Wenyang-Yiqi Granule Suppresses Oxygen-Glucose Deprivation-Induced Cardiomyocyte Autophagy Through Mammalian Target of Rapamycin Activation in H9c2 Cells

Shuibo Gao[1] Xiaofang Yu[2] Lihua Han[2] Hong Wu[1,2]

[1] Laboratory of Cell Imaging, Second School of Clinical Medicine, Henan University of Chinese Medicine, Zhengzhou, Henan, China
[2] Institute of Cardiovascular Disease, Henan University of Chinese Medicine, Zhengzhou, Henan, China

Address for correspondence Hong Wu, MD, Laboratory of Cell Imaging, Second School of Clinical Medicine, Henan University of Chinese Medicine, 6 Dongfeng Road, Zhengzhou, Henan, 450002, China (e-mail: wuhong@hactcm.edu.cn).

CMNP 2023;3:e41–e50.

Abstract

Background Wenyang-Yiqi Granule (WYYQ) is a four-component herbal formula, widely used to treat heart failure in China. It is known to regulate autophagy, but the mechanism(s) are unknown.

Methods H9c2 cells were treated with WYYQ for 24 hours prior to oxygen-glucose deprivation (OGD). Expressions of the autophagy markers Beclin-1 and light chain 3 (LC3) were evaluated via quantitative polymerase chain reaction analysis. Protein levels of Beclin-1, LC3, p62, and mammalian targets of rapamycin (mTOR) were determined by Western blot analysis. Transmission electron microscopy was used to explore the effects of WYYQ on autophagosome formation.

Results Treatment with WYYQ dramatically restrained OGD-induced autophagy, which was characterized by an inhibition of Beclin-1 and increased LC3 mRNA expression. In addition, WYYQ decreased the expression of Beclin-1 and the ratio of LC3-II/LC3-I; however, the abundance of p62 was enhanced at the protein level. Manipulation of the LC3-II/LC3-I ratio, p62 abundance, and autophagosome formation in response to WYYQ were associated with mTOR activity.

Conclusions These findings show that WYYQ plays a protective role during hypoxic-ischemic stress through the suppression of excessive autophagy, which may be partially explained by its effects on mTOR. These data provide novel insight into the cardioprotective effects of WYYQ during cardiomyocyte autophagy.

Keywords
- cardiomyocyte autophagy
- H9c2 cell
- heart failure
- mTOR pathway
- oxygen-glucose deprivation
- Wenyang-Yiqi Granule

Introduction

Heart failure (HF) is a clinical syndrome characterized by the inability of the heart to meet the metabolic demands of the human body.[1,2] Myocardial remodeling is associated with an increased risk of HF. Previous studies have shown that autophagy, a lysosomal-dependent process that degrades intracellularly dysfunctional components, played an intricate role in HF.[3,4] Emerging evidence suggested that autophagy mediates myocardial remodeling through the enhanced activity of lysosomes and upregulation of autophagosome production.[5] Consequently, therapeutic strategies aimed at the regulation of autophagy in HF represent an adaptive response for the protection of cardiomyocytes from hypoxic-ischemic stress.

received
September 7, 2022
accepted after revision
October 20, 2022
article published online
March 14, 2023

DOI https://doi.org/10.1055/s-0043-1764132.
ISSN 2096-918X.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Wenyang-Yiqi Granule (WYYQ) is widely used for the prevention and treatment of HF in China, and it is a compound medication that includes Wutou (Aconiti Radix Lateralis praeparata),[6] Rougui (Cinnamomi Cortex),[7] Huangqi (Astragali Radix),[8] and Renshen (Panax Ginseng).[9] Previous studies showed that WYYQ exerts inhibitory effects on the excessive activation of the renin–angiotensin–aldosterone system (RAAS) in rats with HF.[10] However, the mechanism(s) underlying anti-RAAS over-activation were not defined. RAAS overactivation leads to cellular injury by promoting autophagic activity.[11,12] Furthermore, cardiomyocyte autophagy is modulated by angiotensin II,[13] an activator of RAAS. Therefore, this study speculated that WYYQ inhibits RAAS activity by regulating autophagy in cardiomyocytes.

Changes in the autophagy-related proteins Beclin-1, light chain 3 (LC3), and p62 reflect the status of autophagy in cells.[14,15]A higher LC3-II/LC3-I ratio promotes autophagy dysfunction.[16,17] Autophagy is regulated by the mammalian target of rapamycin (mTOR), a serine/threonine kinase. mTOR acts as a pivotal component of two distinct signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which regulate diverse cellular behaviors.[18] Genetic inhibition of mTORC1 triggers autophagy in yeast and in Drosophila,[19] while starvation-induced autophagy can be inhibited by mTORC1 activation.[20] In addition, mTORC1 modulates autophagy at the transcriptional level by fine-tuning lysosomal genes and their activities.[21] Thus, mTOR signaling has emerged as a target to improve cardioprotection.

The relationship between WYYQ and mTOR signaling in response to HF has been poorly characterized to date. Appropriate interventions using WYYQ may limit myocardial remodeling and regulate autophagy in HF. This study established an oxygen-glucose deprivation (OGD)-mediated in vitro model to mimic autophagic conditions. The pleiotropic effects of WYYQ on the expression of the autophagy-related proteins Beclin-1, LC3, and p62 were examined. Furthermore, the underlying mechanism(s) with which WYYQ modulates mTOR signaling were explored based on autophagic dysfunction.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Sciencell Research Laboratories (Sciencell, Carlsbad, CA, United States). Rapamycin was obtained from Yuanye Bio-Technology (Shanghai, China). CCK-8 was purchased from Dojindo Laboratories (Shanghai, China). Annexin V-FITC-PI Apoptosis Detection Kits were purchased from Becton, Dickinson and Company (BD) (Franklin Lakes, NJ, United States). Antibodies directed against LC3B (sc-398822), mTOR (sc-517464), and p-mTOR (sc-293133) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Horseradish peroxidase-conjugated anti-rabbit IgG antibodies (SA00001-2) and antibodies directed against Beclin-1 (11306-1-AP), p62 (18420-1-AP), and GAPDH (10494-1-AP) were purchased from ProteinTech Biotechnology (Wuhan, China). WYYQ, composed of Wutou (Aconiti Radix Lateralis praeparata), Rougui (Cinnamomi Cortex), Huangqi (Astragali Radix), and Renshen (Panax Ginseng), was purchased from Sichuan Xinlv Pharmaceutical Co., Ltd. (Sichuan, China).

