Semaglutide enhances PINK1/Parkin-dependent mitophagy in hypoxia/reoxygenation-induced cardiomyocyte injury

LIQIN LI[1*], LILI JIN[2*], YAPING TIAN[3] and JUN WANG[1]

[1]Department of Endocrinology, The First Central Hospital of Baoding, Baoding, Hebei 071000, P.R. China;
[2]Department of Cardiology, The First Central Hospital of Baoding, Baoding, Hebei 071000, P.R. China;
[3]Department of Endocrinology, Graduate School of Chengde Medical College, Chengde, Hebei 067000, P.R. China

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Abstract. The present study aimed to explore how semaglutide can help protect the heart from injury caused by hypoxia/reoxygenation (H/R) and to reveal the underlying mechanism. Briefly, AC16 cardiomyocytes were subjected to 8 h of hypoxia followed by 12 h of reoxygenation to simulate H/R. The cells were divided into the following five groups: Normoxia, H/R, H/R + semaglutide, H/R + semaglutide + rapamycin (autophagy inducer), and H/R + semaglutide + 3-methyladenine (3-MA; autophagy inhibitor) groups. Cell viability was examined using a Cell Counting Kit-8 assay, ATP levels were examined using a bioluminescent detection kit, reactive oxygen species (ROS) production was detected using a ROS Assay Kit, and monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-LC3 was assessed using tandem mRFP-GFP fluorescence microscopy, while autophagosomes were observed using transmission electron microscopy. Furthermore, the protein expression levels of autophagy markers (LC3, p62 and Beclin1) and regulators of mitochondrial autophagy [PTEN-induced putative kinase protein-1 (PINK1) and Parkin] were examined using western blot analysis. In AC16 cells, exposure to hypoxia followed by reoxygenation led to an increase in oxidative stress. This condition also induced an increase in autophagy activity, as evidenced by an increase in the number of autophagosomes, elevated LC3-II/LC3-I ratio, and upregulation of p62, Beclin1, PINK1 and Parkin expression compared with those in cells cultured under normoxia. Notably, treatment with semaglutide or rapamycin effectively reversed the H/R-induced oxidative stress, enhanced the changes in autophagy activity, autophagosome levels and elevated LC3BII/LC3BI ratio, and increased the expression levels of Beclin1, PINK1, Parkin and p62 expression. Notably, the use of 3-MA exhibited distinct effects under the same conditions; it exacerbated oxidative stress, decreased autophagy activity and reduced the LC3BII/LC3BI ratio. In conclusion, semaglutide was found to reduce oxidative stress caused by H/R and to increase autophagy via the ROS/PINK1/Parkin/p62 pathway. The present study offers a novel understanding of how semaglutide may protect the heart, and suggests its potential use in the treatment of myocardial ischemia/reperfusion injury.

Introduction

Coronary artery disease (CAD) is the most prevalent form of cardiovascular disease, and the primary medical approach for treating CAD involves timely reperfusion, including percutaneous coronary intervention and coronary artery bypass grafting (1). However, reperfusion can exacerbate cardiac hypoxia/reoxygenation (H/R) injury, leading to increased mortality. Mechanisms such as overproduction of reactive oxygen species (ROS), apoptosis and imbalanced mitochondrial dynamics can contribute to cardiac H/R injury (2). Efforts have been made to understand the cellular and molecular mechanisms of cardiac H/R injury, including the interplay between autophagy and apoptosis (3).

Autophagy is a cellular process that degrades and recycles damaged organelles and proteins, which is critical in regulating cardiomyocyte survival during H/R injury (4). Autophagy maintains cellular homeostasis and promotes cell survival by clearing dysfunctional cellular components (5); however, the exact mechanisms by which autophagy is regulated during H/R injury and how it interacts with other cellular stress responses are not yet fully understood.

The maintenance of cardiac homeostasis depends on the crucial process of eliminating dysfunctional mitochondria through mitophagy (6). The primary regulator of mitophagy is the PTEN-induced putative kinase protein-1 (PINK1)/Parkin pathway, although the FUN14 domain-containing 1 (FUNDC1), Bcl2-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L/NIX) pathways can also control mitophagy. Dysregulated mitophagy is associated with cardiac dysfunction

Correspondence to: Professor Jun Wang, Department of Endocrinology, The First Central Hospital of Baoding, 320 Changcheng North Street, Baoding, Hebei 071000, P.R. China
E-mail: junwangnfm@163.com

*Contributed equally

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related to aging, aortic stenosis, myocardial infarction or diabetes (7). Notably, PINK1-mediated mitophagy serves an important role in maintaining mitochondrial quality control during ischemia/reperfusion injury (7,8).

Semaglutide is a glucagon-like peptide-1 (GLP-1) receptor agonist, and is a promising novel therapeutic agent that has exhibited cardioprotective effects in previous studies (9-12). As a member of the GLP-1 receptor (GLP-1R) agonist class, semaglutide mimics the actions of endogenous GLP-1, which is known to regulate glucose metabolism, stimulate insulin secretion and inhibit glucagon release (13). In addition to its well-documented benefits in managing blood glucose levels in type 2 diabetes, semaglutide has shown potential in protecting the heart from damage caused by ischemia and reperfusion (14). Despite these promising findings, the specific molecular mechanisms underlying the cardioprotective effects of semaglutide remain unclear. To address this, the present study performed experiments using an *in vitro* model of H/R in human AC16 cardiomyocytes. The aim of the current study was to reveal the mechanisms underlying the protective effects of semaglutide and to identify novel therapeutic targets for the treatment of H/R injury, which may advance cardiovascular medicine.

Materials and methods

Cell culture conditions and H/R modeling. Numerous studies (15-17) have adopted the AC16 cell line as an *in vitro* model to investigate cardiac H/R injury. The selection of AC16 cells (18) is attributed to their shared characteristics with primary cardiomyocytes, such as the expression of cardiac-specific markers (16,19,20) and responsiveness to H/R conditions (21), making them a valuable tool for exploring the mechanisms of heart diseases.

Human AC16 cardiomyocytes, sourced from the Cell Resource Center, Peking Union Medical College of China (Beijing, China), were maintained in DMEM (cat. no. D6429-500ML; Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (cat. no. FSP500; Shanghai Excell Biological Technology Co., Ltd.) and 1% penicillin-streptomycin (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.). The cells were incubated at 37˚C in a humidified environment of 95% air and 5% CO_2 (CO_2 incubator; CI-191C; Suzhou Jimei Electronic Co., Ltd.). AC16 cells were exposed to semaglutide (cat. no. HY-114118; MedChemExpress) at concentrations ranging between 0 and 40 mmol/l or DMSO (Beijing Solarbio Science & Technology Co., Ltd.) for 24, 48 and 72 h to identify the non-toxic concentration.

To stimulate H/R injury in myocardial cells, a H/R model was established. Control cells were maintained in standard DMEM conditions, while the experimental group underwent 8 h of hypoxia in a specialized incubator (94% N_2, 5% CO_2 and 1% O_2 at 37˚C), with nitrogen replacing oxygen. Following this hypoxic phase, cells were reoxygenated for 12 h at 37˚C in a normoxic environment (95% air and 5% CO_2). To investigate the impact of semaglutide on H/R injury, 5 mmol/l semaglutide was administered to the experimental group during the reoxygenation phase. Additionally, the autophagy activator rapamycin (0.1 μM; cat. no. HY-10219; MedChemExpress) was used to stimulate autophagy and the autophagy inhibitor 3-methyladenine (3-MA; cat. no. HY-19312; MedChemExpress) was used to suppress autophagy for 48 h at 37˚C in a normoxic environment (95% air and 5% CO_2) during reoxygenation; cells were treated with these to evaluate the influence of autophagy on H/R injury in cardiomyocytes.

