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Full Length Article

# Changes of Beclin-1 and ULK1 in retina of mice model in oxygen-induced retinopathy

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ABSTRACT

*Purpose:* To observe the expression differences and potential effects of autophagy-related Beclin1 (mammalian Atg6) and Uncoordinated-51 like kinase 1 (ULK1) in the oxygen-induced retinopathy (OIR) model.
*Materials and methods:* Thirty-three C57BL/6 mice in OIR model group were exposed to 75 ± 0.5% oxygen from postnatal day-of-life 7 (P7) to P12, and were then brought into normal room environment (relative hypoxia stage) and raised to P17. Thirty-three control mice were kept in a normal room environment. The expression of autophagy in the retina tissue was assessed by Western blot analysis. The thickness and ultrastructural of retina were observed by light microscopy and transmission electron microscope (TEM) on P17.
*Results:* In the hyperoxia stage (P8–P11), the expression of Beclin1, ULK1 and Autophagy 5 (Atg5) in retina showed no significant difference between the OIR model group and the control group. In the relatively hypoxia stage (P14 to P17), however, the protein level of Beclin1, ULK1, and Bcl-2-associated X protein (Bax) were upregulated in the retina of the OIR model group, whereas B-cell lymphoma 2 (Bcl-2) was downregulated. The autophagosomes in the photoreceptors of retina in the OIR mice were observed. The inner-segment/out-segment (IS/OS) layer in OIR model group was thinner than that the control group on P17.
*Conclusions:* The expression of Beclin-1 and ULK1 in retina has changed in the OIR model, and the change of Beclin-1 and ULK1 expression is related to the change of oxygen concentration.

## 1. Introduction

Retinopathy of prematurity (ROP), which afflicts infants vision, is caused by an oxygen-induced damage to the developing retinal vasculature.[1] Exposing the developing vasculature to hyperoxia causes the cessation of growth of retinal blood vessels, followed by their constriction and death.[2] Conventional therapies for ROP are limited to removing vasculature by surgery or intra-vitreal injection of anti-vascular endothelial growth factors (anti-VEGF) antibody.[3] However, the vision of ROP patients over the long term is still poor, and effective treatments for their blinding disorders are lacking. Previous researches have indicated that photoreceptor and post-receptor responses alter significantly years after the preterm days,[4,5] suggesting that not only is the retinal vascularization disrupted, but the retinal cells are also damaged.

We found that the expression of autophagy related gene P62 has changed in retina of the OIR model.[6] Based on previous studies, we speculate that autophagy may affect the function of retinal cells in OIR. Autophagy is an intracellular pathway in which cytoplasmic constituents are delivered to the lysosomal pathway for degradation.[7] When cells are subjected to stress, such as hypoxia, injury, or starvation, autophagy is activated immediately (i.e., an induced autophagy).[8] Under mild stress, autophagic activity is beneficial for cell survival, while severe stress leads to dysregulated autophagy, which further results in massive cell death. Thus, autophagy acts as a "double-edged sword" in cell survival. Recent studies have shown autophagy is involved in photoreceptor survival and death.[9] Autophagy increases in the retinas of diabetic patients; the loss of vision is associated with the changes of autophagy.[10] At present, few studies have addressed the mechanisms of autophagy in OIR models. The knowledge of autophagy's basic function in retinas of OIR patients is still scarce. Beclin1 (mammalian Atg6) and Uncoordinated-51 like kinase 1 (ULK1) are key signaling molecules in the autophagy pathway. In this study, we examined the protein expression of autophagy-related Beclin1 and ULK1 in the retinas of an OIR model to further understand how autophagy maintains retinal function in OIR animal model.

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## 2. Materials and methods

### 2.1. Animals

A total of 66 newborn C57BL/6 mice (Purchased from Shanghai SLAC Laboratory Animal Co.,Ltd, China) were used in the present study. All animal protocols were reviewed and approved by the institutional animal care committee of Zhejiang Chinese Medicine University and were in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research (No. 20180108–01).

