



# Autophagy protects against oxidized low density lipoprotein-mediated inflammation associated with preeclampsia



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## ABSTRACT

**Introduction:** Inflammatory responses play an important role in the pathogenesis of preeclampsia. Recently, the anti-inflammatory role played by autophagy has drawn increasing attention. Our aim was to investigate variations in autophagy in preeclampsia and protection against oxidized low-density lipoprotein (oxLDL)-mediated inflammation by autophagy.

**Methods:** We used immunohistochemistry, immunofluorescence, quantitative real-time PCR, and western blotting to analyze the expression of autophagy proteins (beclin-1 and LC3II/LC3I) in preeclampsia placentas and in JEG-3 cells treated with oxLDL and rapamycin.

**Results:** We found a decreased level of autophagy proteins in preeclampsia placentas, and oxLDL did not induce autophagy in JEG-3 cells. Furthermore, when cells were pretreated with rapamycin, autophagy was activated and expression of inflammatory factors (tumor necrosis factor- $\alpha$  and interleukin-6) induced by oxLDL was downregulated.

**Conclusion:** We conclude that impaired autophagy in preeclampsia has potential to decrease trophoblast protection from oxidative and inflammatory stress, thereby contributing to the pathogenesis of preeclampsia.

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## 1. Introduction

Preeclampsia, a serious hypertensive disorder of pregnancy, is a leading cause of maternal death and a major contributor to maternal and perinatal morbidity. However, the triggering factors and underlying mechanisms responsible for the pathogenesis of preeclampsia are elusive. The leading hypotheses rely strongly on disturbed placental functions. Studies have supported a two-stage model: stage one is inadequate placental perfusion, and stage two is the maternal syndrome resulting from inadequate placental perfusion. The consequences of inadequate perfusion are intermittent hypoxia and the generation of oxidative stress, which lead to the release of antiangiogenic proteins and inflammation [1,2].

In addition to apoptosis, autophagy is another programmed cell death pathway. It is a reparative and life-sustaining process by which cytoplasmic components are sequestered in double-membrane vesicles and degraded on fusion with lysosomes under stress to maintain cellular homeostasis [3]. Autophagy plays

important roles in immunity and inflammation. The beneficial and detrimental effects of immunity and inflammation can be balanced by autophagy, which may protect against infections as well as autoimmune and inflammatory diseases [4]. Recently, autophagy has become a highly researched field in obstetrics, and this process may be essential for preimplantation development beyond the four- and eight-cell stages, and for blastocyst survival [5], extravillous trophoblast functions, invasion, and vascular remodeling [6]. Preeclampsia displays many characteristics, including hypoxia and inflammatory responses, and these are associated with autophagy. However, divergent views on how autophagy varies in pregnancy appear in studies of preeclampsia. A study has suggested excessive activation of autophagy in the placenta of mothers with hypertensive disorders compared with normotensive pregnancies, indicating the involvement of excessive autophagy in the development of the disease [7]. Goldman-Woh et al. used published microarray datasets to analyze differential expression of autophagy pathway genes, and no statistically significant difference in autophagy-associated gene expression was found in preeclamptic placenta samples compared with normal samples [8]. Nakashima et al. reported that impaired autophagy in extravillous trophoblast cells contributes to the pathophysiology of preeclampsia [9].

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Oxidized low density lipoprotein (oxLDL) is the oxidative modification of native LDL in numerous disease states resulting from oxidative stress and vascular endothelial injury. OxLDL binding to its receptor, lectin-like oxidized low density lipoprotein receptor 1, causes endothelial dysfunction and could play a significant role in the pathobiology of atherosclerosis, diabetes, hypertension, and preeclampsia [10]. Previous studies have shown that the cytotoxicity induced by oxLDL differs in diverse cell types. A study of human umbilical vein endothelial cells (HUVECs) suggested that oxLDL increases the autophagic level in a concentration-dependent manner [11]. OxLDL inhibits autophagy in macrophages and smooth muscle vascular cells [12,13]. However, there are only a few studies on the effects of oxLDL in the autophagy of human trophoblasts. Therefore, we aimed to investigate autophagy variations in preeclampsia, the influence of oxLDL on autophagy in the JEG-3 cell line, and whether autophagy can protect against oxLDL-mediated inflammation.

