioteke Corporation) and ABI Prism 7500 system (Funglyn Biotech Incorporated). Relative expression of genes was obtained using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked Immunosorbent Assay TNFα, IL-1β, IL-6, IL-10, IFN-γ, and TGFβ levels were determined utilizing enzyme-linked immunosorbent assay (ELISA) kits (Beyotime Biotechnology, Shanghai, China). ARPE-19 cell supernatant was collected for later use. The samples or standard substances with different concentrations were added into the corresponding wells using a volume of 100 μL per well. The mixture was subjected to incubation at room temperature for a period of 120min. Biotinylated antibody (100 μL/well) was supplemented. The incubation was performed at room temperature for a duration of 60min, followed by the supplementation of 100 μL/well of HRP labeled streptavidin. Then, the mixture was incubated for another 20min at room temperature away from light. Then, 100 μL/well of the color developing agent TMB solution was added. The solution was subsequently incubated at room temperature in dark conditions for a duration of 15-20min. The termination solution (50 μL/well) was added, and the optical density (OD) values were measured at 450 nm.

Immunofluorescence Staining of ARPE-19 Cells The ARPE-19 cells have been inoculated within 24 well plates and incubated for 24h and exposed to H_2O_2 with and without Wort, or with and without transinfected with shATG5. The cells were fixed in 4% paraformaldehyde at room temperature for 20-30min. Permeabilization process was carried out using a solution including 0.3% Triton X-100 in phosphate buffer saline (PBS) for 5min at room temperature. The samples were subjected to blocking at room temperature utilizing a 5% bull serum albumin (BSA) solution for 30min. The primary antibody (diluted with 1% BSA) was added with overnight incubation at 4°C. Subsequently, fluorescein secondary antibody was added with incubation for 30min away from light. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (50 μL) or 4′,6-diamidino-2-phenylindole (DAPI) dyes were then added, and the slides were observed under fluorescence microscope (Olympus IX71).

Knockdown of ATG5 in ARPE-19 Cells Plasmid vector (plenty GFP Puro) targeting human ATG5 (shATG5) and its

negative control (shATG5-NC) based on short hairpin RNA (shRNA) were acquired from GenePharma Co. (Shanghai, China). The shATG5 is processed into siRNA in the cell, effectively causing ATG5 gene specific function loss. During the transfection studies, ARPE-19 cells were cultivated in a 25 cm^2 bottle and maintained in DMEM/F-12 media without the addition of antibiotics. Lipofectamine 2000 was used for transfection, followed by gene expression identification through qRT-PCR and protein expression using western blotting (WB). Three candidates targeting *ATG5* were tested to get the optimal RNA silencing effect (Table 2).

Statistical Analysis GraphPad Prism 6 software (GraphPad Software, Inc, San Diego, CA, USA) was utilized to carry out the statistical analysis. For multiple comparisons of groups, statistical significance was calculated and evaluated by one-way analysis of variance (ANOVA). For comparison of two groups, Student's *t*-test was used. Statistically significant data were provided using means±standard deviation (SD) or normalized to the control group. These data were obtained from at least three separate studies. Significance difference was indicated by $P<0.05$.

RESULTS

ROS Induced by H_2O_2 in ARPE-19 Cells Cell viability significantly decreased in a concentration-dependent manner after treatment with H_2O_2 at concentrations above 100 μmol/L for 24h and 48h ($P<0.05$; Figure 1A). We found a turning point between 400 and 600 μmol/L H_2O_2. Exposure to H_2O_2 concentrations below 400 μmol/L resulted in greater ARPE-19 cell death after 24h than 48h. In contrast, at concentrations more than 600 μmol/L, the results were reversed (Figure 1A). Thus, 200 and 600 μmol/L H_2O_2 were chosen for further low-dose exposure and high-dose exposure experiments, respectively. ROS production showed significant elevation following exposure to 200 μmol/L H_2O_2 and peaked at 12h (Figure 1B). Comparable outcomes were achieved after ARPE-19 cells were treated with 600 μmol/L H_2O_2 (Figure 1C).

H_2O_2-induce Autophagy in ARPE-19 Cells Autophagy in ARPE-19 cells induced by H_2O_2 were determined by the expression of microtubule-associated protein 1A light chain 3 (MAPLC3)-II, MAPLC3-I, and p62 using WB. LC3-II in ARPE-19 cells exposed to 200 μmol/L H_2O_2 significantly elevated within 48h, peaking at 12h (Figure 2A, 2B). p62 exhibited a marked reduction after 3, 12, and 24h of exposure to 200 μmol/L H_2O_2 (Figure 2A, 2C). Similarly, LC3-II significantly increased and p62 decreased within 48h after 600 μmol/L H_2O_2 treatment (Figure 2D-2F). Impressively, WB analysis demonstrated a significant reduction of LC3-II and increase of p62 after exposure to 200 μmol/L H_2O_2 for 7d or longer (Figure 2A-2C). Strikingly, we didn't notice the similar effect in ARPE-19 cells treated with 600 μmol/L H_2O_2 (Figure 2D-2F).

