

Nobiletin ameliorates cardiac impairment and alleviates cardiac remodeling after acute myocardial infarction in rats via JNK regulation

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Abstract

Nobiletin was found to protect against acute myocardial infarction (AMI)-induced cardiac function decline and myocardial remodeling, although the dose–effect relationship and underlying pathways remained unclear. In the current research, different doses of Nobiletin (7.5, 15 and 30 mg/kg/day) were administered to AMI rat model for 21 days. Survival rate, echocardiography, and histological analysis were assessed in vivo. In addition, MTT assay, flow cytometry, and Western blotting were conducted to explore Nobiletin's cytotoxicity and antiapoptotic effect on H9C2 cells. Mechanistically, the activation of MAPK effectors and p38 in vivo was studied. The results showed medium- and high-dose Nobiletin could significantly improve survival rate and cardiac function and reduce the area of infarction and cardiac fibrosis. Medium dose showed the best protection on cardiac functions, whereas high dose showed the best protective effect on cellular apoptosis and histological changes. JNK activation was significantly inhibited by Nobiletin in vivo, which could help to explain the partial contribution of autophagy to AMI-induced apoptosis and the discrepancy on dose–effect relationships. Together, our study suggested that JNK inhibition plays an important role in Nobiletin-induced antiapoptotic effect in myocardial infarction, and medium-dose Nobiletin demonstrated the strongest effect in vivo.

KEYWORDS

acute myocardial infarction, apoptosis, cardiac remodeling, hypertrophy, nobiletin

Abbreviations: AMI, acute myocardial infarction; AMPK, AMP-activated protein kinase; CO, cardiac output; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; LAD, left anterior descending; LVAWd, left ventricular end-diastolic anterior wall thickness; LVAWs, left ventricular end-systolic anterior wall thickness; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDd, left ventricular end-diastolic diameter; LVIDs, left ventricular end-systolic diameter; LVM, left ventricular mass; LVM/BM, left ventricular mass/body mass; LVPWd, left ventricular end-diastolic posterior wall thickness; LVPWs, left ventricular end-systolic posterior wall thickness; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGD, oxygen-glucose deprivation; PI3K, Phosphoinositide 3-kinase; TTC, 2, 3, 5-triphenyl tetrazolium chloride.

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1 | INTRODUCTION

Acute myocardial infarction (AMI) is an imminent threat to public health, as it is one of the leading causes of death globally.¹ Previous studies on animal and cell models suggested that myocardial cell death is modulated by the interplay between oxidative stress, inflammation, apoptosis, and autophagy, which contribute to the progression and prognosis of cardiac ischemic diseases.² Loss of cardiomyocyte induced by ischemia injury is a key factor that results in cardiac remodeling, pathological hypertrophy and life-threatening heart failure.^{3,4} Thus finding novel agents to modulate cell death during ischemia injury is an important therapeutic target that draws constant research attention.

Nobiletin is a polymethoxy flavonoid enriched in citrus, which was reported to have anti-inflammatory,^{5,6} anticancer,⁷ and anti-apoptotic effects.⁵⁻⁷ In the past few years, the protective effects of Nobiletin against ischemic injury were reported in hepatic, renal, and cerebral ischemic diseases.⁸⁻¹⁰ Studies have shown that Nobiletin could alleviate hippocampal injury following cerebral ischemia and improve learning and memory.¹¹⁻¹³ While there are few studies of Nobiletin on myocardial infarction, our previous study found that Nobiletin could protect against myocardial ischemic injury through restoration of autophagic flux, alleviation of apoptosis, and reduction in cardiac remodeling.¹⁴ Interestingly, neither chloroquine (inhibitor of autophagic flux) nor 3-MA (inhibitor of autophagosome formation) could fully reverse the anti-apoptotic effect of Nobiletin (previous study, Figure 4A and B). Moreover, the study showed AMI-induced activation of classical autophagy upstream signaling pathways mTOR, AMPK, and PI3K were unaffected by Nobiletin treatment. Therefore, it is speculated that diverse cellular signaling pathways contribute to the protective effect of NOB.

JNK (c-Jun N-terminal kinase) is an important third-tier kinase in the MAPK pathway and it is at the crosstalk of a wide range of stimuli and regulates a multitude of downstream activities ranging from transcription regulation, cytoskeletal dynamics to apoptosis induction.^{15,16} It plays an important part in apoptosis induction, including extrinsic, mitochondrial, and ER-stress-induced apoptotic pathways, by regulating apoptosis-related gene expression and protein modification.¹⁷⁻²¹ For example, JNK could activate the apoptotic pathway by increasing apoptotic gene expression via c-Jun activation, inhibiting Bcl-2 directly via phosphorylation, or releasing proapoptotic Bad and Bax.¹⁶ Therefore, JNK has received a lot of attention as a research target to develop potential therapy against myocardial ischemic injury.²² The relationship between JNK and autophagy is more complex and maybe context-dependent. Studies have found that JNK could upregulate ATG gene expression,²³ and activated JNK could release Beclin1 from Bcl-2/Beclin1 complex via phosphorylation,²⁴ which induces detrimental autophagy.^{25,26} In this study, the modulatory effect on major MAPK family members JNK, ERK1/2, and p38 MAPK were explored to elucidate the regulation on the upstream pathway by Nobiletin. Furthermore, the dose-effect relationship of the protective effect of Nobiletin was explored by assays.