## 2. Materials and methods

### 2.1. Placenta and patient information

Placental tissues were collected after caesarean delivery from normotensive pregnancies (NP, n = 20) and preeclampsia pregnancies (PE, n = 26). Samples were collected from each placenta and then pooled to obtain one sample per placenta, after which they were placed on ice, transported to the laboratory, processed within 30 min, rinsed in ice-cold phosphate buffered saline (PBS), and then immediately frozen at  $-80^{\circ}\text{C}$  until analysis. This study was approved by the Committee for Ethical Review of Research at Qingdao University, China.

Preeclampsia is defined as a sustained systolic blood pressure of  $\geq 140$  mmHg, or a sustained diastolic blood pressure of  $\geq 90$  mmHg on two separate readings and 24-h urine protein collection with  $\geq 300$  mg in the specimen. Patients with complicated HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome were excluded to avoid clinical phenotypic differences. Normotensive pregnancy is defined as a pregnancy with normal blood pressure ( $<140/90$  mmHg) and no proteinuria [2] (See Table 1).

### 2.2. Immunohistochemistry

Autophagy-related protein beclin-1 forms an early complex that promotes the synthesis and growth of pre-autophagosomal membranes. LC3 is synthesized as proLC3 and integrated into the membranes of autophagosomes. Thus, beclin-1 and LC3 are considered as markers for detecting autophagy [14,15].

Immunohistochemistry was performed using 4  $\mu\text{m}$ -thick sections of samples embedded in paraffin. The sections were incubated for 1 h at  $64^{\circ}\text{C}$ , deparaffinized in xylene, and rehydrated in ethanol and water. The sections underwent antigen retrieval in citrate buffer (pH 6.0) followed by blocking peroxidase activity (Block; Dako, Tokyo, Japan), and then incubated with the appropriate primary antibody diluted in PBS for 1 h at room temperature. Detection was performed using the Envision horseradish peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine color development system (DAKO) for consistent development times between samples. Rabbit polyclonal antibodies against beclin-1 (dilution 1:600; Abcam, USA) and LC3 (dilution 1:2200, Abcam) were used as the primary antibodies. Normal rabbit serum was substituted for primary antibodies with the negative control. Positive sections from the manufacturers were used as positive controls.

**Table 1**

Characteristics of subjects from whom the placentas were obtained.

Characteristic	NP	PE
n	20	26
Maternal age (y)	$32.0 \pm 5.0$	$33.2 \pm 4.1$
BMI ( $\text{kg}/\text{m}^2$ )	$24.1 \pm 3.5$	$25.6 \pm 5.0$
Gestational age at delivery (wk)	$36.2 \pm 2.3$	$35.1 \pm 3.2$
Systolic BP (mmHg)	$120.0 \pm 2.2$	$166.3 \pm 5.8$
Diastolic BP (mmHg)	$74.1 \pm 3.2$	$98.17 \pm 5.5$
Proteinuria (mg/24 h)	$21 \pm 12$	$4201 \pm 300.0$

BP, blood pressure; BMI, body mass index.

### 2.3. Cell culture

The human choriocarcinoma JEG-3 cell line was obtained from the Shanghai Institute for Biological Sciences (Chinese Academy of Sciences). All experiments were performed in complete culture medium to avoid induction of autophagy via the serum starvation pathway. The cell line was maintained in  $\alpha$ -minimum essential medium (41500034; GIBCO, Shanghai, China) containing 10% fetal bovine serum and an antibiotic mixture at  $37^{\circ}\text{C}$  in a humidified atmosphere with 5%  $\text{CO}_2$ .

Cells were treated with various concentrations of oxLDL (25, 50, 100, and 150 mg/l; Yiyuan Biotech, China) for various times (6, 12, 24 and, 48 h), or were pretreated with rapamycin (100 nM; Qcbio Science, China) for 1 h.

### 2.4. Immunofluorescence

To assess the presence and location of the LC3 protein, JEG-3 cells were seeded into 24-well plates and cultured under various conditions. Then, the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton for 5 min, and blocked in 5% bovine serum albumin for 30 min. The cells were then incubated with a rabbit anti-LC3 antibody (diluted 1:500; Abcam ab48394) at  $4^{\circ}\text{C}$  overnight. After washing with PBS, the cells were incubated with CY3-conjugated goat anti-rabbit IgG (diluted 1:500; Jackson, USA) at  $37^{\circ}\text{C}$  for 30 min. The cells were counterstained with 4',6'-diamidino-2-phenylindole (diluted 1:1000; Boster, Wuhai, China) at room temperature for 5 min. Images were captured using a confocal microscope (ZEISS, Germany).