Cell Culture

H9c2 cells, a rat cardiomyocyte cell line, were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in an incubator at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

Oxygen-Glucose Deprivation Treatment

OGD-induced cell injury models were established as previously described.[22] Briefly, H9c2 cells were seeded at a density of 1.0×10^5 cells in 24-well culture plates under normal culture conditions. For OGD treatment, cells at 80 to 90% confluence were switched to serum-free and glucose-free DMEM and were transferred to a tri-gas incubator perfused with 95% N_2 and 5% CO_2 for 6 hours. Subsequently, cells were pretreated with WYYQ at different concentrations (0.4, 0.8, and 1.6 mg/mL) and/or rapamycin (10 nM) for 24 hours prior to establishing the OGD model. Cells that served as the control group were incubated in a standard medium.

Cell Viability Assays

For cell viability assays, H9c2 cells were seeded at a density of 0.7×10^5 cells per well in 96-well plates. After 24 hours, various concentrations of WYYQ (0, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/mL) were applied. Cells were continuously cultured for 24 hours prior to the addition of 10 µL of medium containing CCK-8 reagent for each well. Plates were incubated for 30 h at 37°C in the dark, and the absorption at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer's instructions. Data shown are the average of six wells per group.

Transmission Electron Microscopy Assessments

Transmission electron microscopy (TEM) was performed as previously described.[23] In brief, H9c2 cells were harvested and fixed in 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, United States) in 0.1 M phosphate-buffered saline (pH 7.4) at 4°C for 2 hours. Cells were then fixed in 1% osmium acid at 4°C for 1 hour, dehydrated in gradient ethanol, embedded in epoxy resin 812 mixed with acetone, sliced, and stained with uranyl acetate and lead citrate. Images were obtained via TEM (JEM-1400, JEOL Ltd., Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from H9c2 cells using the Trizol reagent, followed by subsequent cDNA synthesis through

Table 1 PCR primer sequence

Gene	Primer sequence
LC3	Fwd: 5′-CAGATGAAGACACCCTGATTGA-3′
	Rev: 5′-TCCAGACGTTTCAGAGCTAATG-3′
Beclin-1	Fwd: 5′-CAGGAACTCACAGCTCCATTAC-3′
	Rev: 5′-CCATCCTGGCGAGTTTCAATA-3′
β-actin	Fwd: 5′-GAACCCTAAGGCCAACCGTG-3′
	Rev: 5′-AGGCATACAGGGACAACACAG-3′

reverse transcription (1 μg) using RT EasyTM II Kits (FOREGENE, Chengdu, China). Real-time polymerase chain reaction EasyTM-SYBR Green I Kit (FOREGENE, Chengdu, China) and primers listed in ►**Table 1** were used. Fold changes in gene expression were measured as follows: $2^{-\Delta\Delta ct}$, where ΔCt represents the differences in cycle threshold levels between samples.

Western Blot Analysis

Western blots were performed as previously described.[24] Total cellular proteins were extracted from H9c2 cells, and their concentrations were determined using bicinchoninic acid (BCA) kits (Beyotime Biotechnology, Shanghai, China). Lysates were resolved via SDS-PAGE and transferred to PDVF membranes to determine corresponding protein expressions. Membranes (Millipore, Billerica, MA, United States) were probed with primary antibodies directed against LC3 (1: 1000), Beclin-1 (1: 500), p62 (1:1,000), mTOR (1: 1000), and p-mTOR (1: 1000) at 4°C overnight. Membranes were washed and labeled with secondary goat-antimouse or goat-antirabbit antibodies. GAPDH (1:1,000) was probed as the loading control. Blots were visualized using ECL solution using the Bio-Rad Gel Doc XR^+ Imaging System (Bio-Rad, Hercules, CA, United States).

Annexin-V FITC/PI Assay

Cellular apoptotic rates were determined using Annexin-V FITC/PI assay kits as previously described.[25] Briefly, cells pretreated with WYYQ were collected and resuspended in binding buffer (provided with the kit) at 1.0×10^5 cells/mL. Cells were labeled with annexin-V-FITC and propidium iodide (PI) for 20 minutes at room temperature in the dark and were analyzed by flow cytometry (JAZZ; BD Biosciences, San Diego, CA, United States). Data were analyzed using FlowJo 7.6 software (TreeStar, Ashland, OR, United States).

Statistical Analysis

Experiments were performed in six repetitions. Data are presented as the mean ± standard deviation. Statistical differences were determined by one-way analysis of variance followed by *LSD* tests for two-group comparisons amongst multiple comparisons. *p*-Values ≤ 0.05 were considered statistically significant.

Results

Oxygen-Glucose Deprivation Intervention Induces Autophagy in H9c2 Cells

H9c2 cells were exposed to OGD for 0, 3, 6, 9, and 12 hours, then harvested, and the expressions of autophagy-related genes or proteins were assessed. The data showed that the mRNA levels of Beclin-1 markedly increased at 6 hours of OGD compared with levels at 0 hour (►**Fig. 1A**). A significant enhancement in the expression of LC3 mRNA was also observed at 6 hours (►**Fig. 1B**). Western blot analysis showed that the LC3-II/LC3-1 ratio was significantly higher from 3 hours of OGD 3 to 12 hours (►**Fig. 1C**). Moreover, the expression of Beclin-1 was maximally upregulated at 6 hours of OGD (►**Fig. 1C**), while p62 levels were downregulated at 6 hours of OGD (►**Fig. 1C**). According to these data, 6 hours of OGD were selected as an appropriate time point for the induction of autophagy.

Wenyang-Yiqi Granule Increases Cell Viability and Reduces Apoptosis in H9c2 Cells under Oxygen-Glucose Deprivation Challenge

To determine the optimal concentration at which WYYQ protects against OGD-triggered cell injury, the cytotoxicity of WYYQ in H9c2 cells was first assessed following treatments with 0, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/mL WYYQ for 12 and 24 hours via CCK-8 assays. As shown in ►**Fig. 2A**, no cytotoxicity occurred with cumulative concentrations of WYYQ up to 1.6 mg/mL for 12 or 24 hours. However, an obvious decrease in cell viability was observed in cells treated with $\geq$ 3.2 mg/mL WYYQ. Therefore, for subsequent assessments, the three nontoxic doses of 0.4, 0.8, and 1.6 mg/mL WYYQ were selected as low, middle, and high dose groups, respectively.

Next, it was explored whether WYYQ protected against OGD-induced cardiomyocyte apoptosis. The apoptotic cell distribution triggered by OGD was examined in H9c2 cells using annexin V/PI staining. As shown in ►**Fig. 2B**, H9c2 cells were characterized as early apoptotic (annexin V^+/PI^-) or late apoptotic (annexin V^+/PI^+). The OGD challenge induced cell apoptosis in up to 45.12 ± 4.79% of cells, while the apoptotic rates of H9c2 cells treated with WYYQ at concentrations of 0.4, 0.8, and 1.6 mg/mL were 14.14 ± 2.01%, 7.21 ± 0.79%, and 5.93 ± 0.63%, respectively.

