Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2026**; 31(4): 50047
https://doi.org/10.31083/FBL50047

Original Research

Ameliorative Effect of Luteolin on H_2O_2-Induced Mitophagy and Apoptosis in Cardiomyocytes Through Modulation of the AMPK/mTOR Pathway

Zhipeng Yang[1], Xiaolu Liu[1], Daijian Liu[2], Kuiying Ma[1], Ming Zhao[1,*], Yu Wang[2,*]

[1]Department of Cardiology, Affiliated Hospital of Inner Mongolia Minzu University, 028002 Tongliao, Inner Mongolia, China
[2]Key Laboratory of Basic Pharmacology of Ministry of Education, Zunyi Medical University, 563000 Zunyi, Guizhou, China
*Correspondence: langzhe73@163.com (Ming Zhao); shen348@126.com (Yu Wang)
Academic Editor: Natascia Tiso
Submitted: 15 January 2026 Revised: 12 March 2026 Accepted: 17 March 2026 Published: 20 April 2026

Abstract

Background: Luteolin, a natural flavonoid, is an active ingredient in traditional herbs used to treat cardiovascular diseases. However, little is known about the effects of luteolin on oxidative damage in cardiomyocytes, and the underlying mechanisms remain poorly understood. Therefore, this study aimed to investigate the protective effects of luteolin against hydrogen peroxide (H_2O_2)-induced mitophagy and apoptosis in cardiomyocytes. **Methods**: H9c2 cells were exposed to H_2O_2 for 4 h, which caused severe cellular damage accompanied by apoptosis. The protein expression of β-actin, FK506 binding protein 12, mammalian target of rapamycin (mTOR), acetyl-coenzyme A carboxylase alpha (ACC), sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*) (SIRT1), peroxisome proliferator-activated receptor gamma, coactivator 1-alpha (PGC-1α), autophagy-related 5 homolog (*S. cerevisiae*) (ATG5), microtubule-associated protein 1 light chain 3 beta (LC3B), B-cell lymphoma 2 (BCL2)-associated X protein (Bax), B-cell leukemia/lymphoma 2 (Bcl-2), PTEN-induced putative kinase 1 (PINK1), and peroxisome proliferator-activated receptor gamma (PPARγ) was analyzed by Western blotting. Intracellular reactive oxygen species (ROS) levels were assessed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining (DCFH) oxidation staining. The expressions of phosphorylated AMP-activated protein kinase alpha (p-AMPKα), SIRT1, and caspase 8 were evaluated by immunofluorescence. Mitochondrial membrane potential and mitochondrial permeability transition pore (MPTP) opening were assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) staining and an MPTP assay kit, respectively. **Results**: H_2O_2 treatment significantly reduced the viability of H9c2 cardiomyocytes and induced mitochondrial apoptosis. Furthermore, H_2O_2 upregulated the expression of p-AMPK, SIRT1, mTOR, ACC, and PGC-1α, while downregulating PPARγ expression. Concurrently, H_2O_2 activated mitophagy, suggesting involvement of the AMPK/mTOR signaling pathway. Notably, pretreatment with luteolin effectively reversed these H_2O_2-induced alterations by attenuating excessive ROS production, inhibiting MPTP opening, and normalizing the Bcl-2/Bax ratio and caspase 8 expression. Additionally, luteolin suppressed the H_2O_2-induced upregulation of proteins associated with the AMPK/mTOR signaling axis, mitophagy, and apoptosis. **Conclusions**: These findings suggest that luteolin protects H9c2 cells from mitochondria-mediated apoptosis by modulating the AMPK/mTOR signaling pathway and inhibiting excessive mitophagy. Moreover, these results suggest that luteolin has potential as a therapeutic agent for preventing and treating cardiovascular diseases.

Keywords: luteolin; mitophagy; reactive oxygen species; cardiovascular diseases

1. Introduction

Cardiovascular disease (CVD) is a group of diseases that entail the heart or blood vessels. CVD contains coronary artery disease, such as angina pectoris and myocardial infarction. At the cellular level, shortly after ischemia or hypoxia, there is a rapid decrease in the amount of the energy molecule adenosine triphosphate (ATP) in cardiomyocytes. If ischemia continues, cytochrome c released from mitochondria triggers apoptosis, ATP depletion following increased mitochondrial permeability, and ultimately necrosis of cardiomyocytes [1,2]. Oxidative damage to proteins, lipids, and nucleic acids and mitochondrial membrane infiltration is due to excessive accumulation of reactive oxygen species (ROS), which leads to cell death [3]. Excess ROS in turn causes damage to mitochondria and may trigger mitophagy. As an active and highly oxygen-consuming organ, the heart is susceptible to oxidative stress (OS), leading to cell necrosis and apoptosis [4,5]. O_2^- is the ROS initially involved in mitophagy induced by glucose, glutamate, pyruvate, or serum loss. In the presence of superoxide dismutase (SOD), superoxide is rapidly dismutated into hydrogen peroxide (H_2O_2). Compared to O_2^-, H_2O_2 exhibits greater membrane permeability and acts as a key signaling molecule in redox homeostasis, but its accumulation can also lead to oxidative damage [6]. H_2O_2 plays an instrumental factor in cellular oxidation-reduction equilibrium and signal transduction due to its better membrane

permeability. H_2O_2 reacts with transition metal ions such as Fe^{2+} to generate OH, a highly reactive and destructive ROS [7]. Upregulation of OS markers has been shown to predict cardiovascular disease [8]. Therefore, inhibition of OS and cardiomyocyte apoptosis is of great importance for the treatment and prevention of CVD.

Antioxidants have been receiving attention as a new therapeutic strategy for CVD. Luteolin (Lut) is widely distributed in nature and is a natural flavonoid compound that can be isolated from a variety of natural herbs, vegetables, and fruits, with a higher content of whole-leaf cymbidium orchids, chili peppers, wild chrysanthemums, honeysuckle, and perilla. Lut exhibits various pharmacological activities, including anti-neoplastic [9,10], cardiovascular [11,12], anti-inflammatory [13], diabetic [14], hepatoprotective [15], antioxidant [16], and neurological disorders [17]. In addition, Lut inhibits apoptosis in I/R-injured rat cardiomyocytes by regulating the Toll-like receptor 4 (TLR4)/nuclear factor kappa-B (NF-κB)/NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome (TLR4/NF-κB/NLRP3) pathway [18].

Mitophagy is a particular type of autophagy, which is the process of cells breaking down organelles and pathogens that are invading them. Mitophagy is the depolarization of intracellular mitochondria in response to stimuli from internal and external environments, such as ROS, nutrient deficiencies, cellular senescence, etc. Autophagosomes can specifically phagocytose damaged mitochondria and fuse with lysosomes to cause degradation of damaged mitochondria, thus maintaining the stability of the intracellular environment [19]. The mitochondrial marker PTEN induced putative kinase 1 (PINK1) and the microtubule-associated protein 1 light chain 3 beta (LC3B) are usually co-localized in studies to show mitophagy.

It is well known that AMPK and mTOR regulate cellular catabolism and biosynthesis processes through different mechanisms, which together maintain cellular energy balance and metabolic stability [20].

Although attempts have been made to study the pharmacological activity of Lut against CVD, the molecular mechanisms between apoptosis, oxidative stress mitophagy, and Lut remain to be investigated. Elucidating the correlation between these processes may be a mechanism for the treatment of CVD. The paper aimed to demonstrate the molecular mechanism by which Lut affects cardiovascular disease.

2. Experimental Procedures

2.1 Material and Chemicals

Lut (purity >98%) was obtained from Nanjing Jingzhu Bio-technology Co., Ltd. The H9c2 cell line is a rat embryonic cardiomyocyte line that is one of the most commonly used tools for studying myocardial biology and cardiac disease mechanisms. H9c2 cells are widely used in the construction of cardiac disease models, such as myocardial infarction, arrhythmia, and heart failure. Compared with primary cardiomyocytes, H9c2 cells have the advantages of easy accessibility, rapid growth, and stable replication, thus becoming an important tool for cardiomyocyte research. H_2O_2 was purchased from Shandong Anjet High-Tech Disinfection Technology Co., Ltd. Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (SH30243.FS, USA). Fetal bovine serum (FBS 04-001-1A) was purchased from Biological Industries (Israeli). Penicillin-streptomycin solutions were purchased from HyClone (SV30010. USA). Trypsin was purchased from Beijing solarbio science & technology Co., Ltd. (Beijing, China). The mitochondrial permeability transition pore (MPTP C2009S) and Enhanced mitochondrial membrane potential assay kit with JC-1 (C2003S) were purchased from Beyotime Biotechnology (China). The Reactive Oxygen Species Assay Kit was obtained from Beyotime Biotechnology (S0033S China). Beta Actin Polyclonal antibody (β-actin (1:5000, Cat No. 66009-1-Ig)), FK506 binding protein 12-rapamycin associated protein 1 (mTOR (1:5000, Cat No. 66888-1-Ig)), acetyl-Coenzyme A carboxylase alpha (ACC (1:2000, Cat No. 21923-1-AP)), sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae) (SIRT1 (1:2000, Cat No. 13161-1-AP)), peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α (1:5000, Cat No. 66369-1-Ig)), ATG5 autophagy related 5 homolog (S. cerevisiae) (ATG5 (1:2000, Cat No. 10181-2-AP)), microtubule-associated protein 1 light chain 3 beta (LC3B (1:2000, Cat No. 14600-1-AP)), BCL2-associated X protein (BAX (1:2000, Cat No. 50599-2-Ig)), B-cell leukemia/lymphoma 2 (Bcl-2 (1:2000, Cat No. 68103-1-Ig)), PTEN induced putative kinase 1 (PINK1 (1:1000, Cat No. 23274-1-AP)), peroxisome proliferator-activated receptor gamma (PPARγ (1:2000, Cat No. 16643-1-AP)) was obtained from Proteintech Group, Inc (USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Proteintech Group, Inc (USA). Cell Counting Kit-8 (CCK-8) was obtained from (Cat. No.: HY-K0301 MCE, China), The purity of all chemical reagents was at least of analytical grade.