2 | MATERIALS AND METHODS

2.1 | Animals

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and the protocol was approved by the Animal Research Committee of Guangzhou Medical University. Adult male Sprague-Dawley rats (7-8 weeks of age, 200-220 g) were purchased from the Medical Experimental Animal Center of Guangdong Province and kept on a 12-h light/dark cycle under temperature- and humidity-controlled environment for 7 days. All rats received food and water available ad libitum.

All rats were randomly divided into six groups ($n = 54$ per group): (1) Sham group (Sham); (2) AMI group (AMI); (3) AMI+Nobiletin low-dose group (AMI+Nob-L, Nobiletin 7.5 mg/kg/day); (4) AMI+Nobiletin medium-dose group (AMI+Nob-M, Nobiletin 15 mg/kg/day); (5) AMI+Nobiletin high-dose group (AMI+Nob-H, Nobiletin 30 mg/kg/day); and (6) AMI +Enalapril (AMI+Ena, Enalapril 10 mg/kg/day). Rats in Nobiletin and Enalapril groups received Nobiletin (#962600, Sigma-Aldrich) or Enalapril (Merck Sharp & Dohme), respectively, via intraperitoneal injection once every 24 h for 21 days. Rats in the Sham and AMI group were administered with equal volume of saline solution.

2.2 | Rat model of acute myocardial infarction

Animal model of myocardial infarction was induced by left anterior descending (LAD) coronary artery ligation as we previously described.^{14,27} In brief, rats were anesthetized with 2% (v/v) isoflurane in O₂ and connected to a respirator for mechanical ventilation. Body temperature was maintained with a heating pad. After exposure of the heart by thoracotomy and pericardiotomy between the fourth and fifth rib on the left, LAD coronary artery was ligated with a 6-0 silk suture 2 mm below the tip of the left atrial appendage. Proper LAD ligation was confirmed by the whitening of the myocardial tissue distal to the suture and by electrocardiography. Sham animals underwent thoracotomy and pericardiotomy, but not LAD ligation.

2.3 | Measurement of infarction size and fibrosis

After 21 days of LAD ligation, the rats were euthanized via intraperitoneal injection of pentobarbital sodium (200 mg/kg), and the hearts were rapidly removed. The left ventricle including the septum was dissected free and weighed. The myocardial tissue was sectioned for H&E staining and histology as we described previously.¹⁴ Myocardial infarct size was measured by 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining, whereas fibrosis was measured by fixing the cardiac tissue in 4% paraformaldehyde for Masson's staining as previously described.¹⁴ For TTC staining, the left ventricle was sliced transversely into 2-mm-thick slices perpendicular to the axis of the LAD

immediately and incubated in TTC (pH 7.4) for 20 min at 37°C. The tissue from area-at-risk (identified as red tissue staining) was isolated from the infarct area (absence of staining) and the nonrisk region (purple tissue staining) for use in molecular studies. The area of infarction and area of fibrosis were analyzed and quantified by Image-Pro Plus v.5.0 (Media Cybernetics Inc.).

2.4 | H9C2 cell culture

The H9C2 rat cardiac myoblast cell line was acquired from American Type Culture Collection (ATCC), and cultured as previously described.²⁸ Cells were cultured in complete high-glucose DMEM medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin, maintained under an atmosphere of 95% humidified air and 5% (v/v) CO₂ at 37°C. Cells were passed when the monolayer reached 90% confluence. Passages 3–9 were used for study.

2.5 | Establishment of oxygen-glucose deprivation model

As we previously described,¹⁴ cells were subjected to serum-free, glucose-free, and sodium pyruvate-free DMEM (#D5030, Sigma-Aldrich) after rinsing twice with the same medium, and were subjected to hypoxia treatment for 12 h in an anoxia chamber (InVivo 500, Ruskinn Life Science) (94%N₂/5%CO₂/1%O₂) at 37°C. The Nobiletin-treated group was exposed to 20 mM Nobiletin 2 h before oxygen-glucose deprivation (OGD) treatment.

2.6 | Cell viability assay

The cells were seeded in 96-well plates and allowed to recover for more than 24 h before OGD treatment as described earlier. The viability of cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and the absorbance at 570 and 620 nm was determined with a Biorad plate reader.