### 2.5. Quantitative real-time PCR for mRNA expression analysis

Total RNA from placental tissues and cell lysates was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA (500 ng) was used to synthesize cDNA using a PrimeScript RT reagent Kit (Takara, Japan). The cDNA was subsequently amplified with SG Fast qPCR Master Mix (High Rox,  $2 \times$ ; BBI, Canada) using a StepOnePlus instrument (ABI, USA). The primers used are listed in Table 2. PCR conditions were as follows: 30 s at  $95^{\circ}\text{C}$ , followed by 40 cycles of 7 s at  $95^{\circ}\text{C}$ , 10 s at  $57^{\circ}\text{C}$ , and 15 s at  $72^{\circ}\text{C}$ . Relative mRNA expression levels were determined by normalizing the expression of each gene to the  $\beta$ -actin gene using the  $2^{-\Delta\Delta\text{Ct}}$  method [16] relative to the expression in the control group. Normal JEG-3 cells without oxLDL or rapamycin were used as the control group.

### 2.6. Western blotting

Total proteins were extracted in RIPA lysis buffer (Beyotime, Zhejiang, China). After centrifugation, the supernatants were collected to determine the protein concentrations. Equal protein

**Table 2**  
Quantitative real-time PCR primers used in this study.

Gene	Forward	Reverse	Product
LC3	5'-TAGTTGCGTTACACCCCTTCTTG-3'	5'-TCACCTTCACCGTCCAGTTT-3'	144bp
Beclin 1	5'-TGAGGGATGGAAGGGTCTAAG-3'	5'-GCCTGGGCTGGTAAGTAAT-3'	161bp
mTOR	5'-TCACATTACCCCTTCACCA -3'	5'-TCAGCGAGTCTTGCTATTCC-3'	230bp
IL6	5'-GAGGAGACTTGCTGGTGAA-3'	5'-TTGGTCAGGGGTGGTAT-3'	187bp
TNF- $\alpha$	5'-ACATCCAACCTTCCAAACG-3'	5'-GGTGGTCTTGCTTAAAGTTCTA-3'	145bp
$\beta$ -actin	5'-TAGTTGCGTTACACCCCTTCTTG-3'	5'-TCACCTTCACCGTCCAGTTT-3'	151bp

amounts were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked with blocking solution (Beyotime) and incubated overnight with the primary antibody. The membranes were washed and incubated with a secondary antibody (1:500 dilution; Cwbiotech, Beijing, China). Labeled proteins were detected with an enhanced chemiluminescence system (Amersham Life Science, USA). To correct for differences in protein loading, the membranes were washed and re-probed with a monoclonal antibody against human  $\beta$ -actin (1:1000 dilution; Sigma, Shanghai, China). The relative intensities of protein bands were analyzed using a scanner (MSF-300G; Microtek Laboratory, USA). The following antibodies were used: anti-beclin-1 polyclonal antibody (dilution 1:1000; Abcam ab55878) and anti-LC3 polyclonal antibody (dilution 1:1000; Abcam ab48394).