2.2 H9c2 Cell Culture and Grouping

H9c2 cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin, placed in an incubator at 37 °C with 95% humidity and 5% CO_2. Lut (5, 10, and 20 µM) and 5 µM metoprolol treated cells for 24 h. Then cells were divided into control group, H_2O_2-induced model group, Lut-treated group, and metoprolol-treated group. All groups except the normal group were treated with H_2O_2 (700 µM) for 4 h. All cell lines were validated by species-specific PCR and tested negative for mycoplasma.

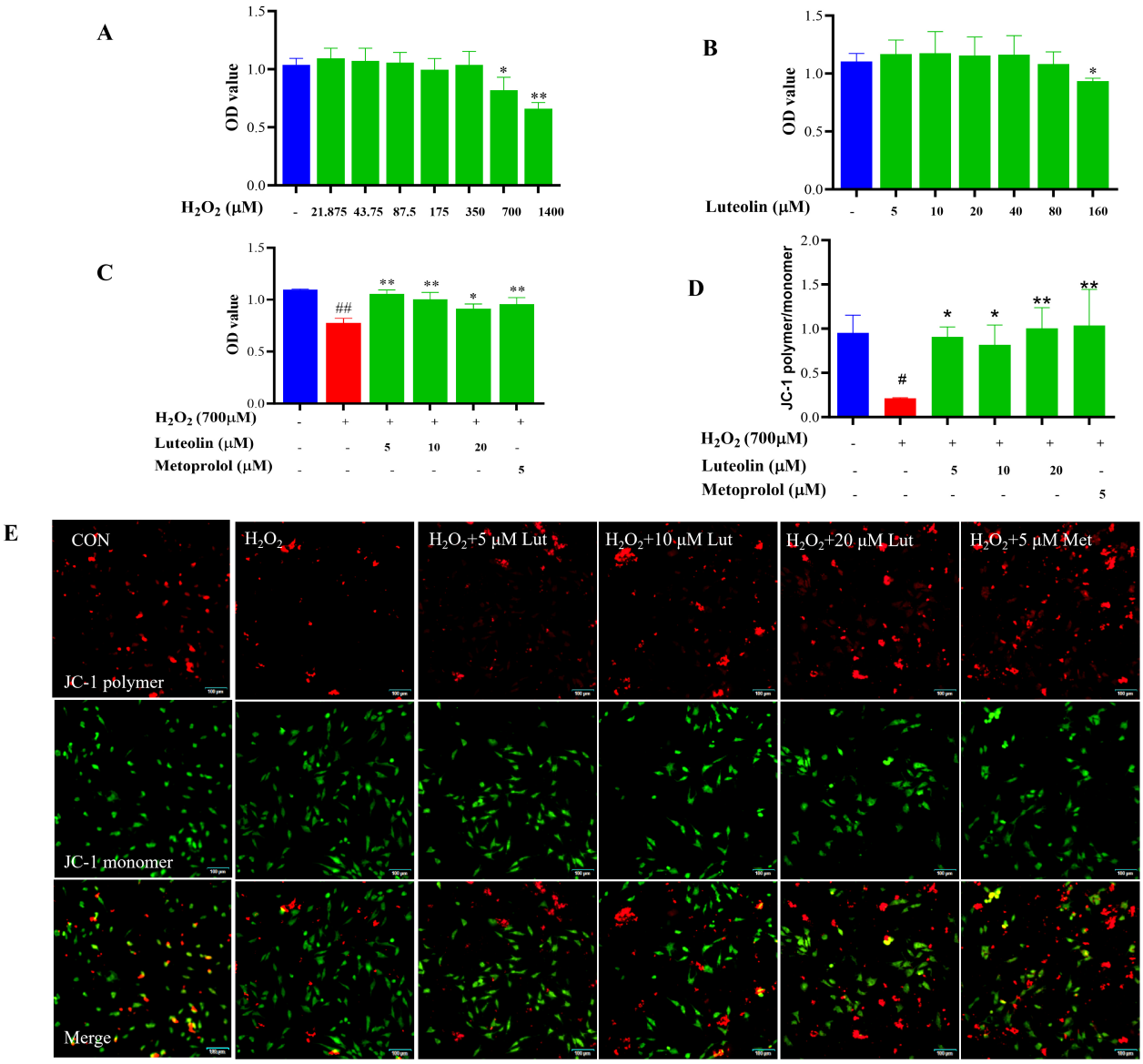

Fig. 1. Effect of Lut on cell survival rate and H_2O_2-induced cytotoxicity. H9c2 cells were treated with 700 μM H_2O_2 for 4 h and incubated with/without Lut/metoprolol for 24 h. (A) Effect of H_2O_2 on Cell Survival Rate. (B) Effect of Lut on Cell Survival Rate. (C) Effect of Lut on H_2O_2-Induced Cytotoxicity. (D,E) Effect of Lut on H_2O_2-Induced cellular MMP by JC-1. Scale bar = 100 μm (applies to all panels in E). *vs MOD, *$p < 0.05$, **$p < 0.01$; #vs CON, #$p < 0.05$, ##$p < 0.01$. +: treated; –: untreated. Lut, luteolin; H_2O_2, hydrogen peroxide; MMP, Mitochondrial Membrane Potential; JC-1, a lipophilic cationic dye used to measure MMP; MOD, model group (cells treated with H_2O_2 only); CON, control group (untreated cells).

2.3 CCK-8 Assay

H9c2 cells in the logarithmic growth phase were selected, washed with PBS, digested with trypsin, and at the end of centrifugation, they were prepared into cell suspension. Cells were counted under an automatic cell counter (Life Technologies, USA) using a cell counting plate. They were sequentially inoculated in 96-well culture plates according to 1.0×10^4/100 μL per well. The 96-well plates were placed in an incubator and after wall attachment, the plates were added 0, 21.875, 43.75, 87.5, 175, 350, 700, and 1400 μmol/L H_2O_2 for 4 h, respectively, and setting up a control group (adding medium without H_2O_2). H9c2 cells were routinely inoculated into 96-well plates overnight, then using 0, 5, 10, 20, 40, 80 and 160 μmol/L Lut culture for 24 h. H9c2 cells were routinely inoculated into 96-well plates overnight, then using 5, 10, 20 μmol/L Lut culture for 24 h. each well was punched into 100 μL containing 10 μL of CCK-8 and then placed into the incubator for 4 h. Cell viability was calculated by detecting OD values using a 450 nm wavelength enzyme marker (Thermo Fisher Scientific, USA).