### 2.2. Experimental protocol of OIR model

Mice were further divided into a control group (n = 33) and an OIR model group (n = 33). The mice in OIR model were established by Smith et al.[11] Briefly, mice were placed into an oxygen-enrichment device (Hangzhou APU Instrument and Equipment Co., Ltd., China) at postnatal day of life 7 (P7) with their nursing mothers. In the device, mice were exposed to 75 ± 0.5% $O_2$ for 5 days (through P12), and were then returned to room environment and raised to 17 days (P17). The control mice were raised in normal room environment for 17 days. Mice in both groups were supplied with standard mouse water and chow. Temperature and humidity were maintained at 25 °C and 75–80%, respectively. Day/night cycles of 12 h were used. After the experiment, all mice were killed with carbon dioxide.

### 2.3. Western blot analysis

Total protein extract of retinas was obtained from mice eyes at P8–P17 (fifty-four eyes in oxygen-exposed group and fifty-four eyes in control group). The protein concentrations were measured using a BCA Protein Assay kit (Beyotime, China). Samples containing 50 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis gels (PAGE) using 10% and 15% polyacrylamide gel, then transferred onto a polyvinylidene difluoride membrane (Bio-Rad, USA). The membrane was blocked in 5% TBS (10 mM Tris; pH 8.0, 150 mM NaCl, 0.5% Tween 20 and 5% fat-free dry milk) for 1 h at room temperature, then probed overnight at 4 °C with specific primary antibodies against Beclin-1, ULK1, Atg5, BCL-2, Bax and β-actin (1:1000; Cell Signaling Technology, USA), and incubated in blocking buffer with β-actin as an internal control. Immunoblots were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, USA). Membranes were developed with the ECL substrate (Pierce, Thermo Scientific, USA). Images of labeled specimens were obtained with a ChemiDoc XRS imagining system (Bio-Rad Laboratories). Quantitation of bands were performed by densitometry using the Image Lab software (Bio-Rad Laboratories) and normalized to the expression of β-actin.

### 2.4. Transmission electron microscopy

Eyes of oxygen-exposed (n = 6) and control group (n = 6) mice were enucleated and fixed immediately in 2% glutaraldehyde and 4% paraformaldehyde on P17. Then the retinas were dissected and post-fixed in 2% aqueous osmium tetroxide for 1.5 h. After embedding in Epon, ultrathin sections were cut at 1 μm and stained in uranyl acetate. The ultrastructure of retinal photoreceptor cells was observed under an electron microscope (Type 1400plus, Tokyo, Japan).

### 2.5. Hematoxylin and eosin (H&E)

On P17, eyes of OIR model (n = 6) and control group (n = 6) were fixed immediately in 1% formaldehyde and 1.25% glutaraldehyde after enucleation for 48 h. Then, they were embedded in Optimum Cutting Temperature compound. Serial sections (5 μm thick) were cut through the cornea, parallel to the optic disc in a sagittal plane. The tissue sections were stained with hematoxylin and eosin (H&E). Under light microscopy, we observed the nuclei of the vascular endothelial cells of the neovascular vessels, which extending beyond the inner limiting membrane of the retina into the vitreous, and the thickness of retinal layers was measured by NDP (View2) software.

### 2.6. Statistics

Statistical analyses were performed using SPSS 18 (SPSS Inc., Chicago, IL, USA). Student's *t*-test and ANOVA was used to determine the significance of differences between the groups. Statistical significance was defined as a $p$ value less than 0.05.

## 3. Results

### 3.1. The protein expression of Beclin1, ULK1 and Atg5 in OIR model

To investigate the autophagy function in retina, the protein levels of Beclin, ULK1 and Atg5 were assessed. Beclin1 in retinas was upregulated in the relative hypoxia phase (Fig. 1A). There was no significant change in the expression of ULK1 and Atg5 between the OIR mice and control mice (Fig. 1B and C). However, it was found that the expression of ULK1 protein was up-regulated at two time points P15 and P17 in the OIR model group. These results indicated that autophagy may be induced after exposure to high oxygen environment.

