Ononin alleviates H_2O_2-induced cardiomyocyte apoptosis and improves cardiac function by activating the AMPK/mTOR/autophagy pathway

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Abstract. Ononin (ON) is an isoflavone with numerous reported bioactivities, including anti-oxidative, anti-inflammatory and neuroprotective effects. Autophagy is a critical homeostatic process in the body that has been reported to closely associate with the apoptotic processes of cardiomyocytes. Using flow cytometry, western blotting, echocardiography and Masson's staining, the present study investigated the effects of ON on H_2O_2-induced cardiomyocyte apoptosis and myocardial infarction, in addition to any potential underlying molecular mechanisms. H_2O_2 treatment reliably induced apoptosis in H9C2 cells. The anti-apoptotic effects of ON were revealed by flow cytometry results and by the downregulation of cleaved-caspase 3. Further investigations indicated that ON may alleviate apoptosis by enhancing autophagy, as evidenced by increased microtubule-associated proteins 1A/1B light chain 3B expression and p62 degradation. Activation of the 5' AMP-activated protein kinase (AMPK)/mTOR pathway was observed after ON administration following H_2O_2-induced cardiomyocyte injury. However, these anti-apoptotic effects mediated by ON were lost after autophagy inhibition by chloroquine or AMPK inhibition by Compound C. Finally, the protective effects of ON on cardiomyocytes *in vitro* could also be observed *in vivo*. A myocardial infarction model was established by ligating the left anterior descending branch of the rat heart. Using echocardiography and Masson's staining, ON was shown to increase the ejection fraction and decrease cardiac fibrosis in rats with myocardial infarction. These results suggest that ON exerts cardioprotective effects by improving autophagy via the AMPK/mTOR signaling pathway.

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Introduction

Cardiovascular disease is one of the leading causes of human mortality worldwide, which is an important social and economic burden (1). The loss of cardiomyocytes during various ischemic heart diseases is considered to be irreversible, thereby contributing to permanent cardiac dysfunction (2). Apoptosis is a type of cell death that is characterized by prominent changes in caspase activation and serves an important role in cardiac injury (3). Therefore, the amelioration of apoptosis and improving cardiomyocyte viability are of particular research interest.

Autophagy is the phagocytosis and subsequent degradation of intracellular proteins or organelles for metabolism (4). Autophagy has been implicated in numerous physiological and pathological processes, including survival, differentiation, cancer, aging and heart disease (5). Emerging evidence has indicated that autophagy exerts a biphasic role in cardiac physiology, whereby excessive or reduced autophagy levels can both result in cardiomyocyte apoptosis (6,7). However, the exact mechanism underlying this remains unclear. mTOR is considered to be a key signaling component among the several proteins that have been reported to regulate autophagy (8). mTOR can directly or indirectly integrate cell signals, such as ATP and hypoxia, to regulate autophagy induction (9,10). In addition, as an important regulatory enzyme of metabolism, increased phosphorylation of 5'-AMP-activated protein kinase (AMPK) was previously found to inhibit autophagy (11).

Isoflavones are particularly abundant in legumes (such as soy, chickpea and red clover) and can also be found in various traditional Chinese medicines (such as Astragali Radix and Puerariae Lobatae Radix) (12,13). Previous studies have reported the protective effects of isoflavone in neuron injury, where it conferred antioxidant and anti-inflammatory properties (14,15). Ononin (ON) is a bioactive isoflavone of legumes, however, to the best of our knowledge, the specific functions and mechanisms of ON in cardiomyocytes have not been previously reported. Therefore, the present study aimed to assess the potential role of ON in H_2O_2-induced H9C2 cell injury and investigate how it could modulate the AMPK/mTOR/autophagy signaling pathway. Additionally, ON was studied in a rat myocardial infarction model to study its possible role in cardiac function.

Materials and methods

Drugs. ON (MedChemExpress), chloroquine (CQ; MedChemExpress) and Compound C (CC; Selleck Chemicals) were dissolved in DMSO (Sigma-Aldrich; Merck KGaA) and diluted with PBS.

Cell culture and treatment. The rat cardiomyocyte H9C2 cell line was purchased from the American Type Culture Collection. Cells were cultured in high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Beyotime Institute of Biotechnology) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck KGaA) in an atmosphere containing 5% CO_2 at 37˚C. The cells were subsequently randomly divided into the following treatment groups: i) Control group; ii) H_2O_2 group, which involved culturing in serum-free DMEM and exposed to H_2O_2 (400 μmol/l) to stimulate cardiomyocyte injury for 4 h at 37˚C; iii) H_2O_2 + ON group, which was treated with H_2O_2 and ON (10 μmol/l) simultaneously for 4 h at 37˚C; iv) H_2O_2 + ON + CC group, which was treated with H_2O_2, ON and CC (10 μM) simultaneously for 4 h at 37˚C; and (v) H_2O_2 + ON + CQ group, which was treated with H_2O_2, ON and CQ (10 μM) simultaneously for 4 h at 37˚C.

Cell viability assay. A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) was used to evaluate cell viability. A total of $1x10^4$ H9C2 cells were plated onto 96-well plates and after treatment with H_2O_2 (400 μmol/l), 10 μl CCK-8 solution was added to each well and incubated at 37˚C for 2 h. Finally, the optical density absorbance of each well was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.) to calculate the relative viability.