Table 2 Sequences of the three shATG5 candidates

shATG5 candidates	Sequences
shATG5-NC	5′-CCGGGCGCGATAGCGCTAATAATTTCT-3′
shATG5-1	5′-GCCATCAATCGGAAACTCATGGAATATCC-3′
shATG5-2	5′-GCCTGTCAAATCATAGTAT-3′
shATG5-3	5′-CCTGAACAGAATCATCCTTAA-3′

Autophagy-related Genes Expression in ARPE-19 Cells Induced by H_2O_2 qRT-PCR was utilized to assess the expression levels of autophagy-associated genes, including *BECN1*, *ATG5*, *ATG7*, *ATG9*, and *p62*. The findings indicated that, compared to the control group, the levels of *BECN1*, *ATG5*, *ATG7*, and *ATG9* genes exhibited a significant elevation following treatment with 200 μmol/L H_2O_2, while the *p62* gene level significantly decreased as expected (Figure 3A, 3C). Comparable outcomes were achieved when ARPE-19 cells were treated with 600 μmol/L H_2O_2 (Figure 3B, 3C). We also noted that the relative variation of all the above genes within the group treated with 600 μmol/L H_2O_2 was greater than that within the group treated with 200 μmol/L H_2O_2 (Figure 3C), revealing that 600 μmol/L H_2O_2 treatment was more effective at inducing autophagy in ARPE-19 cells.

Inflammatory Cytokines Alteration after H_2O_2 treatment by ELISA Compared with the control group, TNFα and IL-6 levels exhibited a significant increase during the treatment period, with the maximum increase observed at 24h 200 μmol/L H_2O_2 exposure (Figure 4A, 4C). Similarly, we detected a significant increase in TNFα and IL-6 release at all the specific time-points following 600 μmol/L H_2O_2 exposure, with the topmost increase detected at 48h (Figure 4B, 4C). IL-1β release was decreased significantly following the addition of 200 μmol/L H_2O_2 for a duration of 24h, while increased significantly when the cells were exposed to 600 μmol/L H_2O_2 for a duration of 48 and 72h (Figure 4). Besides, there were noticeable increases in IFN-γ generation at 72h following 200 μmol/L H_2O_2 exposure, and at 72, 96h after 600 μmol/L H_2O_2 treatment, respectively (Figure 4). The increases of TNFα, IL-6, IL-1β, and IFN-γ were more robust in the 600 μmol/L H_2O_2 treatment than in the 200 μmol/L H_2O_2 (P values not shown; Figure 4C), which was consistent with the autophagy activation by H_2O_2 in ARPE-19 cells (Figure 3C). Both 200 and 600 μmol/L H_2O_2 treatment dramatically decreased IL-10 level at all the specific time-points, except for 96h of 200 μmol/L H_2O_2 treatment (Figure 4). TGFβ release by H_2O_2 treatment in ARPE-19 cells was in a totally different fashion from all the above inflammatory factors, presenting significant increases at all the detected time-points with 200 μmol/L H_2O_2 exposure, yet a remarkable reduction at 48h time-point following 600 μmol/L H_2O_2 treatment. Overall, H_2O_2 treatment contributed to inflammatory damage in ARPE-19 cells by releasing various inflammatory factors in different patterns.

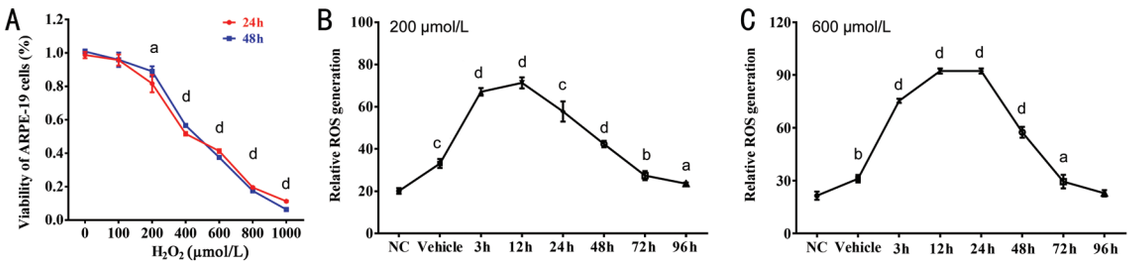

Figure 1 H_2O_2 treatment induces the generation of ROS in ARPE-19 cells A: Cell viability after 24 and 48h of H_2O_2 treatment at varying concentrations versus untreated controls; B, C: ROS levels in ARPE-19 cells exposed to 200 (B) and 600 μmol/L (C) H_2O_2 at different time intervals, relative to untreated cells. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001, [d]*P*<0.0001 *vs* control. H_2O_2: Hydrogen peroxide; ROS: Reactive oxygen species; ARPE-19: Adult retinal pigment epithelial cell line-19; SD: Standard deviation; NC: Control.