### 3.2. The protein expression of Bcl-2 and Bax in OIR model

Next, we evaluated the changes of Bcl-2 and Bax on retinal tissue. Although the expression of Bax in OIR mice increased at first (P8–P9), subsequently the two groups showed no difference. Bax was upregulated again from P14 to P17 in the relative hypoxia phase, (Fig. 2B), and Bcl-2 was upregulated in the hyperoxia phase (P8–P11). However, in the relative hypoxia phase (P15–P17), the protein expression of Bcl-2 was decreased. Taken together, the Bcl-2 protein level in the retinas showed a downregulation tendency from P8 to P17 (Fig. 2A). These results indicate that in the retinas of the OIR mice, the hyperoxia-induced increase of apoptosis is in line with the changes in autophagy activation.

### 3.3. The formation of autophagosomes in OIR model

The autophagosomes in retina were observed. The transmission electron micrographs of retina in the OIR model and control mice were shown in Fig. 3. The ultrastructure of photoreceptor in control mouse were normal (Fig. 3A, D). In the OIR group, the autophagosomes enclosed cytoplasm (Fig. 3B) and the bilayer membrane structure (Fig. 3C) were found in retinal cells. The outer membrane of photoreceptors was sparsely arranged (Fig. 3E) and vacuolated (Fig. 3F). These findings indicated that autophagy occurred in the photoreceptors of OIR mice.

### 3.4. Thicknesses of retinas in the OIR model were greater than in the control

Compared with the mice raised in normal room environment, the structures of retinas in the OIR mice were altered. We measured the preretinal layers and the thickness of retinas in the two groups at P17. Retinal structures appear normal in the control group (Fig. 4A). The oxygen-exposed mice retinas showed edematous changes (Fig. 4B). The vessels protruded into the vitreous at the preretinal layer in OIR mice (Fig. 4C). The thickness of the outer nuclear layer (ONL) increased significantly in the OIR group; however, the thicknesses of inner-segment/outer-segment (IS/OS) in OIR mice were thinner than those in the control group. The total thicknesses of retinas (from nerve fiber layer (NFL) to RPE) in the OIR model group were greater than in the control group, but this difference was not significant (Fig. 4D).

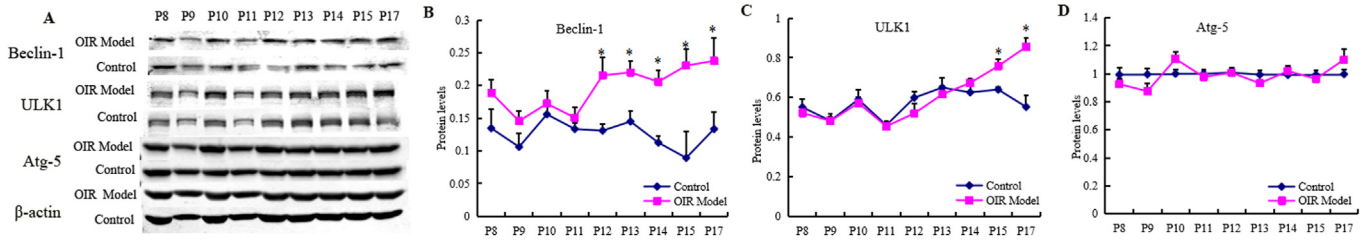


**Fig. 1.** The retinal expression levels of Beclin1, ULK1, and Atg5 in different time assessed by Western blot (A) and the quantification change comparing the two groups (B: Beclin1; C: ULK1; D: Atg5). *, $P < 0.05$. β-actin was used as the loading control.