Flow cytometry assay. The FITC Annexin V Apoptosis Detection Kit (BD Biosciences) was used to evaluate H9C2 cell apoptosis, and the experimental procedures were conducted according to the manufacturer's protocols. After H_2O_2 treatment (400 μmol/l), H9C2 cells were collected, washed twice with PBS and transferred into a culture tube ($5x10^4$ cells/tube) before 5 μl Annexin V-FITC and 5 μl PI were added to the cells and incubated for 15 min at room temperature in the dark. BD FACSCalibur™ flow cytometer (BD Biosciences) and Cell Quest software version 3.1 were used to perform the subsequent apoptosis analysis.

Western blot analysis. Total protein from H9C2 cells or rat heart tissue was lysed using RIPA buffer (Cell Signaling Technology, Inc.) supplemented with protease and phosphatase inhibitors (Cell Signaling Technology, Inc.). Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). Protein lysate samples of 30 μg were separated by 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). The membranes were blocked with 8% non-fat milk at room temperature for 60 min, before being incubated with the following primary antibodies overnight at 4˚C: Cleaved-caspase-3 (1:500; Cell Signaling Technology, Inc., cat. no. 9664), caspase 3 (1:1,000; Cell Signaling Technology, Inc., cat. no. 9662), Bcl2 (1:800; Cell Signaling Technology, Inc., cat. no. 3869), Bax (1:1,000; Cell Signaling Technology, Inc., cat. no. 2772), GAPDH (1:10,000, Abcam, cat. no. ab9385), p62 (1:1,000; Cell Signaling Technology, Inc., cat. no. 5114), LC3-I (1:1,000; Cell Signaling Technology, Inc., cat. no. 4108), LC3-II (1:1,000; Cell Signaling Technology, Inc., cat. no. 4108), actin (1:10,000; Abcam, cat. no. ab20272), phosphorylated (p-) AMPK (1:1,000; Cell Signaling Technology, Inc., cat. no. 4186), AMPK (1:1,000; Cell Signaling Technology, Inc., cat. no. 5832), p-mTOR (1:1,000; Cell Signaling Technology, Inc., cat. no. 5536) and mTOR (1:1,000; Cell Signaling Technology, Inc.; cat. no. 2972). After washing with PBS with 0.1% Tween®-20 three times at 10 min each, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies of goat anti-rabbit IgG (1:3,000; Cell Signaling Technology, Inc., no. 7074) and goat anti-mouse IgG (1:3,000; Cell Signaling Technology, Inc., no. 56970) for 1 h at room temperature. Finally, the blots were visualized using an ECL kit (EMD Millipore) and quantified using the Image Lab software version 4.0 (Bio-Rad Laboratories, Inc.) with GAPDH used as the loading control of Cleaved-caspase-3 and actin used as the loading control of the other proteins.

Myocardial infarction model and ononin treatment. All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Animal Care and Use Committee of Wenzhou Medical University (Cixi, China). Male Sprague-Dawley rats (250-300 g, 6-8 weeks) were obtained from Experimental Animal Center of Wenzhou Medical University. Rats were maintained in climate- and light-controlled room (24±1˚C, relative humidity of 65±15%, 12/12-h light/dark cycle) with *ad libitum* access to food and water for at least 1 week prior to the experiments. Myocardial infarction (MI) was surgically induced as previously described (16). A total of 18 rats were anesthetized via an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and ventilated with a rodent ventilator. Rats were divided into three groups (n=6/group): i) Sham; ii) MI and iii) MI + ON. The heart was then exposed, and the left anterior descending coronary artery was ligated permanently with a 6-0 silk suture in the MI group. The heart was exposed but the left anterior descending coronary artery was not ligated in the sham group. Rats in the treatment groups were intragastric injection of ON (20 mg/kg) (13) daily, whilst the MI group received the same volume of laboratory animal drinking water daily until 28 days after surgery.

Cardiac function assessment. Heart function was assessed using transthoracic echocardiography 28 days after MI induction. Left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were analyzed using Vevo® 2100 workstation software version 3.1.0 (VisualSonics, Inc.).

Masson's trichrome staining. A total of 28 days post-surgery, all rats were euthanized with 20% CO_2 for histological analysis. All heart tissues were quickly excised and dehydrated in a 30% sucrose solution. The hearts were then embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek USA, Inc.) and cut into 7-μm slices. Modified Masson's Trichrome Stain kit (Beijing Solarbio Science & Technology Co., Ltd.) was

