Expression levels of ROS and Atg proteins in the vitreous in rhegmatogenous retinal detachment

Nan Huang¹, Xue-Yan Gao², Jin-Ping Li³, Xing Lu⁴, Hua-Mei Zhu⁵, Kai Dong³

¹Department of Ophthalmology, Xiaoxian People’s Hospital, Suzhou 234000, Anhui Province, China
²Graduate School of Bengbu Medical College, Bengbu 233000, Anhui Province, China
³Department of Ophthalmology, Eye Center, the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui Province, China
⁴Graduate School of Anhui Medical University, Hefei 230001, Anhui Province, China
⁵Hefei Aier Eye Hospital, Hefei 230001, Anhui Province, China

Correspondence to: Kai Dong. Department of Ophthalmology, Eye Center, the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, Anhui Province, China. happysubmission@163.com; Hua-Mei Zhu. Hefei Aier Eye Hospital, Hefei 230001, Anhui Province, China. 821252337@qq.com

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Abstract

● AIM: To detect the concentrations of reactive oxygen species (ROS), transient receptor potential mucin-1 (TRPML1), and autophagy-related (Atg) proteins (LC3-I, LC3-II, and Beclin1) in vitreous humor of patients with simple rhegmatogenous retinal detachment (RRD).

● METHODS: RRD patients enrolled as the RRD group, and patients with idiopathic macular hole (IMH) and idiopathic macular epiretinal membrane (IMEM) were enrolled as control group. The levels of ROS, TRPML1, LC3-I, LC3-II, and Beclin1 in vitreous humor of patients in the RRD and control groups were detected by enzyme-linked immunosorbent assay (ELISA).

● RESULTS: The RRD group included 28 eyes 28 patients and had a higher concentration of ROS in vitreous humor (631.86±18.05 vs 436.34±108.22 IU/mL, P<0.05). The ROS level in patients with a wide retinal detachment (RD) extent (RD range ≥1/2) was higher than that with a narrow RD extent (RD range<1/2, P<0.05). ROS concentration was negatively correlated with RD time (r=-0.46, P=0.01).

The expression levels of LC3-I and Beclin1 significantly decreased in RRD (P<0.05), but there were no correlations with the RD time, RD extent, or macular involvement.

● CONCLUSION: In eyes with RRD, the concentration of ROS in vitreous humor increases and the expression levels of Atg proteins decrease, reflecting possibly that autophagy is inhibited.

● KEYWORDS: autophagy-related proteins; reactive oxygen species; vitreous body; retinal detachment

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INTRODUCTION

Retinal detachment (RD) is the separation between the neurosensory retina and the retinal pigment epithelium[1-2]. Although surgery corrects RD 81%-98% of the time[3], the visual acuity of most patients is poor due to the death of photoreceptor cells[4-5]. Chen et al[6] showed that autophagy plays an important role in the death of photoreceptor cells. Autophagy is a highly conserved lysosomal pathway by which eukaryotic cells decompose intracellular substances and a necessary process of internal environmental balance. Through autophagy, intracellular components are recycled or used to generate energy[7]. More than 41 autophagy-related (Atg) proteins have been identified, among which LC3-II and Beclin1 are considered markers of autophagy activation[8-9]. Some scholars have proposed that the separation of the neurosensory retina from the retinal pigment epithelium reduces the supply of oxygen and nutrients in the outer segment of the photoreceptor, leading to the relative hypoxia of the photoreceptor cell layer and the production of reactive oxygen species (ROS) due to these stress stimuli[10] (Figure 1).

In our previous studies, the expression of ROS in the rat rhegmatogenous retinal detachment (RRD) model was increased, autophagy was promoted by rapamycin, and the expression levels of Atg proteins, such as LC3 and Beclin1, were increased, which eliminated excessive ROS and protected photoreceptor cells[11]. A later study found that increasing...
Regulate autophagy. By PI3KC3, and it plays different roles in adaptor protein formation to be an indicator of the localization and activity of PI3P catalyzed autophagy-related 14 L, UVRAG, AMBRA1, and Rubicon. Its detection hydrolase. Beclin1 can interact with many identified proteins, such as mitochondrial damage, excessive accumulation of reactive oxygen species, and activation of the transcription factors HIF-1, p53, FOXO3, and NRF2, stimulating the transcription of BNIP3 and NIX, TIGAR, LC3 and BNIP3 and p62, respectively. The endoplasmic reticulum stress sensor PERK can also be induced, whose downstream effector can induce autophagy gene expression. After autophagy is activated, LC3-I fuses with a lysosome, its components are degraded by lysosome hydrolase. Beclin1 can interact with many identified proteins, such as autophagy-related 14 L, UVRAG, AMBRA1, and Rubicon. Its detection can be an indicator of the localization and activity of PI3P catalyzed by PI3KC3, and it plays different roles in adaptor protein formation to regulate autophagy.