Wenyang-Yiqi Granule Downregulates Oxygen-Glucose Deprivation-Induced Autophagy in H9c2 Cells

The mRNA levels of Beclin-1 and LC3, both autophagy-promoting factors, were upregulated by the OGD challenge compared with the control group (►**Fig. 3A-B**). WYYQ treatment for 24 hours prior to the OGD challenge significantly downregulated Beclin-1 and LC3 at the mRNA level when compared with the OGD-treated group (►**Fig. 3A-B**). The protein levels of Beclin-1 were enhanced compared with those of the control group (►**Fig. 3C**), which was consistent with the mRNA expression of autophagy-associated Beclin-1 genes. The ratio of LC3-II/LC3-I was also enhanced in the OGD-treated group. In addition, the protein levels of p62, an

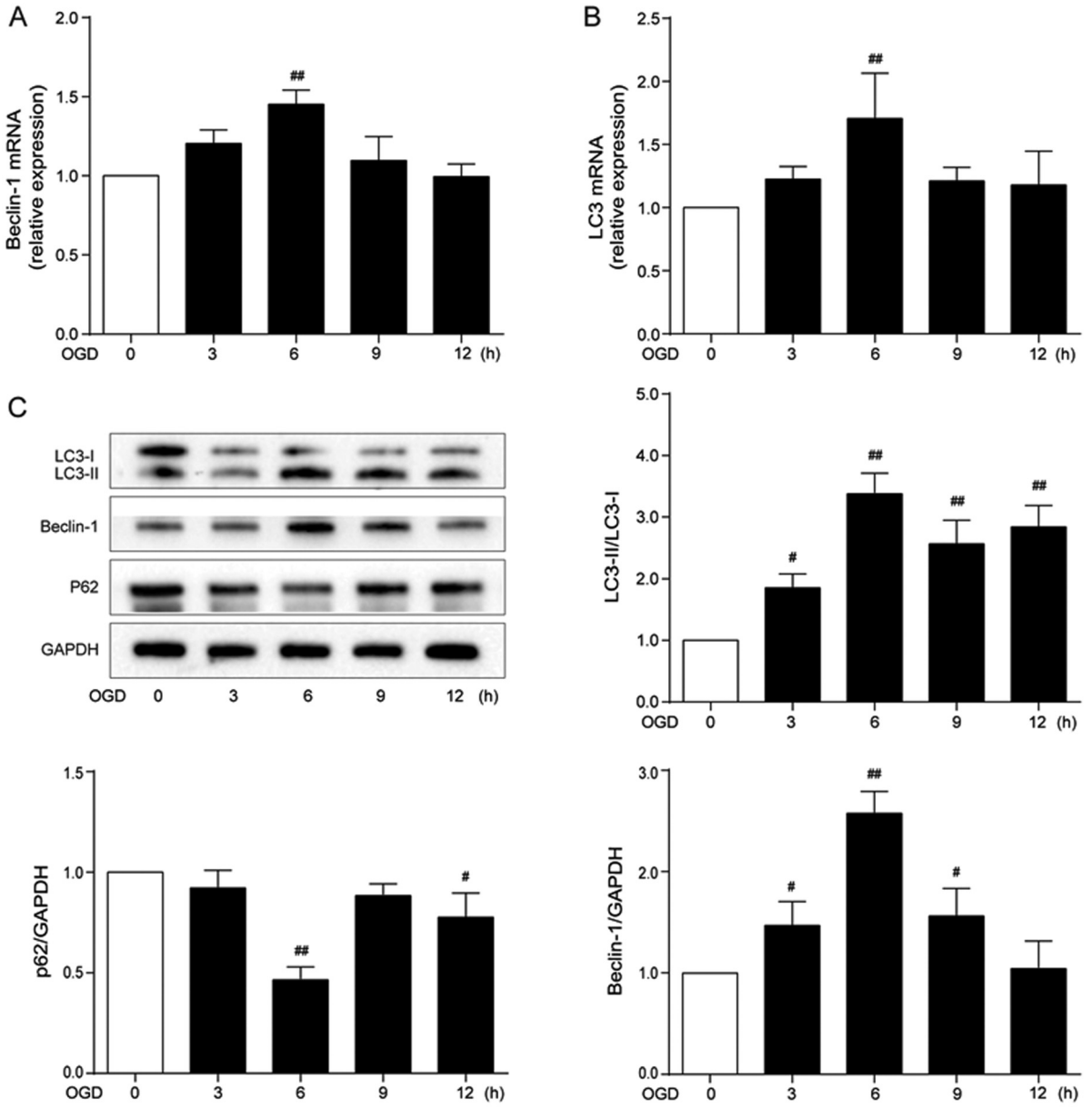

Fig. 1 Autophagy induction in response to oxygen-glucose deprivation in H9c2 cells. Cells were subjected to oxygen-glucose deprivation (OGD) for 0, 3, 6, 9, and 12 hours. (**A**) Beclin-1 and (**B**) LC3 mRNA levels were measured by qPCR. (**C**) Protein levels of Beclin-1, LC3, and p62 were assessed via Western blot analysis. Data are the mean ± SD. $n = 6$. $^{\#}p < 0.05$ versus control, $^{\#\#}p < 0.01$ versus control. SD, standard deviation; qPCR, quantitative polymerase chain reaction.

inhibitory of autophagy, were remarkably reduced in OGD-treated H9c2 cells when compared with those of the control and WYYQ-treated H9c2 cells that demonstrated increased p62 expression (►**Fig. 3C**). Taken together, these data suggested that WYYQ reduced OGD-induced autophagy in H9c2 cells and that 1.6 mg/mL WYYQ was the optimal level to inhibit autophagy.

Wenyang-Yiqi Granule Suppresses Autophagosome Production in Response to Rapamycin

TEM was used to evaluate the ultrastructure of H9c2 cells undergoing autophagy. As shown in ►**Fig. 4**, there was a trend toward increased autophagy induced by OGD in H9c2 cells when compared with control cells. In addition, the formation of autophagosomes was suppressed by treatment with WYYQ. In addition, rapamycin accompanied by OGD resulted in an increase in autophagy processes. The increased number of autophagosomes was suppressed by pretreatment with WYYQ.