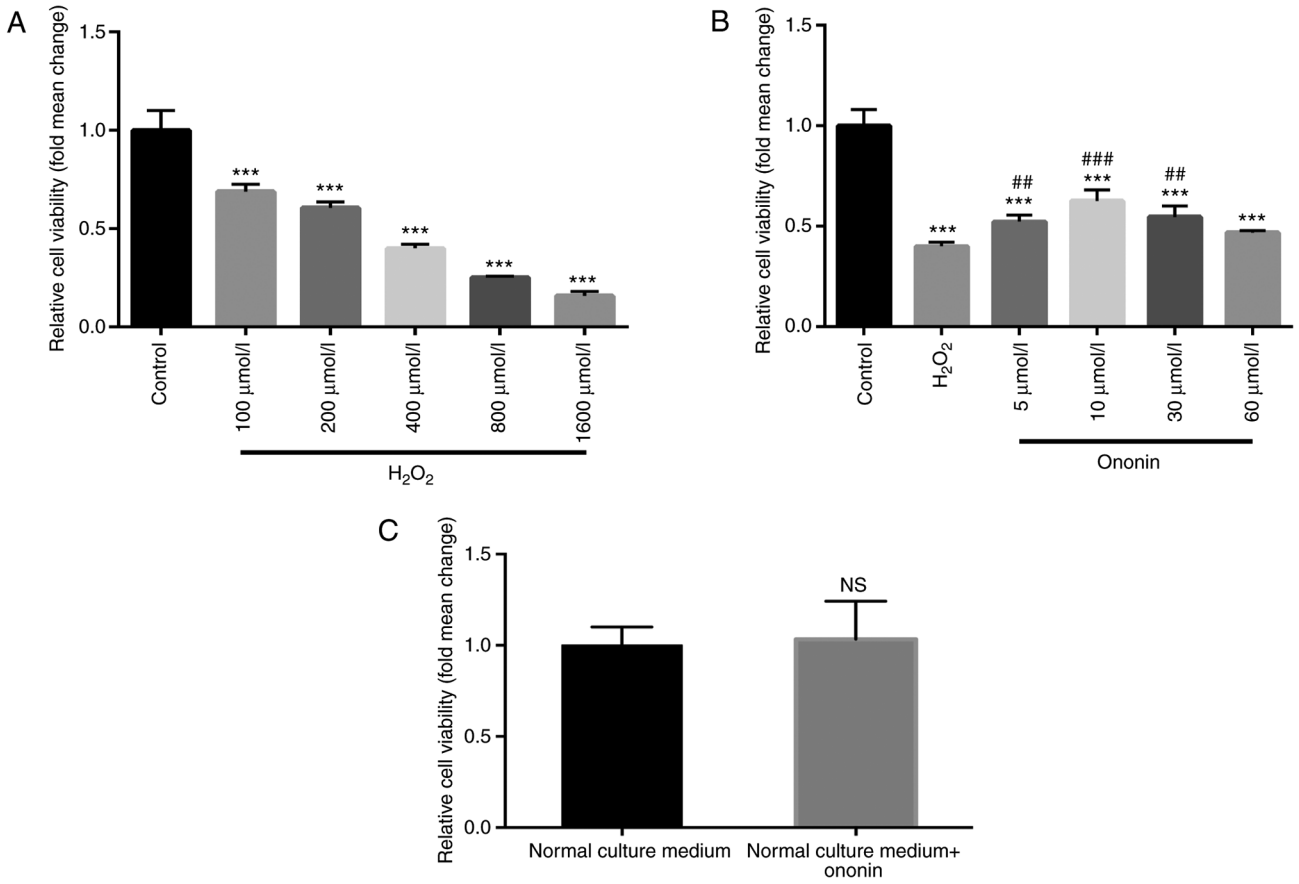

Figure 1. ON enhances the viability of H9C2 cardiomyocytes following H_2O_2-induced injury. (A) CCK-8 viability assays of H9C2 cardiomyocytes treated with different concentrations of H_2O_2 (100, 200, 400, 800 and 1,600 μmol/l) and (B) co-treated with increasing concentrations of ON (5, 10, 30 and 60 μmol/l) and H_2O_2 (400 μmol/l) were performed. ***P<0.001 vs. Control; $^{##}$P<0.01 and $^{###}$P<0.001 vs. H_2O_2. (C) CCK-8 viability assays of H9C2 cardiomyocytes with or without ON treatment (10 μmol/l) were performed. n=5 for all groups. ON, ononin; CCK-8, Cell Counting Kit-8; NS, not significant.

used to examine infarct size according to the manufacturer's instructions. A light microscope (Leica Microsystems GmbH) was used to capture images (x1 magnification). Following Masson's staining, myocardial fibers appear red, while collagen fibers appear blue. The proportion of the infarct area was quantified using Imaging Pro Plus software 6.0 (Media Cybernetics, Inc.) and calculated as follows: Infarct heart area (%)=(endocardial length + epicardial length of the infarcted area/endocardial length + epicardial length of whole left ventricle) x100%.

Statistical analysis. All data are presented as the mean ± SEM. All the cell experiments were repeated ≥ three times. GraphPad Prism 6.0 software (GraphPad Software, Inc.) was used for graph generation and data analysis. Student's t-test was performed to analyze differences between two groups. Differences among > three groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

ON improves cell viability in H_2O_2-induced H9C2 cardiomyocyte injury. As indicated by the results of CCK-8 assays, H_2O_2 induced a concentration-dependent reduction in H9C2 cardiomyocyte viability compared with that in the control group (Fig. 1A). Due to its ability to induce a 50% decrease in cell viability, the 400 μmol/l dose of H_2O_2 was selected for subsequent experiments. Treatment with suitable concentrations of ON (5, 10 or 30 μmol/l) significantly ameliorated the H_2O_2-induced reductions in H9C2 cell viability (Fig. 1B) with 10 μmol/l ON exerting the optimal protective effects. Therefore, 10 μmol/l ON was chosen for subsequent experiments. An excess of ON (60 μmol/l) did not induce significant protective effects (Fig. 1B). To rule out the possibility that ON may increase the viability of the cardiomyocytes without H_2O_2 treatment, ON (10 μmol/l) was added to the H9C2 cell culture medium but did not affect cell viability (Fig. 1C). These results indicate the cardioprotective effects of ON against H_2O_2-induced H9C2 cell injury.