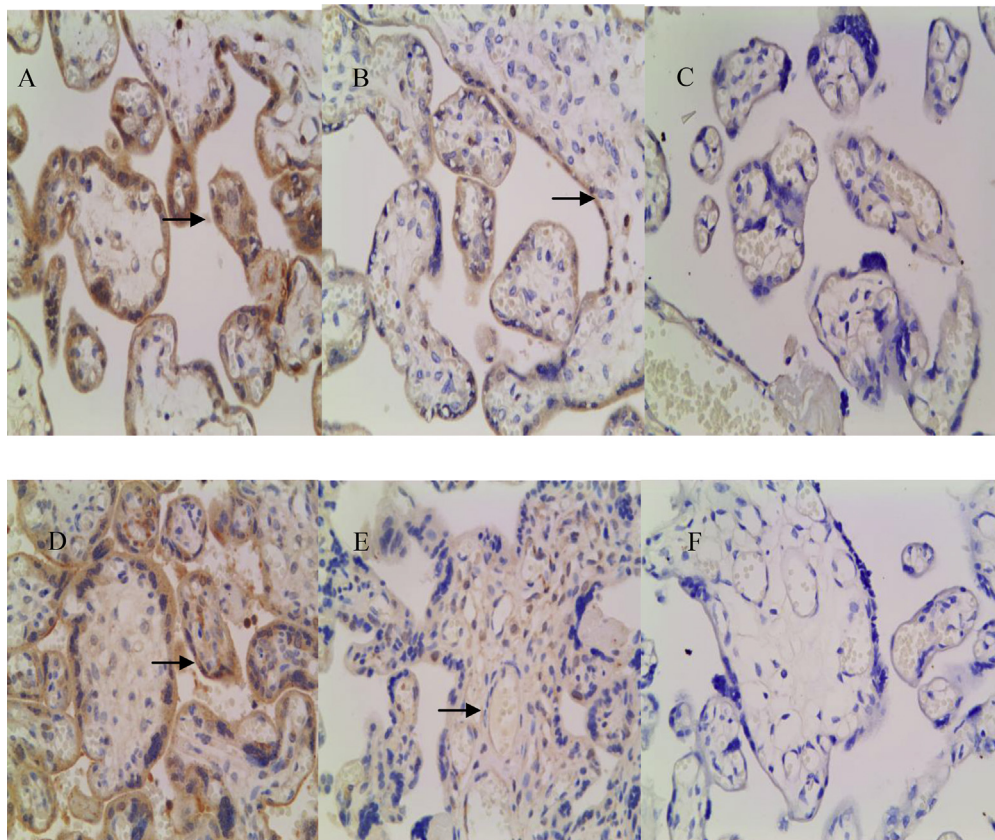
### 2.7. Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences 13.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean  $\pm$  SE. A *t*-test was used to determine statistical significance. A probability level of less than 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Comparison of autophagy levels between normotensive and preeclampsia pregnancies

Immunohistochemical analyses showed that beclin-1 was mainly localized in cytotrophoblasts, while LC3 was localized in



**Fig. 1.** Immunoreactivities of beclin-1 and LC3 in placentas from normotensive pregnancies (NP) compared with preeclampsia pregnancies (PE). Immunohistochemical analyses showed that beclin-1 is mainly localized in cytotrophoblasts, while LC3 is localized in cytotrophoblasts and syncytiotrophoblasts. Beclin 1 and LC3 immunoreactivities were dense in placentas from NP pregnancies compared with PE pregnancies.

- A: Beclin-1 expression in cytotrophoblasts from NP (arrows).  
 B: Beclin-1 expression in cytotrophoblasts from PE (arrows).  
 C: Negative control for the beclin-1 experiment.  
 D: LC3 expression in cytotrophoblasts from NP (arrows).  
 E: LC3 expression in cytotrophoblasts from NP (arrows).  
 F: Negative control for the LC3 experiment.

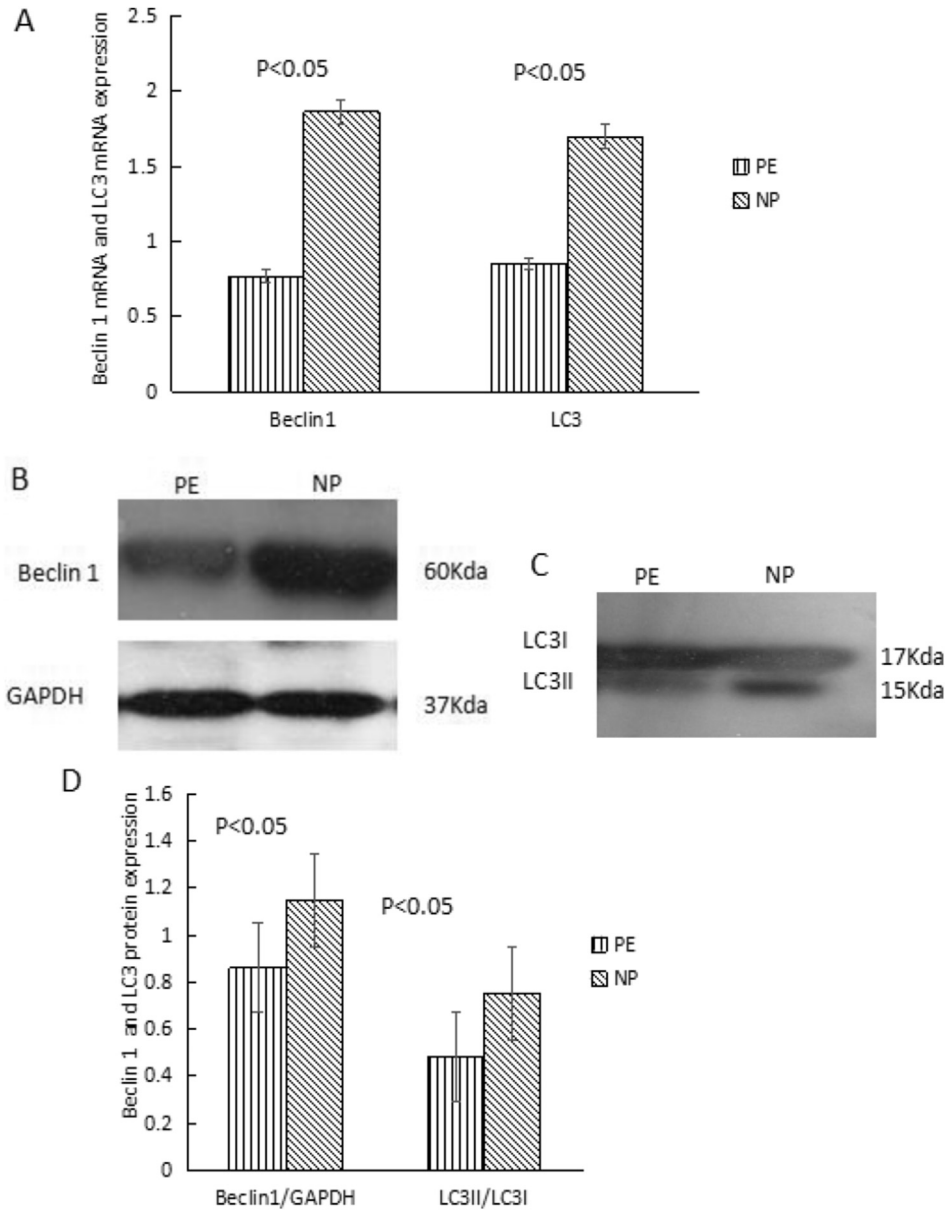
cytotrophoblasts and syncytiotrophoblasts. Beclin 1 and LC3 immunoreactivities were dense in placentas from normotensive pregnancies compared with those in preeclampsia pregnancies (Fig. 1).

