

Protective effects of luteolin-7-O-glucoside against starvation-induced injury through upregulation of autophagy in H9c2 Cells

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Summary

Cardiomyocyte nutrient deprivation is a common clinical event that mediates various cardiac ischemic processes and is associated with autophagy activation and cell survival or death. Luteolin-7-O-glucoside (LUTG) was one of the flavonoid glycosides isolated from *Dracocephalum tanguticum*. Previous research had showed that LUTG pretreatment had significant protective effects against doxorubicin-induced cardiotoxicity. However, whether LUTG could protect cardiomyocytes from starvation-induced injury was not clear. In this study, cardioprotection and mechanisms of LUTG against starvation-induced injury were investigated *in vitro*. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) assay showed starvation-induced autophagy is a homeostatic and protective response for H9c2 cell survival. LUTG could protect against injury induced by starvation in H9c2 cells. Acridine orange (AO) staining showed that pretreatment with LUTG enhanced lysosomal autophagy. Western blotting indicated that LUTG enhanced autophagy by down-regulating the expression of phospho-extracellular signal regulated kinase1/2 (p-ERK), phospho-protein kinase B (p-Akt) and phospho-mammalian target of rapamycin (p-mTOR). These results suggest that LUTG might act as a promising therapeutic agent for preventing starvation-induced cardiotoxicity by upregulation of autophagy through the Akt/mTOR and ERK signal pathway.

Keywords: Luteolin-7-O-glucoside (LUTG), starvation, autophagy, extracellular signal regulated kinase (ERK), mammalian target of rapamycin (mTOR)

1. Introduction

Cardiomyocyte nutrient deprivation is a common clinical event that mediates various cardiac ischemic processes such as coronary heart disease and heart failure (1,2). In response to starvation, autophagy is upregulated that provides an internal source of nutrients for energy generation and, thus promotes survival (3,4). Autophagy is a process of self-cannibalization in which double-

membrane autophagosomes sequester organelles or portions of cytosol and fuse with lysosomes for breakdown by resident hydrolases (5,6). The resulting breakdown products can be recycled back to the cytosol for reuse during starvation, and are used to generate energy and to build new proteins and membranes (5,6). Autophagy may function primarily as a cytoprotective mechanism, to maintain nutrient and energy homeostasis during starvation conditions (7,8). However, activation of autophagy can also be harmful: excessive autophagy might cause undesirable cell death (9,10). Additional studies are needed to clarify whether autophagy activation serves as an adaptive or maladaptive cardiac response to starvation.

Autophagy is involved in the pathological process of many cardiovascular diseases, and more and more drugs are being studied that can regulate autophagy

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(2,11). Regulation of autophagy may be an effective mechanism for drug therapy. *Dracocephalum tanguticum* Maxim (Labiatae), a perennial herb distributed in the western region of China, has been used traditionally as folk medicine with a wide range of effects such as anti-hypoxia activity and less toxic side effects for treating gastritis, hepatitis, dizziness, rheumatoid arthritis, and scabies. The herb can effectively scavenge oxygen free radicals to reduce lipid peroxidation and regulate calcium ion stability, and thus protect the myocardial cell from injury. In searching for cardioprotective agents from these natural products, *Dracocephalum tanguticum* Maxim was investigated. We have reported the isolation, structure elucidation and evaluation of antioxidant, and cytoprotective activity of 17 flavonoids along with their preliminary structure-activity relationships (12). The results have demonstrated that flavonoids with OH groups at 3', 4'-position in the B-ring, and a double bond between C-2 and C-3 were necessary for their protective effects against doxorubicin (DOX)-induced cardiotoxicity. LUTG, luteolin-7-O- β -D-glucopyranoside, was isolated from the plants of *Dracocephalum tanguticum* Maxim which had this structure, and the previous study had shown that among all of the tested compounds, LUTG exhibited both a strong antioxidative effect and high protective activity against DOX-induced toxicity. Further investigation found LUTG could decrease DOX-induced death of H9c2 cells, reduce creatine kinase and lactate dehydrogenase levels, and inhibit the elevated intracellular $[Ca^{2+}]$ concentration. LUTG showed a cardioprotective effect by inhibiting the DOX-induced intracellular level of ROS and apoptosis (12,13). Furthermore, pretreatment with LUTG did not decrease the antineoplastic activity of DOX (12). Taken together, LUTG may act as a promising therapeutic agent for preventing the cardiotoxicity induced by DOX. Whether LUTG could protect against starvation-induced cardiomyocyte injury and the underlying mechanisms are not clear.