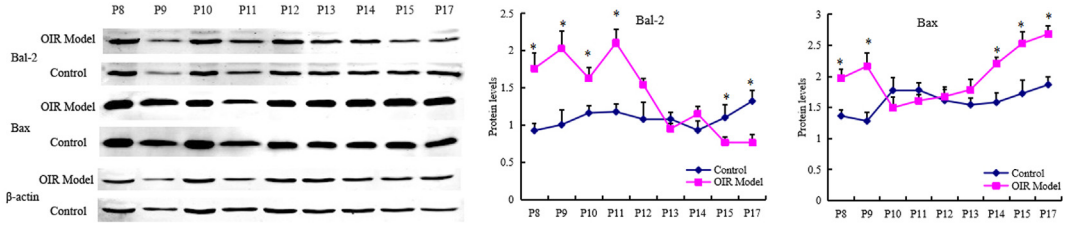


**Fig. 2.** The retinal expression levels of Bal-2 and BAX in different time assessed by Western blot (A) and the quantification change comparing the two groups (B: Bcl-2; C: Bax).*, $P < 0.05$. β-actin was used as the loading control.

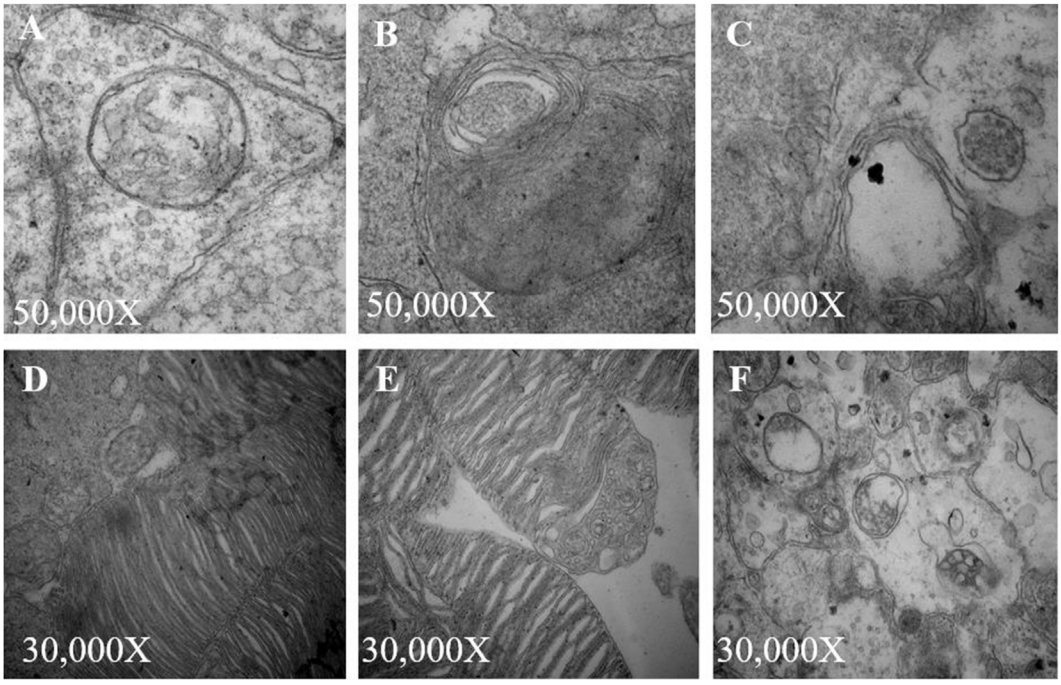


**Fig. 3.** Observation of ultrastructure of retina by Transmission electron microscopy.
The ultrastructure of retina in OIR mice at P17: Autophagic event were encountered in the mouse retina under TEM. Retinal ultrastructure appeared normal in the control group (A, D). In the OIR group (B, C, E, F): Autophagosomes enclosed cytoplasm (B). Bilayer membrane structure (C). The outer membrane of photoreceptor cells was sparsely arranged (E) and vacuolated (F).