Wenyang-Yiqi Granule Reduces Cardiomyocyte Autophagy Through Regulating the Mammalian Targets of Rapamycin Pathway

As shown in ►**Fig. 4**, WYYQ suppressed the rapamycin-induced increase of autophagosomes in response to OGD. Then, it was further investigated whether WYYQ could reduce autophagy-related gene or protein expression through regulation of the mTOR pathway. The results

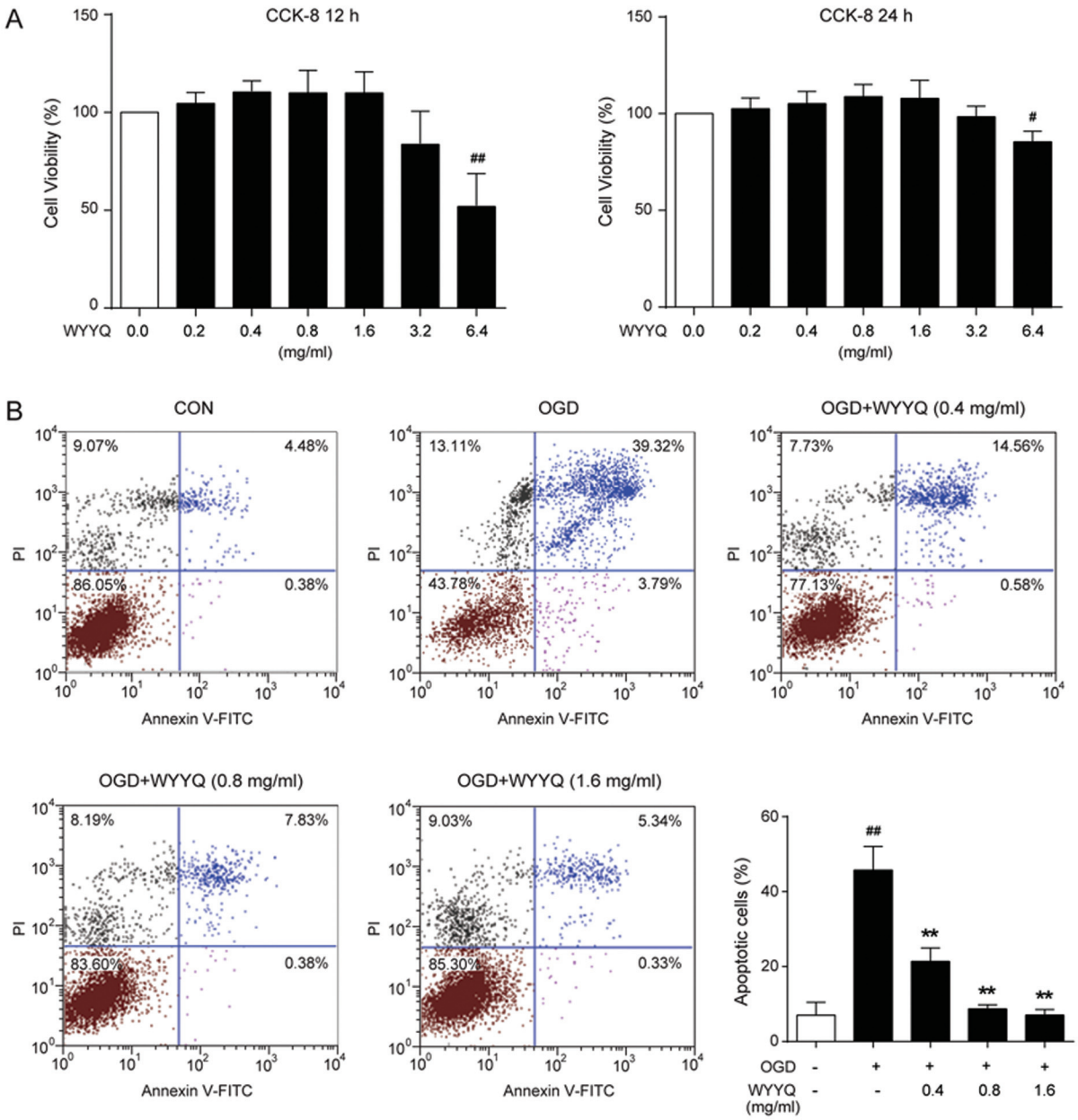

Fig. 2 Effects of WYYQ on cell viability and apoptosis induced by oxygen-glucose deprivation in H9c2 cells. (**A**) Cell viability detected by MTT following the treatment of WYYQ (0, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/mL). (**B**) Apoptotic rates of H9c2 cells detected by flow cytometry with Annexin-V FITC/PI staining. Data are the means ± SD. $n = 6$. $^{\#}p < 0.05$ versus control, $^{\#\#}p < 0.01$ versus control, and $^{**}p < 0.01$ versus OGD. SD, standard deviation; WYYQ, Wenyang-Yiqi Granule.

showed that rapamycin, an autophagy activator, increased the mRNA levels of Beclin-1 and LC3, which were significantly reduced by treatment with 1.6 mg/mL WYYQ (►**Fig. 5A and B**). Similarly, WYYQ at 1.6 mg/mL decreased the expression of Beclin-1 and the ratio of LC3-II/LC3-I at the protein level (►**Fig. 5C**). WYYQ also increased the expression of p62, which was decreased in H9c2 cells treated with rapamycin and OGD. Moreover, OGD resulted in a significant decrease in the phosphorylation of mTOR, which was abrogated by WYYQ (►**Fig. 5C**). Rapamycin also decreased mTOR phosphorylation; however, WYYQ did not reverse the decrease in phosphorylation in H9c2 cells treated with rapamycin, indicating that WYYQ mediated its inhibitory effects on autophagy through the mTOR pathway (►**Fig. 5C**).

Discussion

This study employed an OGD-induced cell model, which is an accepted model of autophagy. OGD exposure significantly induced autophagy and apoptosis in H9c2 cells. Treatment with WYYQ inhibited the increase in Beclin-1 and LC3, reduced the LC3-II/LC3-I ratio, decreased autophagosome formation, and enhanced p62 abundance through activating the mTOR pathway. For the first time,

