# Resveratrol inhibits autophagy in HTR-8/SVneo model by alleviating trophoblast oxidative stress

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**Research**

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# Abstract

### Background

The normal function of the placenta at each time stage of pregnancy is essential to a successful outcome. Placental dysfunction and increased oxidative stress and autophagy are the cause of a series of severe pregnancy complications. Resveratrol is a potent antioxidant that has shown beneficial effects in many diseases. We aim to investigate whether excessive autophagy is associated with oxidative stress in the trophoblast oxidative stress model. Resveratrol was taken to clarify its role in excessive autophagy of placental trophoblasts.

### Methods

We established an in vitro model of oxidative stress by exposing the human first-trimester extravillous trophoblast cell line HTR-8/SVneo to $H_2O_2$. Levels of autophagy-related proteins (LC3, Beclin-1, p53 and mTOR) were detected by western blot.

### Results

Treatment with resveratrol significantly ameliorated $H_2O_2$-induced cytotoxicity, morphological damage, oxidative stress and autophagy. Mechanistically, resveratrol restored the levels of autophagy-related proteins including LC3-II, Beclin-1 and p53, mTOR that were dysregulated by $H_2O_2$.

### Conclusions

Resveratrol may protect human trophoblasts against $H_2O_2$-induced oxidative stress by reducing excessive autophagy, thus ensuring the normal biological functions of trophoblasts.


# Highlights

- Excessive autophagy is associated with oxidative stress in trophoblast oxidative stress model
- Resveratrol plays an important role in regulating excessive autophagy of placental trophoblasts.

# 1. Introduction

The normal function of the placenta at each time stage of pregnancy is essential to a successful outcome. Any change in the placenta may affect the health of the mother and the fetus. The normal functioning of trophoblast proliferation and invasion is essential for blastocyst implantation, placenta formation, and an appropriate mother–fetus relationship.

Oxidative stress plays a vital role in the pathogenesis of various reproductive diseases during pregnancy. So, inhibiting oxidative damage and apoptosis induced by oxidative stress is an important intervention strategy for pregnancy diseases [1]. Oxidative stress reflects the imbalance between the intracellular

reactive oxygen species (ROS) levels and the antioxidant defense system. Oxidative damage in the placenta leads to inflammation and apoptosis, and the resulting cellular debris is released into the maternal circulation. These placenta-derived factors then act on the maternal endothelium leading to systemic endothelial dysfunction [2]. Therefore, reducing placental oxidative stress is a feasible strategy to ensure maternal and fetal health.

Autophagy is one of the essential mechanisms for maintaining steady-state cellular stress responses [3]. It may play a vital role in protecting trophoblast cells in the early placenta's anoxic and nutrient-limited environment. However, excessive autophagy may destroy cell structures and induce cell death. Autophagy markers have been detected in the placenta, indicating that this process is involved in every gestation [4]. Impaired autophagy at the maternal-fetal interface decreases the placental cells' ability to scavenge the excess ROS, resulting in oxidative stress that contributes to the pathophysiology of several pregnancy-related diseases [5].

Resveratrol is a natural nonflavonoid polyphenol that mainly comes from grapes, berries, peanuts, and other plants. It exerts anti-inflammatory, antitumor, and antioxidation effects but can also improve metabolic diseases. Resveratrol can effectively remove superoxide, help regulate apoptosis in various pathophysiological and biological processes, and be used to treat chronic diseases. Previous studies have suggested that resveratrol's beneficial effects can be attributed to its antioxidant properties [6, 7]. Many studies have shown that resveratrol can be safely administered throughout the pregnancy cycle and effectively counter oxidative stress during pregnancy[8].

Although resveratrol regulates autophagy in different cell types, it is unclear whether its protective effects on trophoblasts under oxidative stress are mediated by autophagy. Therefore, this study aimed to investigate the potential impact and underlying mechanisms of resveratrol on $H_2O_2$ treated human trophoblasts. We are focused on whether resveratrol can affect antioxidative stress and autophagy regulation in placental trophoblasts, which is of great significance for resveratrol's clinical application, especially in the promotion application of reproductive medicine.