## 4. Discussion

The development of ROP is accompanied by abnormal proliferation of blood vessels. But in clinical work, after injecting anti-neovascularization drugs in the vitreous cavity, the vision of ROP patients over the long term is still poor. We speculate that in addition to vascular lesions, the retinal cells in ROP are also dysfunctional. Therefore, we focused on the changes of autophagy of retinal cells. In this study, we observed the expression changes of Beclin1 and ULK1 in retinal cells during hyperoxia (the first stage of OIR; from P8–P12) and relative hypoxia (the second stage of OIR; from P13–P17).

ULK1 has been suggested to function in the initial stage of the autophagy pathway. ULK signaling controls autophagosome formation in conjunction with key regulatory factors such as Beclin1 and Atgs. ULK1 was positioned to function upstream of the Beclin1 pathway.[12,13] In the OIR mice, retinas were characterized by high protein levels of ULK1 from

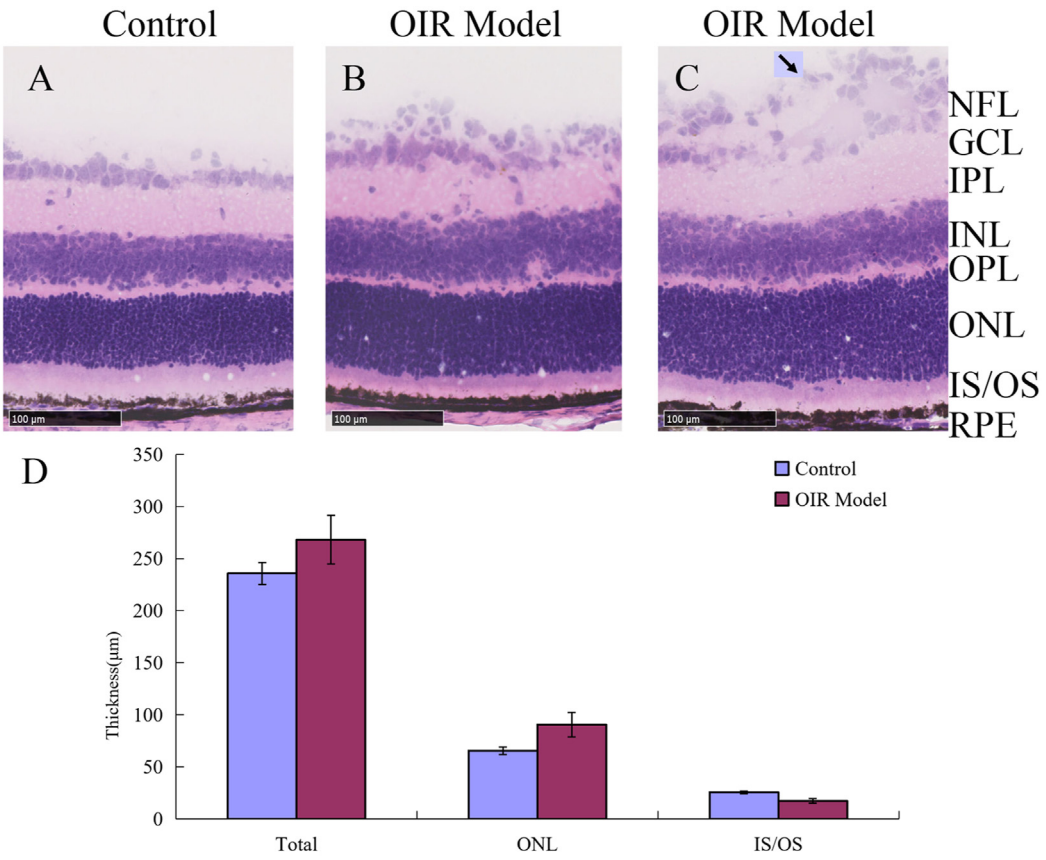


**Fig. 4.** Pathological changes of retinal tissues in the retinopathy of prematurity (OIR) group. Retinal structures appear normal in the control group (A). Retinas of OIR model mice showed edematous changes, especial the ONL, while the thickness of IS/OS layer in the OIR model decreases significantly (B, D). The vessels protrude into the vitreous at the preretinal layer in OIR mice (C, arrows). *, $P < 0.05$.