Assessment of cell viability. According to the manufacturer's instructions, cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay (cat. no. CA1210; Beijing Solarbio Science & Technology Co., Ltd.). This assay was utilized to evaluate the cytotoxicity of different concentrations of semaglutide on AC16 cell viability in a dose- and time-dependent manner, both under normoxic conditions and H/R conditions.

AC16 cells were incubated with 10 μl CCK-8 for 2 h in the dark. The absorbance was measured at 450 nm using a microplate reader (ReadMax 1200; Shanghai Flash Spectrum Biotechnology Co., Ltd.).

ATP level determination. Intracellular ATP levels were measured using a bioluminescent detection kit (ATP Assay Kit; cat. no. E-BC-F002; Elabscience®; Elabscience Bionovation Inc.) according to the manufacturer's instructions. A total of $2x10^6$/ml cells were used and luminescence intensities were recorded with a multi-mode microplate reader (ReadMax 1200; Shanghai Shanpu Laser Technology Co., Ltd.).

ROS measurement. ROS production was assessed using the ROS Assay Kit (cat. no. S0033S; Beyotime Institute of Biotechnology), which relies on measuring the fluorescence intensity of dihydroethidium and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). After treatment, the cells ($2x10^6$/ml) were incubated with 10 μM/l DCFH-DA for 20 min at 37˚C in the dark, washed three times with PBS and were then observed under a fluorescence microscope (Nikon Eclipse ti2; Nikon Corporation).

Transmission electron microscopy. AC16 cells ($2x10^6$/ml) were collected by centrifugation at 1,000 x g for 3 min at 4˚C the culture medium was removed, and the cells were fixed with 2.5% glutaraldehyde electron microscope fixative (cat. no. G1102; Wuhan Servicebio Technology Co., Ltd.) at 4˚C for 2-4 h. Subsequently, the samples were fixed with 1% osmium tetroxide (cat. no. 18456; Ted Pella, Inc.), dehydrated using a series of ethanol and propylene oxide solutions, embedded and cut into 60-80-nm sections. The sections were then stained with uranium-lead double staining (2% uranium acetate and 2.6% lead citrate) for 15 min at room temperature. After final washes, the grids were blotted dry and left to dry overnight at room temperature. The embedding molds were The cells were embedded in acetone and SPI-Pon 812 Epoxy Resin, then the embedding molds were placed in an oven at 60˚C for 48 h for polymerization. Cells were finally observed under a transmission electron microscope (JEM-1200; Hitachi, Ltd.).

Monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-LC3 assay and confocal microscopy. AC16 cells were seeded at a density of $1x10^5$ cells/well in 24-well plates and were then incubated overnight at 37˚C in a 5% CO_2 environment. To ensure optimal virus infection, the cell confluence

rates were maintained between 30 and 50%, taking into account cell size and growth rate variations. The mRFP-GFP-LC3 adenoviral vectors used in this assay were obtained from Hanbio Biotechnology Co., Ltd. This assay relies on the differential pH stability of GFP and RFP. Specifically, enhanced GFP fluorescence is quenched in acidic lysosome conditions (pH <5), whereas mRFP fluorescence remains unaffected. The merged green and red images reveal autophagosomes as yellow puncta and autolysosomes as red puncta. An increase in yellow and red puncta indicates enhanced autophagic flux, whereas an increase solely in yellow puncta or a decrease in both suggests autophagic flux blockage. Infection with the aforementioned adenoviral vectors ($1x10^9$ TU/ml) was carried out according to the manufacturer's guidelines, with an optimal multiplicity of infection of 50 determined through preliminary experiments, with the cells being infected for 36 h. LC3 puncta were visualized using a confocal microscope [ICX41; Sunny Optical Technology (Group) Company Limited] and were quantitatively analyzed using ImageJ software (version 4.20; National Institutes of Health) and GraphPad Prism software 19.0 (Dotmatics).

Western blot analysis. Protein lysates were extracted from cells using RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) and the protein concentration was determined using a BCA Protein Assay Kit (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.). Proteins (30 μg/lane) were then separated by SDS-PAGE on 8-10% gels (cat. no. ZS305; Beijing Zomanbio International Biogene Technology Co., Ltd.) and were transferred onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk (cat. no. D8340; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h, then incubated with primary antibodies at 4˚C overnight. The following primary antibodies were used: Sequestosome 1/p62 (1:1,000; cat. no. PM045; MBL International Co.), Beclin1 (1:1,000; cat. no. PD017; MBL International Co.), LC3B (1:1,000; cat. no. WL01506; Wanleibio Co., Ltd.), PINK1 (1:1,000; cat. no. DF7742; Affinity Biosciences), Parkin (1:1,000; cat. no. AF0235; Affinity Biosciences) and β-actin (1:5,000; cat. no. EM21002; HUABIO). The blots were then probed with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (1:10,000; cat. nos. ab97080 and ab97040; Abcam) for 60 min at 37˚C. After secondary antibody incubation, the PVDF membrane was immersed in TBS-0.05% Tween-20 and shaken for 45 min; this was repeated three times. For visualization, ECL reagents A and B (cat. no. P10300; Suzhou Xinsaimei Biotechnology Co., Ltd.) were mixed equally and set aside. Subsequently, plastic wrap was placed on the exposure platform, the membrane was blotted dry and placed on the wrap, the ECL solution was applied and left to react for 1 min, and the blots were visualized. The optical density values of the target bands were analyzed using a gel image processing system (SH-523; Shenhua Science Technology Co., Ltd.) to semi-quantify the blots. All experiments were performed in triplicate.

Statistical analysis. Data were analyzed using SPSS 22.0 statistical software (IBM Corp.). The measurement data are presented as the mean ± SEM. One-way ANOVA was conducted to compare multiple groups, followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of various concentrations of semaglutide on AC16 cell viability. The optimal concentration of semaglutide varies according to cell line and experimental setup. Although previous studies have evaluated its concentration in other cell lines, such as H9C2 cells (14,22), the concentration needed for treating AC16 cells remains to be further explored. In the initial stage, a CCK-8 assay was performed to assess the dose-dependent cytotoxicity of different concentrations of semaglutide on AC16 cell viability under normoxic conditions (Fig. 1). AC16 cells were exposed to DMSO or to semaglutide at concentrations ranging between 0 and 40 μmol/l for 0 (Fig. 1A), 24 (Fig. 1B), 48 (Fig. 1C) and 72 h (Fig. 1D). As the concentration of semaglutide increased, the proliferation rate of AC16 cells decreased. For subsequent experiments, non-toxic concentrations of semaglutide (1.25, 2.5, 5, and 10 mmol/l) were selected for cell treatment.