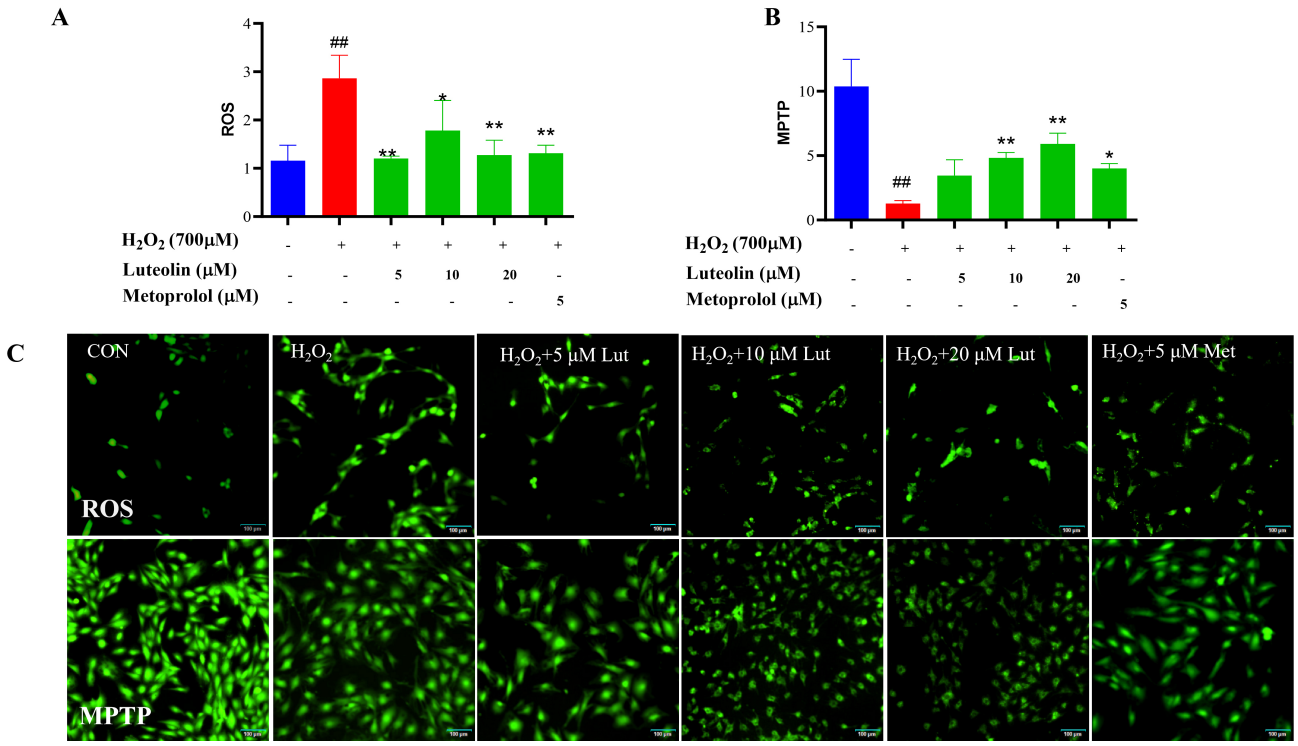

Fig. 2. Effect of Lut on H_2O_2-induced Apoptosis. H9c2 cells were treated with 700 μM H_2O_2 for 4 h and incubated with/without Lut/metoprolol for 24 h. (A) The effect of Lut on intracellular ROS levels induced by H_2O_2. (B) The effect of Lut on H_2O_2-induced intracellular MPTP levels. (C) Representative fluorescence images of ROS and MPTP under different treatment conditions. Scale bar = 100 μm (applies to all panels in C). * vs MOD, *$p < 0.05$, **$p < 0.01$; #vs CON, ##$p < 0.01$. +: treated; –: untreated. ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore.

2.4 Detection of Intracellular ROS Levels

H9c2 cells were seeded in 96-well black clear-bottom plates at a density of 1.0×10^4 cells per well and incubated overnight. After pretreatment with luteolin (5, 10, 20 μM) or metoprolol (5 μM) for 24 h, cells were exposed to 700 μM H_2O_2 for 4 h to induce oxidative stress. Subsequently, cells were washed with PBS and incubated with 10 μM DCFH-DA probe in serum-free medium at 37 °C for 30 min in the dark. After incubation, cells were washed three times with PBS to remove excess probe, and intracellular ROS levels were immediately observed using a fluorescence microscope (OLYMPUS, Japan) at excitation/emission wavelengths of 488/525 nm. Fluorescence intensity was quantified to assess ROS production. The fluorescence intensity was quantified using ImageJ software (NIH, USA) and expressed as Mean Fluorescence Intensity.

2.5 Determination of Enhanced Mitochondrial Transmembrane Potential (ΔΨm)

Treat the cells according to the method under 2.3, 1 mL of 1×JC-1 DCFH-DA solution was added to each well and incubated for 20 min. The supernatant was discarded and washed twice with JC-1 staining buffer in the dark. Fluorescence microscope (OLYMPUS, Japan) examined cells labeled with JC-1.

2.6 Detected Opening of the MPTP

Treat the cells according to the method under 2.3, add Calcein AM staining solution according to the instructions of the MPTP Assay Kit, and incubate for 30 min at 37 °C in an incubator away from light. At the end of the incubation, replace with fresh pre-warmed culture medium at 37 °C and incubate at 37 °C away from light for 30 min. Then, Cells with the addition of detection buffer were observed under a fluorescence microscope. The fluorescence intensity was quantified using ImageJ software (NIH, USA) and expressed as Mean Fluorescence Intensity.

2.7 Immunofluorescence Assay for p-AMPKα, SIRT1, and Caspase8 Levels in H_2O_2-Induced Apoptosis

First, cells were fixed by adding 4% paraformaldehyde for 1 hour at 4 °C; then cells were incubated with 0.3% Triton X-100 (PBS preparation) for 15 minutes and incubated with 5% BSA for 30 minutes. The corresponding primary antibody was added to each well, incubated overnight at 4 °C, and the secondary antibody was added the next day. Finally, incubate with DAPI for 5 minutes and observe under a fluorescence microscope.

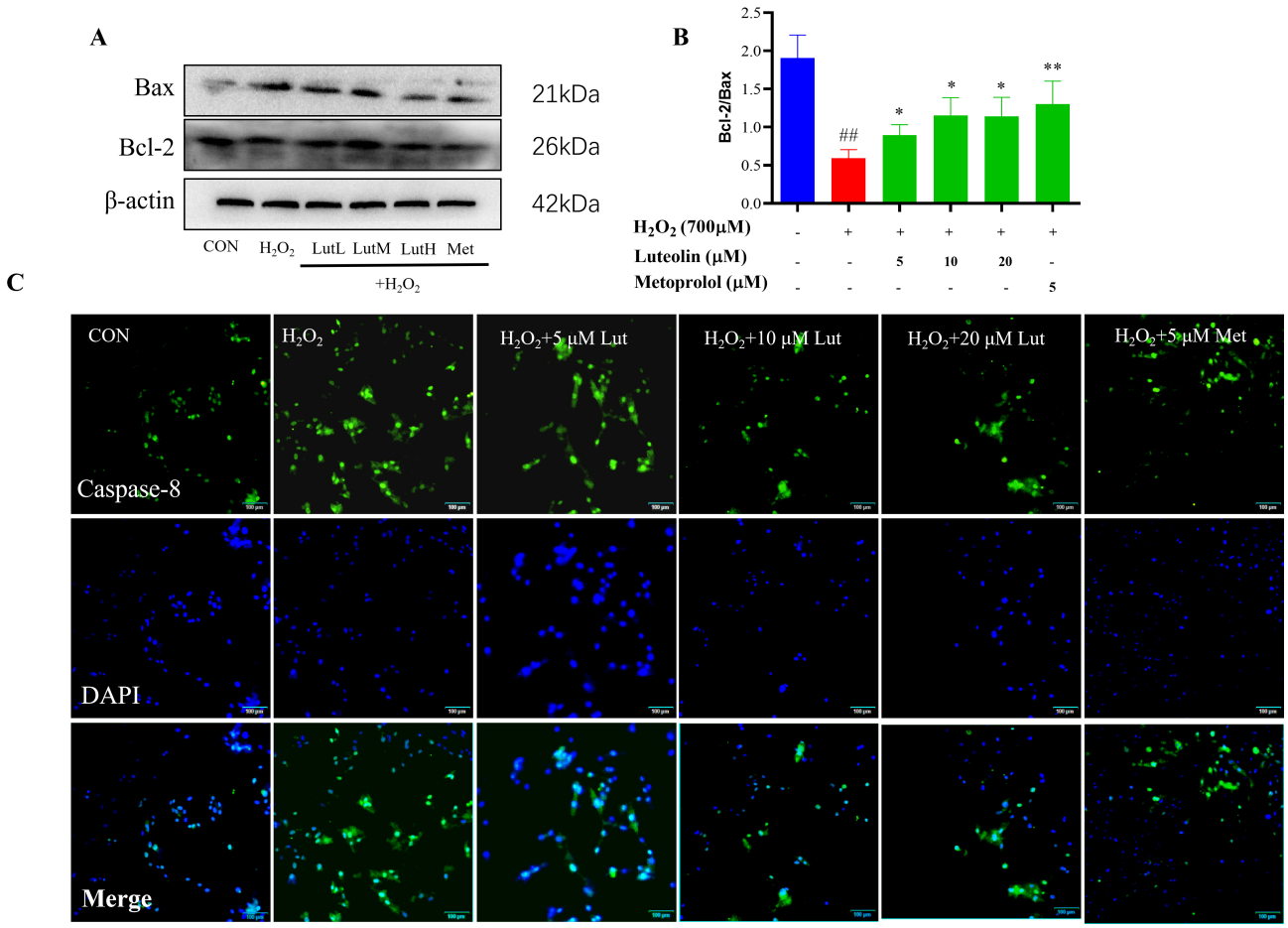

Fig. 3. Effect of Lut on H_2O_2-induced Apoptosis. H9c2 cells were treated with 700 μM H_2O_2 for 4 h and incubated with/without Lut/metoprolol for 24 h. (A,B) The expression of Bcl-2/BAX was analyzed by Western blot. (C) The expression of Caspase8 was analyzed by immunofluorescence. Scale bar = 100 μm (applies to all panels in C. *vs MOD, *$p < 0.05$, **$p < 0.01$; #vs CON, ##$p < 0.01$. +: treated; –: untreated. Bcl-2, B-cell leukemia/lymphoma 2; BAX, B-cell lymphoma 2 (BCL2)-associated X protein.