## 2. Materials And Methods

## 2.1 Cell culture

Dr. Charles Graham, Queen’s University of Canada provided the HTR-8/SVneo cell line. The cells were cultured in DMEM/F12 (HyClone, USA) with 10% FBS (Gibco, USA), and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco), and were maintained in a humidified incubator with 5% $CO_2$ at 37 °C.

## 2.2 Establishment of an oxidative stress model of HTR-8/SVneo cells[9]

Cultured cells were cultured in a 37°C, 5% $CO_2$ incubator plated on a six-well culture plate at a density of $1 \times 10^5$ cells per well. After 24 hours, $H_2O_2$ (Sigma-Aldrich, USA) was added at concentrations of

300 μmol/L to the culture, with a control group being set up at the same time. All groups were cultured for an additional 3 hours under constant experimental conditions.

## 2.3 Detection of autophagy protein expression in HTR-8/SVneo cells by Western blotting

Resveratrol was added at 50 μmol/L to the oxidative stress model for 8 hours (Preliminary experiments have been done). Cultured cells were harvested with a rubber scraper and washed twice with cold phosphate-buffered saline (PBS). Cell pellets were lysed and kept on ice for at least 20 min in RIPA lysis buffer (Millipore, USA), with phenylmethylsulphonyl fluoride and protease inhibitors cocktail (Thermo Scientific, USA). The lysates were cleared by centrifugation, and the supernatants were collected. The BCA assay quantified proteins, and loading buffer 5X was added to the proteins, which were incubated for 5 min at 95 °C. Then, proteins were loaded on an SDS-PAGE polyacrylamide gel, transferred to Immobilon-P PVDF membrane (Millipore), probed with the appropriate primary antibodies, and detected by chemiluminescence (ECL, Thermo Scientific). Images were then acquired with Image-Lab software (Bio-Rad, USA). Image analysis of western blots was performed with Image-Lab analyzer software.

## 2.4 Observation of autophagy protein in HTR-8/SVneo cells by immunofluorescence

For immunofluorescence studies, after adding resveratrol, $5 \times 10^5$ cells were seeded in 24-well plates on glass coverslips. Transfected cells grown on glass coverslips were fixed for 30 min in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Promega) in PBS buffered saline for 30 min. After washing with TBS-0.1% Triton X-100 (TBSTx), nonspecific binding sites were blocked with TBSTx-5% BSA for 60 min. Then cells were applied sequentially with a 1:25 dilution of rabbit polyclonal antibody anti- Beclin-1: green, 1:50 dilution of rabbit polyclonal antibody anti- LC3: green at 4 °C overnight, and a 1:200 dilution of goat anti-rabbit IgG at room temperature for 60 min in the dark. For negative control, PBS was added instead of the primary antibody. Further, immunofluorescence was visualized under a laser-scanning confocal microscope (Leica, China, SP5).

## 2.5 Reagents and antibodies

Beclin-1 (SAB1305637), LC3 (SAB1305638), p53 (SAB1306006), mTOR (SAB4501038) were obtained from Sigma Aldrich (CA, USA) and dissolved in ethanol and DMSO, respectively. ACTIN antibody was purchased from Sigma-Aldrich (G9545). Goat anti-rabbit IgG antibody was purchased from Abcam (ab150077, UK).

## 2.6 Statistical analyses

All data are expressed as mean ± SD, obtained from more than three independent experiments, and analyzed by GraphPad Prism 8.0 (GraphPad Software, CA, USA). Statistically significant differences (*$P$< 0.05, **$P$< 0.01) were examined using the Student's $t$-test and one-way ANOVA.

# 3. Result

### 3.1 Resveratrol protects trophoblasts from $H_2O_2$-induced toxicity

The effects of resveratrol were then tested on HTR-8/ SVneo cells treated with $H_2O_2$ as above, which revealed a significant protective effect at 50 μmol/L, with no further increase seen in cell viability with higher doses (Fig. 1A). Therefore, 50 μmol/L resveratrol was used in subsequent experiments. Resveratrol treatment attenuated the shrinkage and floatation induced by $H_2O_2$ in the HTR-8/SVneo cells (Fig. 1B).