**Transient receptor potential mucin-1 (TRPML1) expression could also activate autophagy and protect photoreceptor cells**[12]. However, the role autophagy of in photoreceptor cells in RRD eyes is still not clear. By detecting the levels of Atg proteins and ROS in vitreous humor, this study preliminarily explores the intracellular autophagy in eyes with RRD to lay a foundation for protecting photoreceptor cells and improving postoperative visual acuity by intervening against ROS and autophagy.

**SUBJECTS AND METHODS**

**Ethical Approval** This study followed the guidelines of the Declaration of Helsinki and was approved by the Hospital Ethics Review Committee of the First Hospital of the University of Science and Technology of China (No.2020-N-(H)-086). The patients or their families agreed and signed the informed consent forms.

**Subjects and Specimen Sources** From December 2020 to May 2021, 44 patients with simple RRD, idiopathic macular hole (IMH), or idiopathic macular epiretinal membrane (IMEM) who underwent vitrectomy in the First Affiliated Hospital of University of Science and Technology of China were enrolled[13-14], including 25 male patients and 19 female patients aged 30-74 (57.64±10.16y). Patients’ demographics data are summarized in Table 1. Patients with previous vitreoretinal surgery history, penetrating injury, uveitis, aphakia, age-related macular degeneration, diabetic retinopathy, or uncontrolled glaucoma were excluded[15-16]. After eye anesthesia, standard 23-G vitrectomy was performed through the flat part of ciliary body, the perfusion catheter was closed, and the vitreous in the central axis was excised. A small amount of vitreous humor (approximately 0.4 mL) was collected in a sterile Eppendorf tube, and the patient’s age, sex, diagnosis, eye disease history, medical history, preoperative visual acuity, and specimen collection date were recorded[17].

The collected vitreous humor was centrifuged at 10 000 rpm for 6min in a high-speed cryo-centrifuge (EPPENDORF, Hamburg, Germany) at 4°C to remove particles. The supernatant (0.3 mL) was transferred to a sterile Eppendorf tube and then immediately stored in a freezer (EPPENDORF, Hamburg, Germany) at -80°C for later use[18].

**Measurement of Retinal Detachment Extent** The RD area was measured with a wide-angle laser fundus camera (OPTOS, Dunfermline, UK). After dividing the image into quadrants with the fovea macula as the center, the sum of quadrants including any RD area was taken as the RD extent[18].

**Detection Method** The collected specimens were taken out of the refrigerator 60min in advance and naturally warmed to room temperature. Dilutions of samples and standards were prepared according to the instruction manual of enzyme-linked immunosorbent assay (ELISA) kit (Jianglai Biotechnology, Shanghai, China). The sample to be tested (10 μL) and sample diluent (40 μL) were added together, and then the mixture was incubated at 37°C for 30min. Enzyme-labeled reagent (50 μL) was added to each well after washing the mixture, and color developed at 37°C in the dark for 15min. Stopping solution (50 μL) was added to each well to terminate the reaction. Finally, the optical density of each well was measured in sequence at 450 nm wavelength. The experiment done in three biological replicates[19].

**Data Processing and Statistics** The experimental data are expressed as mean±standard deviation, and all the experimental data were statistically processed using GraphPad Prism 8.0 (GraphPad, California, USA). The unpaired t-test

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value (Groups)</th>
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<tbody>
<tr>
<td>Gender</td>
<td>Male 25, Female 19</td>
</tr>
<tr>
<td></td>
<td>Age (y) 57.64±10.16</td>
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**RRD**: Rhegmatogenous retinal detachment.
was run to statistically analyze the ELISA results of the two groups. Pearson’s correlation coefficient between the factors in the RRD group was calculated. \( P < 0.05 \) indicated a statistically significant difference.