2.8 Western Blot Analysis

Cells were lysed with RIPA cell lysis buffer containing 1% PMSF, placed on ice for 30 min, and centrifuged at 12,000 g and 4 °C for 10 min. Protein samples were prepared based on protein concentration by adding the upsampling buffer and boiling for 5 min. Samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane, which was blocked with 5% skimmed milk for 1.5 h. The membrane was incubated with primary antibody, overnight for 4 °C, followed by incubation with HRP-conjugated secondary antibody for 1.5 h. Finally, the protein bands were exposed to the ECL ultrasensitive luminescent developer.

2.9 Molecular Docking Analysis

Obtain the 2D structure of the small molecule by Pubchem (https://pubchem.ncbi.nlm.nih.gov/) and convert it to a mol2 file in Chemdraw3D for backup; query the database in PDB (https://www.rcsb.org/) to obtain the live 3D structure file of the target, such as AMPKα (PDB: 2h6d) [21], SIRT1 (PDB: 4kxq) [22], ATG5 (PDB: 4tq1) [23], LC3B (PDB: 7elg) [24], PINK1 (PDB: 7t4n) [25] and mTOR (PDB: 8ppz) [26]. Finally, MOE is used for molecular docking and result visualization

2.10 Statistical Analyses

Data were processed and statistically analyzed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. For comparisons between two groups, statistical significance was determined using a two-tailed Student's *t*-test. For multiple group comparisons, one-way analysis of variance (ANOVA) was employed, followed by Tukey's post hoc test for pairwise comparisons among all groups, or Dunnett's post hoc test when comparing multiple treatment groups against a single control group. All experiments were independently repeated at least three times, and data are presented as mean ± standard deviation (SD). A value of $p < 0.05$ was considered statistically significant.

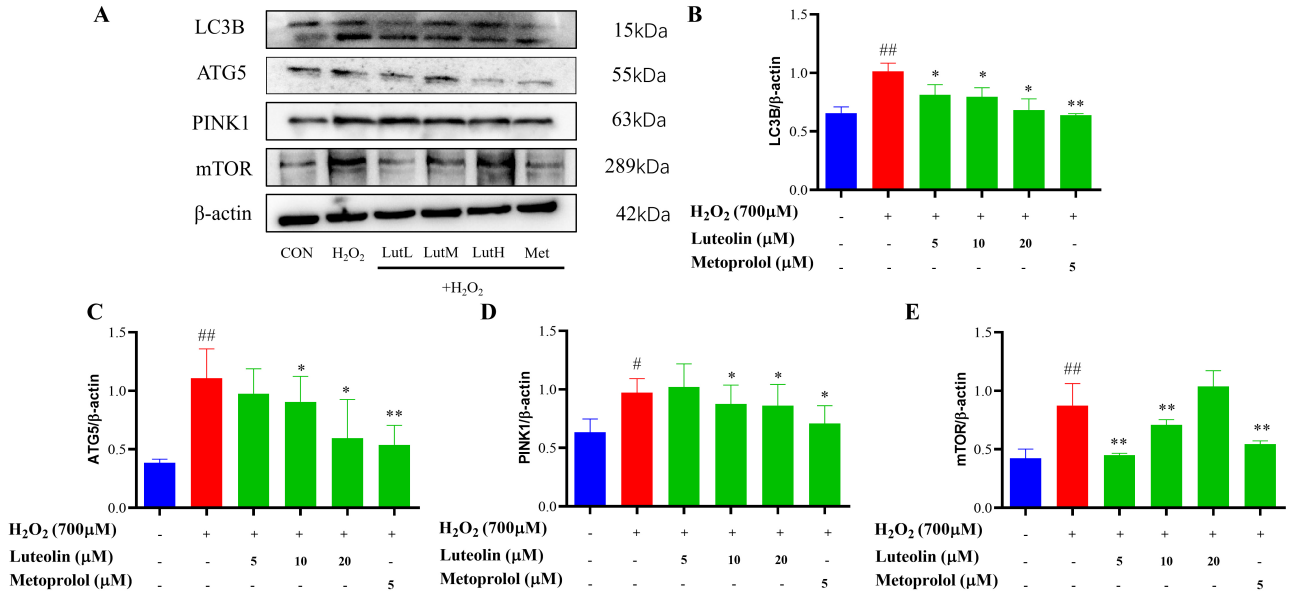

Fig. 4. Effects of Lut on the expression of mTOR and mitophagy. H9c2 cells were treated with 700 μM H_2O_2 for 4 h and incubated with/without Lut/metoprolol for 24 h. (A–E) The expression of mTOR, ATG5, PINK1, and LC3B was analyzed by Western blot. *vs MOD, *$p < 0.05$, **$p < 0.01$; #vs CON, #$p < 0.05$, ##$p < 0.01$. +: treated; –: untreated. mTOR, mammalian target of rapamycin; ATG5, Autophagy Related 5; PINK1, PTEN induced putative kinase 1.

3. Result

3.1 Effects of Lut on Cell Viability and H_2O_2-Induced Cytotoxicity

As the concentration of H_2O_2 increased in Fig. 1A, the cell survival rate gradually decreased. For comprehensive consideration, H_2O_2 with a moderate degree of cellular damage was chosen to establish a cardiomyocyte injury model by treating with H_2O_2 at 700 μmol/L for 4 h. As shown in Fig. 1B, the optimal dosing concentration was screened from Lut at concentrations of 5, 10, and 20 μM. As shown in Fig. 1C, Lut/metoprolol (5 μM) pretreatment showed significant protection against H_2O_2-induced Cytotoxicity.

3.2 Lut Attenuated H_2O_2-Induced Apoptosis and Ameliorates MMP Decline

Decrease in Mitochondrial Membrane Potential (MMP) causes early apoptosis, and in this study, JC-1 fluorescent probe was used to detect MMP in cells. Compared with the control group, the red/green fluorescence ratio of the cells in the model group was decreased, i.e., MMP was decreased; compared with the model group, the red/green fluorescence ratio of the cells in the Lut group was increased, i.e., MMP levels were increased, as shown in Fig. 1D and Fig. 1E.

3.3 Lut Attenuated Intracellular ROS Generation and MPTP Opening

ROS generated by H_2O_2 could promote cellular damage. Compared with the control group, intracellular DCFH-DA probe green fluorescence signal was enhanced and intracellular ROS levels were elevated in the model group, as shown in Fig. 2A. The intracellular ROS levels were reduced in the Lut (5, 10, 20 μM) group compared with the model group. Elevated levels of ROS in mitochondria ultimately lead to the release of cytochrome C and a decrease and loss of MMP. As shown in Fig. 2B, the fluorescence intensity became weaker in the model group compared with the control group and increased significantly after treatment with Lut ($p < 0.01$). Representative fluorescence images (Fig. 2C) showed that Lut reversed H2O2 induced ROS accumulation and MPTP opening.

3.4 Lut Increased the Ratio of Bcl-2/BAX and Decreased the Expression of Caspase8

Apoptosis caused activation of the Bcl-2/BAX and Caspase protein families. In this study, the expression of Bcl-2/BAX and Caspase8 proteins was examined. After the cells were treated with H_2O_2, the ratio of Bcl-2/BAX was decreased and Caspase8 was enhanced, but pretreatment with Lut, the ratio of Bcl-2/BAX was elevated and the expression of Caspase8 was down-regulated (Fig. 3).

3.5 Lut Inhibited mTOR and Mitophagy Related Protein

We further elucidated the mechanism by which Lut attenuates H_2O_2-induced apoptosis, by examining the mitophagy signaling pathway. Exposure to H_2O_2 increased the protein level of mTOR and activation of mitophagy related expression of ATG5, PINK1, and LC3B. We interpreted this overactivation as a detrimental, pro-death re-