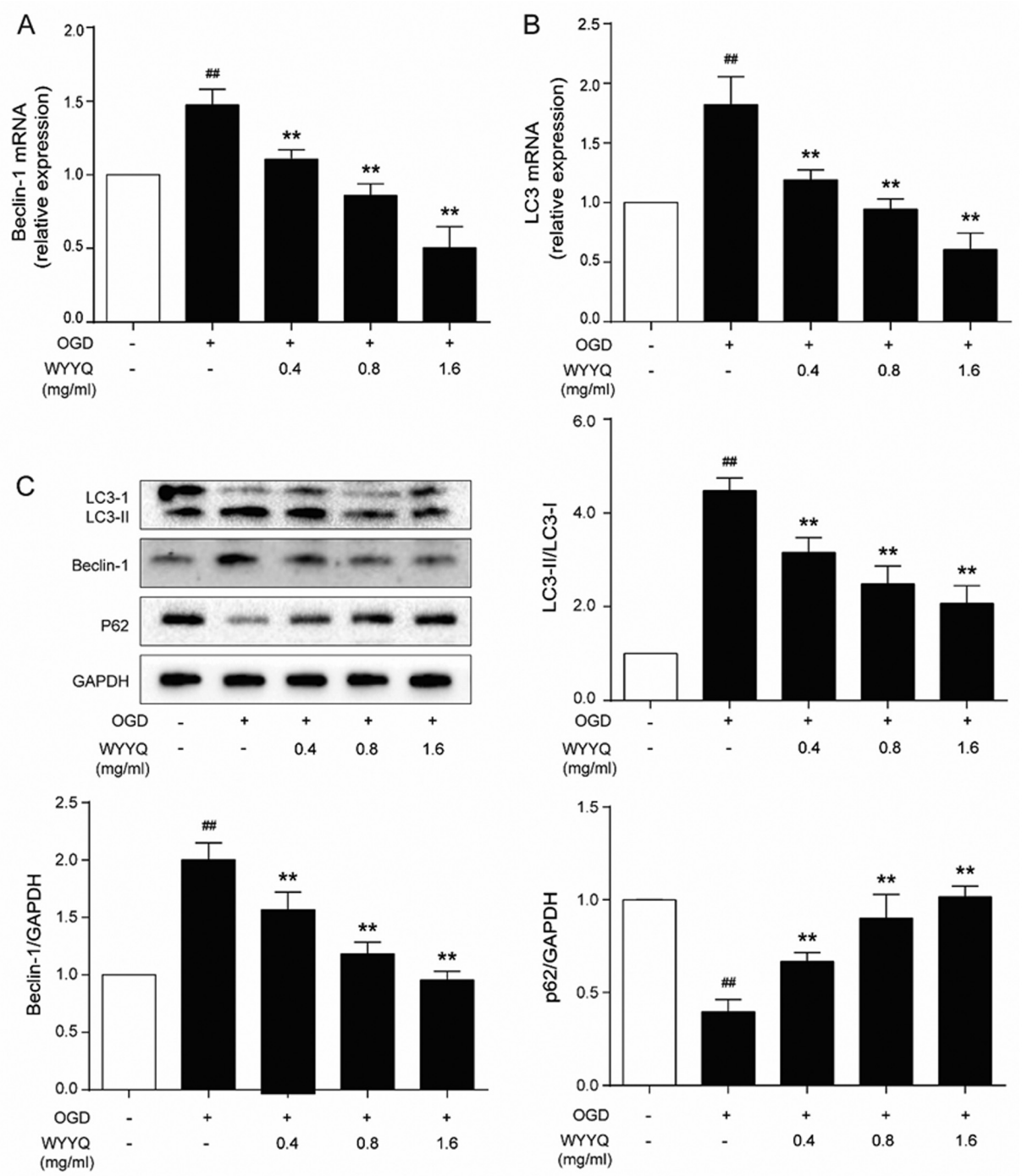

Fig. 3 WYYQ treatment reduces autophagy in response in H9c2 cells treated with OGD. (**A**) Beclin-1 and (**B**) LC3 gene expression assessed by qPCR; (**C**) levels of Beclin-1, LC3, and p62 assessed through Western blot. Data are the means ± SD. $n = 6$. $^{\#\#}p < 0.01$ versus control and $^{**}p < 0.01$ versus OGD. OGD, oxygen-glucose deprivation; qPCR, quantitative polymerase chain reaction; SD, standard deviation; WYYQ, Wenyang-Yiqi Granule.

these results showed the inhibitory effects of WYYQ on cardiomyocyte autophagy under OGD exposure, thus indicating that WYYQ exerted protective effects against HF, typically accompanied by cardiomyocyte damage driven by hypoxic-ischemic stress.

Autophagy has evolved as a process in which the body degrades and absorbs its own components, including long-lived proteins and damaged cellular components. Autophagy is disturbed in pathological conditions. HF is characterized by cardiac remodeling, which includes the accumulation of compensatory structural and functional alterations to alleviate ventricular wall stress.[26] Autophagy occurs in hypoxic cells[27] and organs during cardiovascular diseases, such as HF,[28] suggesting its stimulation under ischemic conditions. The data of the present study showed that autophagy are activated following OGD exposure in H9c2 cells. WYYX played a positive role in cell protection through the down-regulation of autophagic activity. A previous study reported

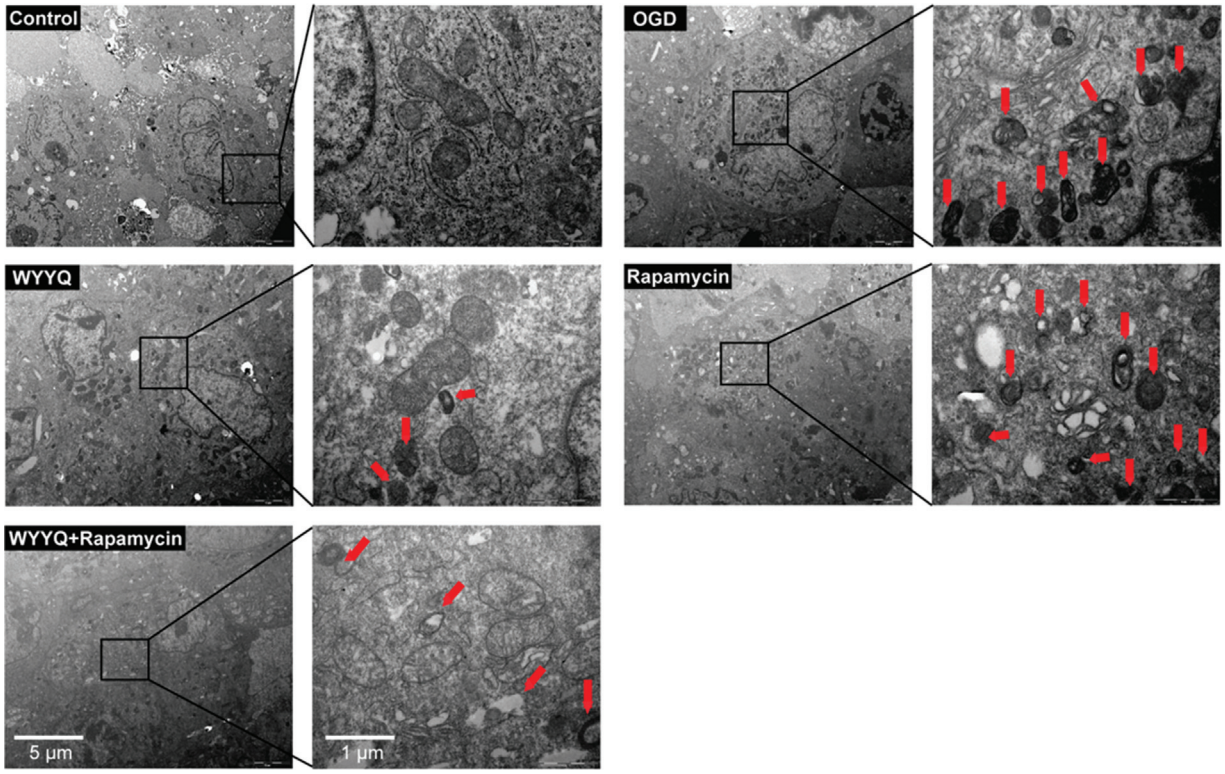

Fig. 4 Ultrastructural alterations in H9c2 cells by TEM. The black boxed area shows representative autophagic vacuoles in H9c2 cells (x4,000 magnification, scale bar: 5 μm). Arrows indicate the double membranes of autophagic vacuoles (x30,000 magnification, scale bar: 1 μm). TEM, transmission electron microscopy.