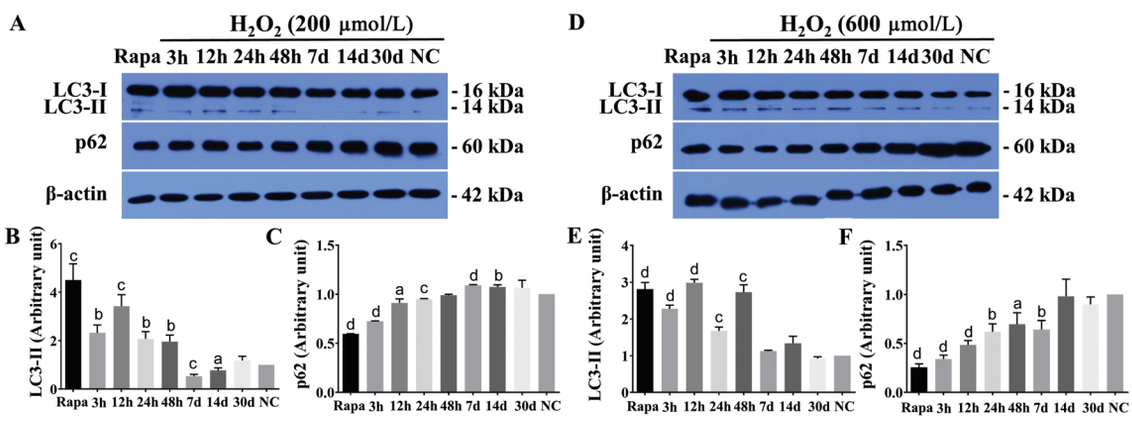

Figure 2 H_2O_2 treatment activates autophagy of ARPE-19 cells A: Representative WB images detecting LC3, p62, and β-actin (loading control) of ARPE-19 cells treated with 200 μmol/L H_2O_2 at various time points; B, C: Quantification of LC3-II (B) and p62 (C) relative to control; D: Representative WB images detecting LC3, p62, and β-actin in ARPE-19 cells treated with 600 μmol/L H_2O_2 at various time points; E, F: Quantification of LC3-II (E) and p62 (F) relative to control. Statistical significance: [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001, [d]*P*<0.0001 *vs* control. H_2O_2: Hydrogen peroxide; WB: Western blot; ARPE-19: Adult retinal pigment epithelial cell line-19; Rapa: Rapamycin; SD: Standard deviation; NC: Control.

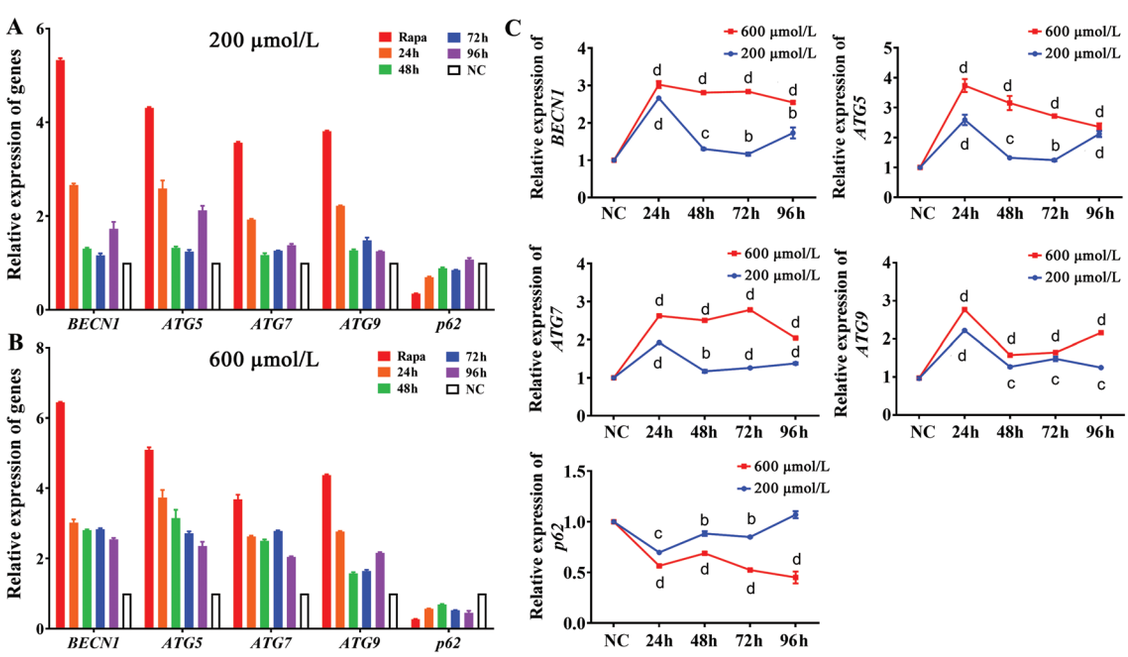

Figure 3 Autophagy related genes *BECN-1*, *ATG5*, *ATG7*, *ATG9* and *p62* were detected by qRT-PCR A, B: Relative expression of *BECN1*, *ATG5*, *ATG7*, *ATG9*, and *p62* in ARPE-19 cells at different time points post-treatment with 200 μmol/L (A) and 600 μmol/L (B) H_2O_2; C: Expression dynamics of *BECN1*, *ATG5*, *ATG7*, *ATG9*, and *p62* normalized to untreated controls. Statistical significance is indicated by [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001, [d]*P*<0.0001 *vs* control. qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; ARPE-19: Adult retinal pigment epithelial cell line-19; H_2O_2: Hydrogen peroxide; SD: Standard deviation; NC: Control.