The mammalian target of rapamycin (mTOR) plays a central role in autophagy by integrating the class I PI3K signaling and amino acid-dependent signaling pathways (10,14). Autophagy is induced by nutrient starvation through the inhibition of mTOR, resulting in translocation of the mTOR substrate complex from the cytosol to certain domains of the endoplasmic reticulum (ER) or closely attached structures (15,16). Otherwise, activation of the phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) pathway, by expressing an active form of Akt, or expressing a constitutively active form of 3-phosphoinositide-dependent protein kinase 1 (PDK1), has an inhibitory effect on autophagy (10,17). The mTOR signaling pathway is critical because of its ability to integrate the information from nutrient, metabolic and hormonal signals (10,18). Besides, amino acids inhibit the rapidly accelerated fibrosarcoma-mitogen activated

protein kinase kinase1 (Raf1)/mitogen-activated protein kinase kinase1/2 (MEK1/2)/extracellular signal regulated kinase1/2 (ERK1/2) signaling cascade, leading to inhibition of autophagy (10).

In the present study, we investigated the effects and the related mechanisms of LUTG against starvation-induced cardiotoxicity using H9c2 cells *in vitro*. The results demonstrated that LUTG could prevent cardiomyocytes from starvation-induced injury and the protective effects may be related to upregulation of autophagy through the PI3K/Akt and ERK pathway.

2. Materials and Methods

2.1. Materials and chemicals

LUTG was isolated from *Dracocephalum tanguticum* Maxim by Prof. Ren in our school (12). In the present study, LUTG was dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* assay and stored at a concentration (100 mM) that was diluted with Dulbecco's modified Eagle's medium (DMEM), and a solvent control with DMSO was performed at no more than 2% (v/v).

DMEM and Earle's Balanced Salt Solution (EBSS) were from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Tianjin Biotechnology Development Center (Tianjing, China). Bicinchoninic acid (BCA) protein assay kit and radio immunoprecipitation assay (RIPA) buffer were from Beyotime Institute of Biotechnology (Beijing, China). Antibodies against p-Akt and Akt were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Antibody against P44/42-ERK1/2, ERK1/2, P-PTEN, PTEN, P-mTOR, mTOR, P-Beclin1, Beclin1, LC3B and β -actin were from Cell Signaling Technology (Boston, USA). Antibodies against horseradish peroxidase (HRP)-conjugated secondary antibody were from ZSGB-BIO (Beijing, China). 3-Methyladenine (3-MA) was from Millipore (Massachusetts, USA). Phenylmethylsulfonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) and other chemicals were from Sigma.

2.2. Cell Culture

Rat cardiac H9c2 cells (ATCC Rockville, MD, USA) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 μ g/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere (5% CO₂-95% air). The cells were fed every 1-2 days, and detached using 0.05 trypsin/0.02% EDTA when they reached about 80-90% confluence. Then cells seeded at an appropriate density according to each experimental design, and the cells used were in the exponential phase of growth before exposure to drugs in all experiments.

2.3. Cell treatment with LUTG

H9c2 cells were seeded at an appropriate density. Following overnight adherence, cells preincubated with or without LUTG (10 and 20 μM) for 24 h followed by starvation (incubation with EBSS) for another 24 h. The concentration of LUTG was used according to the result of the *in vitro* cell proliferation assay. After this incubation, cells were harvested after trypsin digestion by centrifugation (1000 rpm \times 5 min) and parameters were measured as described in materials and methods.

2.4. Cell viability *in vitro*

H9c2 cells were seeded in 96-well plates at a density of 5,000 cells/well. Following overnight adherence, cells were incubated with LUTG (5, 10, 20, 40 and 80 μM) in DMEM supplemented with 10% fetal bovine serum at 37°C for 24 h followed by incubation with EBSS for another 0, 3, 6, 12, and 24 h, and cell viability was determined by MTT assay. Cells were treated with MTT solution (final concentration, 0.5 mg/mL) for 4 h. The supernatants were removed carefully, followed by the addition of 100 μL DMSO to each well to dissolve the precipitate. The absorbance was measured at 570 nm in a microplate reader (Synergy HT).