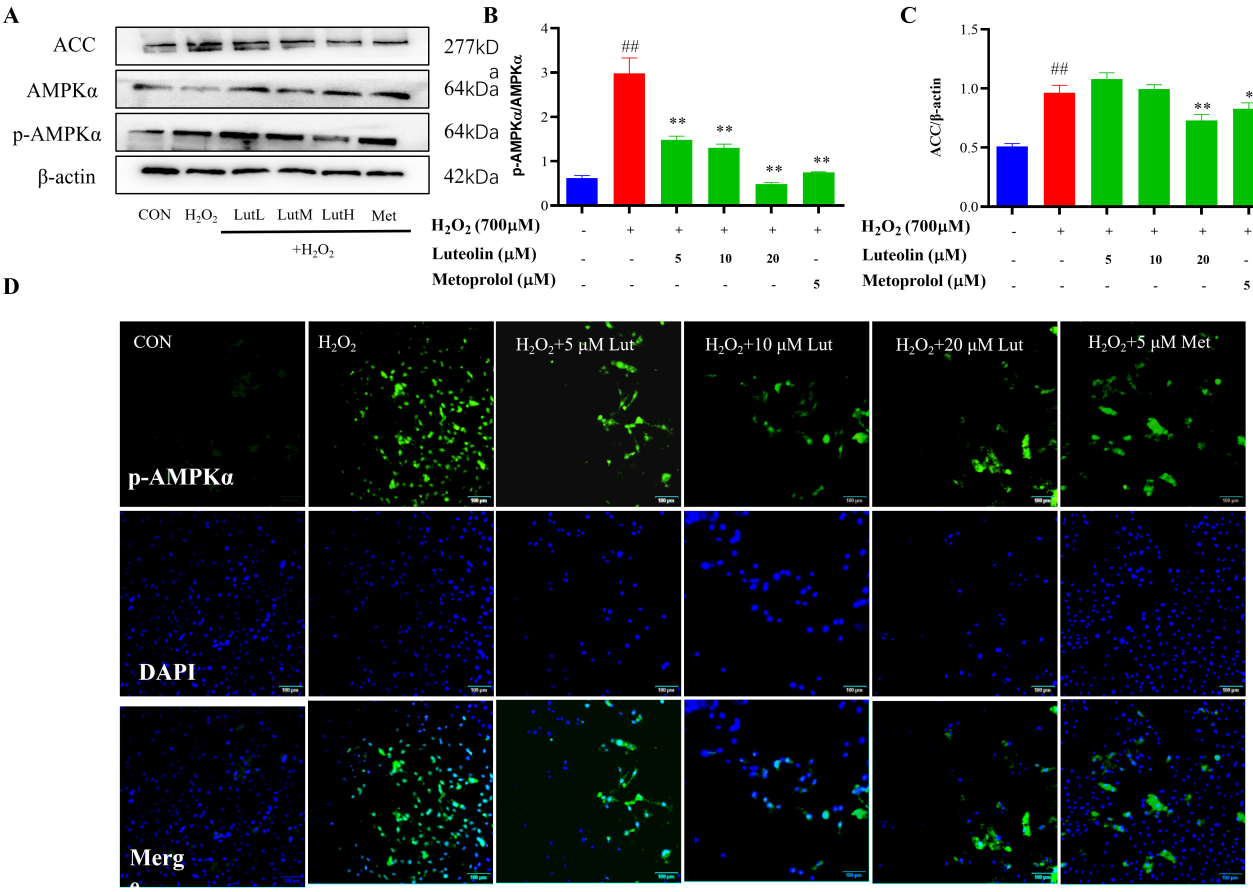

Fig. 5. Effects of Lut on the expression of AMPK/ACC signaling pathway. H9c2 cells were treated with 700 μM H_2O_2 for 4 h and incubated with/without Lut/metoprolol for 24 h. The expression of β-actin was measured as an internal control. (A–C) The expression of p-AMPKα, AMPKα and ACC were analyzed by Western blot. (D) The expression of p-AMPKα was analyzed by immunofluorescence.Scale bar = 100 μm (applies to all panels in D. *vs MOD, *$p < 0.05$, **$p < 0.01$; #vs CON, ##$p < 0.01$. +: treated; –: untreated. AMPK, AMP-activated protein kinase; ACC, acetyl-Coenzyme A carboxylase alpha.

sponse that contributes to H_2O_2-induced cardiomyocyte injury. Treatment with Lut reversed these changes, significantly decreasing the levels of ATG5, PINK1, and LC3B-II. This suggests that the protective effect of Lut is associated with suppressing this pathological overactivation of mitophagy. The involvement of the mTOR pathway was also evident, as H_2O_2 increased mTOR expression, which was restored by Lut treatment, aligning with the known role of the mTOR in stress-induced mitophagy (Fig. 4).

3.6 Lut Regulated AMPK Signaling Pathway

In this study, Lut decreased the levels of p-AMPKα/AMPKα and ACC (Fig. 5A). Immunofluorescence technique found Lut inhibited p-AMPKα in H9c2 cells treated with H_2O_2 (Fig. 5B). The results were further confirmed by Western blot analysis of ACC (Fig. 5C) and immunofluorescence staining of p-AMPKα (Fig. 5D).

The levels of PPARγ were decreased, whereas the expression of SIRT1, PGC-1α was increased in H9c2 cells treated with H_2O_2. Lut increased the levels of PPARγ and decreased the levels of SIRT1 and PGC-1α (Fig. 6A–D). Immunofluorescence technique found Lut decreased expression of SIRT1 in H9c2 cells treated with H_2O_2 (Fig. 6E).

The results showed that the binding energies of Lut with AMPKα, SIRT1, ATG5, LC3B, PINK1 and mTOR were –6.4, –5.5, –5.5, –5.4, –5.6 and –5.8 kcal/mol (Fig. 7).

4. Discussion

In recent years, natural compounds have been recognized as promising agents for the treatment of cardiovascular-related diseases. Many Chinese herbal medicines, such as polyphenols and flavonoids, have been shown to have antioxidant and anti-inflammatory effects and to be effective in modulating oxidative stress [27,28], leading to therapeutic effects. Among them, Lut is widely distributed in nature, having been named because it was initially isolated from the leaves, stems, and branches of the herb Resedaodorata L., and it can be isolated from a variety of natural herbs, vegetables, and fruits. The an-

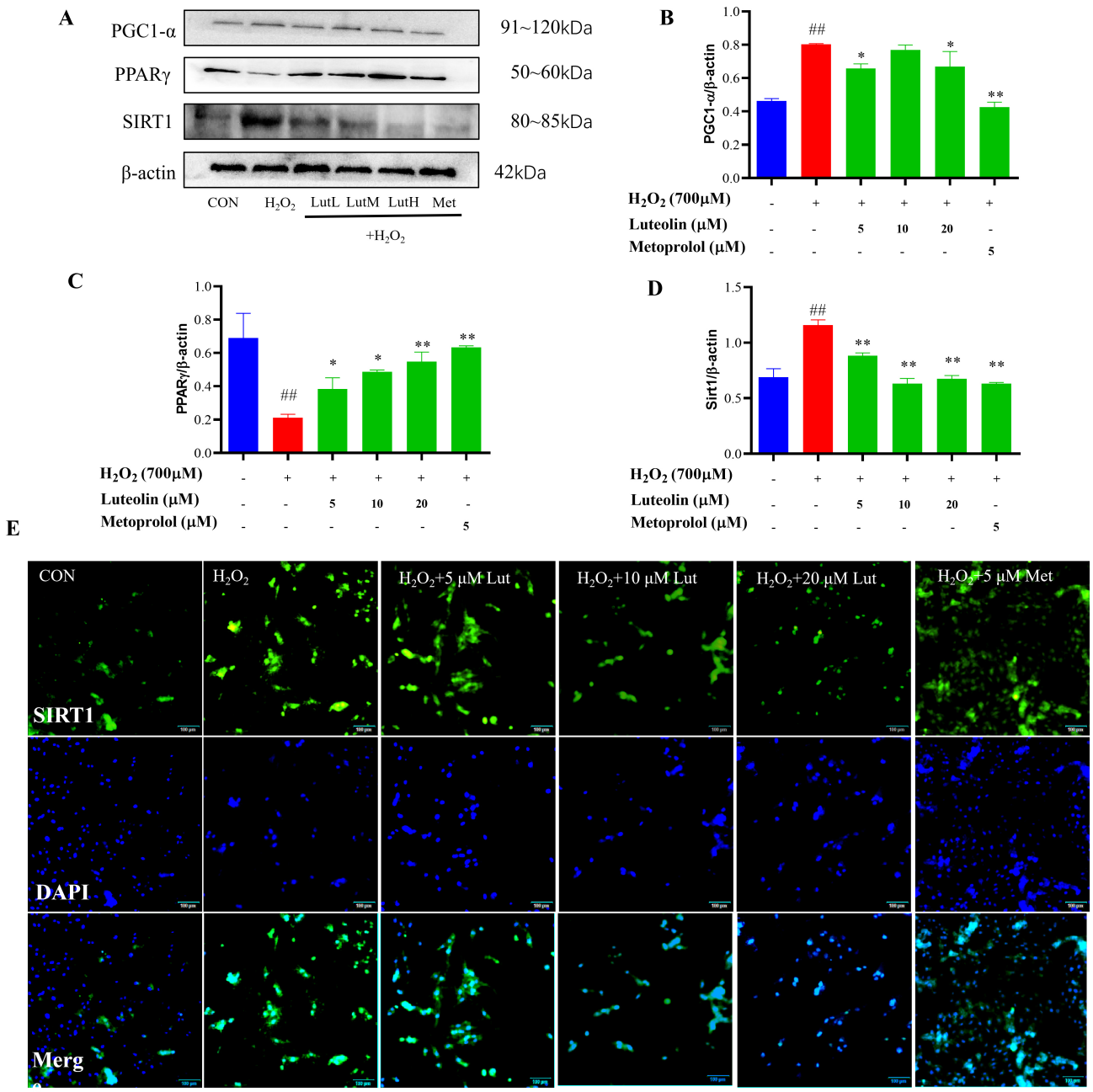

Fig. 6. Effects of Lut on the expression of SIRT1, PGC-1α, PPARγ. H9c2 cells were treated with 700 μM H_2O_2 for 4 h and incubated with/without Lut/metoprolol for 24 h. (A–D) The expression of SIRT1, PGC-1α, and PPARγ were analyzed by Western blot. (E) The expression of SIRT1 was analyzed by immunofluorescence.Scale bar = 100 μm (applies to all panels in E. *vs MOD, *$p < 0.05$, **$p < 0.01$; #vs CON, ##$p < 0.01$. +: treated; –: untreated. SIRT1, sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*); PGC-1α, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; PPARγ, peroxisome proliferator-activated receptor gamma.