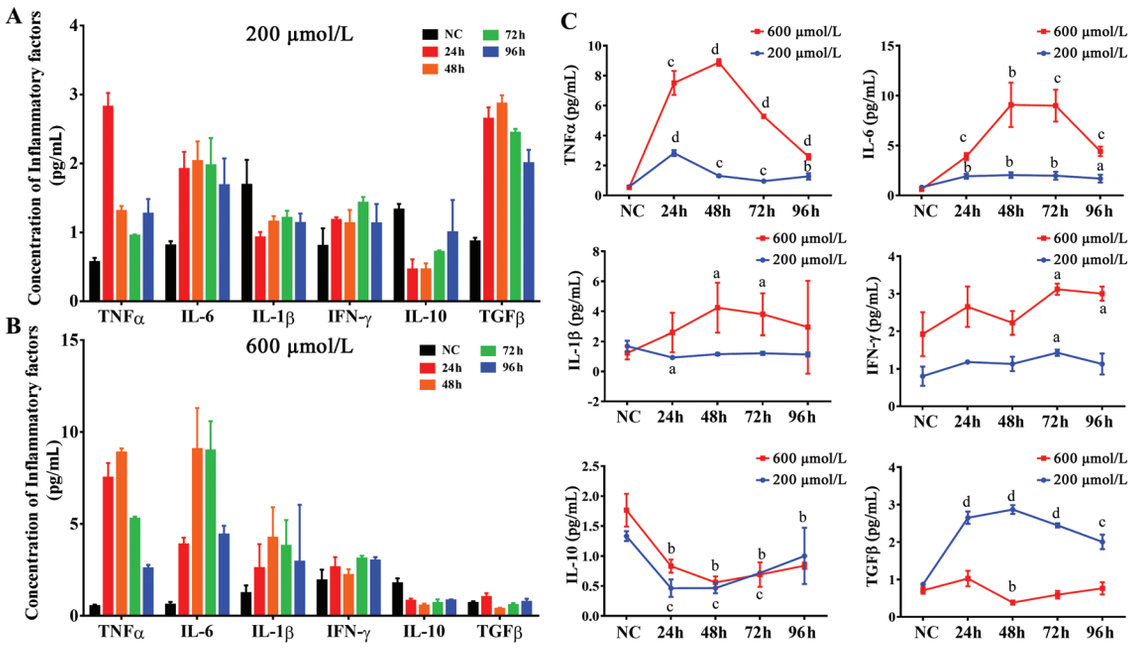

Figure 4 ELISA analysis of inflammatory cytokine release in ARPE-19 cells post-H_2O_2 exposure A, B: TNFα, IL-6, IL-1β, IFN-γ, IL-10, and TGFβ levels at different time points after 200 (A) and 600 μmol/L (B) H_2O_2 treatment; C: Variations of inflammatory cytokines TNFα, IL-6, IL-1β, IFN-γ, IL-10, and TGFβ following H_2O_2 treatment relative to untreated controls. Statistical significance: $^aP<0.05$, $^bP<0.01$, $^cP<0.001$, $^dP<0.0001$ *vs* control. ELISA: Enzyme-linked immunosorbent assay; TNFα: Tumor necrosis factor α; IL: Interleukin; TGFβ: Transforming growth factor β; IFN-γ: Interferon-γ; NC: Control.

Effects of Autophagy Inhibition on Cell Viability after H_2O_2 Treatment *BECN1* and *ATG5* genes were lowered by Wort and improved by Rapa. When ARPE-19 cells were cultured with 200 and 600 μmol/L H_2O_2 for 24h, *BECN1* and *ATG5* levels increased significantly (Figure 5A and 5B). Similarly, *BECN1* and *ATG5* gene expression levels after oxidative stress were inhibited by Wort and promoted by Rapa (Figure 5A, 5B). Compared with H_2O_2 alone, BECN1 and ATG5 protein levels of ARPE-19 cells were significantly reduced after H_2O_2 combined with Wort for 24h (Figure 5C, 5D). Likewise, immunofluorescence (IF) staining showed that BECN1 exhibited a significant increase in ARPE-19 cells exposed to 200 and 600 μmol/L H_2O_2 for 24h, respectively, whereas the increase of BECN1 was considerably suppressed by Wort (Figure 5E). CCK-8 analysis showed that ARPE-19 cells exposed to both H_2O_2 and Wort experienced a more severe loss of viability than those exposed to H_2O_2 alone (Figure 5F). Moreover, the apoptosis rate of H_2O_2-treated ARPE-19 cells was further intensified after autophagy was additionally inhibited for 24h (Figure 5G).

Knockdown of *ATG5* was applied to inhibit autophagy. shATG5-1 was the most effective at downregulating ATG5 expression after transfection with different shATG5s (Figure 6A), leading to the selection of shATG5-1 for further experiments. Besides, after treatment with 200 and 600 μmol/L H_2O_2 for 24h, ATG5 up-regulation was significantly inhibited in ATG5-knockdown ARPE-19 cells (Figure 6B). We next confirmed that ATG5 protein levels were considerably inhibited under oxidative stress induced by 200 and 600 μmol/L H_2O_2 when autophagy was blocked through transfection of shATG5 in ARPE-19 cells, as assessed by WB (Figure 6C, 6D) and IF staining analysis (Figure 6E). Moreover, autophagy blockage through shATG5-mediated *ATG5* knockdown remarkably decreased ARPE-19 cell viability at 48 and 72h after H_2O_2 treatment (Figure 6F). Similarly, TUNEL staining showed that *ATG5*-knockdown ARPE-19 cells exhibited higher susceptibility 24h following treatment with 200 and 600 μmol/L H_2O_2 (Figure 6G).

Reduced Autophagy on ROS and Inflammatory Factors in ARPE-19 Cells under Oxidative Stress We further explored whether oxidative stress-caused ROS level and cytokine release could be modulated by regulating autophagy. It was demonstrated that ROS generation, after cells were exposed to 200 and 600 μmol/L H_2O_2 for 24h, was further promoted by Wort, yet noticeably restrained by Rapa (Figure 7A). In addition, we detected a significant increase in TNFα release in the 200 and 600 μmol/L H_2O_2+Wort treatment groups compared to the H_2O_2 treatment group, while Rapa significantly inhibited the increase in TNFα release after 24h H_2O_2 exposure (Figure 7B). Additionally, the release of IL-6 under H_2O_2 treatment followed the same pattern as TNFα in response to modulation of autophagy (Figure 7C). Surprisingly, modulated autophagy had an opposite effect on TGFβ release, significantly restraining the increased release