2.5. Cell cycle measurement

H9c2 cells were cultured in 6-well plates and then starved for 0, 3, 6, 12, 24 h respectively. Cells were harvested and washed, and fixed in cold 70% ethanol overnight, and then suspended in 1 mL propidium iodide (PI) solution (50 $\mu\text{g}/\text{mL}$ DNase-free RNase A) for 30 min. Cell cycle was analyzed using a FACScan Flow Cytometer (Becton Dickinson, New Jersey, USA). The percentages of cells in G0/G1, S and G2/M phases were determined using ModFit LT software 3.0 (Varity Software House, Topsham, USA).

2.6. Detection of intracellular acid phosphatase

The activity of acid phosphatase in the lysosomes increased when the lysosomal degradation (autophagy) was enhanced. To detect the intracellular acid phosphatase, acridine orange (AO) was utilized as an intracellular indicator of acidic phosphatase (19). In this study, H9c2 cells were seeded overnight in 24-well plates, and pretreated without or with LUTG at concentrations of 5, 10 and 20 μM for 24 h, followed by incubation with EBSS for another 12 h. The cells were fixed using 95% ethanol solution for 15 min. H9c2 cells were washed using phosphate buffered saline (PBS) and further incubated with 0.01% AO (500 μL) for another 15 min. The cells were washed 2 times with PBS before being observed and photographed under the fluorescence microscope (IX-7, Olympus, Tokyo, Japan) (400 \times).

2.7. Western blotting analysis

After H9c2 cells were cultured in 6-well plates and treated with conditioned media as indicated, cells were collected and lysed with RIPA buffer on ice for 30 min. The suspension was centrifuged at 13,000 g for 15 min at 4°C, and the supernatant was collected. Protein concentration in total cell lysate was measured using the BCA protein assay kit with bovine serum albumin (BSA) as standard. The other supernatants were stored at -80°C for Western blotting analysis.

After addition of sample loading buffer, protein samples were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) (Millipore Corporation, Massachusetts, USA) in Tris-glycine buffer. The membrane was blocked with 5% (w/v) non-fat dry milk in 20 mM Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 2 h, followed by incubation with the appropriate primary antibodies at 4°C overnight and then washed three times and exposed to HRP-conjugated secondary antibodies in TBST containing 5% non-fat dry milk for 1 h at room temperature. The primary antibodies included cyclin D1, p21, p-Beclin1, Beclin1, microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B, LC3B), p-Akt1/2/3, Akt1/2/3, phosphatase and tensin homolog deleted on chromosome ten (PTEN), p-PTEN, p44/42-ERK1/2, ERK1/2, p-mTOR, mTOR and β -actin. The membranes were washed with TBST three times, and the antigen-antibody bands were detected using an enhanced chemiluminescence reagent kit (Millipore, Massachusetts, USA) and quantified by densitometry using a ChemiDoc XRS+image analyzer (Bio-Rad, California, USA).

2.8. Statistical analyses

Data are described as the mean \pm S.E.M. and analyzed by one-way ANOVA. A *p* value < 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS/Win13.0 software (SPSS, Inc., Chicago, IL).

3. Results

3.1. Effect of starvation on cell viability

To analyze the effects of starvation on the proliferation of H9c2 cells, cell viability was evaluated with the MTT assay. As shown in Figure 1, cell viability was decreased by starvation in a time-dependent manner. There was nearly a 40% decrease in cell viability after 12 hours of starvation compared with the control group (*p* < 0.01).

3.2. Effect of starvation on cell cycle

After H9c2 cells were incubated in starvation medium

for different time durations, cell cycle was measured using flow cytometry and the expression of cell cycle proteins p21 and cyclin D1 were detected by Western blotting. As shown in Figure 2A and 2B, under starvation conditions, the number of myocardial cells in S phase decreased; G0/G1 phase cells increased significantly compared with the normal group. This indicated that starvation induced myocardial cell cycle arrest in the G0/G1 phase, and prevented them from moving to the S phase transformation. As shown in Figure 2C, the

expression of p21 and cyclin D1 continued to decline with time of starvation. This indicated that starvation blocked the myocardial cell cycle by inhibiting the expression of p21 and cyclin D1.