tioxidant activity of the Lut molecule is mainly attributed to the 3′,4′-dihydroxyl structure (catechol moiety) in the phenolic B-ring, which is essential for the activity of the flavonoid molecule. On the pyran C ring of the flavonoid, a 2,3-double bond is conjugated to a 4-oxo function (i.e., the 1,4-pyranone moiety), which disperses the unpaired electrons on the A, B, and C rings and forms a more stable phenoxyl radical. This may bring better antioxidant activity for flavonoids [29]. Therefore, we utilized its antioxidant activity to protect the mitochondria of cardiomyocytes by effectively reducing the excess ROS produced in H_2O_2-induced H9c2 apoptosis through mitophagy.

Oxidative stress results from excessive accumulation of intracellular oxygen free radicals and related metabolites.

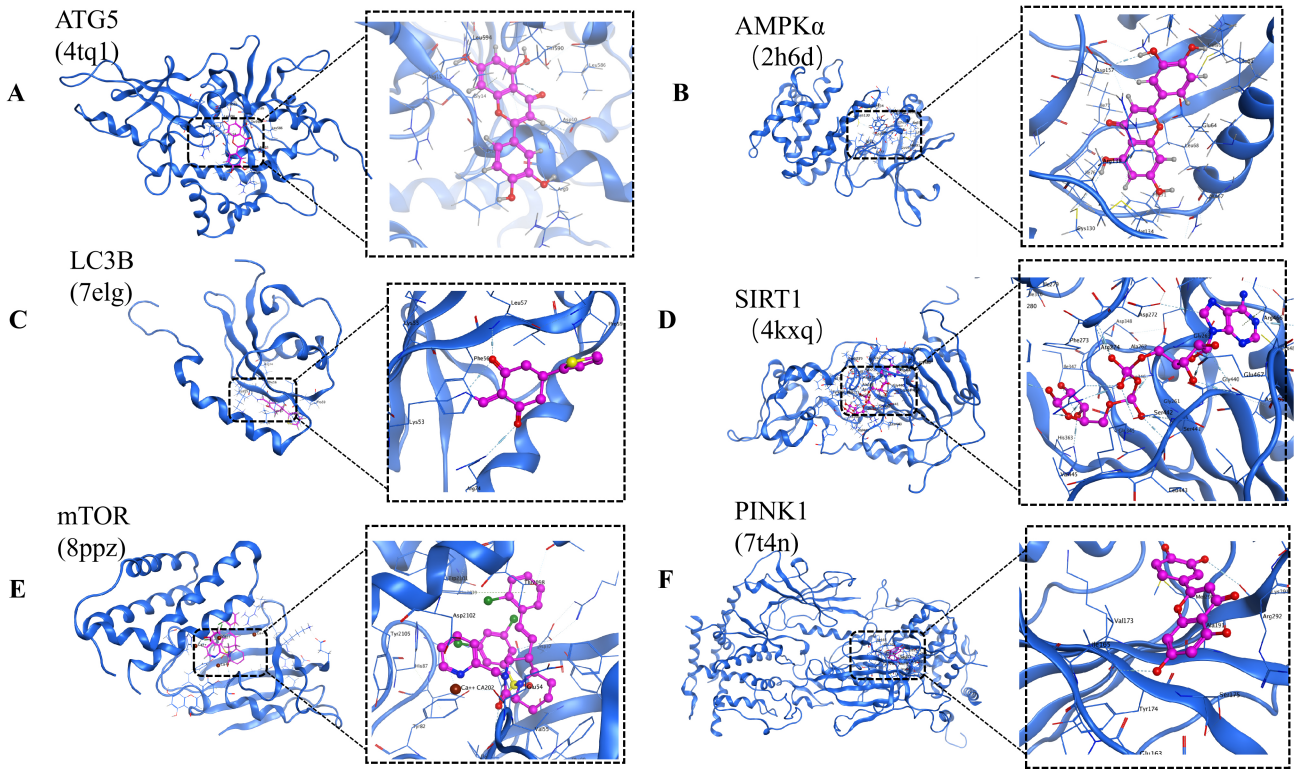

Fig. 7. Docking results of Lut with ATG5 (PDB: 4tq1), AMPKα (PDB: 2h6d), LC3B (PDB: 7elg), SIRT1 (PDB: 4kxq), mTOR (PDB: 8ppz) and PINK1 (PDB: 7t4n). (A–F) Key residues in binding site surrounding Lut.

Among them, mitochondria are the primary source of ROS in mammals and increased mitochondrial ROS production underlies cardiovascular and many other diseases [30]. Lut reduced intracellular ROS levels in H9c2 cells induced by the H_2O_2. Lut pretreatment significantly ameliorated oxidative stress in H9c2 cardiomyocytes, suggesting that Lut could be a candidate for cardiovascular disease. Peroxidative damage to mitochondria can accelerate the opening of the MPTP, triggering pro-apoptotic factors such as cytochrome C emission [31]. Peroxidative damage to mitochondrial membranes can trigger the opening of the mitochondrial permeability transition pore (MPTP), leading to the release of pro-apoptotic factors such as cytochrome c into the cytosol [32]. The Bcl-2 protein family serves as a critical checkpoint in the mitochondrial apoptotic pathway. The anti-apoptotic protein Bcl-2 preserves mitochondrial integrity, while the pro-apoptotic protein Bax promotes mitochondrial outer membrane permeabilization [33]. The ratio of Bcl-2 to Bax is often used as an indicator of apoptotic susceptibility [34]. In our study, H_2O_2 treatment significantly decreased the Bcl-2/Bax ratio and increased Caspase8 expression, indicating that the mitochondrial apoptotic pathway was engaged. Notably, treatment with luteolin (Lut) reduced intracellular ROS, restored $\Delta\Psi$m, elevated the Bcl-2/Bax ratio, and decreased Caspase8 expression. These findings indicate that Lut protects H9c2 cardiomyocytes from H_2O_2-induced injury by suppressing mitochondria-mediated apoptosis.

Mitochondria are multitasking organelles that are crucial for maintaining a balance of multiple cellular pathways, including ATP production, redox signaling, and programmed cell death [35]. Mitochondria play an instrumental regulatory role during I/R injury [36–38]. Liu *et al.* [39] concluded that treatment with lignocaine could activate the AMPK/mTOR pathway through upregulation of SIRT3, thereby inhibiting inflammatory response and apoptosis, promoting neurogenesis, and ameliorating neurological deficits after ischemia in rats after CIRI. In the present study, p-AMPKα/AMPKα protein level and mTOR were significantly increased in H9c2 cells treatment with H_2O_2. Notably, Lut pretreatment reversed these changes, suggesting that its cardioprotective effect involves the modulation of the AMPK/mTOR signaling pathway. Although AMPK is known for negatively regulating mTORC1 signal activity (mainly through phosphorylation of Raptor or TSC2), it does not directly degrade or inhibit the synthesis of total mTOR protein [40]. The simultaneous increase of p-ampk and total mTOR in this model reflects the biphasic adaptive response of cells to severe oxidative stress. On the one hand, the mitochondrial dysfunction and ROS burst induced by H2O2 will trigger a rapid energy crisis, which will lead to the strong phosphorylation and activation of AMPK. This activation is a classic protective mechanism that restores ATP levels by shifting cellular metabolism from anabolism to catabolism [41]. On the other hand, the up regulation of total mTOR level under H2O2 stress suggests that there is

a compensatory survival promoting mechanism. The latest research evidence shows that ROS can up regulate mTOR expression at the transcriptional level by activating NF-κB or Nrf2 pathway, or by inhibiting proteasome degradation [42,43]. Luteolin treatment can restore the expression of p-ampk-mtor. By reducing oxidative stress, luteolin can re balance the disordered cell homeostasis by regulating the level of p-ampk and the expression of mTOR. AMPK not only influences the transcription of PGC-1α but also indirectly activates the SIRT1 deacetylase by increasing intracellular NAD^+ levels [44]. With the increase of p-AMPK protein expression in H_2O_2-induced apoptosis, the expression of its downstream SIRT1 proteins increased accordingly, and SIRT1 activation further activated PGC-1α, which contributed to the decrease in the expression level of PPARγ, However, these effects were suppressed by pretreatment with Lut. Luteolin increased PPARγ expression (Fig. 6D), which may contribute to cardioprotection through lipid metabolism regulation; however, the relationship between this effect and the observed changes in SIRT1/PGC-1α requires further mechanistic investigation. Activation of AMPK enables ACC response and participates in the regulation of energy metabolism [45]. ACC plays a critical role in fatty acid synthesis, and it is the rate-limiting enzyme in the de novo synthesis of fatty acids [46]. In our study, we found Lut could inhibit the expression of ACC in H9c2 cells treatment with H_2O_2.