ON alleviates apoptosis in H_2O_2-induced H9C2 cardiomyocytes. Flow cytometry assays (Fig. 2A, B and D) demonstrated that H_2O_2 (400 μmol/l) could induce apoptosis in H9C2 cardiomyocytes. The apoptosis-promoting effect of H_2O_2 was confirmed by results from the western blot assays, which was evidenced by the significant increase in cleaved-caspase 3 levels (Fig. 2E and F) and significant down-regulation in the Bcl2/Bax ratio (Fig. 2G and H) compared with those in the control group. After ON administration

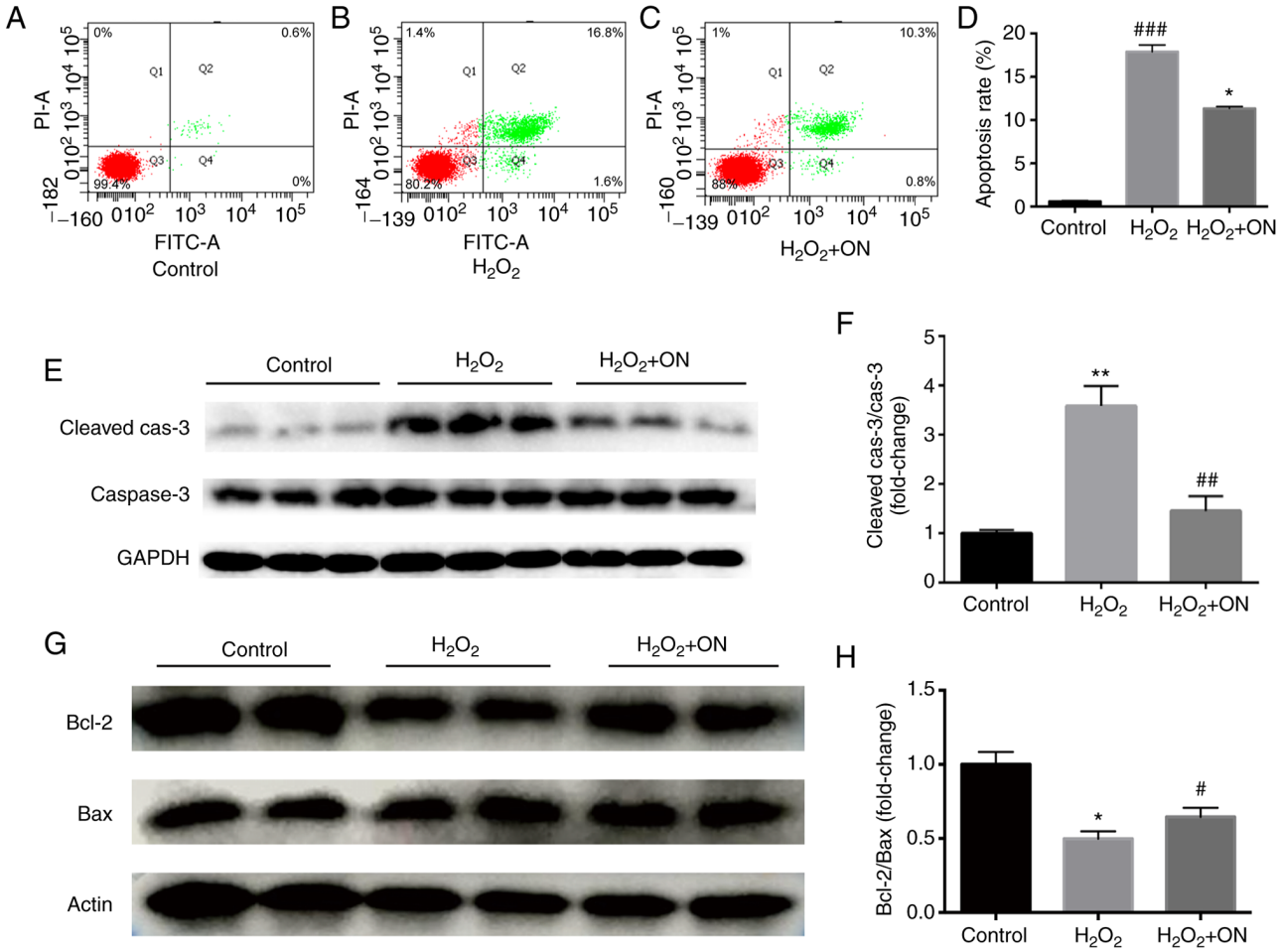

Figure 2. ON decreases H_2O_2-induced apoptosis in H9C2 cardiomyocytes. Representative flow cytometry scatter plots in the (A) control, (B) H_2O_2 and (C) H_2O_2 + ON groups to show the apoptotic levels. (D) Quantitative analysis of the flow cytometry scatter plots. $^{###}$P<0.001 vs. Control; *P<0.05 vs. H_2O_2. (E) Western blotting assays of cleaved-caspase 3 and caspase 3 in the three groups, which was (F) quantified. (G) Western blotting assays of Bcl2 and Bax in the three groups, which was (H) quantified. *P<0.05 and **P<0.01 vs. Control; $^{#}$P<0.05 and $^{##}$P<0.01 vs. H_2O_2. n=3 for all groups. ON, ononin.