3.3. Effect of starvation on myocardial cell autophagy

To investigate the effect of starvation on myocardial cell autophagy, the expression of p-Beclin1 and LC3B were detected by Western blotting. As shown in Figure 3A, the expression of p-Beclin1 was upregulated, while the expression of LC3B decreased with a longer starvation duration. Autophagy inhibitor 3-MA was used to detect the role of autophagy in the cells under normal and starvation conditions, and validated whether autophagy is conducive to the survival of cells. As shown in Figure 3B, 3-MA and starvation could also decrease the viability of H9c2 cells ($p < 0.01$). Furthermore, 3-MA could more significantly inhibit the viability of H9c2 cells under starvation conditions (compared with starvation, $p < 0.01$). It indicated that autophagy was a protective mechanism for normal cells and cells under starvation.

3.4. Effect of starvation on the expression of Akt and ERK proteins

After H9c2 cells were incubated in starvation medium

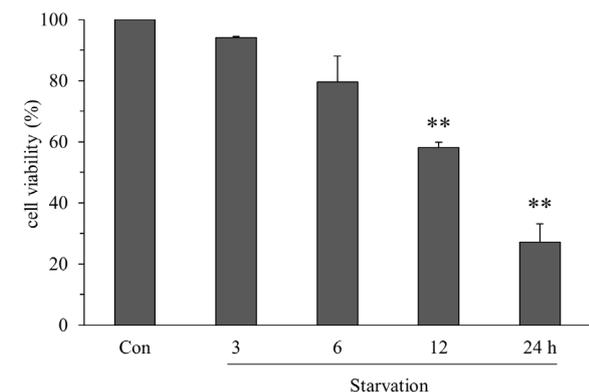


Figure 1. Starvation decreased cell viability in a time-dependent manner. H9c2 cells were incubated in starvation medium (EBSS) for 0, 3, 6, 12, or 24 h respectively. Cell viability was quantified using the MTT assay. ** $p < 0.01$ compared with control.