P15 to P17, indicating an induction in the autophagic process in the retinas of the OIR model. Moreover, the protein levels of Beclin1 were upregulated from P12 to P17, which means ULK1 may combine with Beclin1 for the multiple autophagy components to form autophagosomes in the retinal cells.

As previously demonstrated, anti-apoptotic Bcl-2 inhibits Beclin-1 dependent autophagy.[14] Under conditions of stress, Bcl-2 is displaced from Beclin1 and Bax to induce autophagy and apoptosis, respectively. In contrast, under conditions of extreme starvation, Bcl-2 is dissociated from Bax, and promotes apoptosis.[15] In the present study, the hyperoxia stress possibly disrupted the bonding between Bcl-2 and Beclin1 to induce autophagy. In the hypoxia stage, Bax may have promoted caspase-mediated cleavage of Beclin-1, which induced apoptosis. Studies have shown that hyperoxia can induce autophagy in retinal cell, the autophagic functions as an adaptive response to stress.[16] We also found that the expression of autophagy was upregulated after the mouse retinal tissue was exposed to hyperoxia. Moreover, the retina in mice is characterized by increased apoptosis markers at the phase of relative hypoxia; at that time, cells begin to die.[17,18] These results were in line with our finding of the increased apoptosis in the late phase of OIR. The Atg5 cleavage fragment could promote nuclear fragmentation, and cleaved Atg5 directly induces apoptosis but not autophagy.[15] However, in our study, the protein expression of Atg5 showed no significant difference between the hyperoxia phase and the hypoxia phase.

We also found many autophagosomes in OIR model. Especially in the photoreceptor, the outer membrane of photoreceptor cells were sparsely arranged and vacuolated. Moreover, the whole thickness of retina increased. The IS/OS layer was thinner than that of the control mice at P17. Moreover, the retinas of the OIR model mice became edematous, suggesting that the photoreceptor of OIR model may be damaged. Excessive autophagy is an important pathological feature of ocular diseases.[19,20] The present results are consistent with the supposition that an enhancement of autophagy in OIR mice may result in a decreased function of the protective mechanism that is important to retinal cell survival in response to injury.

Previous studies indicated that oxygen-induced retinal apoptosis in a retinopathy model decreased from P13 to P15, and then remained constant until P17.[21,22] The present results showed that in the initial stage of hyperoxia (from P8 to P9) the expression of Bax in retinal cells increased, then the expression decreased slightly (from P10 to P13), but the difference was not statistically significant. From P14 to P17, the expression of Bax increased again. We speculated that the sudden entry into hyperoxia environment led to an increased stress response of Bax expression. In addition, the expression of Bcl-2 in retinal cells was upregulated in the hyperoxia phase (from P8 to P11), then decreased in the relative hypoxia phase (P15–P17), and showed a downregulated tendency. These results indicate that in the retinas of the OIR mice, an increased oxygen-induced expression of the apoptotic markers is paralleled by the autophagy activation.

## 5. Conclusions

Beclin-1 and ULK1 plays an important role in the pathophysiology of retinal cells in the OIR model. Inhibiting excessive autophagy activity in retinal tissue may help alleviate the severity of OIR.

## Study approval

All animal protocols were reviewed and approved by the institutional animal care committee of Zhejiang Chinese Medicine University and

were in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

## Author contributions

The authors confirm contribution to the paper as follows: Conception and design of study: WJ, LFF; Data collection: WJ, DE; Analysis and interpretation of results: WJ, ZYL; Drafting the manuscript: WJ, LFF; All authors reviewed the results and approved the final version of the manuscript.

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## Declaration of completing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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