(10 μmol/l), compared with those in the H_2O_2 group, both the number of apoptotic cells (Fig. 2C and D) and the level of apoptosis-associated protein cleaved-caspase 3 were significantly decreased (Fig. 2E and F), whilst the Bcl2/Bax ratio was significantly increased (Fig. 2G and H). These results suggest that ON may alleviate apoptosis to promote cardiomyocyte viability.

Ononin suppresses H_2O_2-induced H9C2 cardiomyocyte apoptosis by enhancing autophagy. Autophagy serves an important role in cardiomyocyte injury and restoration in several cellular models (17). To determine the effects of ON on autophagy in H_2O_2-treated H9C2 cardiomyocytes, the expression of autophagy-associated proteins LC3-II and p62 was estimated after ON treatment. Compared with that in the H_2O_2 group, the H_2O_2 + ON group showed a significant decrease in p62 expression (Fig. 3A and B) and a significant increase in LC3-II expression (Fig. 3A and C), suggesting that ON can enhance autophagy in H_2O_2-treated H9C2 cells. Moreover, CQ (10 μM), an autophagy inhibitor, significantly increased the levels of p62 and LC3 II compared with those in the H_2O_2 + ON group (Fig. 3A-C). Furthermore, compared with that in the H_2O_2 + ON group and the H_2O_2 group, CQ significantly reversed the anti-apoptotic effects of ON (Fig. 3D), further suggesting the autophagy modulatory role of ON in the cardiomyocytes following H_2O_2 challenge.

ON promotes autophagy via the AMPK/mTOR pathway. The AMPK/mTOR signaling pathway serves an important role in autophagy (18). Therefore, it was hypothesized that ON may also engage autophagy through this pathway. CC, a specific AMPK inhibitor, was used to test the present hypothesis in H9C2 cells. Compared with that in the H_2O_2 group, ON treatment significantly increased the p-AMPK/AMPK ratio (Fig. 4A and B) whilst significantly diminishing the p-mTOR/mTOR ratio (Fig. 4A and C). After treatment with CC (10 μM) in the H_2O_2 group, compared with those in the H_2O_2 + ON group, the effects of ON on p-AMPK/AMPK (Fig. 4A and B), p-mTOR/mTOR (Fig. 4A and C), p62 (Fig. 4A and D) and LC3-II (Fig. 4A and E) were significantly reversed. However, the protective effects of ON on cell viability against H_2O_2 were lost after the AMPK/mTOR pathway was inhibited by CC (Fig. 4F).

Subsequently, the p-AMPK/AMPK ratio was also measured in the rat heart tissues. Compared with that in the MI group, ON significantly increased the p-AMPK/AMPK ratio, consistent with the *in vitro* results (Fig. 4G and H). These results suggest that ON can enhance autophagy through the p-AMPK/mTOR signaling pathway to improve viability and alleviate apoptosis.