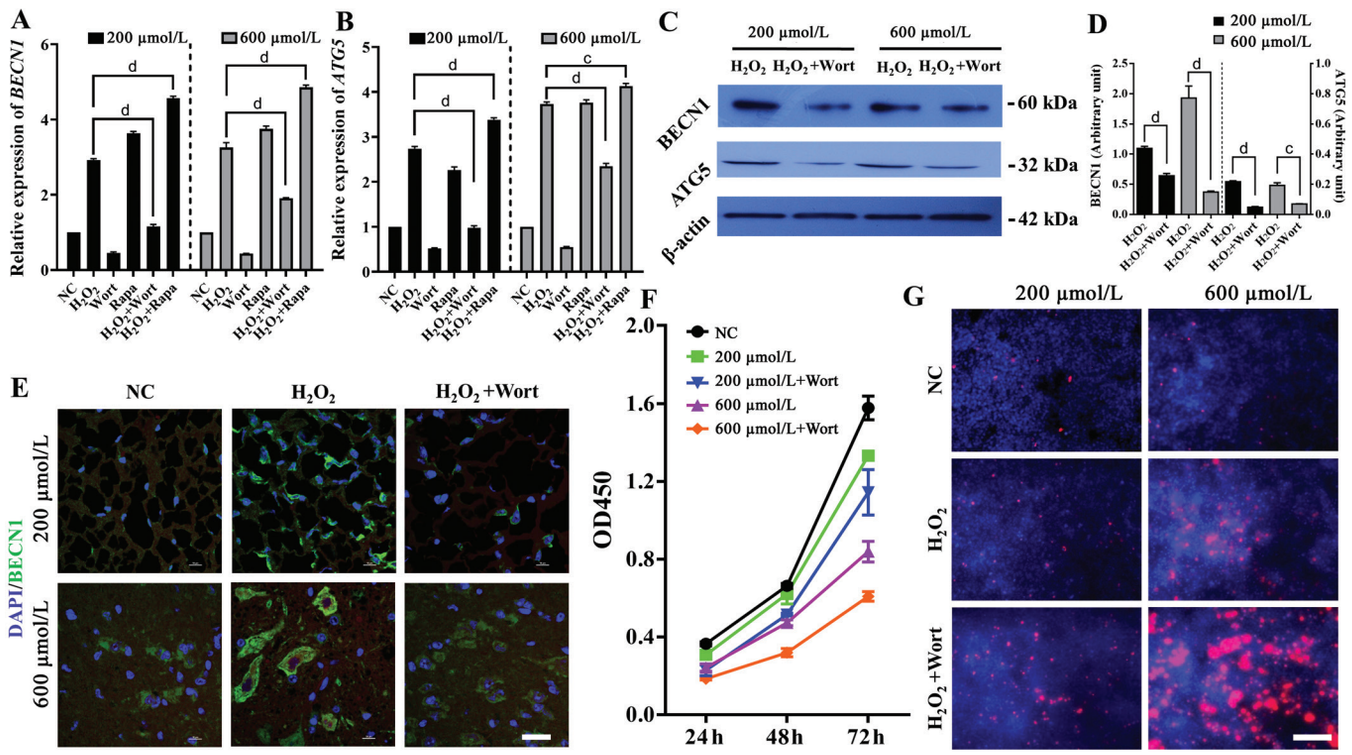

Figure 5 Inhibited autophagy by Wort affects cell viability of ARPE-19 cells after H_2O_2 treatment A, B: Relative expression of *BECN1* (A) and *ATG5* (B) following 24h treatment with H_2O_2, Wort, Rapa, H_2O_2+Wort, and H_2O_2+Rapa; C, D: Protein expression of BECN1 and ATG5 after 24h H_2O_2 treatment with or without Wort, represented by WB (C) and quantified (D) from three independent experiments; E: Representative IF images of BECN1 (green) and DAPI (blue) treated with H_2O_2±Wort for 24h. Scale bar: 20 μm; F: Cell viability with Wort under H_2O_2 treatment. H_2O_2 200 μmol/L *vs* H_2O_2 200 μmol/L +Wort: $P_{24h\text{-}72h}$=0.0087, 0.0239, 0.0512, respectively; H_2O_2 600 μmol/L *vs* H_2O_2 600 μmol/L+Wort: $P_{24h\text{-}72h}$=0.0077, 0.0005, 0.0024, respectively. G: Representative TUNEL staining of ARPE-19 cells exposed to H_2O_2±Wort for 24h. Scale bar: 100 μm. Statistical significance: ^cP<0.001, ^dP<0.0001. Wort: Wortmannin; ARPE-19: Adult retinal pigment epithelial cell line-19; H_2O_2: Hydrogen peroxide; Rapa: Rapamycin; SD: Standard deviation; WB: Western blot; IF: Immunofluorescence; DAPI: 4′,6-diamidino-2-phenylindole; CCK-8: Cell counting kit-8; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; NC: Control.

with Wort and noticeably promoting it with Rapa (Figure 7D). Collectively, autophagy has been observed to play a protective role in mitigating ROS generation by oxidative stress, as well as in the secretion of inflammatory factors in ARPE-19 cells.