The concentration of semaglutide used in the present study was carefully selected after extensive experimentation and a review of the literature (14,23). Subsequently, a series of dose-response experiments were conducted to determine the optimal concentration range that would allow the effective investigation of the biological effects of semaglutide in the H/R model. The CCK-8 assay was used to examine the effects of different concentrations of semaglutide on AC16 cell viability after H/R (Fig. 2). The CCK-8 assay demonstrated that, in AC16 cells treated with semaglutide + H/R, cell viability increased with the increasing concentration of semaglutide. The highest cell viability was observed when the concentration of semaglutide was 5 mmol/l. However, when the concentration of semaglutide reached 10 mmol/l, the cell viability was reduced. Additionally, based on the present observations, the association between semaglutide treatment and cell viability was most significant at the 48-h time point, indicating a robust response to treatment at this duration. Therefore, 5 mmol/l semaglutide was chosen, and cells were treated for 48 h for all subsequent experiments. In the current study, the H/R model was established using AC16 cells, which is consistent with previous studies (21,24). The concentration of semaglutide administered and the duration of its application were aligned with the parameters reported by Wu *et al* (23).

The cells were divided into the following five groups: Normoxia, H/R, H/R + semaglutide (5 mmol/l), H/R + semaglutide (5 mmol/l) + rapamycin (autophagy inducer), and H/R + semaglutide (5 mmol/l) + 3-MA (autophagy inhibitor).

ATP production. The exposure of AC16 cardiomyocytes to H/R led to a significant decrease in ATP levels compared with those in the normoxia control group; however, compared with the H/R group, treatment with semaglutide + H/R reversed this effect, resulting in a substantial increase in ATP levels (Fig. 3). Additionally, compared with the H/R + semaglutide group, further enhancement of ATP content was observed when rapamycin was combined with semaglutide. Conversely,

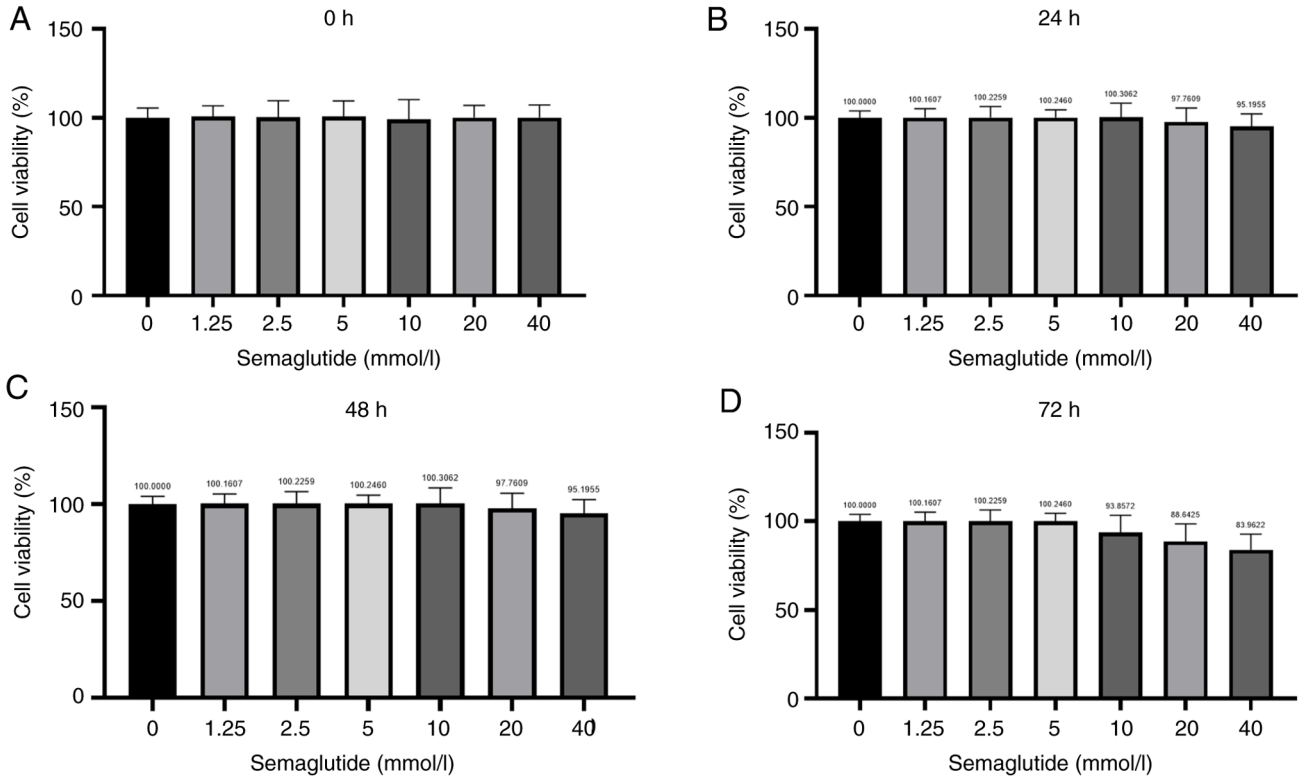

Figure 1. A Cell Counting Kit-8 assay was performed to assess the dose-dependent cytotoxicity of different concentrations of semaglutide on AC16 cell viability under normoxic conditions. AC16 cells were incubated with varying doses of semaglutide (0, 1.25, 2.5, 5, 10, 20 and 40 mmol/l) for (A) 0, (B) 24, (C) 48 and (D) 72 h. The experiments were repeated three times.

the addition of 3-MA, an autophagy inhibitor, resulted in a marked reduction in ATP levels.

ROS levels. Subsequently, the present study explored the impact of semaglutide on the regulation of oxidative stress. A notable increase in ROS levels was detected in cells exposed to H/R compared with those in the control group (Fig. 4). By contrast, treatment with semaglutide resulted in a significant reduction in ROS fluorescence intensity, indicating its ability to protect against hypoxia-induced oxidative stress. Additionally, compared with in the H/R + semaglutide group, the introduction of rapamycin further decreased ROS levels, while treatment with 3-MA led to an increase in ROS fluorescence intensity.

Autophagosome accumulation analyzed by transmission electron microscopy. The present study investigated the impact of semaglutide on autophagosome formation using transmission electron microscopy. The observations using transmission electron microscopy revealed an increase in the presence of autophagosomes in AC16 cells exposed to H/R compared with those in control cells (Fig. 5). Treatment with semaglutide further amplified the number of autophagosomes in H/R-exposed cells, suggesting that semaglutide may provide protective effects against hypoxic damage by enhancing autophagy. Co-administration of semaglutide and rapamycin led to a substantial augmentation in autophagosome formation, as characterized by more autophagosomes, compared with semaglutide alone, further supporting the stimulatory effect of these compounds on autophagy. Conversely, the addition of 3-MA to semaglutide-treated H/R cells resulted in a partial reduction in autophagosome accumulation, underscoring the inhibitory effect of 3-MA on autophagy.

Analysis of autophagy by confocal fluorescence microscopy. To further validate the impact of semaglutide on autophagy in cardiomyocytes, tandem GFP-mRFP-LC3 adenovirus microscopy, a method commonly used to study autophagic flux, was performed to observe the progression of autophagy. The quenching of GFP fluorescence in a lysosomal acid environment, while mRFP fluorescence remains stable, allows for the identification of the formation of autophagosomes and autolysosomes through the presence of yellow puncta (GFP/mRFP) and red puncta (mRFP). Using the mRFP-GFP-LC3 adenoviral assay to monitor autophagosome dynamics, it was observed that semaglutide exerted a protective effect on cells against hypoxia-induced damage by activating autophagy. Compared with in the H/R group, the semaglutide + H/R group exhibited a significant increase in the number of mRFP-GFP-LC3-positive autophagosomes. Furthermore, when compared with the H/R + semaglutide group, the rapamycin-treated group showed a marked elevation in autophagosome count, whereas the 3-MA-treated group demonstrated a significant reduction in autophagosome number (Fig. 6).