Mitophagy acts as a double-edged sword in the maintenance of cellular homeostasis [47]. Under physiological conditions, it serves as a quality control mechanism to eliminate damaged mitochondria. However, excessive mitophagy can lead to mitochondrial depletion and energy crisis, ultimately triggering apoptosis [48]. The PINK1/Parkin pathway is a central regulator of mitophagy [49]. In healthy mitochondria, PINK1 is continuously cleaved and degraded. Upon mitochondrial damage, the loss of mitochondrial membrane potential stabilizes PINK1 on the outer mitochondrial membrane, where it recruits Parkin and initiates the autophagic clearance of damaged mitochondria [50]. Our results showed that exposure to H_2O_2 significantly increased the expression of mitophagy-related proteins, including ATG5, PINK1, and LC3B, indicating excessive activation of mitophagy. Notably, treatment with Lut reversed these changes, significantly decreasing the levels of ATG5, PINK1, and LC3B. These findings suggest that the cardioprotective effect of Lut is associated with suppressing this pathologically overactivated mitophagy.

5. Limitations of the study

Despite the promising findings, several limitations should be acknowledged. First, this study was conducted exclusively using an in vitro H_2O_2-induced oxidative stress model in H9c2 cardiomyocytes, which does not fully recapitulate the complex pathophysiology of myocardial I/R injury *in vivo*. Therefore, the observed protective effects of Lut require further validation in appropriate animal models, and our ongoing work includes such in vivo studies.

6. Conclusion

Luteolin protects H9c2 cardiomyocytes from H_2O_2-induced injury by modulating the AMPK/mTOR signaling pathway, inhibiting excessive mitophagy, and thereby reducing mitochondria-mediated apoptosis.

Availability of Data and Materials

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher upon reasonable request.

Author Contributions

ZY: Methodology, Resources, Writing – original draft; XL: Data curation, Formal analysis; DL: Methodology, Resources; KM: Data curation, Resources; MZ: Formal analysis, Funding acquisition, Writing – review & editing; YW: Conceptualization, Writing – review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

We would like to express our gratitude to all those who helped us during the writing of this manuscript. Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This work has been supported by Natural Science Foundation of Inner Mongolia (NO: 2023LHMS08082), Education Institutions in Inner Mongolia Autonomous Region (NO: NJZY22456). Science and Technology Department of Inner Mongolia Scientific Research Fund Project (2021GG0279).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Juricic S, Klac J, Stojkovic S, Beleslin B, Tesic M, Jovanovic I, *et al*. Molecular and Cellular Mechanisms of Myocardial Ischemia and Reperfusion Injury: A Narrative Review. Cells. 2026; 15: 265. https://doi.org/10.3390/cells15030265.

[2] Wang K, Zhu Q, Liu W, Wang L, Li X, Zhao C, *et al*. Mitochondrial apoptosis in response to cardiac ischemia-reperfusion injury. Journal of Translational Medicine. 2025; 23: 125. https://doi.org/10.1186/s12967-025-06136-8.

[3] Zou R, Shi W, Tao J, Li H, Lin X, Yang S, *et al*. SIRT5 and post-translational protein modifications: A potential therapeu-

tic target for myocardial ischemia-reperfusion injury with regard to mitochondrial dynamics and oxidative metabolism. European Journal of Pharmacology. 2018; 818: 410–418. https://doi.org/10.1016/j.ejphar.2017.11.005.

[4] Bugger H, Pfeil K. Mitochondrial ROS in myocardial ischemia reperfusion and remodeling. Biochimica et Biophysica Acta. Molecular Basis of Disease. 2020; 1866: 165768. https://doi.org/10.1016/j.bbadis.2020.165768.

[5] Liang J, Wu M, Chen C, Mai M, Huang J, Zhu P. Roles of Reactive Oxygen Species in Cardiac Differentiation, Reprogramming, and Regenerative Therapies. Oxidative Medicine and Cellular Longevity. 2020; 2020: 2102841. https://doi.org/10.1155/2020/2102841.

[6] Imlay JA. The Barrier Properties of Biological Membranes Dictate How Cells Experience Oxidative Stress. Molecular Microbiology. 2025; 123: 454–463. https://doi.org/10.1111/mmi.15353.

[7] Madamanchi NR, Runge MS. Redox signaling in cardiovascular health and disease. Free Radical Biology & Medicine. 2013; 61: 473–501. https://doi.org/10.1016/j.freeradbiomed.2013.04.001.

[8] Jelinek HF, Muteir I, Al-Aubaidy H. Hierarchical random forest model, inflammation and oxidative stress as predictors of the atherogenic index of plasma and diabetes progression. Scientific Reports. 2025; 15: 35381. https://doi.org/10.1038/s41598-025-19289-9.

[9] Liu T, Xu J, Yan HL, Cheng FC, Liu XJ. Luteolin Suppresses Teratoma Cell Growth and Induces Cell Apoptosis via Inhibiting Bcl-2. Oncology Research. 2019; 27: 773–778. https://doi.org/10.3727/096504018X15208986577685.

[10] Mohi-Ud-Din R, Mir RH, Wani TU, Alsharif KF, Alam W, Albrakati A, *et al*. The Regulation of Endoplasmic Reticulum Stress in Cancer: Special Focuses on Luteolin Patents. Molecules (Basel, Switzerland). 2022; 27: 2471. https://doi.org/10.3390/molecules27082471.

[11] Hu Y, Zhang C, Zhu H, Wang S, Zhou Y, Zhao J, *et al*. Luteolin modulates SERCA2a via Sp1 upregulation to attenuate myocardial ischemia/reperfusion injury in mice. Scientific Reports. 2020; 10: 15407. https://doi.org/10.1038/s41598-020-72325-8.

[12] Qi Y, Fu S, Pei D, Fang Q, Xin W, Yuan X, *et al*. Luteolin attenuated cisplatin-induced cardiac dysfunction and oxidative stress *via* modulation of Keap1/Nrf2 signaling pathway. Free Radical Research. 2022; 56: 209–221. https://doi.org/10.1080/10715762.2022.2067042.

[13] Caporali S, De Stefano A, Calabrese C, Giovannelli A, Pieri M, Savini I, *et al*. Anti-Inflammatory and Active Biological Properties of the Plant-Derived Bioactive Compounds Luteolin and Luteolin 7-Glucoside. Nutrients. 2022; 14: 1155. https://doi.org/10.3390/nu14061155.

[14] Han M, Lu Y, Tao Y, Zhang X, Dai C, Zhang B, *et al*. Luteolin Protects Pancreatic β Cells against Apoptosis through Regulation of Autophagy and ROS Clearance. Pharmaceuticals (Basel, Switzerland). 2023; 16: 975. https://doi.org/10.3390/ph16070975.

[15] Zaki MSA, Abadi AM, El-Kott AF, Mohamed G, Alrashdi BM, Eid RA, *et al*. Protective efficacy of luteolin against aflatoxinB1-induced toxicity, oxidative damage, and apoptosis in the rat liver. Environmental Science and Pollution Research International. 2023; 30: 52358–52368. https://doi.org/10.1007/s11356-023-26085-3.

[16] Ding T, Yi T, Li Y, Zhang W, Wang X, Liu J, *et al*. Luteolin attenuates lupus nephritis by regulating macrophage oxidative stress via HIF-1α pathway. European Journal of Pharmacology. 2023; 953: 175823. https://doi.org/10.1016/j.ejphar.2023.175823.

[17] Tan XH, Zhang KK, Xu JT, Qu D, Chen LJ, Li JH, *et al*. Luteolin alleviates methamphetamine-induced neurotoxicity by suppressing PI3K/Akt pathway-modulated apoptosis and autophagy in rats. Food and Chemical Toxicology: an International Journal Published for the British Industrial Biological Research Association. 2020; 137: 111179. https://doi.org/10.1016/j.fct.2020.111179.

[18] Li JP, Yan R, Ma PL, Fu P, Tian HT, Wang LL. Effects of luteolin in different doses on the cardiomyocyte apoptosis in rats with myocardial ischemia reperfusion. Journal of Biological Regulators and Homeostatic Agents. 2020; 34: 2311–2315. https://doi.org/10.23812/20-560-L.

[19] Basak B, Holzbaur ELF. Mitochondrial damage triggers the concerted degradation of negative regulators of neuronal autophagy. Nature Communications. 2025; 16: 7367. https://doi.org/10.1038/s41467-025-62379-5.

[20] Smiles WJ, Ovens AJ, Kemp BE, Galic S, Petersen J, Oakhill JS. New developments in AMPK and mTORC1 cross-talk. Essays in Biochemistry. 2024; 68: 321–336. https://doi.org/10.1042/EBC20240007.