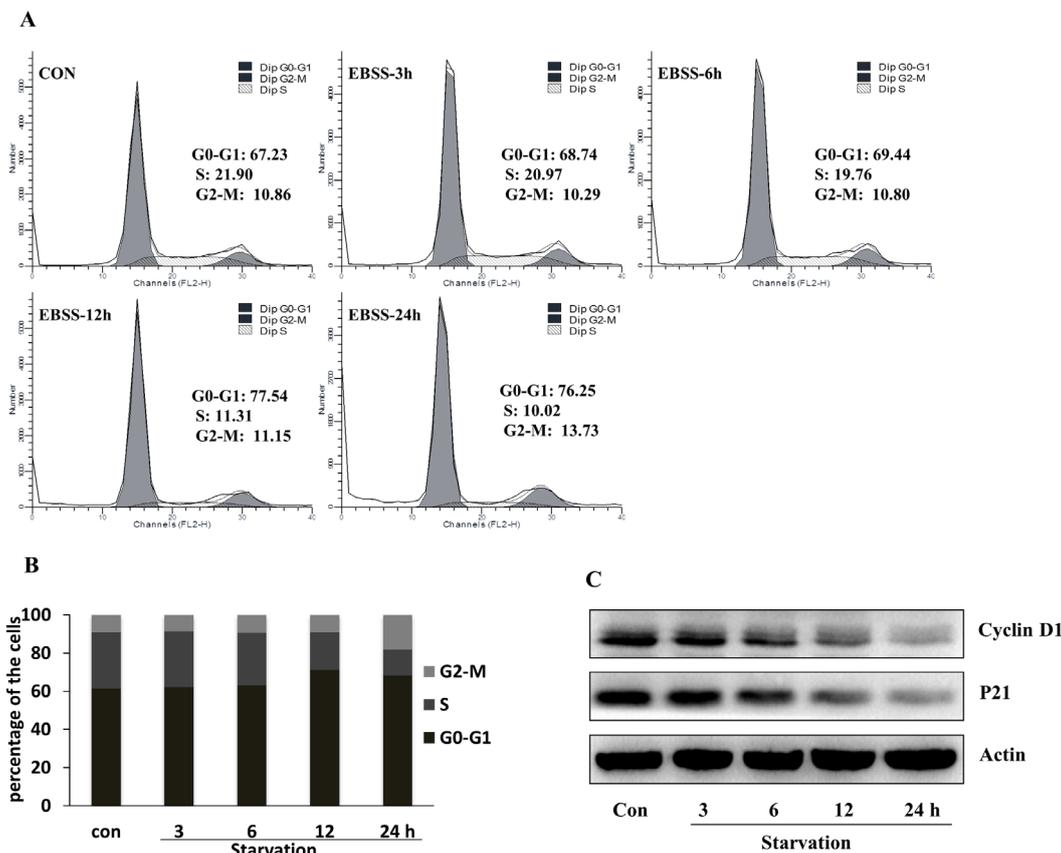


Figure 2. Starvation inhibited the cell cycle. H9c2 cells were incubated in starvation medium for 0, 3, 6, 12, or 24 h respectively, the cell cycle was detected by flow cytometry (A) and the expression of p21 and cyclin D1 were detected by Western blotting (B).

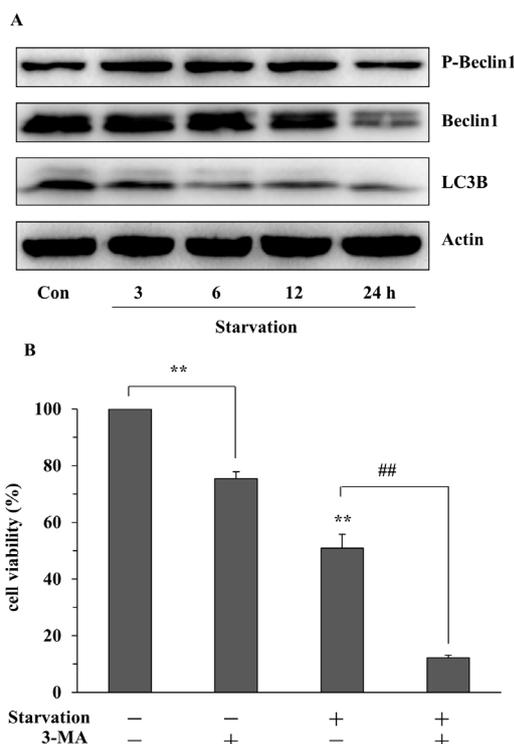


Figure 3. Effects of starvation on autophagy and cell viability. H9c2 cells were incubated in starvation medium for 0, 3, 6, 12, or 24 h respectively, and the expression of autophagy-associated proteins was detected by Western blotting (A). H9c2 cells co-treated with or without 3-MA were incubated in normal or starvation medium. Cell viability was quantified using the MTT assay (B). ** $p < 0.01$ compared with normal group. ### $p < 0.01$ compared with starvation group.

for different durations, the expression of Akt, mTOR, PTEN and ERK were detected by Western blotting. As shown in Figure 4, starvation pretreatment could decrease the expression of p-Akt and p-mTOR. The expression of p-ERK under starvation for 3 h was down-regulated slightly, and was up-regulated after 3 h, but the expression of p-PTEN was not changed. These results indicated that starvation-induced autophagy might be regulating the expression of p-Akt, p-mTOR, and p-ERK.

3.5. Effect of LUTG on cardiomyocyte injury induced by starvation

MTT assay was used to detect the protective effect of LUTG on cell injury induced by starvation. As shown in Figure 5, LUTG (5, 10, and 20 μM) could significantly increase cell viability and protect the H9c2 cells from injury induced by starvation ($p < 0.05$ and $p < 0.01$).

3.6. Effect of LUTG on cardiomyocyte autophagy induced by starvation

To investigate the effect of LUTG on autophagy, the expression of LC3B was detected by Western blotting and autophagy inhibitor 3-MA was used to detect whether the protective effect of LUTG was related to

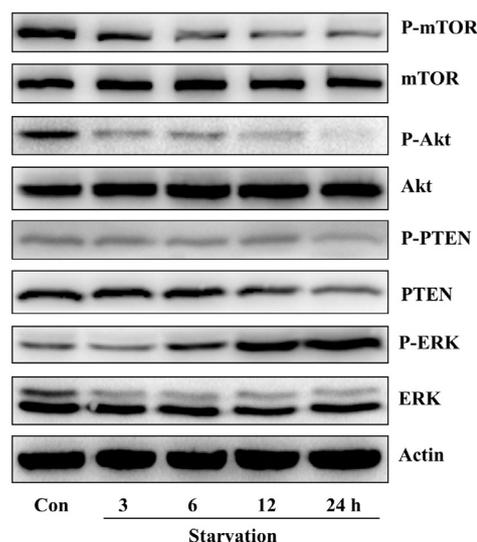


Figure 4. The expression of Akt and ERK proteins in starved H9c2 cells. H9c2 cells were incubated in starvation medium for 0, 3, 6, 12, or 24 h respectively, the expression of Akt and ERK proteins were detected by Western blotting.