A. Viability of HTR-8/SVneo cells pre-treated with 300 μmol/L $H_2O_2$ for 3 h followed by stimulation with varying doses of resveratrol (10, 50, 100, 200 μmol/L) for 8 h. (*$P < 0.05$, **$P < 0.01$). B. Representative images showing morphological changes induced by $H_2O_2$ and resveratrol in HTR-8/SVneo cells (200 ×magnification).

### 3.1 Expression levels of autophagy protein and the effects of resveratrol on the expression levels of autophagy protein in the oxidative stress model of HTR-8/SVneo cells as assessed by Western blotting

The autophagy activity in the HTR-8/SVneo cell oxidative stress model can be reflected by the level of autophagy-related proteins LC3, Beclin-1, p53 and mTOR. LC3 has two subtypes, LC3I and LC3II, the transformation of LC3I into LC3II increases when cell autophagy occurs. Therefore, LC3II a commonly used autophagy marker, so Western blotting was used in the present study to detect the expression of LC3II and Beclin-1 in the control and model groups. Fig. 2 showed that these two proteins' expression levels in the oxidative stress model of HTR-8/SVneo cells treated with $H_2O_2$ increased significantly ($P < 0.01$). Meanwhile, p53 and mTOR are also classical molecular markers of autophagy. Fig. 2 showed that these two proteins' expression levels in the oxidative stress model of HTR-8/SVneo cells treated with $H_2O_2$ decreased significantly ($P < 0.01$).

The effects of resveratrol on the expression levels of autophagy protein in the oxidative stress model of HTR-8/SVneo cells were investigated using Western blotting, which revealed that resveratrol significantly reduced the levels of LC3, Beclin-1, p53 and mTOR compared with the model group ($P < 0.01$) (Fig. 2). Since the expression levels of LC3 and Beclin-1 are useful markers for autophagy, we performed a Western blot analysis. Compared with the model group, the expression level of LC3-II, Beclin-1 protein was further reduced by resveratrol treatment ($P < 0.01$), the expression level of p53 and mTOR protein was further increased by resveratrol treatment ($P < 0.01$).

In the HTR-8/SVneo cell oxidative stress model (p53: A, mTOR: B, LC3-II: C, Beclin-1: D) (*$P < 0.05$, **$P < 0.01$) Compared with the control group, the expression levels of LC3-II, Beclin-1 in the model group were increased. The expression levels of p53, mTOR in the model group were decreased. Compared with the $H_2O_2$ group, the expression levels of LC3-II, Beclin-1 in the model group were decreased. The expression levels of p53, mTOR in the model group were increased.

### 3.2 Expression of autophagy in oxidative stress by immunofluorescence

As shown in Fig. 3, To confirm that LC3 and Beclin-1 are autophagic central proteins expressed in the $H_2O_2$-induced oxidative stress model of HTR-8/SVneo cells, we labeled LC3 and Beclin-1 in cells with red fluorescence and labeled their nuclei with blue fluorescence (using DAPI staining). The distribution of LC3 and Beclin-1 in cells was observed using laser scanning confocal microscopy. In the HTR-8/SVneo cell oxidative stress model, many fluorescence spots were evident in the cytoplasm, which indicated an elevation of the autophagy bodies. The expression level of autophagy regulatory protein increased significantly. In contrast, there were only weak fluorescent spots in the control group. The fluorescence intensity can reflect the autophagy level, and so the above results show that not only oxidative stress but also a high level of autophagy expression were present in the placenta trophoblasts.

We used it to investigate the expression of autophagy-related protein expression. Western blotting results (Fig. 3) showed that the expression of LC3, Beclin-1 in the oxidative stress model of HTR-8/SVneo cells treated with $H_2O_2$, was significantly increased than in the control group ($P < 0.01$). But the expression of LC3, Beclin-1 in the oxidative stress model of HTR-8/SVneo cells treated with resveratrol, was significantly decreased than in the model group ($P < 0.01$).

In the oxidative stress model of HTR-8/SVneo cells, many fluorescent spots appeared in the cytoplasm, indicating an increase in autophagosomes in the cytoplasm and the expression of autophagic regulatory proteins. Fewer fluorescence spots were evident in the control group. The fluorescence intensity, to some extent, reflects the autophagy level. A: Rabbit anti-LC3 monoclonal antibody (red) and DAPI staining were used to display nuclear (blue) immunolabeling. Scale: 50 mm. B: Nuclear DAPI staining (blue) with rabbit anti-Beclin-1 monoclonal antibody (red) immunostaining microscopy. Scale: 50 mm.