that excessive autophagic activity triggers the release of apoptotic-related factors, resulting in apoptotic responses,[29] in which Beclin-1 and Bcl-2 play a role in the crosstalk between autophagy and apoptosis. The present study found enhanced apoptotic rates under OGD conditions, which was consistent with these studies. WYYQ also lowered apoptotic rates upon the comparison of the effects under OGD conditions. This suggested that WYYQ manipulated the cellular metabolic demands by regulating both the autophagic and apoptotic activity induced by nutrient deficiency.

mTOR plays a central role in diverse cellular processes, including cell growth and metabolism.[30] mTOR is actively associated with physiological and pathological responses that occur during cardiovascular diseases, such as HF,[30] with its downregulation triggering the pathological development of cardiomyopathy with HF.[31] The present study showed that mTOR activity was downregulated, while autophagy was upregulated following OGD exposure in H9c2 cells. Furthermore, the impaired activity of mTOR and the enhanced autophagy induced by OGD was reversed by WYYQ. WYYQ had an inhibitory effect on OGD-induced autophagy, suggesting that it played a role in the protection of cardiomyocytes against pathological lesions because of starvation or hypoxia. A challenging view indicated that mTOR activation showed diverse dysfunctional effects, including exasperated pathological hypertrophy or misfolded protein accumulation[32]; however, the inhibition of mTOR activity, particularly the selective inhibition of mTORC1, in addition to the enhancement of autophagy, resulted in reduced cardiovascular damage in response to pressure overload or chronic ischemic injury.[33,34] Autophagy plays a dual role in impaired cardiac tissue, namely, autophagy is cardioprotective, but its attenuation or over-stimulation is harmful to cardiomyocytes, depending on the timing and degree of autophagic activity.[35] Therefore, therapeutic strategies focused on manipulating autophagy in HF patients should consider these effects to fine-tune the autophagic process for optimal benefit.

Accumulating evidence has shown that Chinese herbs used for the treatment of cardiovascular diseases function through the adjustment of autophagic responses. Qiliqiangxin has been shown to inhibit myocardial inflammation and cardiomyocyte death and abrogate autophagy in mouse models of pressure overload.[36] Similarly, both Xuefu Zhuyu decoction and modified Yi Qi decoction showed beneficial effects on cardiac function by lowering the expression of the autophagic proteins Beclin-1 and LC3.[37,38] In contrast, the traditional Chinese medicine Tongxinluo promoted autophagy through the activation of the mitogen-activated protein kinase/ERK, thus showing a protective role in cardiac microvascular endothelial cells during ischemic injury.[39] Orientin extracted from *Polygonum orientale* L protected myocardial cells against hypoxic injury through the induction of autophagy.[40] It remains a matter of debate whether the fine-tuning of autophagic responses regulated by Chinese herbs is beneficial. This study demonstrated that WYYQ protected against cell injury and inhibited OGD-induced autophagy enhancement through the activation of mTOR. Future studies will be