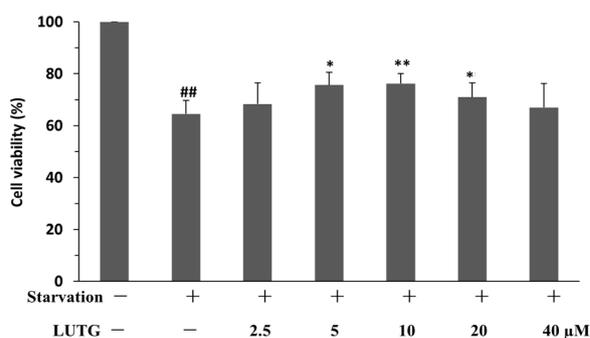


Figure 5. Effects of LUTG on cell viability induced by starvation in H9c2 cells. Cells were incubated without or with LUTG (2.5, 5, 10, 20, and 40 μM) for 24h, followed by incubation with EBSS for another 12h. After this incubation, cell viability was determined with the MTT assay. Values are represented as mean \pm S.E.M. ($n = 6$). ### $p < 0.01$ compared with the normal group; * $p < 0.05$, ** $p < 0.01$ compared with starvation group.

autophagy. As shown in Figures 6A and 6B, LUTG could increase the expression of LC3B and cell viability compared with starvation. 3-MA could reduce the protective effect of LUTG on cell injury induced by starvation. This indicated that LUTG could protect H9c2 cells against starvation-induced injury through enhancing cell autophagy.

To confirm the effect of LUTG on autophagy, AO staining was used to detect lysosomal degradation by measuring the activity of acid phosphatase. AO as an intracellular indicator emits red fluorescence in acidic lysosomes, and displays green fluorescence in neutralized cytosol and nuclei. As shown in Figure 6C, intracellular eosinophilic granules increased under starvation conditions and LUTG further increased the number of red eosinophilic granules in the cell. This indicated that LUTG enhanced lysosomal degradation,