Fig. 2 shows that the normotensive pregnancies had significantly elevated beclin 1 and LC3 expression compared with preeclampsia pregnancies at the level of mRNA and protein, respectively. LC3 displayed two bands on western blots, namely LC3II and LC3I. LC3 is converted to LC3I by autophagy-related proteases. Upon induction of autophagy, LC3I is further processed into LC3II and then integrated into the membranes of autophagosomes; thus, the relative expression ratio for LC3II to LC3I is

considered to be a marker for autophagy detection [15].

3.2. Autophagy induction in JEG-3 cells by cotreatment with rapamycin and oxLDL

Exposure to oxLDL has been found to increase the expression of beclin-1 and LC3-II in HUVECs and macrophages (J774A.1) at 100 mg/l for 6 h [17,18]. Based on this observation, we investigated whether autophagy could be induced in trophoblasts (JEG-3 cell line) by oxLDL as a single treatment. Confocal microscopy images showed that neither oxLDL treatment at 25, 50, 100, or 150 mg/l for 6 h or varying the oxLDL treatment time (100 mg/l for 6, 12, 24, or



**Fig. 2. Beclin 1 and LC3 mRNA and protein expression in the placentas from the NP group compared with the PE group.** The NP group had significantly elevated beclin 1 expression levels compared with the PE groups at the level of mRNA and protein, respectively. LC3 shows two bands on western blots, namely LC3II and LC3I. The relative expression ratio between LC3II and LC3I (LC3II/LC3I) was elevated in the NP group. A: Beclin 1 and LC3 mRNA expression in placentas. B: Western blot of Beclin 1 in placentas. C: Western blot of LC3 in placentas. D: Beclin 1 and LC3 protein expression in placentas. NP, normotensive pregnancies. PE, preeclampsia pregnancies.



48 h) could induce autophagy in the JEG-3 cells.

Autophagy is regulated by different signal pathways. One of the classic pathways is PI3K/Akt/mTOR. The PI3K/Akt/mTOR signaling pathway is a central pathway involved in autophagy through the regulation of cell growth, motility, protein synthesis, cell metabolism, cell survival, and cell death in response to various stimuli [19]. Phosphorylation of Akt is up-regulated following activation of PI3K, and mTOR can integrate upstream activating signals through the PI3K/Akt/mTOR pathway and become phosphorylated, which inhibits autophagy [20]. Rapamycin is a specific inhibitor of mTOR and was among the first mTOR-targeted therapeutics to enter the clinic [21].