# 4. Discussion

Embryo formation is a complex process of synergistic action between embryo and mother. The successful implantation of human embryo, placental formation, embryo growth and development are closely related to the proliferation, differentiation and invasion function of trophoblast. The normal proliferation and biological function of trophoblast are prerequisites for successful pregnancy[10].

HTR-8/SVneo cell line is considered the closest study model to trophoblast cells derived from early human pregnancy (8 ~ 10 weeks) [11]. The HTR-8/SVneo human trophoblast cell line applied in the present study is more similar to primary trophoblasts and normal human physiological conditions. Therefore, we established an in vitro model of oxidative stress by exposing the human trophoblast cell line HTR-8/SVneo cells to $H_2O_2$.

The in-depth study on the regulation of trophoblast oxidative stress and autophagy will be beneficial to elucidate the pathogenesis of a class of primary trophoblastic diseases such as abortion, preeclampsia, fetal growth restriction, and provide new therapeutic targets for the treatment of these diseases.

It is becoming increasingly clear that autophagy and oxidative stress are interrelated activities that are essential for fetal development and parturition. Further investigations of their interrelationships at

different gestational stages will lead to an improved understanding of the mechanisms by which alterations in their interactions contribute to placental pathology, pregnancy disorders, and premature delivery.

Autophagy is a lysosomal-mediated intracellular degradation system that serves as a primary cell maintenance mechanism via which unnecessary or dysfunctional cell components are degraded [12]. Many experimental and clinical studies have shown that oxidative stress is closely related to autophagy [13–15]. Oxidative stress products are ROS, $H_2O_2$ and other reactive oxygen species clusters, and a large proportion of ROS can promote oxidative stress-induced cell damage and apoptosis [16].

In early pregnancy, autophagy induction preserves trophoblast function in the low oxygen and placental nutrient environment. Inadequate regulation of the ROS-autophagy axis leads to abnormal autophagy activity and contributes to the development of preeclampsia and intrauterine growth restriction. ROS-autophagy interactions are altered at the end of gestation and participate in the initiation of parturition at term.

ROS has been copiously reported as early inducers of autophagy. Thus, while ROS results in autophagy induction to maintain trophoblast viability and activity in the first trimester, its increased intensity near term coupled with activation of pro-inflammatory mediators and release of danger signals results in its contribution to the inhibition of autophagy near term. Excessive levels of inadequately opposed oxidative stress and increased or impaired autophagy are significant contributors to pregnancy complications. The increased induction of autophagy in association likely contributes to the development of a restricted growth fetus. These findings strongly indicate that elevated oxidative stress increases autophagy in preeclamptic placentas[17, 18]. The dysregulation of autophagy activity contributes to the pathology of multiple diseases[18–25].

There is also evidence [26, 27] that autophagy and oxidative stress play an essential role in pregnancy [28]. Under normal circumstances[29–32], trophoblast autophagy is suppressed at a basal level. In hypoxic, oxidative stress, infection, hormone stimulation, and other conditions, the autophagy level is much improved. In the early stage of normal pregnancy, autophagy caused by placental hypoxia also produces certain reactive oxygen species and increases oxidative stress [33].

Intracellular ROS levels, especially $H_2O_2$, are significantly elevated in starvation environments, inducing autophagy [34, 35]. These oxidation conditions are necessary for autophagy to occur, so the use of antioxidants inhibits the autophagy-lysosome formation and subsequent protein degradation [36, 37].

Therefore, it is crucial to study the effects of oxidative stress and autophagy on trophoblast biological function. Studies [38] have shown that excessive autophagy will lead to HTR-8/SVneo invasion and angiogenesis disorders, indicating that autophagy is essential for human extra-villi's normal function trophoblast. This study provides a new idea for trophoblast autophagy pathogenesis and pregnancy-related diseases under oxidative stress.

The autophagy activity, growth arrest, and apoptosis of cells are enhanced under induction by $H_2O_2$ [39].