PINK1/Parkin pathway activation. The protein levels of Parkin and PINK1 were significantly increased in cells exposed to H/R compared with those in the control group (Fig. 7). Additionally, treatment with semaglutide resulted in further enhancement of the expression levels of Parkin

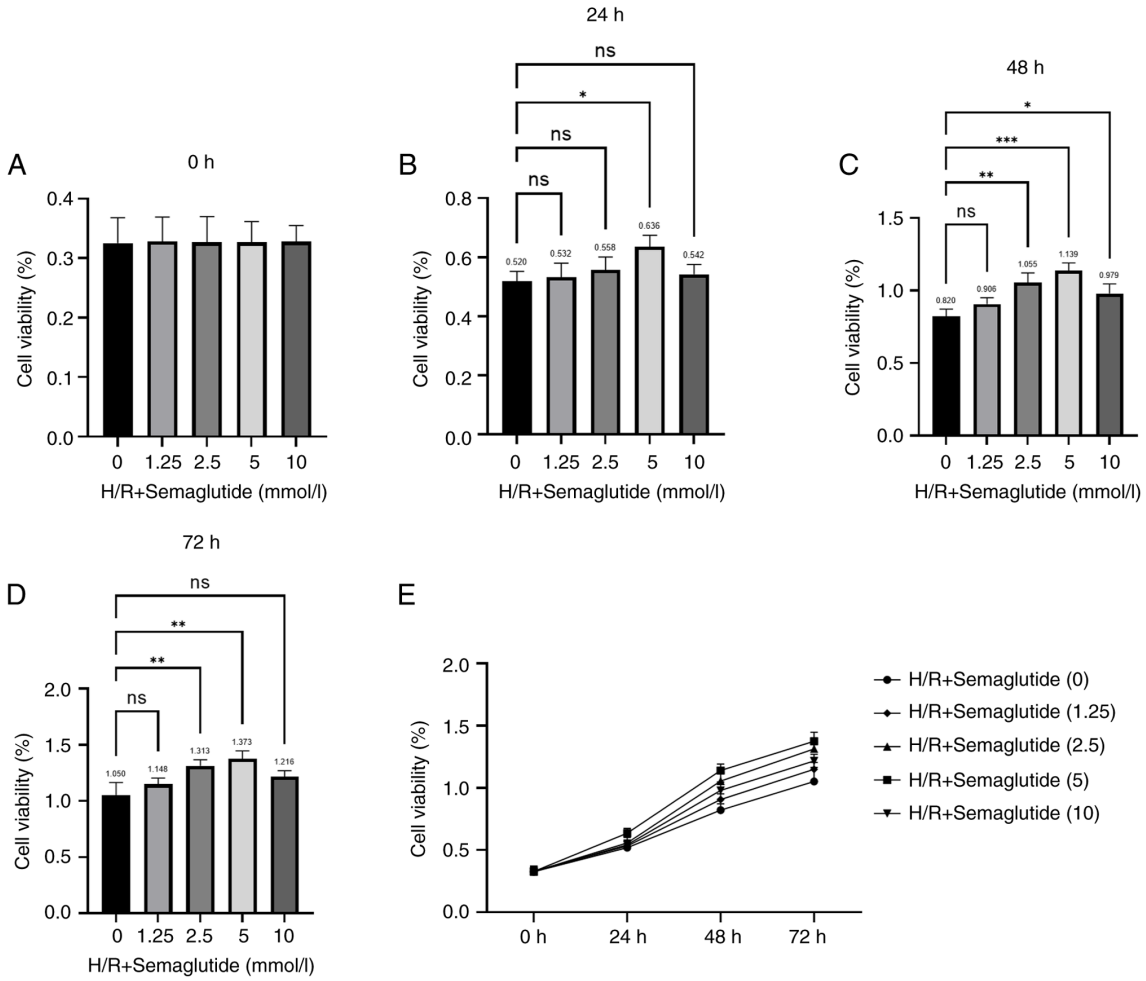

Figure 2. Effects of different concentrations of semaglutide on cell viability under H/R conditions. The AC16 cells were divided into the following groups: Normoxia group, H/R group and H/R + semaglutide groups (1.25, 2.5, 5, or 10 mmol/l), and cell viability was examined using a Cell Counting Kit-8 assay after (A) 0, (B) 24, (C) 48 and (D) 72 h. Semaglutide was safe up to a concentration of 5 mmol/l. (E) Treatment with 5 mmol/l semaglutide for 48 h was the optimal treatment used for subsequent experiments. The experiments were repeated three times. *P<0.05, **P<0.01, ***P<0.001. H/R, hypoxia/reoxygenation; ns, not significant.

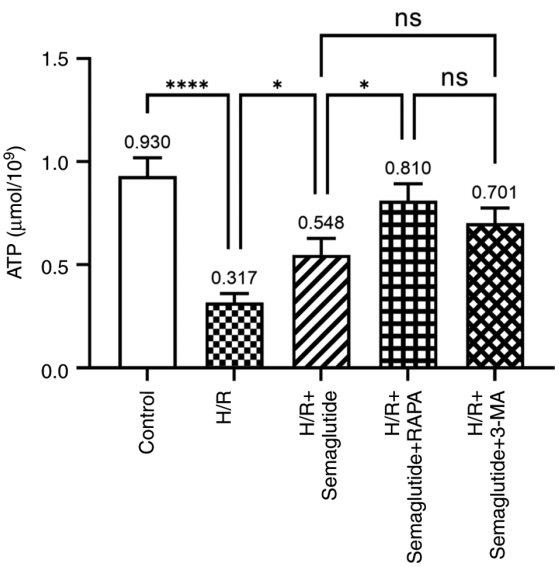

Figure 3. Comparative analysis of ATP content across different treatment groups. Semaglutide increased the levels of ATP. The experiments were repeated three times. *P<0.05, ****P<0.0001. RAPA, rapamycin; 3-MA, 3-methyladenine; H/R, hypoxia/reoxygenation; ns, not significant.

and PINK1, indicating its potential role in activating the PINK1/Parkin pathway. These findings offer valuable insights into the mechanisms responsible for the cardioprotective effects of semaglutide. Compared with in the H/R + semaglutide group, the protein expression levels of Parkin and PINK1 were significantly higher in the rapamycin group, whereas they were significantly reduced in the 3-MA group.

Autophagy markers. The present study investigated whether H/R triggered autophagy in cardiomyocytes. Under normoxic conditions, western blot analysis revealed minimal signaling for autophagy activation; however, following H/R, autophagy markers were significantly increased in cardiomyocytes, as indicated by the elevated LC3B-II/LC3B-I ratio, along with increased expression levels of the autophagy markers Beclin1 and p62 (Fig. 8). Furthermore, treatment with semaglutide significantly enhanced the expression levels of these proteins, thereby underscoring its pivotal role in promoting autophagy. Notably, compared with in the H/R + semaglutide group, rapamycin treatment led to even higher levels of autophagy marker expression, whereas 3-MA reduced their expression levels.