**RESULTS**

**Clinical Data**  Forty-four eyes of 44 patients were included in this study. Two groups of patients were formed as follows: a control group consisting of patients who underwent vitrectomy for the management of IMH or IMEM, and RRD group consisting of patients with RRD. Table 1 shows the patient’s demographic data in the groups. The differences in age between the groups were not statistically significant (Table 1).

**Comparison of ROS Expression Between the Two Groups**  The ROS level in the RRD group (631.86±18.05 IU/mL, \( P=0.00 \); A) decreased. The LC3-II expression (5335.77±463.79; D) was not significantly different from that of the control group (5257.55±1695.82 pg/mL, \( P=0.17 \)). The transient receptor potential mucin-1 expression (5.56±0.72) was not significantly different from that of the control group (7.59±1.25 ng/mL, \( P=0.33 \); E). RRD: Rhegmatogenous retinal detachment.

**Comparison of Expression Levels of Atg Proteins Between the Two Groups**  The levels of LC3-I (3018.99±365.47 pg/mL, \( P=0.00 \); B) and Beclin1 (4.60±1.37 ng/mL, \( P=0.01 \); C) decreased. The LC3-II expression (5335.77±463.79; D) was not significantly different from that of the control group (5257.55±1695.82 pg/mL, \( P=0.17 \)). The transient receptor potential mucin-1 expression (5.56±0.72) was not significantly different from that of the control group (7.59±1.25 ng/mL, \( P=0.33 \); E). RRD: Rhegmatogenous retinal detachment.

**Correlations Between the RD Extent and the Levels of ROS and Atg Proteins in the RRD Group**  The ROS level in patients with a large RD extent (RD range ≥1/2, 640.60±26.79) was significantly higher than that in patients with a small RD extent (RD range<1/2, 615.29±6.28 IU/mL, \( P=0.02 \); A). The LC3-I levels in patients with a large RD extent (RD range ≥1/2, 3102.87±449.35) was not significantly different from that in patients with a small RD extent (RD range<1/2, 1720.57±255.31 pg/mL, \( P=0.40 \); B). Beclin1 level was not significantly different between patients with a large RD extent (RD range ≥1/2, 4.52±1.29) and patients with a small RD extent (RD range<1/2, 4.73±0.01 ng/mL, \( P=0.06 \); C).

**Figure 2**  Expression levels of reactive oxygen species, LC3-I, LC3-II, Beclin1, and transient receptor potential mucin-1 after RRD. After RRD, the reactive oxygen species level in vitreous humor increased significantly (631.86±18.05 IU/mL, \( P=0.00 \); A). The expression levels of LC3-I (3018.99±365.47 pg/mL, \( P=0.00 \); B) and Beclin1 (4.60±1.37 ng/mL, \( P=0.01 \); C) decreased. The LC3-II expression (5335.77±463.79; D) was not significantly different from that of the control group (5257.55±1695.82 pg/mL, \( P=0.17 \)). The transient receptor potential mucin-1 expression (5.56±0.72) was not significantly different from that of the control group (7.59±1.25 ng/mL, \( P=0.33 \); E). RRD: Rhegmatogenous retinal detachment.

**Figure 3**  Comparisons between retinal detachment (RD) range, macular involvement and levels of reactive oxygen species and Atg proteins. After rhegmatogenous RD, the reactive oxygen species level in patients with a large RD extent (RD range ≥1/2, 640.60±26.79) was significantly higher than that in patients with a small RD extent (RD range<1/2, 615.29±6.28 IU/mL, \( P=0.02 \); A). The LC3-I levels in patients with a large RD extent (RD range ≥1/2, 3102.87±449.35) was not significantly different from that in patients with a small RD extent (RD range<1/2, 1720.57±255.31 pg/mL, \( P=0.40 \); B). Beclin1 level was not significantly different between patients with a large RD extent (RD range ≥1/2, 4.52±1.29) and patients with a small RD extent (RD range<1/2, 4.73±0.01 ng/mL, \( P=0.06 \); C).
Correlations Between RD Macular Involvement and the Levels of ROS and Atg Proteins in the RRD Group
The ROS level in patients with macular involvement (648.71±1.19) was not significantly different from that in patients without macular involvement (611.41±2.40 IU/mL, P=0.65; Figure 4A). The LC3-I level in patients with macular involvement (3314.75±69.70) was not significantly different from that in patients without (2314.00±338.82 pg/mL, P=0.30; B). The Beclin1 level in patients with macular involvement (4.30±2.37) was not significantly different from that in patients without (3.99±0.76 ng/mL, P=0.44; C).