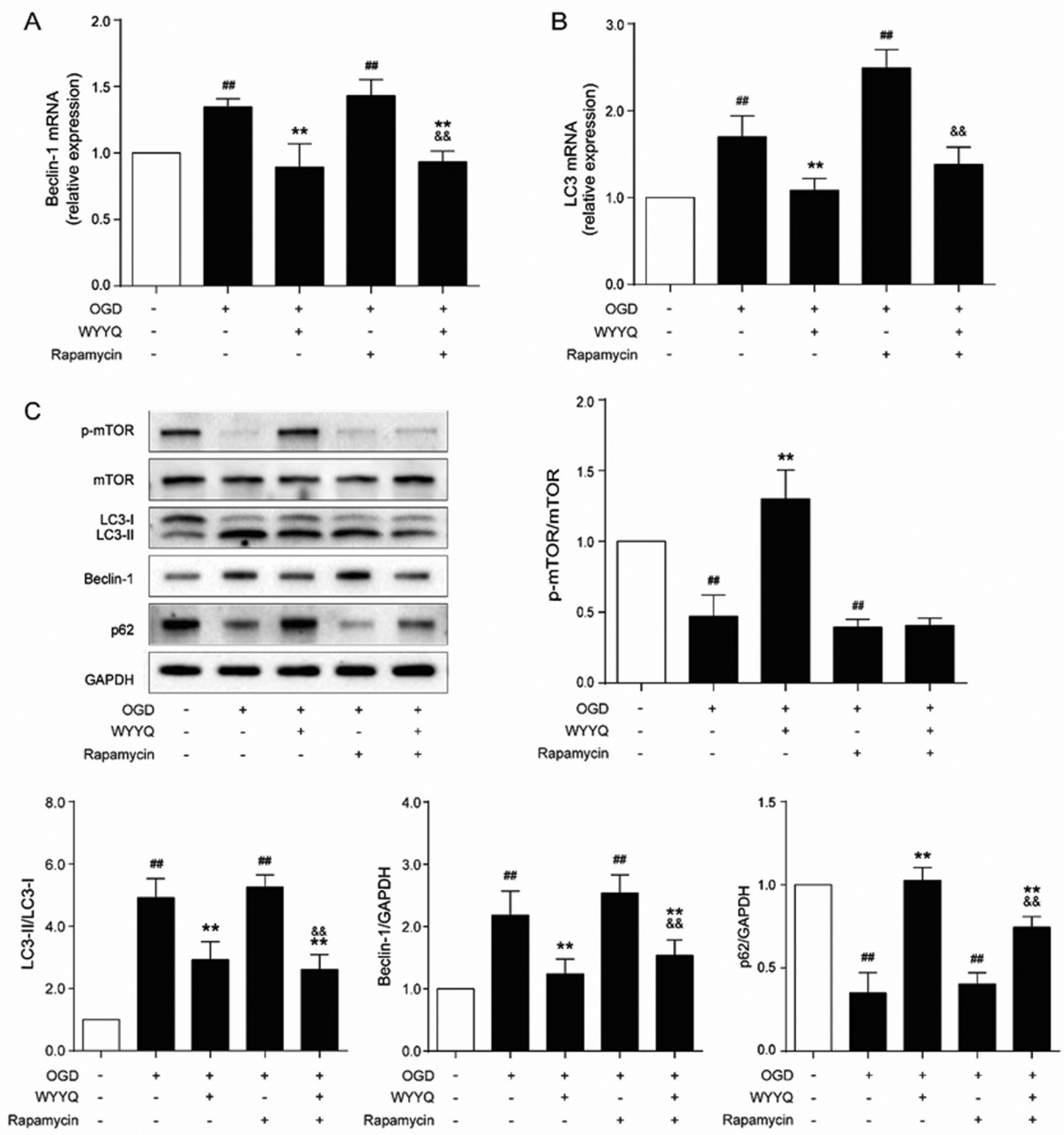

Fig. 5 WYYQ suppresses cardiomyocyte autophagy through regulating mTOR signaling. (**A**) Beclin-1 and (**B**) LC3 gene expressions assessed via qPCR; (**C**) levels of p-mTOR, mTOR, LC3-II/LC3-I, Beclin-1, and p62 assessed through Western blot analysis. Data are the mean ± SD. $n = 6$. $^{\#\#}p < 0.01$ versus control, $^{**}p < 0.01$ versus OGD, and $^{\&\&}p < 0.01$ versus OGD and rapamycin. mTOR, mammalian targets of rapamycin; OGD, oxygen-glucose deprivation; qPCR, quantitative polymerase chain reaction; SD, standard deviation; WYYQ, Wenyang-Yiqi Granule.

required to accurately investigate the autophagic activity and mechanism(s) underlying the function of WYYQ on cardiomyocyte autophagy during HF progression.

Various ingredients of WYYQ have been shown to protect cardiomyocytes in vivo. Cinnamaldehyde, extracted from Lauraceae, can reduce the high glucose-induced oxidative damage of cardiomyocytes through the TRPA1/Nrf2 pathway in a db/db diabetic mouse model and inhibit the production of ROS and autophagy through the TLR4-NOX4 pathway to ameliorate LPS-induced cardiac dysfunction in rats.[41] Astragaloside IV, obtained from Huangqi (Astragali Radix), attenuated the ischemia-reperfusion injury of rat myocardial cells via energy regulation mechanism or CaSR/ERK1/2 and related apoptosis signaling pathways.[42] It has also been reported that Astragalus polysaccharide regulates oxidative stress through the MAPK pathway or down-regulated the expression ATF6- and PERK-related factors during ER stress, thus reducing cardiomyocyte apoptosis during diabetic cardiomyopathy.[43,44] Ginsenoside Rb3, an active component of *Renshen (Panax Ginseng)*, regulates the energy metabolism of cardiomyocytes by activating the PPARα pathway in a mouse HF model to reduce apoptosis.[45] The present study

investigated the antiautophagy effects of WYYQ on cardiomyocytes in vitro. Interactions of WYYQ with the myocardium in vivo warrant further investigations in future studies.

Conclusions

In summary, this study showed that WYYQ exerts an inhibitory effect on OGD-induced autophagy. WYYQ significantly decreased the expressions of Beclin-1 and LC3 but increased the abundance of p62 following OGD exposure, in addition to the formation of autophagosomes, which were associated with the activation of the mTOR pathway. Pharmacological manipulation of autophagy represents a potential therapeutic intervention for the treatment of cardiovascular diseases.

CRediT Authorship Contribution Statement

L.H. and H.W. were responsible for conceptualization, methodology, and formal analysis. S.G., X.Y., and H.W. were responsible for funding acquisition, writing original draft, and writing—review and editing.

Funding

This work was funded by the National Natural Science Foundation of China (81373610, 81673800).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

We thank all the staff at the Laboratory of Cell Imaging at the Henan University of Chinese Medicine for their valuable contributions. We are grateful to have had the opportunity to present our initial data in an abstract at the annual meeting of Experimental Biology in 2019 (https://www.fasebj.org/doi/abs/10.1096/fasebj.2019.33.1_supplement.471.28).

References

1 Papanagnou ED, Gumeni S, Sklirou AD, et al. Autophagy activation can partially rescue proteasome dysfunction-mediated cardiac toxicity. Aging Cell 2022;21(11):e13715
2 Cui X, Wang Y, Liu H, Shi M, Wang J, Wang Y. The molecular mechanisms of defective copper metabolism in diabetic cardiomyopathy. Oxid Med Cell Longev 2022;2022:5418376
3 Li Z, Wang J, Yang X. Functions of autophagy in pathological cardiac hypertrophy. Int J Biol Sci 2015;11(06):672–678
4 Gatica D, Chiong M, Lavandero S, Klionsky DJ. Molecular mechanisms of autophagy in the cardiovascular system. Circ Res 2015;116(03):456–467
5 Peng L, Zhuang X, Liao L, et al. Changes in cell autophagy and apoptosis during age-related left ventricular remodeling in mice and their potential mechanisms. Biochem Biophys Res Commun 2013;430(02):822–826
6 Yang M, Ji X, Zuo Z. Relationships between the toxicities of *Radix aconiti* lateralis preparata (Fuzi) and the toxicokinetics of its main diester-diterpenoid alkaloids. Toxins (Basel) 2018;10(10):391
7 Sun L, Liu LN, Li JC, et al. The essential oil from the twigs of Cinnamomum cassia Presl inhibits oxytocin-induced uterine contraction in vitro and in vivo. J Ethnopharmacol 2017;206:107–114
8 Shahzad M, Shabbir A, Wojcikowski K, Wohlmuth H, Gobe GC. The antioxidant effects of *Radix astragali* (*Astragalus membranaceus* and related species) in protecting tissues from injury and disease. Curr Drug Targets 2016;17(12):1331–1340
9 Li J, Liu S, Wang J, et al. Fungal elicitors enhance ginsenosides biosynthesis, expression of functional genes as well as signal molecules accumulation in adventitious roots of Panax ginseng C. A. Mey. J Biotechnol 2016;239:106–114
10 Han L, Wang Z, Chai S, Xing G, Wang H. Effects of comparison of warming yang prescription with invigorating qi prescription on renin, angiotensin II, and idosterone at different time phase in rats with heart failure after myocardial infarction. CJTCMP 2015;30(11):4103–4105
11 Dai DF, Rabinovitch P. Mitochondrial oxidative stress mediates induction of autophagy and hypertrophy in angiotensin-II treated mouse hearts. Autophagy 2011;7(08):917–918
12 Ichihara A. (Pro)renin receptor and autophagy in podocytes. Autophagy 2012;8(02):271–272
13 Porrello ER, Delbridge LM. Cardiomyocyte autophagy is regulated by angiotensin II type 1 and type 2 receptors. Autophagy 2009;5(08):1215–1216
14 Zheng S, Han F, Shi Y, Wen L, Han D. Single-prolonged-stress-induced changes in autophagy-related proteins beclin-1, LC3, and p62 in the medial prefrontal cortex of rats with post-traumatic stress disorder. J Mol Neurosci 2017;62(01):43–54
15 Masuda GO, Yashiro M, Kitayama K, et al. Clinicopathological correlations of autophagy-related proteins LC3, beclin 1 and p62 in gastric cancer. Anticancer Res 2016;36(01):129–136
16 Ma LL, Ma X, Kong FJ, et al. Mammalian target of rapamycin inhibition attenuates myocardial ischaemia-reperfusion injury in hypertrophic heart. J Cell Mol Med 2018;22(03):1708–1719
17 Hang P, Zhao J, Su Z, et al. Choline inhibits ischemia-reperfusion-induced cardiomyocyte autophagy in rat myocardium by activating Akt/mTOR signaling. Cell Physiol Biochem 2018;45(05):2136–2144
18 Weinberg MA. RES-529: a PI3K/AKT/mTOR pathway inhibitor that dissociates the mTORC1 and mTORC2 complexes. Anticancer Drugs 2016;27(06):475–487
19 Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 1998;273(07):3963–3966
20 Watanabe-Asano T, Kuma A, Mizushima N. Cycloheximide inhibits starvation-induced autophagy through mTORC1 activation. Biochem Biophys Res Commun 2014;445(02):334–339
21 Settembre C, Fraldi A, Medina DL, Ballabio A. Signals from the lysosome: a control centre for cellular clearance and energy metabolism. Nat Rev Mol Cell Biol 2013;14(05):283–296
22 Chiang MH, Liang CJ, Liu CW, et al. Aliskiren improves ischemia- and oxygen glucose deprivation-induced cardiac injury through activation of autophagy and AMP-activated protein kinase. Front Pharmacol 2017;8:819
23 Guan ZF, Zhang XM, Tao YH, et al. EGb761 improves the cognitive function of elderly db/db$^{-/-}$ diabetic mice by regulating the beclin-1 and NF-κB signaling pathways. Metab Brain Dis 2018;33(06):1887–1897
24 Wu H, Gao S, Fu M, Sakurai T, Terakawa S. Fucoidan inhibits Ca2+ responses induced by a wide spectrum of agonists for G-protein-coupled receptors. Mol Med Rep 2018;17(01):1428–1436
25 Wu H, Gao H, Gao S, et al. A Chinese 4-herb formula, Yiqi-Huoxue granule, alleviates H_2O_2-induced apoptosis by upregulating uncoupling protein 2 in H9c2 cells. Phytomedicine 2019;53:171–181
26 Moliner-Abós C, Rivas-Lasarte M, Pamies Besora J, et al. Sacubitril/valsartan in real-life practice: experience in patients with advanced heart failure and systematic review. Cardiovasc Drugs Ther 2019;33(03):307–314
27 Ghosh R, Gillaspie JJ, Campbell KS, Symons JD, Boudina S, Pattison JS. Chaperone-mediated autophagy protects cardiomyocytes against hypoxic-cell death. Am J Physiol Cell Physiol 2022;323(05):C1555–C1575