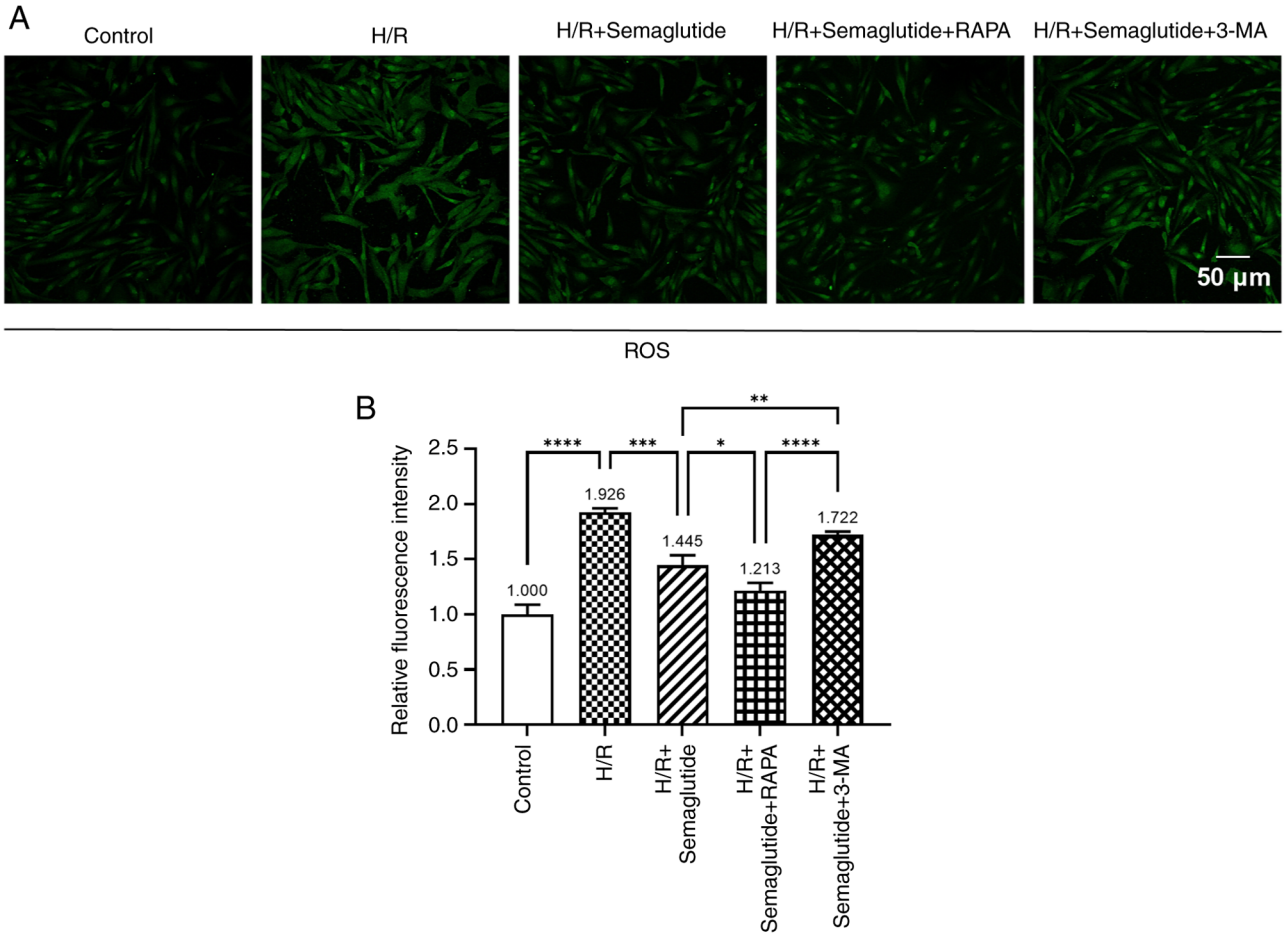

Figure 4. Comparative analysis of ROS levels in different treatment groups. (A) ROS levels were detected by fluorescence microscopy (scale bar, 50 μm). (B) Quantitative analysis of ROS in different groups. The experiments were repeated three times. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. RAPA, rapamycin; 3-MA, 3-methyladenine; H/R, hypoxia/reoxygenation; ROS, reactive oxygen species.

Discussion

Extensive prospective studies (25-27) have indicated that GLP-1R agonists offer cardioprotective benefits for individuals with type 2 diabetes; these benefits include improved cardiac function and myocardial blood flow, and reduced cardiovascular events, all-cause mortality and progression of renal disease. Additionally, GLP-1 has positive effects on blood pressure, cholesterol levels and blood glucose regulation. Notable options in the category of GLP-1R agonists, include liraglutide, semaglutide, albiglutide and dulaglutide (9,28-31). Insights from the SUSTAIN 1-7 trials have indicated that semaglutide effectively improves glycemic control and promotes weight loss, reducing cardiovascular risk in high-risk individuals (32).

The novelty of the present study lies in investigating the protective effects of semaglutide on cardiomyocytes undergoing H/R injury and its relationship with autophagy. While previous research (33,34) has primarily examined the anti-diabetic properties of semaglutide, to the best of our knowledge, its potential role in cardioprotection has not been thoroughly explored. Limited research has investigated the mechanisms underlying the cardioprotective effects of semaglutide. A previous study demonstrated that semaglutide can improve cardiac function, and reduce inflammation and oxidative stress in obese mice, preventing lipid peroxidation and cardiac impairment (35). Li *et al* (36) demonstrated that semaglutide protected against exercise-induced myocardial injury in rats by activating the AMP-activated protein kinase (AMPK) pathway, enhancing autophagy, decreasing ROS production and reducing inflammation-related proteins. However, there is limited research (14) focusing on the effects of semaglutide on H/R-induced myocardial damage.

The present study investigated the protective effects of semaglutide in a model of cardiac H/R. Pathological conditions can cause mitochondria to generate elevated amounts of ROS, inflicting macromolecular damage, and affecting cellular homeostasis and function in several organs, including the heart (37). Superoxide is the major type of ROS that regulates autophagy (38); specifically, by promoting macroautophagy, superoxide facilitates the protection of cardiac myocytes from ischemia/reperfusion injury (39). Firstly, the present study demonstrated that H/R exposure led to a marked increase in oxidative stress in AC16 cells, as evidenced by elevated ROS production. This finding is consistent with the results of a previous report that indicated that oxidative stress serves a pivotal role in myocardial H/R injury (40). Notably, treatment with semaglutide effectively reduced ROS levels, indicating its antioxidant properties. Additionally, semaglutide increased ATP content, which is crucial for sustaining cellular energy