DISCUSSION
Although anatomical reduction can be achieved after surgery, most RRD patients’ visual acuity does not recover due to the death of photoreceptor cells. This was a prospective clinical study in patients with RRD, IMH, or IMEM. By detecting the levels of ROS and Atg proteins in vitreous humor, we studied the death mode of photoreceptor cells and its influencing factors in RRD eyes. The ROS concentration in vitreous of RRD patients increased, and negatively correlated with RD time. However, with the prolongation of RRD, the downward trend of ROS is not obvious. While the expression levels of LC3-I and Beclin1 decreased, which revealed that autophagy of photoreceptor cells was inhibited in RRD eyes.

When cells are stressed in an anoxic environment, ROS accumulation can result in oxidative damage, leading to mitochondrial dysfunction and cell damage. Many mechanisms of oxidative stress in the retina can lead to the death of photoreceptor cells. The molecular regulation mechanisms between ROS and autophagy are also diverse. In the nucleus, when responding to ROS production, the transcription factors hypoxia-inducible factor (HIF)-1, p53, forkhead box O3 (FOXO3), and nuclear factor erythroid 2-related factor 2 (NRF2) are sequentially activated to stimulate the transcription of BCL2/adenovirus E1B 19-kDa–interacting protein 3 (BNIP3) and NIX, TP53-induced glycolysis and apoptosis regulator (TIGAR), LC3 and BNIP3, and p62, respectively. Endoplasmic reticulum stress sensor protein kinase R-like endoplasmic reticulum kinase (PERK) can also be induced, whose downstream effector can induce autophagy...
gene expression. In the cytoplasm, ROS may also affect the formation of autophagosome membranes by regulating Atg4 activity. In turn, autophagy can reduce ROS levels through other pathways, such as the chaperone-mediated autophagy pathway, mitophagy pathway, and p62 delivery pathway. After autophagy is activated, LC3-I can bind with phosphatidylethanolamine to form LC3-II, which is recruited to the autophagosome membrane. After the autophagosome fuses with a lysosome, its components can be degraded by lysosome hydrolase. Under relatively unstable, transient, or other specific conditions, Beclin1 can interact with many identified proteins, such as Atg14 L, UV radiation–associated gene (UVRAG), autophagy and beclin 1 regulator 1 (AMBRA1), and Rubicon. It can be detected as an indicator of the localization and activity of phosphatidylinositol 3-phosphate (PI3P) catalyzed by class III phosphatidylinositol 3-kinase (PI3KC3), and it plays different roles in adaptor protein formation to regulate autophagy. Growing evidence shows that autophagy is related to neurodegenerative diseases. However, whether autophagy is protective or destructive varies from disease to disease and is controversial. Ma et al. showed that mesenchymal stem cell–derived exosomes can prevent photoreceptor loss in RRD eyes by inhibiting inflammatory cytokine induction and upregulating autophagy, and can increase the survival of photoreceptor cells in RRD eyes. This finding is consistent with our method to promote autophagy and protect photoreceptor cells through rapamycin. Liu et al. adopted 3-methyl adenine (3-MA), a class III inhibitor of PI3K activity and autophagosome formation, to downregulate autophagy, but their results instead showed that it could reduce HeLa cell death.

In our previous study, TRPML1 in the animal RRD model activated autophagy. However, in this study, TRPML1 expression in RRD eyes was not significantly different from that of the control group. Interestingly, in our study of a rat RRD model, TRPML1 expression was lower than the control level on the 3rd day of RRD, this discrepancy might be related to the course of RRD.

In this study, the expression levels of LC3-I and Beclin1 decreased, revealing that decreased autophagy might cause the continuous renewal and destruction of nonfunctional proteins and organelles, destroy the balance of intracellular environment, and promote cell death. Although the LC3-II expression was similar between the RRD group and the control group, which might be because that the expression of vitreous protein could not fully reflect the changes of retinal tissue. This study preliminarily confirms that excessively accumulated ROS after in eyes with RRD in the human body can result in photoreceptor cell damage, and autophagy in RRD photoreceptor cells is inhibited, however, the specific underlying molecular mechanisms are still not clear and warrant further study.

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