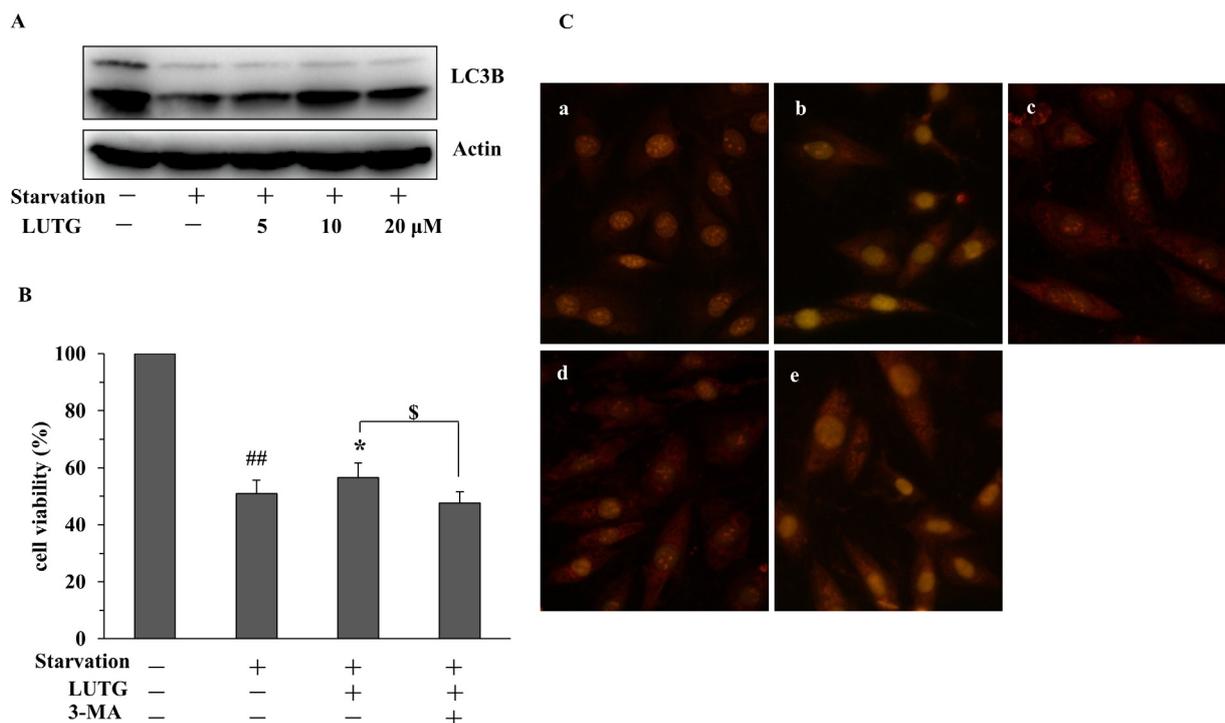


Figure 6. Effect of LUTG on cell autophagy. (A) Western blotting. Cells were incubated without or with LUTG (5, 10, and 20 μM) for 24 h, followed by incubation with EBSS for another 12 h. After this incubation, the expression of LC3B was detected by Western blotting. (B) MTT assay. H9c2 cells pretreated with or without 3-MA and LUTG (10 μM) for 24 h, and incubated in starvation medium for 12 h. Cell viability was detected by MTT assay. ##*p* < 0.01 compared with the normal group; ***p* < 0.01 compared with starvation group; \$*p* < 0.05 compared with LUTG group. (C) Lysosomal degradation. H9c2 cells were pretreated without or with LUTG for 24 h, followed by incubation with EBSS for another 12 h. Morphology of H9c2 cells was assessed using a fluorescence microscope (×400) equipped with quick imaging system without/with AO staining. (a) Control; (b) Starvation; (c) 5 μM LUTG plus starvation; (d) 10 μM LUTG plus starvation; (e) 20 μM LUTG plus starvation.

that is, the enhancement of autophagy.

3.7. Effect of LUTG on the expression of p-Akt, p-mTOR, and p-ERK proteins

To investigate the protective mechanisms of LUTG on H9c2 cell injury induced by starvation, the expression of p-mTOR, p-ERK and p-Akt proteins was detected by Western blotting. As shown in Figure 7, under starvation conditions, the expression of p-ERK increased; and the expression of p-mTOR, p-Akt, and p21 decreased. LUTG upregulated the expression of p21, inhibited the expression of p-ERK and further down regulated the expression of p-Akt and p-mTOR. This indicated that LUTG protected against injury induced by starvation in H9c2 cells by regulating the Akt/mTOR and ERK signaling pathways.

4. Discussion

Autophagy is a conserved cellular pathway that controls protein and organelle degradation, and has essential roles in survival, development and homeostasis (3,10). It is rapidly regulated by nutrient starvation, growth factor withdrawal, or oxidative damage. Because cardiac myocytes are terminally differentiated, cellular degradation *via* autophagy plays an important role in

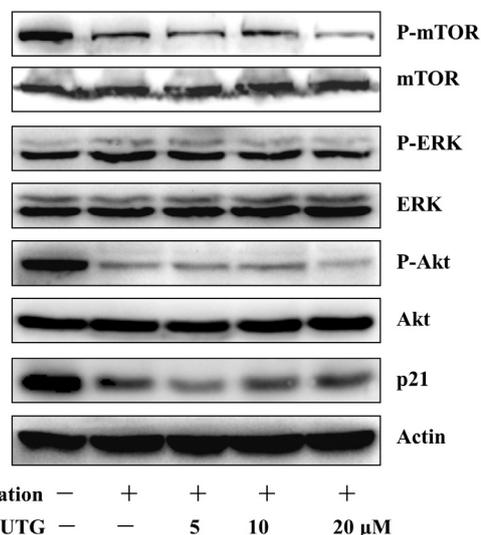


Figure 7. Effects of LUTG on the expression of p-Akt, p-ERK, p-mTOR and p21 in H9c2 cells. After 24 h of treatment without or with LUTG (5, 10, and 20 μM), cardiomyocytes were starved for another 24 h. The expression of p-Akt, p-ERK, p-mTOR, and p21 was detected by Western blotting.

the homeostasis of cardiac cells (20,21). In addition, cardiomyocyte nutrient deprivation is a common clinical event that mediates various cardiac ischemic

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