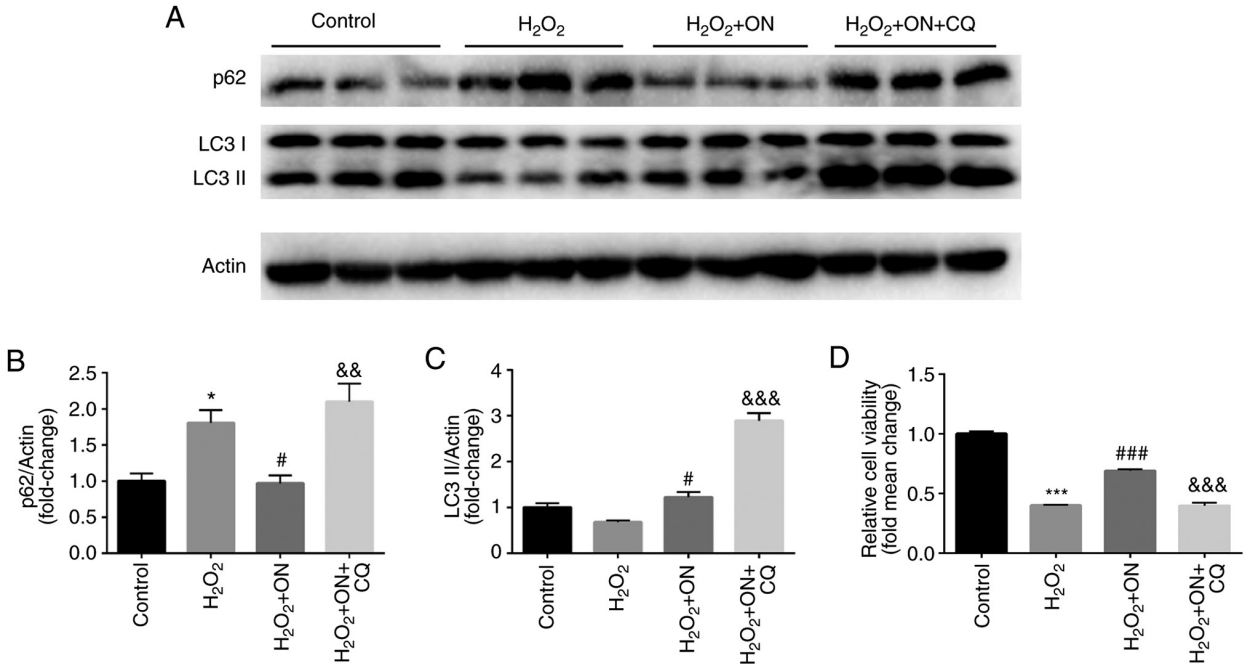

Figure 3. ON improves autophagy in H_2O_2-treated H9C2 cardiomyocytes. (A) Representative western blotting assay images of p62, LC3-I and LC3-II in the control, H_2O_2, H_2O_2 + ON and H_2O_2 + ON + CQ groups. Quantification of (B) p62 and (C) LC3-II protein levels. (D) Cell Counting Kit-8 viability assays in the four different groups were performed. *P<0.05 and ***P<0.001 vs. Control; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. H_2O_2; $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. H_2O_2 + ON. n=3 for all groups. ON, ononin; CQ, chloroquine.

ON treatment results in recovery of cardiac function after MI. To assess whether the anti-apoptotic effect of ON *in vitro* could result in favorable cardiac recovery *in vivo*, echocardiography was performed 28 days after MI induction. Compared with that in the MI group, ON delivery significantly improved cardiac function, as evidenced by the significantly improved LVEF and LVFS (Fig. 5A and B). To verify the improvement in cardiac function induced by ON, the heart tissue sections were examined histologically on day 28 after MI induction by Masson's trichrome staining to visualize the infarct size (Fig. 5C). Compared with that in the MI group, the ON group showed a significantly decreased infarct size (Fig. 5D). These results supported the notion that ON treatment could reduce the infarct area to preserve function.

Discussion

Stress-induced apoptosis contributes to cardiomyocyte loss (19). Therefore, inhibiting and reversing this process may prove to be an effective way to treat heart diseases (20,21). In the present study, the potential effects of ON in H_2O_2-induced H9C2 cardiomyocyte apoptosis were assessed. Increasing attention is being paid on exploring naturally-occurring extracts for drug development due to minimal side effects (22). As a dietary nutrient, isoflavones are widely abundant in legumes and exhibit antioxidant, anti-inflammatory and immunoregulatory effects (13). ON is a major bioactive isoflavone that can regulate oxidative stress, inflammation and immunity (15,23,24). Because of the close associations among oxidative stress, inflammation, immunity and apoptosis, it was speculated that ON can serve a role in H_2O_2-induced H9C2 cell injury (25). In the present study, H_2O_2 reliably induced H9C2 cardiomyocyte apoptosis, which was previously demonstrated (26). Following ON treatment, cell viability improved, and the apoptosis rate decreased, indicating the cardioprotective effects of ON in oxidative stress-induced cardiomyocyte apoptosis. Furthermore, ON increased the ejection fraction and decreased cardiac fibrosis in rats with MI confirmed its potential cardioprotective function.

To the best of our knowledge, the anti-apoptotic role of ON in cardiomyocytes has not been previously reported. It was speculated that autophagy may be involved in this process. Autophagy is involved in different models of cell death, including apoptosis, pyroptosis, necroptosis and necrosis (27,28). Emerging evidence has indicated that autophagy can regulate the apoptosis signaling pathway (29). Although autophagy can initiate apoptosis in cells exposed to an external stimuli, this does not mean that increased autophagy is harmful. Reducing autophagic levels can decrease apoptosis whereas and cell viability can also be improved by increasing autophagy (30). Therefore, autophagy is important for the maintenance of homeostasis and is beneficial to cells at appropriate operation levels. For example, a previous study reported that increased autophagy can promote nerve cell survival under nutrient deficiency (31). The present results demonstrated that ON increased autophagy, as observed by increased expression levels of the autophagy-associated protein markers p62 and LC3-II. Moreover, after treatment with the autophagy inhibitor CQ, ON lost its cardioprotective effects, as indicated by results from the CCK-8 assays. This suggests that ON alleviated H9C2 cardiomyocyte apoptosis at least partially by promoting autophagy.

The full signaling pathway profile that can regulate autophagy remain poorly understood, despite this process being precisely modulated (32). As a key molecule, mTOR integrates upstream signaling to inhibit the induction of autophagy (33). PI3K/AKT and MAPK/ERK1/2 signaling