LC3 is an essential indicator of the severity of autophagy, it appears in two forms in the cytoplasm: LC3I and LC3II. When autophagy occurs, LC3I is converted to LC3II. Therefore, LC3II was used as a molecular marker of autophagy, and the level of LC3II was used to reflect the degree of autophagy [40]. Besides, Beclin-1, the first mammalian autophagy protein identified as a novel Bcl-2-interacting protein. It plays a vital role in the autophagic process's critical step, namely, autophagosome formation [41]. Subsequent studies have demonstrated that this landmark protein is essential for autophagy[42]. Activation of the kinase activity of the Beclin-1 promotes autophagosome maturation [43].

Results from a previous study indicated LC3 and Beclin-1, two important autophagy-related proteins, found to be significantly overexpressed under $H_2O_2$ influence [cite:31]. In consistence with previous studies [44], our findings also showed that Beclin-1, LC3-Ⅱ protein expression was up-regulated in autophagy. The Western blot results obtained in the present study clearly showed that the oxidative stress model of HTR-8/SVneo placental trophoblasts induced autophagy by enhancing autophagy and increasing the level of LC3II ($P < 0.01$). The protein level of Beclin-1 also increased significantly ($P < 0.01$), which further indicated that autophagy was enhanced. Compared with the control group, Beclin-1, LC3-Ⅱ protein expression was up-regulated, and laser confocal immunofluorescence results were the same. Meanwhile, the protein expression level of p53 and mTOR was lower than that of the control group. ($P < 0.01$)

Autophagy is a relatively conservative metabolic pathway that involves many physiological processes and the regulatory mechanisms are very complex. p53 is a significant factor in regulating autophagy.

p53 is an important regulator of autophagy, and its regulation of autophagy is an important part of the stress response of cells stimulated by the external environment [45]. And p53 in the cytoplasm has a negative regulatory effect on autophagy, inhibiting autophagy occurrence [46–50]. p53 proteins can inhibit the activity of AMP-activated protein kinases (AMP-activated protein kinase, AMPK), while AMPK further inhibits the role of mammalian rapamycin target proteins [51]. mTOR activation can inhibit autophagy [46].

mTOR is an atypical serine/threonine-protein kinase receptor for amino acids and ATP, a family member of phosphatidylinositol kinase-associated protein kinase (PIKK).. activation of mTOR enhances phosphorylation of autophagy-related protein complexes and inhibits autophagy. mTOR has a gating effect during autophagy and its activity is critical for autophagosome formation and maturation. It is a negative regulator of autophagy, and many signaling pathways of autophagy go through mTOR pathways [52].

Our findings indicate that $H_2O_2$ can induce oxidative stress and enhance the autophagy activity of HTR-8/SVneo placenta trophoblasts. Since activated autophagy persists throughout the process and overlaps with apoptosis, it is harmful to cell growth and development, consistent with previous research findings. Oxidative stress enhances trophoblasts and endothelial cells' autophagy, affecting the invasion of

trophoblasts and placental blood vessels' formation. Our study has shown that $H_2O_2$ can promote the activation of autophagy in trophoblast cells. However, the mechanism underlying autophagy and apoptosis and a cascade relationship need to be clarified further.

Resveratrol is a natural non-flavone polyphenolic compound with antioxidant, anti-aging and other effects [53, 54]. Resveratrol can modulate mTOR activity. But the protective effect of resveratrol on placental trophoblast was less reported. Hence, the current study took HTR-8-SV/neo cells as the research object to explore the specific mechanism of resveratrol on excessive autophagy induced by oxidative stress in trophoblast cells.

Many pharmacological effects of resveratrol are related to its regulatory effect on autophagy. Currently, resveratrol is an effective autophagy regulator that exerts the role of inducing autophagy or inhibiting autophagy in different cells by regulating autophagy-related signaling pathways[55, 56].

Cell death, preceded by autophagy, plays a positive role in physiologic conditions, depending on the cell environment [57]. We took different major autophagy-related proteins, such as p53, mTOR, Beclin-1 and LC3, which are regulatory proteins of autophagy that participate in the key steps underlying the occurrence of autophagy [58]. As observed by WB and immunofluorescence results, resveratrol treatment can significantly reduce the expression level of Beclin-1 in an oxidative stress model and delays the transformation of LC3I to LC3II ($P < 0.01$), which is the gold standard of autophagy [59]. Resveratrol treatment can also significantly increase p53 and mTOR expression in an oxidative stress model ($P < 0.01$). This further proves the down-regulation effect of autophagy.