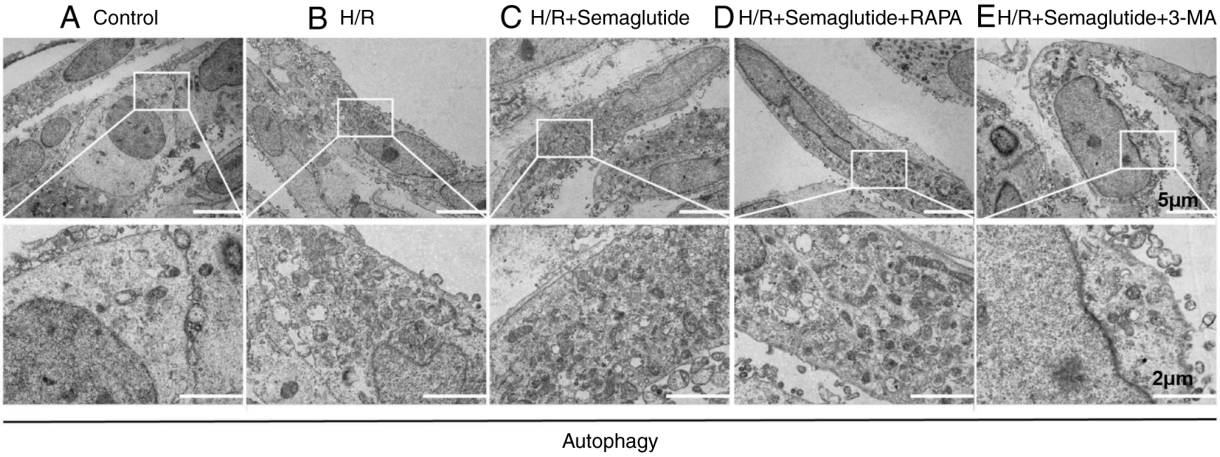

Figure 5. Transmission electron microscopy reveals autophagosome accumulation in AC16 cells treated with semaglutide under H/R conditions. (A) Control group. (B) Electron microscopy revealed that the number of autophagosomes was increased in the H/R group compared with that in the control group. (C) A substantial elevation in autophagosomes was also observed in the H/R + semaglutide group compared with in the H/R group. (D) Number of autophagosomes was increased following the administration of RAPA and (E) decreased following the administration of 3-MA. Top row: Scale bars, 5 μm; magnification, x2,500. Bottom row: Scale bars, 2 μm; magnification, x20,000. The experiments were repeated three times. RAPA, rapamycin; 3-MA, 3-methyladenine; H/R, hypoxia/reoxygenation.

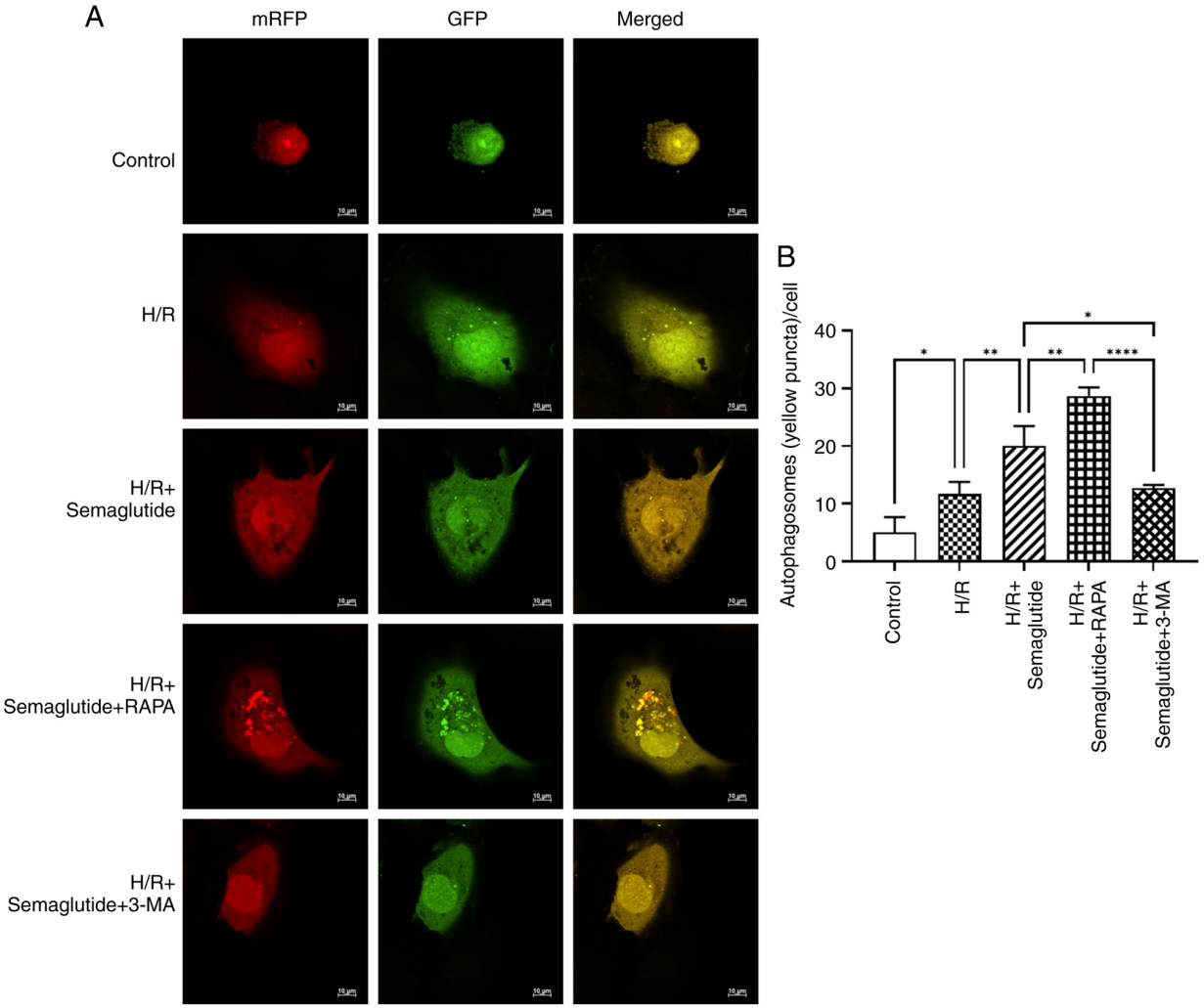

Figure 6. Confocal microscopy analysis of mRFP-GFP-LC3-labeled autophagosomes in AC16 cardiomyocytes under H/R conditions in different groups. (A) AC16 cells were infected with GFP-mRFP-LC3 adenoviral vectors for 36 h and were then subjected to different treatments during reoxygenation. Both green and red fluorescence changes were observed using a confocal microscope. In the images, yellow dots indicate the presence of unfused autophagosomes, while red dots represent fusion events, signifying the formation of autolysosomes. (B) Number of GFP-LC3 and mRFP-LC3 puncta per cell in different groups was quantified. The experiments were repeated three times. *P<0.05, **P<0.01, ****P<0.00. RAPA, rapamycin; 3-MA, 3-methyladenine; H/R, hypoxia/reoxygenation; mRFP, monomeric red fluorescent protein; GFP, green fluorescent protein.