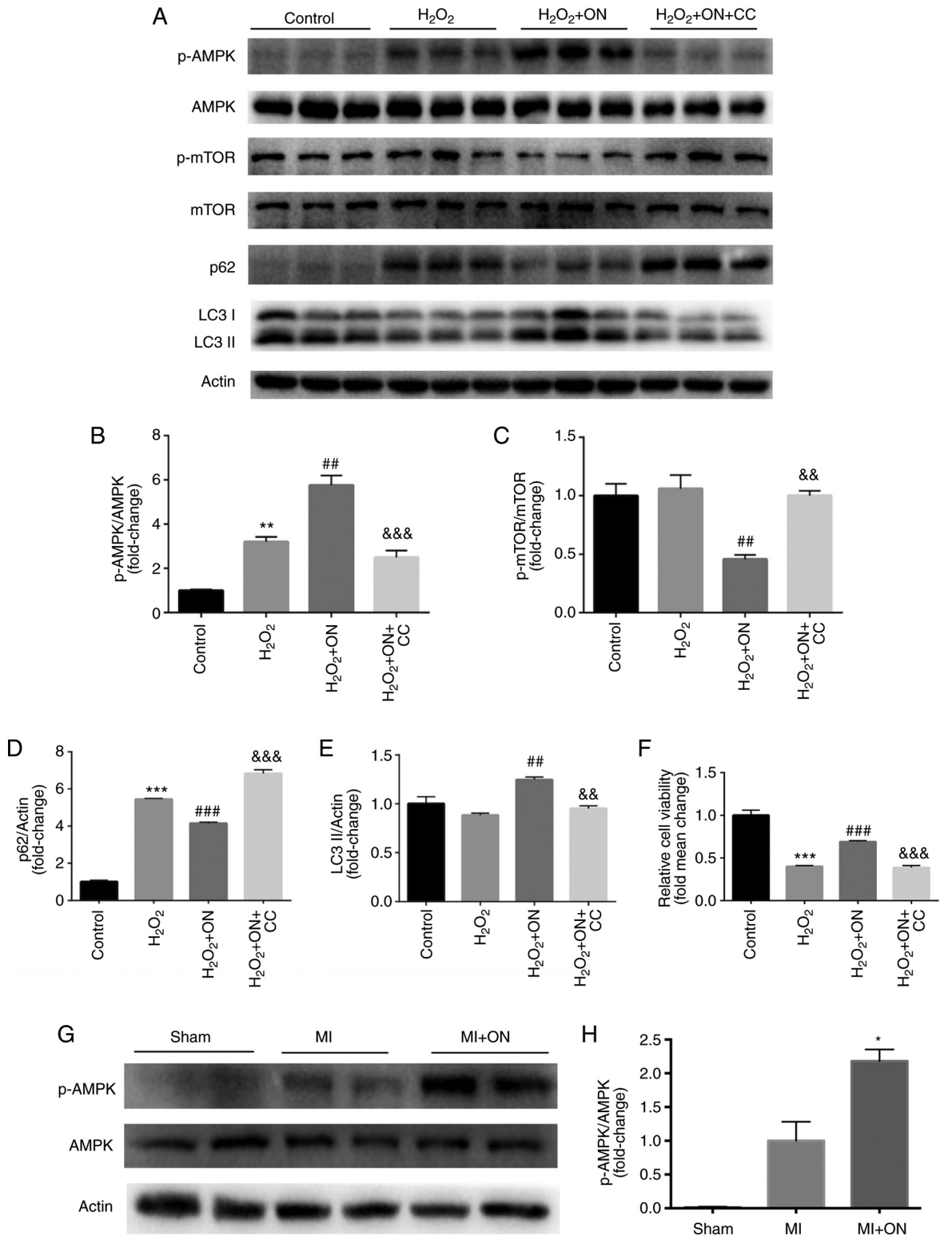

Figure 4. ON promotes autophagy via the AMPK/mTOR signaling pathway. (A) Representative western blotting images of p-AMPK, AMPK, p-mTOR, mTOR, p62, LC3-I and LC3-II in the control, H_2O_2, H_2O_2 + ON and H_2O_2 + ON + CC groups. Quantification of (B) p-AMPK/AMPK, (C) p-mTOR/mTOR, (D) p62 and (E) LC3-II. (F) Cell Counting Kit-8 viability assays in the four different groups were assessed. **P<0.01 and ***P<0.001 vs. Control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2; $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. H_2O_2 + ON. (G) Representative western blotting images of p-AMPK and AMPK in rat heart tissues isolated from the sham, MI and MI + ON groups, which were (H) quantified. *P<0.05 vs. MI group. The *in vitro* western blots were repeated 3 times. n=3 animals/group for *in vivo* western blots. ON, ononin; p-, phosphorylated; CC, Compound C; MI, myocardial infarction; AMPK, 5' AMP-activated protein kinase.

can activate the mTOR pathway to inhibit autophagy, whereas AMPK and p53 signaling negatively regulate mTOR to promote autophagy (34,35). The increased LC3-II and reduced p62 expression after ON treatment indicated that autophagy was upregulated, which was caused by the downregulation of mTOR phosphorylation. The present study also quantified the expression of upstream signaling components (p-AKT) of p-mTOR (data not shown). However, only p-AMPK levels