In conclusion, we have shown that resveratrol treatment can significantly decrease the levels of LC3 and Beclin-1, increase the level of p53 and mTOR autophagy protein in an HTR-8/SVneo human placental trophoblast oxidative stress model. Here we found that resveratrol can significantly reduce the oxidative stress damage of HTR-8/SVneo cells treated with $H_2O_2$. Resveratrol may partly play a protective role by activating mTOR/ autophagy and attenuating the cells' autophagy response.

The findings of this study may provide a useful therapeutic target for the pathogenesis of pregnancy-related diseases. Offering new insight into validating the therapeutic potential of resveratrol in the stage of pregnancy. However, it must be emphasized that the precise molecular mechanisms and direct molecular targets of resveratrol regulating autophagy remain unclear, thus requiring further investigation. With this increased knowledge, novel protocols can then be developed to improve pregnancy outcomes.

# Declarations

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**Consent for publication:** Not applicable.

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**Author contributions**

LI Meihe: Conceptualization, Data curation, Writing- Original draft preparation. KANG Minchao, AN Peng: Methodology, Software. DANG Huimin: Visualization, Investigation. XU Xin: Supervision, Writing- Reviewing and Editing

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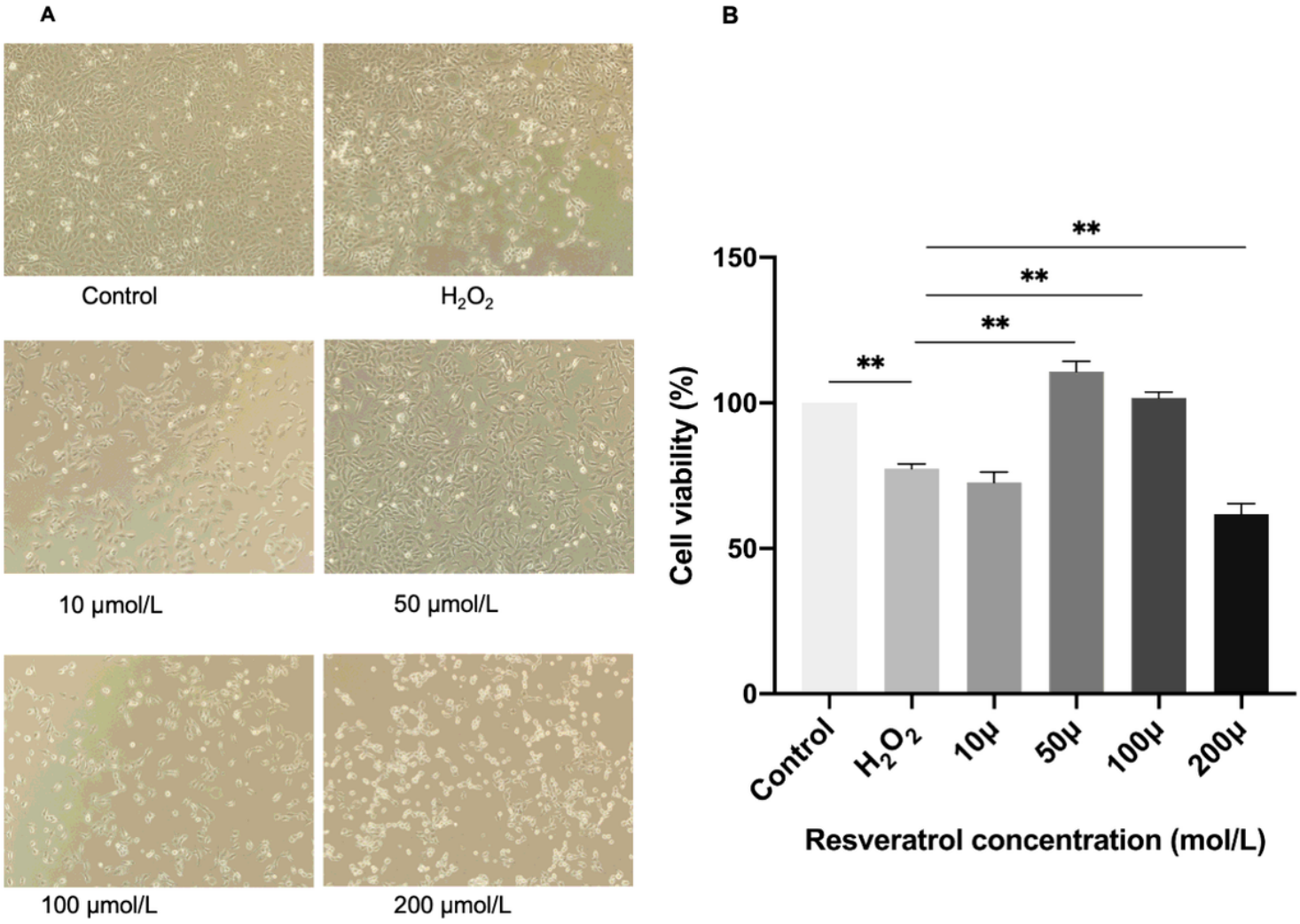
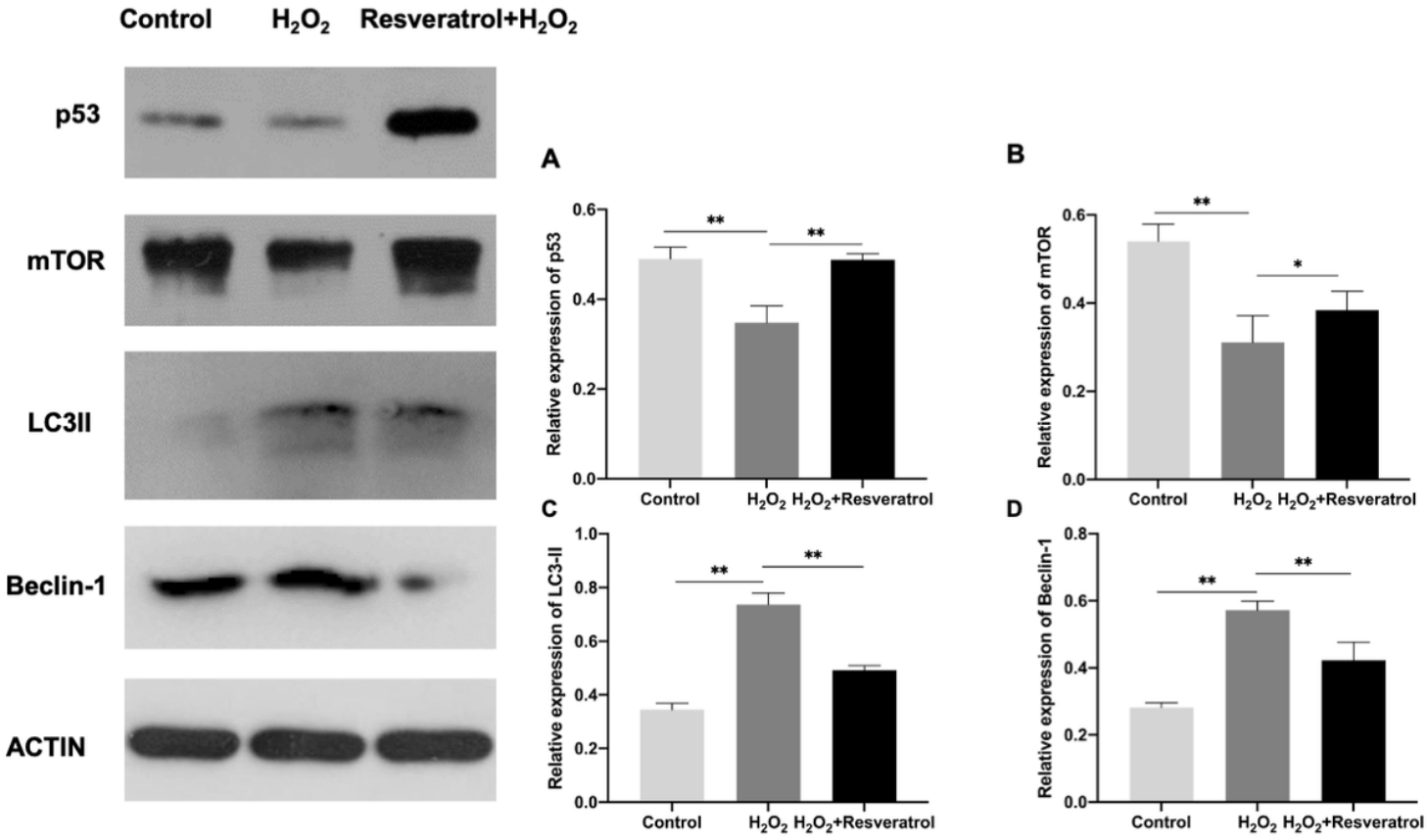
# Figures