28 Wang S, Yao T, Deng F, et al. LncRNA MALAT1 promotes oxygen-glucose deprivation and reoxygenation induced cardiomyocytes injury through sponging miR-20b to enhance beclin1-mediated autophagy. Cardiovasc Drugs Ther 2019;33(06):675–686
29 Calis S, Dogan B, Durdagi S, et al. A novel BH3 mimetic Bcl-2 inhibitor promotes autophagic cell death and reduces in vivo Glioblastoma tumor growth. Cell Death Discov 2022;8(01):433
30 Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. Cell 2017;168(06):960–976
31 Schüttler D, Clauss S, Weckbach LT, Brunner S. Molecular mechanisms of cardiac remodeling and regeneration in physical exercise. Cells 2019;8(10):1128
32 Shende P, Plaisance I, Morandi C, et al. Cardiac raptor ablation impairs adaptive hypertrophy, alters metabolic gene expression, and causes heart failure in mice. Circulation 2011;123(10):1073–1082
33 Ramos FJ, Chen SC, Garelick MG, et al. Rapamycin reverses elevated mTORC1 signaling in lamin A/C-deficient mice, rescues cardiac and skeletal muscle function, and extends survival. Sci Transl Med 2012;4(144):144ra103
34 Wang H, Hua J, Chen S, Chen Y. SERPINB1 overexpression protects myocardial damage induced by acute myocardial infarction through AMPK/mTOR pathway. BMC Cardiovasc Disord 2022;22(01):107
35 Zhu H, Tannous P, Johnstone JL, et al. Cardiac autophagy is a maladaptive response to hemodynamic stress. J Clin Invest 2007; 117(07):1782–1793
36 Zou Y, Lin L, Ye Y, et al. Qiliqiangxin inhibits the development of cardiac hypertrophy, remodeling, and dysfunction during 4 weeks of pressure overload in mice. J Cardiovasc Pharmacol 2012;59 (03):268–280
37 Shi X, Zhu H, Zhang Y, Zhou M, Tang D, Zhang H. XuefuZhuyu decoction protected cardiomyocytes againsthypoxia/reoxygenation injury by inhibiting autophagy. BMC Complement Altern Med 2017; 17(01):325
38 Yu X, Zhao XD, Bao RQ, Yu JY, Zhang GX, Chen JW. The modified Yi qi decoction protects cardiac ischemia-reperfusion induced injury in rats. BMC Complement Altern Med 2017;17 (01):330
39 Cui H, Li X, Li N, et al. Induction of autophagy by Tongxinluo through the MEK/ERK pathway protects human cardiac microvascular endothelial cells from hypoxia/reoxygenation injury. J Cardiovasc Pharmacol 2014;64(02):180–190
40 Liu L, Wu Y, Huang X. Orientin protects myocardial cells against hypoxia-reoxygenation injury through induction of autophagy. Eur J Pharmacol 2016;776:90–98
41 Zhao H, Zhang M, Zhou F, et al. Cinnamaldehyde ameliorates LPS-induced cardiac dysfunction via TLR4-NOX4 pathway: the regulation of autophagy and ROS production. J Mol Cell Cardiol 2016; 101:11–24
42 Yin B, Hou XW, Lu ML. Astragaloside IV attenuates myocardial ischemia/reperfusion injury in rats via inhibition of calcium-sensing receptor-mediated apoptotic signaling pathways. Acta Pharmacol Sin 2019;40(05):599–607
43 Chen W, Sun Q, Ju J, et al. Effect of *Astragalus* polysaccharides on cardiac dysfunction in db/db mice with respect to oxidant stress. BioMed Res Int 2018;2018:8359013
44 Sun S, Yang S, An N, et al. Astragalus polysaccharides inhibits cardiomyocyte apoptosis during diabetic cardiomyopathy via the endoplasmic reticulum stress pathway. J Ethnopharmacol 2019; 238:111857
45 Chen X, Wang Q, Shao M, et al. Ginsenoside Rb3 regulates energy metabolism and apoptosis in cardiomyocytes via activating PPARα pathway. Biomed Pharmacother 2019;120:109487