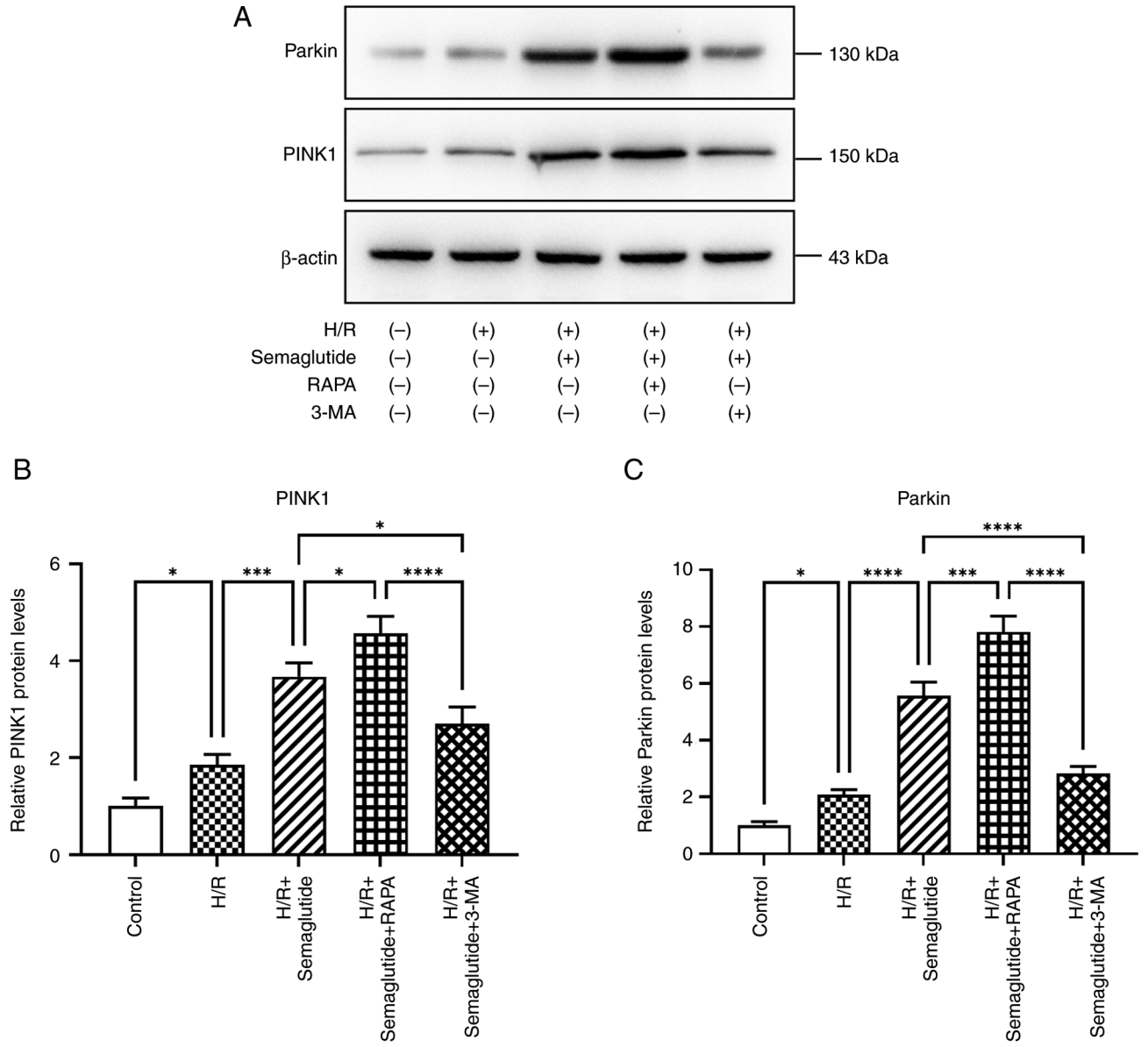

Figure 7. Modulation of Parkin and PINK1 protein expression induced by semaglutide in AC16 cells subjected to H/R. (A) Representative western blotting images of PINK1 and Parkin. β-actin was used as the loading control. Optical density values of the target bands were analyzed using a gel image processing system. Semi-quantitative analysis of (B) PINK1 and (C) Parkin. The experiments were repeated three times. *P<0.05, ***P<0.001, ****P<0.0001. RAPA, rapamycin; 3-MA, 3-methyladenine; H/R, hypoxia/reoxygenation; PINK1, PTEN-induced putative kinase protein-1.

under hypoxic conditions, suggesting improved mitochondrial function and energy metabolism.

Autophagy has emerged as a crucial process for maintaining cardiac homeostasis and response to various stresses, such as H/R injury (41). Autophagy, also known as 'self-eating', is a highly conserved catabolic process responsible for degrading and recycling cellular components, thereby ensuring cellular health and survival (42). The role of autophagy in cardiac diseases is complex (43). On the one hand, autophagy can protect cardiomyocytes from damage by eliminating dysfunctional organelles and toxic protein aggregates (44). On the other hand, dysregulation of autophagy has been linked to the development of various cardiac disorders (45). Therefore, understanding the mechanisms that regulate autophagy in cardiomyocytes is essential for the development of novel therapeutic strategies.

Among the well-recognized autophagy pathways, the PINK1/Parkin pathway, as well as other pathways such as the FUNDC1, BNIP3 and BNIP3L/NIX pathways, are notable. The PINK1/Parkin pathway is triggered in response to mitochondrial damage, resulting in the recruitment of Parkin to impaired mitochondria and subsequent mitophagy; the PINK1/Parkin pathway is the most clearly defined pathway for mitochondrial autophagy (46). When the mitochondrial membrane potential becomes depolarized, PINK1, which is anchored to the mitochondrial outer membrane, will recruit the Parkin protein from the cytoplasm to the mitochondrial outer membrane. Following this, Parkin can enlist the autophagy receptor p62 to facilitate the development of autophagy lysosomes (8,47).

The present study indicated an increase in autophagy activity in H/R-exposed cells, characterized by an elevated LC3-II/LC3-I ratio and increased number of autophagosomes.