DISCUSSION

Investigations have established that autophagy deficiency in RPE cells plays an essential role in AMD incidence and development[22]. Herein, we detected dynamic changes of autophagy in ARPE-19 cells exposed to H_2O_2. Short-time exposure can increase autophagic activity, and the rising trend of autophagic activity will reduce progressively with the duration of exposure. Moreover, the higher the H_2O_2 concentration, the faster the trend's reduction, indicating that chronic-continuous oxidative stress may inhibit autophagy. Our data are in accordance with the results of previous studies. Mitter *et al*[18] exposed ARPE-19 cells to H_2O_2 and measured changes in autophagic activity, showing that autophagy was stimulated in the acute phase but reduced by chronic oxidative stress. A similar phenomenon was confirmed in the Sod2 knockdown mouse model: autophagic activity increases in the early stage but is impaired at later stages of AMD in this model. Golestaneh *et al*[23] suggested that RPE cells from AMD donors showed a decreased level of autophagy flux compared with RPE cells from healthy age-matched controls. One explanation for this phenomenon may be that oxidative stress causes oxidative damage of cells, destroys the molecular structure of cells, and leads to the aggregation of impaired proteins and macromolecules within cells. At this time, the autophagy level rises to remove these oxidized wastes and reduce the oxidative damage of cells. However, as the oxidative damage continues, cell damage worsens, and cell viability decreases. Accordingly, the level of intracellular autophagy also decreases correspondingly and cannot clear the increasing amount of impaired intracellular wastes. Based on this, we consider that autophagic process may be impaired and insufficient in the late stage of AMD.

We further found that the activity of autophagy under oxidative stress was inhibited by Wort or *ATG5* knockdown. Moreover, we found that ROS production induced by oxidative stress decreased after upregulating autophagy with Rapa and

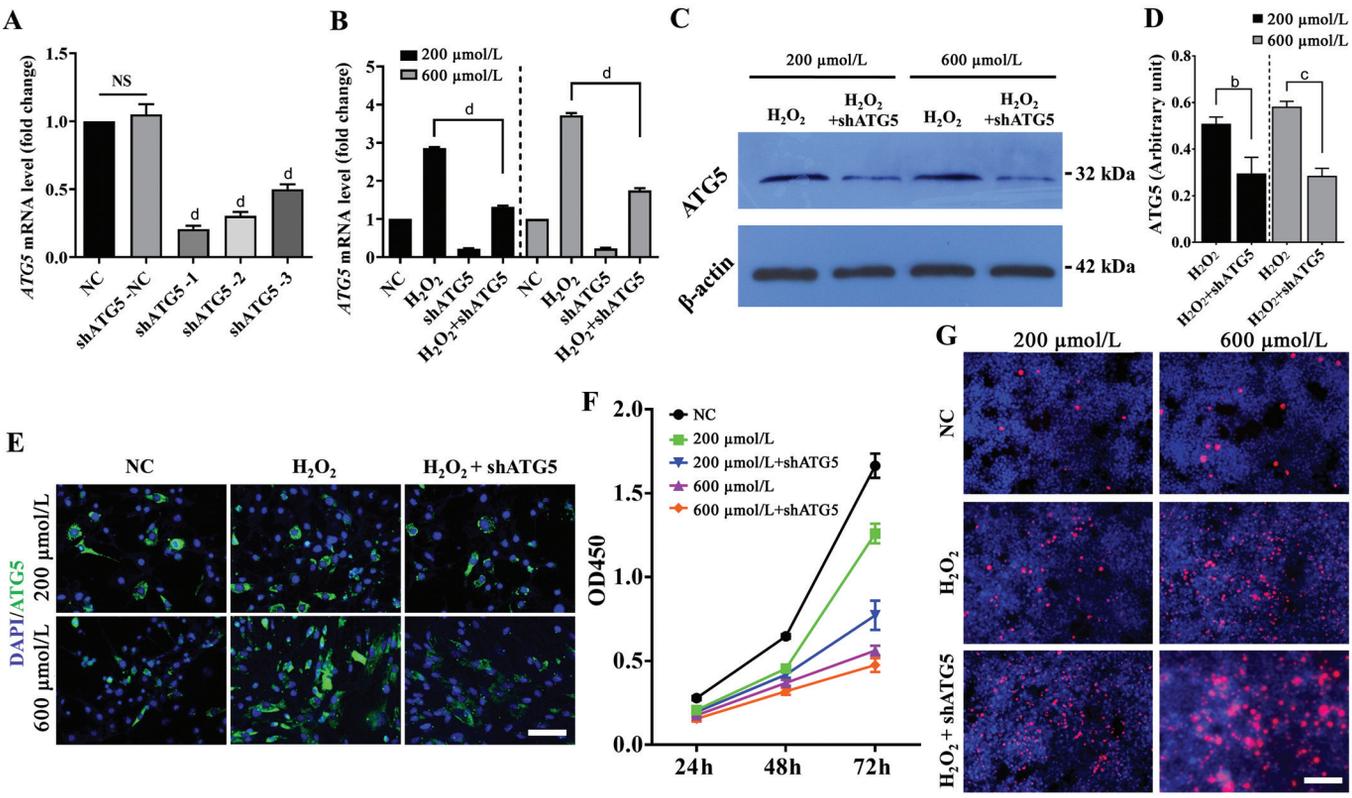

Figure 6 Reduced autophagy by *ATG5* knockdown conferred ARPE-19 cells more susceptible to H_2O_2 induced cell injury A: Decreased *ATG5* mRNA after shRNA transfection, normalized to control; B: Relative *ATG5* mRNA expression treated with H_2O_2, shATG5 and H_2O_2+shATG5 for 24h; C, D: Detection of ATG5 protein expression following 200 and 600 μmol/L H_2O_2 treatment with and without shATG5 transfection, shown by WB (C) and quantified (D) from three independent experiments; E: Representative IF images of ATG5 (green) and DAPI (blue) after H_2O_2 treatment±shATG5 transfection, scale bar: 50 μm; F: Cell viability in *ATG5* knockdown ARPE-19 cells under H_2O_2 exposure; H_2O_2 200 μmol/L *vs* H_2O_2 200 μmol/L+shATG5: $P_{24h\text{-}72h}$=0.106, 0.0344, 0.0013, respectively; H_2O_2 600 μmol/L *vs* H_2O_2 600 μmol/L+shATG5: $P_{24h\text{-}72h}$=0.123, 0.034, 0.0404, respectively; G: Representative TUNEL staining images of ARPE-19 cells with *ATG5* knockdown after 24h of H_2O_2 treatment; Scale bar: 100 μm. Statistical significance: ^bP<0.01, ^cP<0.001, ^dP<0.0001 *vs* control. ARPE-19: Adult retinal pigment epithelial cell line-19; H_2O_2: Hydrogen peroxide; SD: Standard deviation; WB: Western blot; IF: Immunofluorescence; DAPI: 4′,6-diamidino-2-phenylindole; CCK-8: Cell counting kit-8; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; NC: Control.