When the cells were pretreated with rapamycin for 1 h and then exposed to oxLDL, the puncta from LC3 labeling was apparent. To explore the time point that induced autophagy in JEG-3 cells by oxLDL and rapamycin cotreatment, we used confocal microscopy to monitor immunofluorescence induced by oxLDL (100 mg/l) and rapamycin (100 nM). Cells were treated with both rapamycin and oxLDL for various times (0, 6, 12, 24, and 48 h). We found strong positive staining for LC3. Immunofluorescence images showed normal morphology in the JEG-3 cells treated with both rapamycin and oxLDL for 6 and 12 h, and an enlarged nucleus at 24 and 48 h. Therefore, we chose 6 h as the optimal time point for the autophagy experiments. Fig. 3.

### 3.3. Autophagy suppresses the inflammation induced by oxLDL in JEG-3 cells

We detected the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and

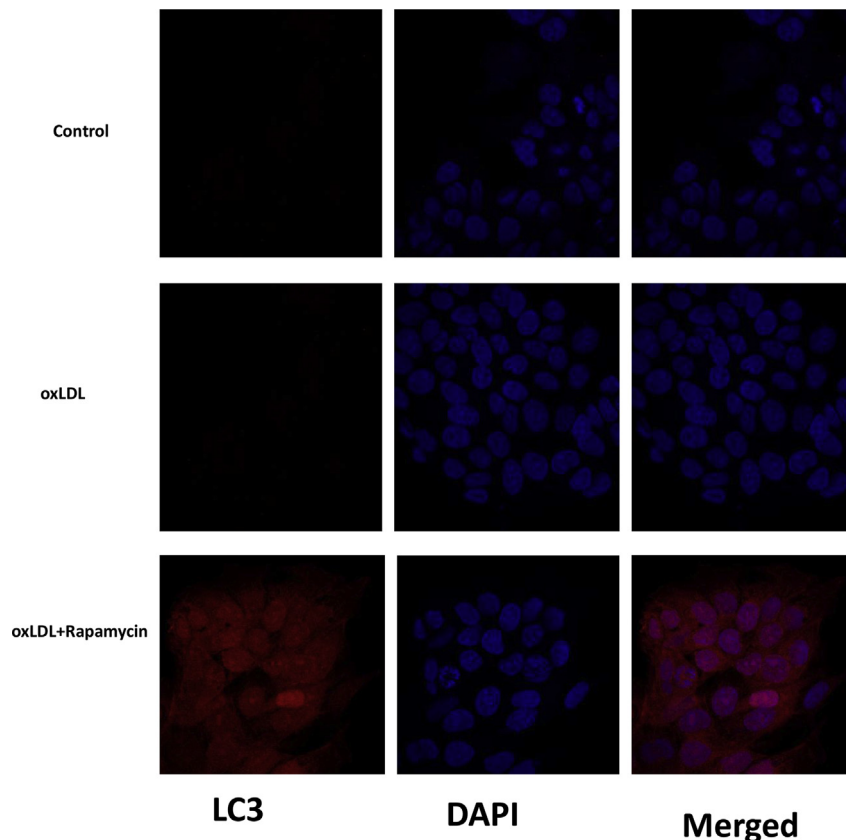
interleukin-6 (IL-6) induced by treatment with various concentrations of oxLDL (25, 50, 100, and 150 mg/l) for 6 h. The results showed that oxLDL at 50 and 100 mg/l produced an obvious increase in TNF- $\alpha$  and IL-6 levels. Furthermore, the expression of mTOR was not affected by 100 mg/l of oxLDL. Therefore, we chose 100 mg/l of oxLDL as the appropriate concentration to use.

Cells were pretreated with rapamycin (100 nM) for 1 h and then exposed to 100 mg/l of oxLDL for 6 h. We found significant increases in the LC3II/LC3I ratio and expression of beclin-1 as well as remarkable decreases in the levels of TNF- $\alpha$  and IL-6. Fig. 4.