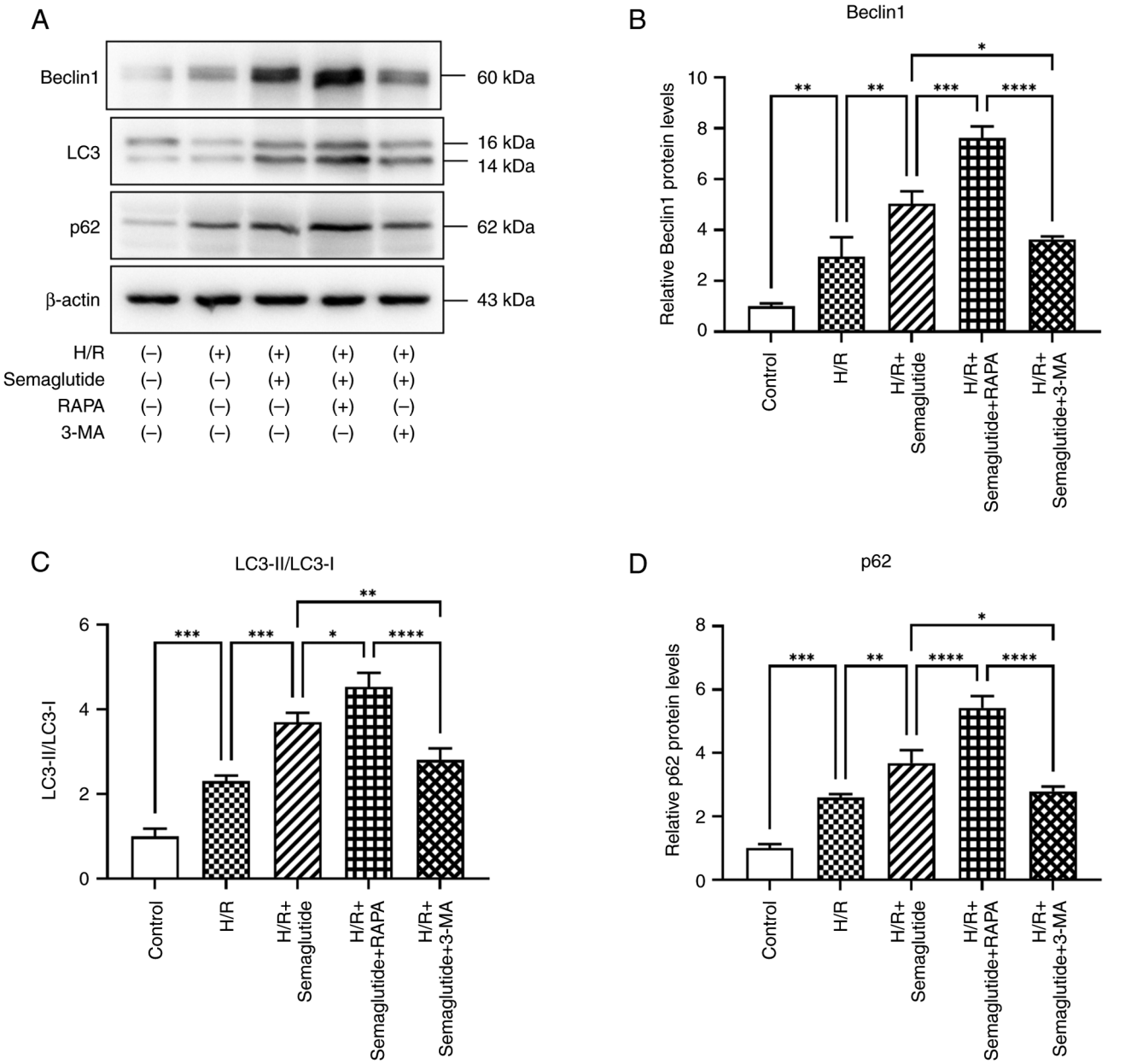

Figure 8. Western blot analysis of autophagy-related proteins in AC16 cells following treatment with semaglutide under H/R conditions. (A) Western blotting indicated the expression levels of Beclin1, LC3 and p62. β-actin was used as an internal control. Optical density values of the target bands were analyzed using a gel image processing system. Semi-quantitative analysis of (B) Beclin1, (C) LC3-II/LC3-I and (D) p62. The experiments were repeated three times. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. RAPA, rapamycin; 3-MA, 3-methyladenine; H/R, hypoxia/reoxygenation.

The present study demonstrated that semaglutide treatment further enhanced the H/R-induced changes in autophagy activity, autophagosome levels and LC3-II/LC3-I ratio, suggesting a regulatory role of semaglutide in autophagy. Additionally, this effect was strengthened by rapamycin, an autophagy inducer, and weakened by 3-MA, an autophagy inhibitor.

The present results demonstrated that H/R induced oxidative stress, increased ROS generation and activated the PINK1/Parkin pathway. Semaglutide treatment further upregulated PINK1 and Parkin expression, suggesting that semaglutide may promote mitophagy through this pathway. However, the lack of a PINK1/Parkin knockout in the present study limited our ability to conclusively attribute the observed effects to a single pathway. Other pathways, including the AMPK pathway or alternative pathways, may also serve roles in the biological processes studied. Without genetic manipulation to confirm their involvement, the primary or exclusive pathway remains unclear. Thus, the present findings should be cautiously interpreted, recognizing the potential for multiple interacting pathways and the need for further research.

Some studies (48,49) have shown that an increase in the LC3-II/LC3-I ratio is accompanied by a decrease in p62 levels, whereas the present study observed an increase in p62 levels in the semaglutide group undergoing H/R, despite the elevated LC3B-II/LC3B-I ratio, which is indicative of impaired autophagic flux. The interplay between p62, a recognized autophagy substrate protein, and autophagic activity is complex. Under typical physiological conditions (50), active autophagy results in the degradation of p62 within lysosomes, leading to decreased p62 levels; however, an intriguing paradox emerges

under conditions of oxidative stress or toxin exposure, where autophagic activation coexists with elevated p62 levels (51,52). This suggests that p62 may serve as a stress-responsive protein (53), which is upregulated in response to oxidative challenges. Upon oxidative stress, nuclear factor erythroid 2-related factor binds directly to an antioxidant response element in the p62 promoter to induce its expression. Given the complexity of the relationship between p62 levels, autophagic activity and cellular stress responses, a comprehensive analysis of autophagic flux is essential for accurately assessing cellular autophagic activity. This analysis should include detailed examinations of the various stages of autophagosome formation, lysosome fusion and substrate degradation. By assessing these stages, a more thorough understanding of the autophagic processes within the cell may be gained. Moreover, a detailed investigation into the degradation of p62, which primarily occurs through autophagy, is crucial.

Semaglutide might influence autophagic activity by interacting with key regulators of autophagy, including mTOR, AMPK or unc-51-like autophagy activating kinase 1. Studying the impact of semaglutide on these autophagy-related proteins and their downstream effects on p62 degradation would further elucidate its role in autophagic flux. This approach will provide deeper insights into the mechanisms by which semaglutide modulates autophagic activity and its potential therapeutic implications.

The findings of the present study indicated that semaglutide may confer protective effects against H/R-induced cardiomyocyte injury by modulating autophagy via the ROS/PINK1/Parkin pathway. Given that both excessive and deficient autophagy can precipitate pathological states, precise regulation of this process is paramount. Consequently, elucidating the optimal timing and methodologies for inhibiting or activating these pathways is of utmost importance, particularly in striking a balance between autophagy-induced cell death and cell survival.

Notably, the present study has some limitations. Firstly, conclusions obtained from investigations in only one cell line are limited and we fully acknowledge the limitations of relying solely on the AC16 cell line to study cardiac H/R. Heart tissue *in vivo* exhibits a high degree of complexity and heterogeneity, which the AC16 cell line may not fully capture as a single cell type. To more comprehensively understand the biological mechanisms involved in the cardiac H/R process, it is necessary to consider using different cell lines or combining animal experiments with *in vitro* studies. Furthermore, the cell experiments should include H/R with RAPA or 3-MA alone, as these groups could provide further insights into the autophagy process. However, given the constraints of the current study, including resource limitations and the need to balance experimental complexity with feasibility, the current grouping strategy was used. Notably, previous studies (14,54), such as Zhu *et al* (14) have demonstrated the expression of GLP-1Rs in cardiomyocytes in control, H/R, H/R + saline and H/R + semaglutide groups, and have investigated the mechanisms by which GLP-1R agonists such as semaglutide exert their cardioprotective effects. In the present study, GLP-1R protein levels were not measured in the control, H/R or H/R + semaglutide groups; we aim to address this limitation by measuring GLP-1R levels in future studies.

In conclusion, the present study demonstrated that semaglutide may protect AC16 cardiomyocytes against H/R-induced injury by reducing oxidative stress and modulating autophagy, particularly through the ROS/PINK1/Parkin/p62 pathway. These findings offer novel insights into the mechanisms of the cardioprotective effects of semaglutide and suggest its potential therapeutic application in myocardial ischemia/reperfusion injury. Future studies are required to further explore the precise mechanisms of action of semaglutide and its clinical implications in the treatment of myocardial ischemia/reperfusion injury.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LQL, LLJ and JW contributed to the research design, manuscript drafting and overall manuscript revision process. YPT performed the data analysis. LQL took the lead in writing the manuscript, while JW and LLJ provided substantial revisions. LQL, LLJ, YPT and JW all participated in data acquisition, analysis and interpretation. LQL and LLJ oversaw the research program, reviewed the manuscript, and confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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