increased after culturing with Wort or after *ATG5* knockdown to downregulate autophagy. A previous study showed that autophagy has a certain function in regulating the state of RPE cells under oxidative stress[23]. To confirm the claim, we detected cellular function and quality under oxidative stress by regulating the autophagic level. The results showed that the cell death rate increased significantly after inhibition of autophagy. Taken together, our current results illustrated a bidirectional relationship between autophagy and oxidative stress, as oxidative stress can stimulate autophagy and enhanced autophagy can, in turn, reduce oxidative stress. In contrast, autophagy deficiency may aggravate oxidative damage to cells and result in cell death. Thus, in RPE cells, autophagy may be crucial for oxidative stress resistance.

Research has shown that chronic inflammation contributes to the development of cancer[24], Alzheimer's disease[25], and AMD, in which oxidative damage is always considered to be the primary trigger. Numerous studies have indicated that oxidative stress can induce inflammatory responses during the development of AMD. Local inflammation can promote the formation of drusen, the degeneration of photoreceptor and RPE cells, the rupture of Bruch's membrane, and the formation of CNV, all of which play a crucial role in the incidence and progression of both dry and wet AMD[26].

Inflammatory factors can be divided into pro-inflammatory factors such as TNFα, IL-6, IL-1β, IFN-γ, and anti-inflammatory factors such as IL-10, and TGFβ. They are released simultaneously and also maintain a dynamic balance in response to immune inflammation[14]. It will cause inflammatory damage or even death of cells if the balance is broken. Thus, we distinguished the dynamic release of the inflammatory factors in ARPE-19 cells cultured with H_2O_2 and found that concentrations of pro-inflammatory factors TNFα, IL-6, IL-1β, and IFN-γ were significantly increased at certain time points. In contrast, the concentration of the anti-inflammatory factors IL-10 was dramatically decreased.

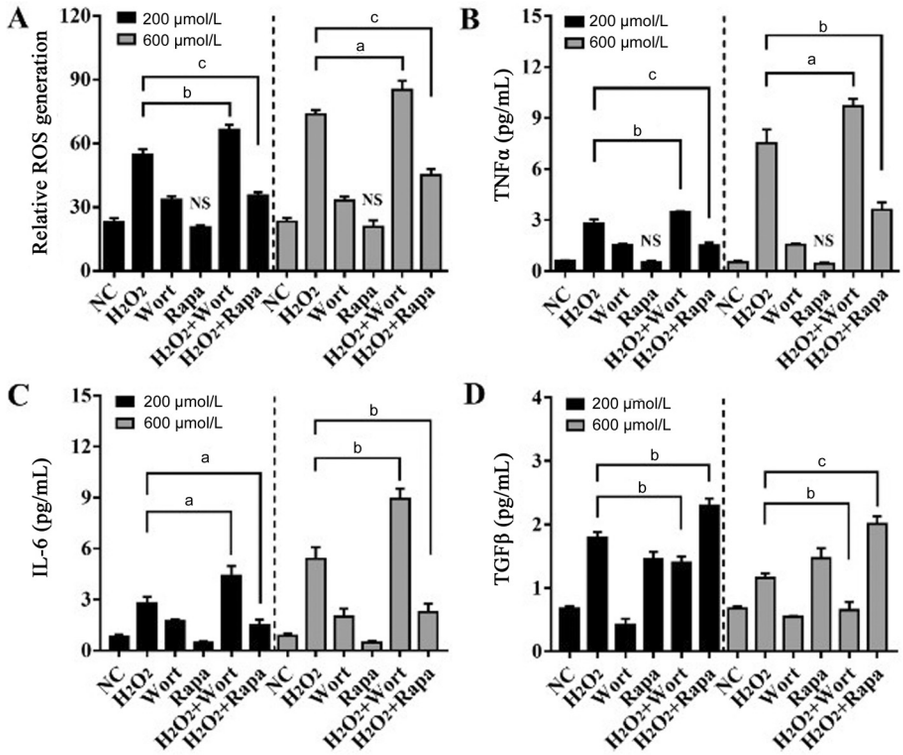

Figure 7 Impact of autophagy modulation on ROS and inflammatory factors release in ARPE-19 cells under oxidative stress A: Intracellular ROS levels after 24h treatment with H_2O_2, Wort, Rapa, H_2O_2+Wort, and H_2O_2+Rapa; B, C: Pro-inflammatory factors release of TNFα (B) and IL-6 (C) was augmented by H_2O_2 and further increased by Wort, but reduced by Rapa; D: Release of anti-inflammatory cytokine TGFβ induced by H_2O_2, was enhanced by Rapa and suppressed by Wort. Statistical significance from three independent experiments: $^aP<0.05$, $^bP<0.01$, $^cP<0.001$. ROS: Reactive oxygen species; TNFα: Tumor necrosis factor α; IL: Interleukin-6; TGFβ: Transforming growth factor β; ARPE-19: Adult retinal pigment epithelial cell line-19; Wort: Wortmannin; Rapa: Rapamycin; H_2O_2: Hydrogen peroxide; SD: Standard deviation; NC: Control.