## 4. Discussion

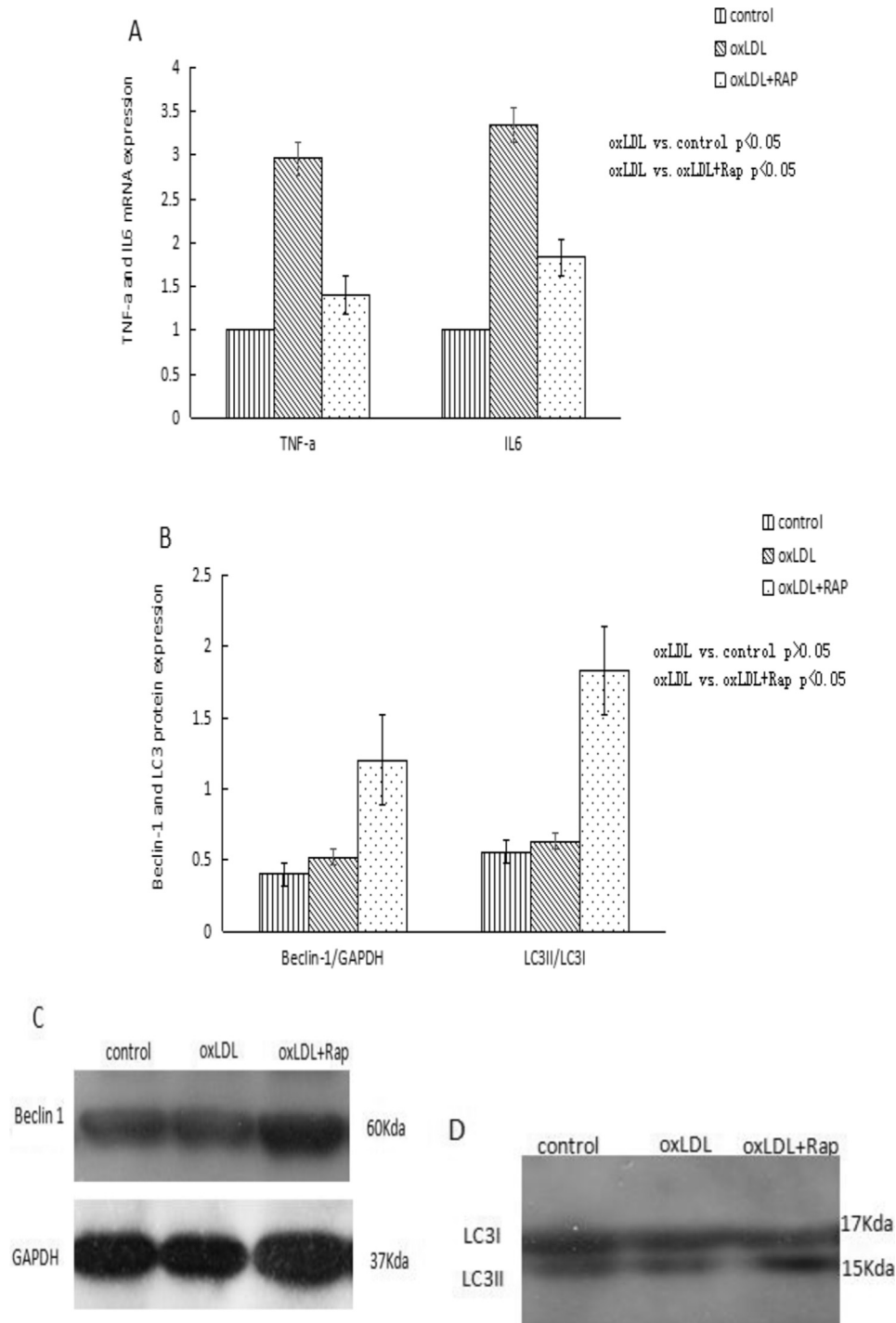
In the present study, our confocal microscopy images showed that oxLDL could not induce autophagy in JEG-3 cells, while in the cells pretreated with rapamycin and then exposed to oxLDL the puncta of LC3 labeling was evident. Furthermore, we found that the expression levels of TNF- $\alpha$  and IL-6 increased following oxLDL treatment, but this effect was abrogated when autophagy was induced in the JEG-3 cells by rapamycin pretreatment. Our data suggest, therefore, that autophagy protects trophoblasts against oxLDL-mediated inflammation.

OxLDL is generated from low density lipoproteins under oxidative stress. OxLDL increases the level of autophagy in HUVECs [22] and inhibits autophagy in macrophages and vascular smooth muscle cells [23,24]. However, the effect of oxLDL on autophagy in trophoblasts is obscure. Bilban and co-workers found that chorionic carcinoma cell lines could be used preferentially for studies on epithelial biology, cell motility and invasion [25]; therefore, we



**Fig. 3. Confocal microscope images showing autophagy induction in JEG-3 cells exposed to oxLDL and rapamycin as indicated by LC3-labeled puncta.**

Confocal microscopy images showed that neither the concentration of oxLDL (25, 50, 100, or 150 mg/L for 6 h) nor the treatment time (100 mg/L for 6, 12, 24, or 48 h) resulted in autophagy induction in JEG-3 cells. When cells were pretreated with rapamycin for 1 h and then exposed to oxLDL for 6 h, the puncta of LC3 labeling was apparent.