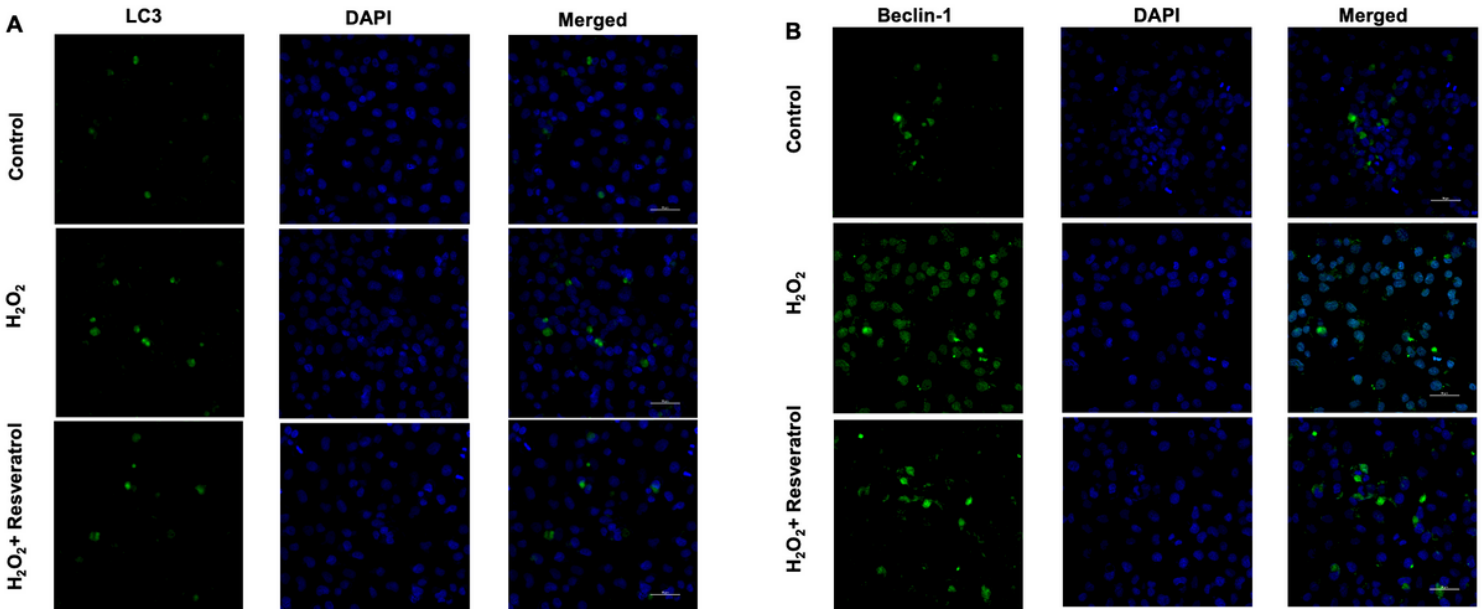

## Figure 1

Effects of H2O2 on the viability and morphology of HTR-8/SVneo cells treated with/out resveratrol




## Figure 2

Effects of H2O2 on the levels of autophagy protein in HTR-8/SVneo cells and the effects of resveratrol on intracellular inflammasome and autophagosome levels




## Figure 3

Autophagy-related protein expression in HTR-8/SVneo cell