Meanwhile, TGFβ release showed a significant increase only with 200 μmol/L H_2O_2 exposure, yet showed a remarkable reduction at 48h following 600 μmol/L H_2O_2 treatment. Hence, the imbalance between anti-inflammatory and pro-inflammatory factors in ARPE-19 cells cultured with H_2O_2 might contribute to cellular injury to certain extent. Evidence has accumulated that these pro-inflammatory factors are capable of participating in the formation of CNV by stimulating VEGF secretion, promoting subretinal fibrosis, as well as inducing the progression of GA[27-28]. In addition, anti-inflammatory factors including TGFβ and IL-10 have been proven to be important promoters of immune homeostasis and tolerance. They are involved in angiogenesis, the inflammatory response, vascular fibrosis, and immune response in the pathogenesis of AMD[29]. Therefore, the release, imbalance, and potential interaction among anti-inflammatory and pro-inflammatory factors under oxidative stress may ultimately lead to the inflammatory deterioration of ARPE-19 cells.

Based on the protective characteristics of autophagy and the previous studies on the interaction between autophagy and oxidative stress, we speculate that autophagy may also have a significant regulatory function in immune inflammation. Therefore, we use autophagy inducers and inhibitors to regulate autophagy, and observe the change of inflammatory factors, ROS generation and cell viability. The data showed that cell apoptosis was obviously increased after downregulating autophagy. In addition, the production of oxidative stress-induced pro-inflammatory factors TNFα and IL-6 exhibited a significant reduction following upregulation of autophagy by Rapa, and significantly increased through downregulation of autophagy by Wort. Moreover, the release of TGFβ exhibited the opposite change to TNFα and IL-6 following H_2O_2 treatment under regulated autophagy. IL-6 is a multifunctional cytokine, which is also involved in multiple processes of the incidence and progression of AMD, including inflammatory immune responses, initiation of CNV, and subretinal fibrosis[30], and is significantly related to GA, the size, and activity of CNV[27]. Besides, TNFα can promote CNV formation *via* upregulating the production of VEGF[28]. Furthermore, TGFβ is a major regulator for fibrosis and an anti-inflammatory regulator as well, which participates in the pathological processes of AMD, such as angiogenesis and subretinal fibrosis[31]. Other studies have shown that the loss of TGFβ signaling can lead to retinal degeneration and an increases in CNV[32-33]. Meanwhile, the level of ROS was elevated by inhibiting autophagy and reduced by increasing

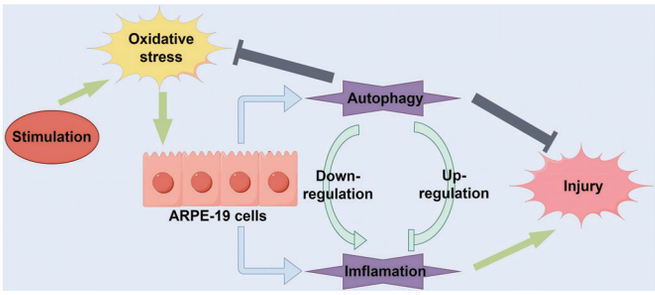

Figure 8 Interplay between oxidative stress, inflammation and autophagy in ARPE-19 cells Upon exposure to harmful stimuli, ARPE-19 cells undergo oxidative stress, leading to an increase in ROS, production of inflammatory factors and subsequent cellular injury. Oxidative stress acts as a double-edged sword, where it can both stimulate and be mitigated by autophagy. Autophagy, in turn, protects cells by reducing ROS and modulating inflammation. Enhanced autophagy suppresses pro-inflammatory cytokines and promotes anti-inflammatory factors, preserving cellular homeostasis. Conversely, inhibited autophagy intensifies inflammation and exacerbates cellular damage. Collectively, the figure underscores the intricate balance between oxidative stress, autophagy, and inflammation and suggests targeting autophagy as a therapeutic strategy to counteract oxidative stress-induced inflammation and cell injury in ARPE-19 cells. ARPE-19: Adult retinal pigment epithelial cell line-19; ROS: Reactive oxygen species.

autophagy. Our data suggested that autophagy activation can not only inversely lower ROS generation but also decrease the generation of pro-inflammatory factors stimulated by oxidative stress and augment the levels of anti-inflammatory factors in ARPE-19 cells. Nevertheless, the WB demonstrated a reduced autophagy level after exposure to 200 μmol/L H_2O_2 for 7d and more, yet we didn't get a similar effect in ARPE-19 cells treated with 600 μmol/L H_2O_2; the potential mechanisms of which need to be explored in further studies.

In addition, several limitations existed in our study. First, the present study was conducted only *in vitro* experiments. *In vivo* experiments using AMD mouse models or donor retina from AMD patients should be conducted to further investigate and confirm the results. Second, the signaling pathway of autophagy regulating the release of inflammatory factors was not elucidated, this is also the direction of our further research. Moreover, for clinical applications, we hypothesize that developments of pharmacology or miRNAs that regulate autophagy may provide putative future treatment options for AMD.

Conclusively, as described in Figure 8, our results suggested a mutual influence between autophagy and oxidative stress, oxidative stress promotes autophagy, while enhanced autophagy mitigates oxidative stress. Moreover, this study found that autophagy can alleviate inflammatory damage caused by oxidative stress, and regulation of autophagy was able to protect ARPE-19 cells from the inflammatory injury. Therefore, regulation of autophagy could offer novel strategies and ideas for future intervention in the progression of AMD.

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