[21] Littler DR, Walker JR, Davis T, Wybenga-Groot LE, Finerty PJ, Jr, Newman E, *et al*. A conserved mechanism of autoinhibition for the AMPK kinase domain: ATP-binding site and catalytic loop refolding as a means of regulation. Acta Crystallographica. Section F, Structural Biology and Crystallization Communications. 2010; 66: 143–151. https://doi.org/10.1107/S1744309109052543.

[22] Davenport AM, Huber FM, Hoelz A. Structural and functional analysis of human SIRT1. Journal of Molecular Biology. 2014; 426: 526–541. https://doi.org/10.1016/j.jmb.2013.10.009.

[23] Kim JH, Hong SB, Lee JK, Han S, Roh KH, Lee KE, *et al*. Insights into autophagosome maturation revealed by the structures of ATG5 with its interacting partners. Autophagy. 2015; 11: 75–87. https://doi.org/10.4161/15548627.2014.984276.

[24] Fan S, Yue L, Wan W, Zhang Y, Zhang B, Otomo C, *et al*. Inhibition of Autophagy by a Small Molecule through Covalent Modification of the LC3 Protein. Angewandte Chemie (International Ed. in English). 2021; 60: 26105–26114. https://doi.org/10.1002/anie.202109464.

[25] Gan ZY, Callegari S, Cobbold SA, Cotton TR, Mlodzianoski MJ, Schubert AF, *et al*. Activation mechanism of PINK1. Nature. 2022; 602: 328–335. https://doi.org/10.1038/s41586-021-04340-2.

[26] Deutscher RCE, Meyners C, Repity ML, Sugiarto WO, Kolos JM, Maciel EVS, *et al*. Discovery of fully synthetic FKBP12-mTOR molecular glues. Chemical Science. 2025; 16: 4256–4263. https://doi.org/10.1039/d4sc06917j.

[27] Thangavel P, Puga-Olguín A, Rodríguez-Landa JF, Zepeda RC. Genistein as Potential Therapeutic Candidate for Menopausal Symptoms and Other Related Diseases. Molecules (Basel, Switzerland). 2019; 24: 3892. https://doi.org/10.3390/molecules24213892.

[28] Martiniakova M, Babikova M, Mondockova V, Blahova J, Kovacova V, Omelka R. The Role of Macronutrients, Micronutrients and Flavonoid Polyphenols in the Prevention and Treatment of Osteoporosis. Nutrients. 2022; 14: 523. https://doi.org/10.3390/nu14030523.

[29] Ahmadi SM, Farhoosh R, Sharif A, Rezaie M. Structure-Antioxidant Activity Relationships of Luteolin and Catechin. Journal of Food Science. 2020; 85: 298–305. https://doi.org/10.1111/1750-3841.14994.

[30] Kervella M, Bertile F, Bouillaud F, Criscuolo F. The cell origin of reactive oxygen species and its implication for evolutionary trade-offs. Open Biology. 2025; 15: 240312. https://doi.org/10.1098/rsob.240312.

[31] Belosludtsev KN, Belosludtseva NV, Dubinin MV. Diabetes Mellitus, Mitochondrial Dysfunction and Ca^{2+}-Dependent Permeability Transition Pore. International Journal of Molecu-

lar Sciences. 2020; 21: 6559. https://doi.org/10.3390/ijms21186559.

[32] Cong J, Li JY, Zou W. Mechanism and treatment of intracerebral hemorrhage focus on mitochondrial permeability transition pore. Frontiers in Molecular Neuroscience. 2024; 17: 1423132. https://doi.org/10.3389/fnmol.2024.1423132.

[33] Silva JPN, Pinto B, Silva PMA, Bousbaa H. BCL-2 and BCL-xL in Cancer: Regulation, Function, and Therapeutic Targeting. International Journal of Molecular Sciences. 2026; 27: 1123. https://doi.org/10.3390/ijms27021123.

[34] Majeed NS, Salam AA, Farhan SR, Abdulrazzaq SA, Menon SV, Kaur M, *et al*. Multifaceted Roles of Bcl-2 Family Proteins: Regulatory Roles in Apoptosis, Physiological Functions, and Therapeutic Potential. Current Medical Science. 2025; 45: 1319–1335. https://doi.org/10.1007/s11596-025-00124-1.

[35] Liu F, Lu J, Manaenko A, Tang J, Hu Q. Mitochondria in Ischemic Stroke: New Insight and Implications. Aging and Disease. 2018; 9: 924–937. https://doi.org/10.14336/AD.2017.1126.

[36] Yao L, Chen H, Wu Q, Xie K. Hydrogen-rich saline alleviates inflammation and apoptosis in myocardial I/R injury via PINK-mediated autophagy. International Journal of Molecular Medicine. 2019; 44: 1048–1062. https://doi.org/10.3892/ijmm.2019.4264.

[37] Zhang W, Chen C, Wang J, Liu L, He Y, Chen Q. Mitophagy in Cardiomyocytes and in Platelets: A Major Mechanism of Cardioprotection Against Ischemia/Reperfusion Injury. Physiology (Bethesda, Md.). 2018; 33: 86–98. https://doi.org/10.1152/physiol.00030.2017.

[38] He Y, Ren S, Liu C, Zheng X, Zhu C. Targeting mitochondrial quality control for myocardial ischemia-reperfusion injury. Mitochondrion. 2025; 84: 102046. https://doi.org/10.1016/j.mito.2025.102046.

[39] Liu S, Su Y, Sun B, Hao R, Pan S, Gao X, *et al*. Luteolin Protects Against CIRI, Potentially via Regulation of the SIRT3/AMPK/mTOR Signaling Pathway. Neurochemical Research. 2020; 45: 2499–2515. https://doi.org/10.1007/s11064-020-03108-w.

[40] Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, *et al*. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Molecular Cell. 2008; 30: 214–226. https://doi.org/10.1016/j.molcel.2008.03.003.

[41] Kim AD, Kang KA, Piao MJ, Kim KC, Zheng J, Yao CW, *et al*. Cytoprotective effect of eckol against oxidative stress-induced mitochondrial dysfunction: involvement of the FoxO3a/AMPK pathway. Journal of Cellular Biochemistry. 2014; 115: 1403–1411. https://doi.org/10.1002/jcb.24790.

[42] Santos AL, Sinha S, Lindner AB. The Good, the Bad, and the Ugly of ROS: New Insights on Aging and Aging-Related Diseases from Eukaryotic and Prokaryotic Model Organisms. Oxidative Medicine and Cellular Longevity. 2018: 1941285. https://doi.org/10.1155/2018/1941285.

[43] Mishra P, Paital B, Jena S, Swain SS, Kumar S, Yadav MK, *et al*. Possible activation of NRF2 by Vitamin E/Curcumin against altered thyroid hormone induced oxidative stress via NFκB/AKT/mTOR/KEAP1 signalling in rat heart. Scientific Reports. 2019; 9: 7408. https://doi.org/10.1038/s41598-019-43320-5.

[44] Chen J, Liu B, Yao X, Yang X, Sun J, Yi J, *et al*. AMPK/SIRT1/PGC-1α Signaling Pathway: Molecular Mechanisms and Targeted Strategies From Energy Homeostasis Regulation to Disease Therapy. CNS Neuroscience & Therapeutics. 2025; 31: e70657. https://doi.org/10.1111/cns.70657.

[45] Li S, Shi Y, Liu P, Song Y, Liu Y, Ying L, *et al*. Metformin inhibits intracranial aneurysm formation and progression by regulating vascular smooth muscle cell phenotype switching via the AMPK/ACC pathway. Journal of Neuroinflammation. 2020; 17: 191. https://doi.org/10.1186/s12974-020-01868-4.

[46] Wang Y, Yu W, Li S, Guo D, He J, Wang Y. Acetyl-CoA Carboxylases and Diseases. Frontiers in Oncology. 2022; 12: 836058. https://doi.org/10.3389/fonc.2022.836058.

[47] Wang Q, Sun Y, Li TY, Auwerx J. Mitophagy in the pathogenesis and management of disease. Cell Research. 2026; 36: 11–37. https://doi.org/10.1038/s41422-025-01203-7.

[48] Tian S, Zhang Y, Liu C, Zhang H, Lu Q, Zhao Y, *et al*. Double-edged mitophagy: balancing inflammation and resolution in lung disease. Clinical Science (London, England : 1979). 2025; 139: 1047–1072. https://doi.org/10.1042/CS20256705.

[49] Narendra DP, Youle RJ. The role of PINK1-Parkin in mitochondrial quality control. Nature Cell Biology. 2024; 26: 1639–1651. https://doi.org/10.1038/s41556-024-01513-9.

[50] Park S, Kozhushko N, Wight TH, Whitworth AJ. Insights into PINK1/Parkin function and dysfunction from Drosophila models. The Biochemical Journal. 2025; 483: BCJ20253459. https://doi.org/10.1042/BCJ20253459.