**Fig. 4.** The expression of TNF- $\alpha$  and IL-6 decreased and the level of LC3II/LC3I and beclin-1 increased significantly when the cells were pretreated with rapamycin and oxLDL. We detected that 100 mg/L of oxLDL markedly increased TNF- $\alpha$  and IL-6 levels and that mTOR expression was not affected by 100 mg/L of oxLDL. Cells were pretreated with rapamycin (100 nM) for 1 h and then exposed to 100 mg/L of oxLDL for 6 h. We found significant increases in the ratio of LC3II to LC3I and expression of beclin-1 as well as decreases in the levels of TNF- $\alpha$  and IL-6.

A: mRNA expression of TNF- $\alpha$  and IL-6.

B: protein expression of Beclin 1 and LC3.

C: Western blot of beclin 1.

D: Western blot of LC3.

used JEG-3 cells as a trophoblast model. Hence, our data represent the first demonstration of the effect of oxLDL on autophagy in JEG-3 cells. Our data showed that neither various concentrations of oxLDL nor various treatment times could induce autophagy in the JEG-3 cells. However, when we used rapamycin to selectively block

mTOR for 1 h before exposing the trophoblasts to oxLDL, the puncta of LC3 labeling was observed.

In the exaggerated systemic inflammatory response of pre-eclampsia [26], excessive TNF- $\alpha$  can specifically inhibit trophoblast migration and integration [27,28]. While clinical management of

preeclampsia does not currently include anti-inflammatory agents, current research is focusing on protection against inflammation via autophagy. Therefore, the novelty of the present study is underscored by finding that oxLDL induced high levels of TNF- $\alpha$  and IL-6 in trophoblasts and that autophagy induction protected against this inflammatory response.

Autophagy, an intracellular process, maintains viability and optimal cellular functions. Cells subjected to this process become engulfed in a structure called an autophagosome and are ultimately degraded by fusion with a lysosome in response to starvation, hypoxia, inflammation, or infection. The released amino acids, fatty acids, and monosaccharides become available for renewed synthesis of macromolecules [4]. One of the possible biological roles for autophagy in the placenta is protection of trophoblasts from the apoptosis induced by hypoxia or nutritional deprivation [29]. Preeclampsia is a major cause of maternal and infant morbidity and mortality. Its pathogenesis is generally accepted as insufficient trophoblast invasion of the maternal endometrium and inadequate remodeling of the maternal spiral arteries. These impairments lead to elevated levels of hypoxia and oxidative stress, and the induction and persistence of inflammation [1]. Such adverse conditions might be expected to induce autophagy; however, it is not clear how variations in autophagy regulation influence preeclampsia. A previous study suggested that sera from women with preeclampsia contain an inhibitor of autophagy [30]. In the present study, we have provided evidence that the expression of LC3 and beclin-1 is decreased in the preeclampsia placenta compared with normotensive pregnancies, as assessed by immunohistochemistry, western blotting and qPCR. These results suggest there is impaired autophagy in the placenta during preeclampsia.

Many researchers have observed that oxLDL levels are increased in the serum of women with preeclampsia compared with normal controls [31–33], and that OxLDL binding to LOX-1 causes endothelial dysfunction and this may contribute to preeclampsia [10,34]. Additionally, the results of the present study have shown that oxLDL can induce high levels of TNF- $\alpha$  and IL-6 in trophoblasts. Altogether, we conclude that oxLDL-mediated inflammatory responses may contribute to preeclampsia. Nevertheless, autophagy induction protected against this inflammatory response in the trophoblasts, but a decreased level of beclin-1 and LC3 in the preeclampsia placenta was observed. Therefore, we conclude that impaired autophagy in preeclampsia might result in decreased protection for trophoblasts from oxidative and inflammatory stress, and this may contribute to the pathogenesis of preeclampsia. Future studies should focus on therapeutic interventions for patients with preeclampsia. How to switch on the protective effects of autophagy is a strategy worth pursuing and a challenge for clinicians.

### Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Autophagy protects against oxidized low density lipoprotein-mediated inflammation associated with preeclampsia."

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