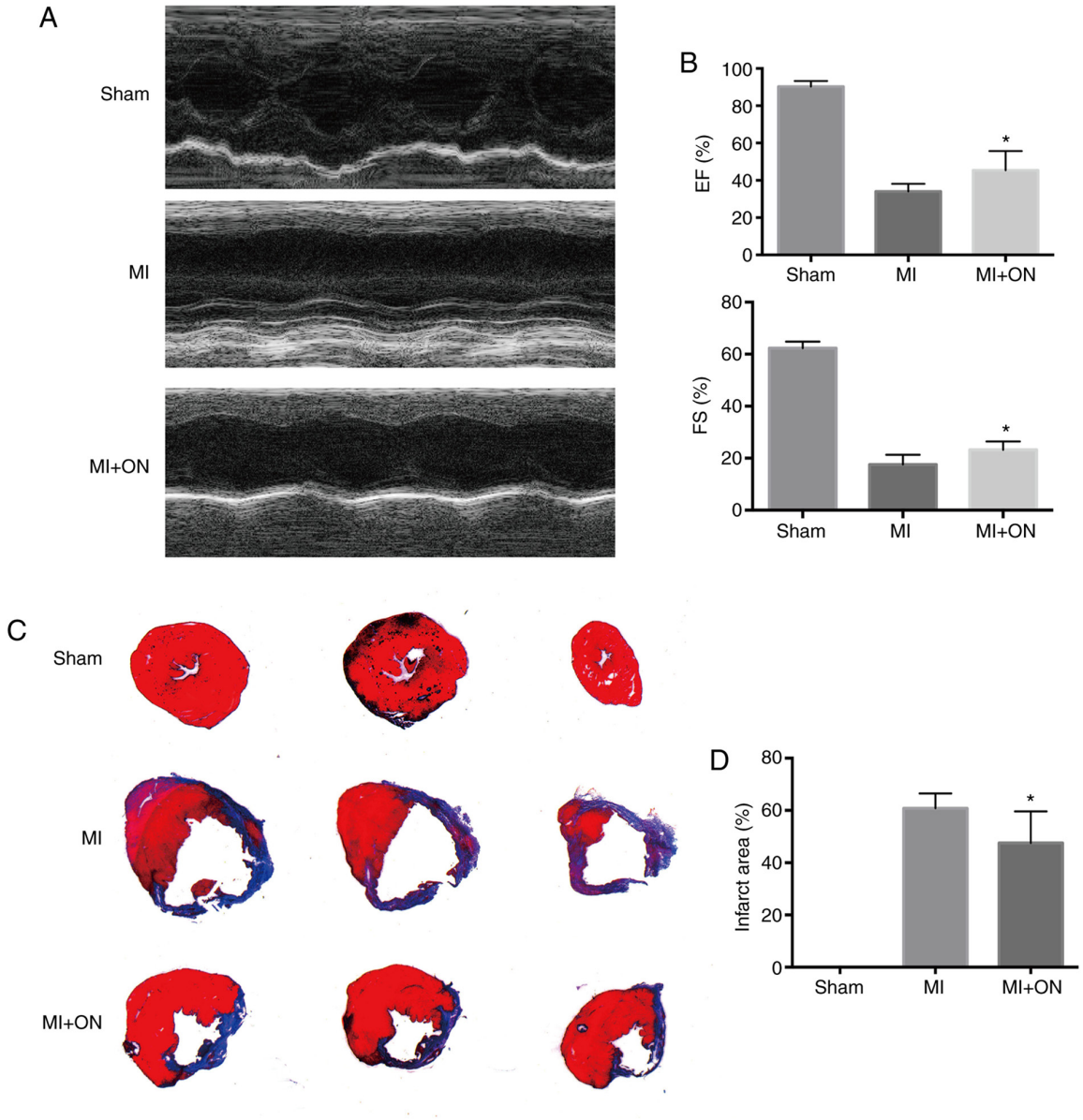

Figure 5. ON improves cardiac functional recovery and reduces cardiac fibrosis after MI. (A) Representative echocardiography images of the sham, MI and MI + ON groups. (B) Left ventricular EF and FS were measured by echocardiography. n=6 animals per group. *P<0.05 vs. MI. (C) Representative Masson's trichrome staining images in the sham, MI and MI + ON groups. (D) Quantitative analysis of infarct size. n=6 animals per group. *P<0.05 vs. MI group. MI, myocardial infarction; ON, ononin; EF, ejection fraction; FS, fraction shortening.

were firmly enhanced after ON treatment. The benefits of p-AMPK stimulation have been previously observed in many cardiovascular diseases, including MI and ischemia reperfusion injury (36-38). In the present study, AMPK activation inhibited the phosphorylation of mTOR, leading to an increase in autophagy. After treatment with the p-AMPK inhibitor CC, ON lost its protective effects as demonstrated by the CCK-8 assays, suggesting that ON can improve cell viability and reduce apoptosis through the AMPK/mTOR/autophagy signaling pathway after H_2O_2-induced cardiomyocyte injury. However, the protective effect of ON on cardiomyocytes and its potential mechanisms was only reported *in vitro* in the present study. The functional properties of ON should be studied further *in vivo* before it can be applied for clinical use. By developing a MI model and evaluating the role of ON in cardiac function and myocardial fibrosis, the present study determined the protective effects of ON further to provide a novel approach for the clinical treatment of MI.

In conclusion, the present study identified a protective role of ON on cardiomyocytes and cardiac function. This effect may be mediated through the AMPK/mTOR/autophagy pathway. However, the specific mechanisms of ON in regulating cardiac function *in vivo* require further exploration.

Acknowledgements

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RP designed the experiments and wrote the manuscript. QZ and JW performed the experiments and analyzed the data. RP and QZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee of Wenzhou Medical University (Cixi, China; approval no. 2019635).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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