2.7 | Detection of cell apoptosis by flow cytometry

The Annexin V-FITC/PI Apoptosis Detection Kit (#556547, BD Pharmingen) was used to measure apoptotic cell population according to the manufacturer's instruction. In brief, after Nobiletin pretreatment at different concentrations and OGD exposure, cells were trypsinized, washed twice with cold PBS and re-suspended in binding buffer. Then the cells were incubated with Annexin V-FITC and PI working solution in the dark at room temperature for 15 min. Cellular fluorescence was measured by flow cytometer (FACSAria; Beckman-Coulter Inc.) and the data were analyzed by FlowJo v.7.6 (TreeStar Inc.). Each experiment was repeated at least three times.

2.8 | Western blot analysis

The tissue from area-at-risk was homogenized with cold RIPA lysis buffer with protease inhibitor cocktail (Sigma) before centrifugation and separation of supernatant. Protein concentration was determined by BCA assay (Pierce). An equal amount of protein extracts (20 µg) from each sample was electrophoretically separated by 10% SDS polyacrylamide gels and electro-transferred to polyvinylidene difluoride (PVDF) membrane (Roche). The membranes were blocked with 5% BSA and incubated overnight at 4°C with primary antibodies against p-JNK (#4668, Cell Signaling Technology), JNK (#9252, Cell Signaling Technology), p-p38 MAPK (#4511, Cell Signaling Technology), p38 (#8690, Cell Signaling Technology), p-ERK1/2 (#4370, Cell Signaling Technology), ERK1/2 (#4695, Cell Signaling Technology), and GAPDH (SC-48166, Santa Cruz Biotechnology) at a working dilution of 1:1000. Membranes were washed three times with TBST and incubated with secondary antibody at a dilution of 1:5000 for 1 h at room temperature. After sufficient washing, blots were developed by 20X LumiGLO Reagent (Cell Signaling Technology). The band intensity was recorded by densitometry and analyzed with ImageJ (NIH). All the experiments were conducted at least three times.

2.9 | Statistical analysis

All data in this study were analyzed using GraphPad Prism v.5.0 (GraphPad Software Inc.). Quantitative data were represented as Mean±SEM. Statistical comparisons were analyzed using one-way ANOVA with Tukey's post hoc test. Differences with $p < .05$ were considered statistically significant.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,²⁹ and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.³⁰

3 | RESULTS

3.1 | Nobiletin improves survival rate and ameliorates cardiac function deterioration in AMI rats

Male Sprague–Dawley rats were subjected to LAD ligation to establish AMI model. 24 h after the procedure, low, medium, or high dose of Nobiletin (7.5, 15 or 30 mg/kg/day) was administered intraperitoneally once per day for 21 days. Enalapril is an angiotensin-converting enzyme inhibitor (ACE-I) that was found to have cardioprotective and antihypertrophic effect in clinical use,^{31–33}

and was administered as a positive drug control (10 mg/kg/day, i.p.). The Kaplan–Meier survival analysis suggested that AMI+Nob-M ($p = .0424$), AMI+Nob-H ($p = .2890$), and AMI+Ena ($p = .0458$) groups have better prognosis compared to AMI group (Figure 1A). Cardiac functions measured by transthoracic echocardiography (Figure 1B and C) also suggested that in AMI+Nob-M, AMI+Nob-H, and AMI+Ena groups, left ventricular internal diameter in systole (LVIDs), left ventricular anterior wall thickness at end-systole (LVAWs) were significantly improved compared to AMI group. Both end-systolic volume (ESV) and end-diastolic volume (EDV) were increased in AMI rats (Figure S1), although both Nobiletin and Enalapril exerted a protective effect on EDV, the overall results were statistically insignificant (Table S1). On the other hand, medium- and high-dose Nobiletin significantly alleviated ESV increase, leading to improved left ventricular fractional shortening (LVFS), left ventricular ejection fraction (LVEF) and cardiac output (Figure 1C), although LVPWd, LVPWs, and LVAWd showed no significant improvement (Figure S1). In addition to the improved left ventricular systolic function, Nobiletin treatment also significantly reduced AMI-induced left ventricular mass

(LVM) increase (Figure 1C), suggesting alleviation of cardiac hypertrophy. In terms of the dose-dependency of Nobiletin, medium dose exhibited better protective effect on survival rate, LVEF, LVFS, and cardiac output than high-dose treatment, although the result is statistically insignificant.

These results were consistent with our previous study that Nobiletin is protective against acute cardiac infarction in rat model. Furthermore, the findings suggested that medium-dose Nobiletin exhibited better protective effect on animal survival, cardiac function preservation, and alleviation of hypertrophy.

3.2 | Nobiletin alleviates myocardial infarction and fibrosis in AMI rats

To investigate the protective effect of Nobiletin on cardiac infarction and fibrosis after ischemic injury, myocardial tissues were stained by HE staining, TTC staining, and Masson's staining, respectively, at the end of the 21-day treatment (Figure 2A and B). HE staining showed

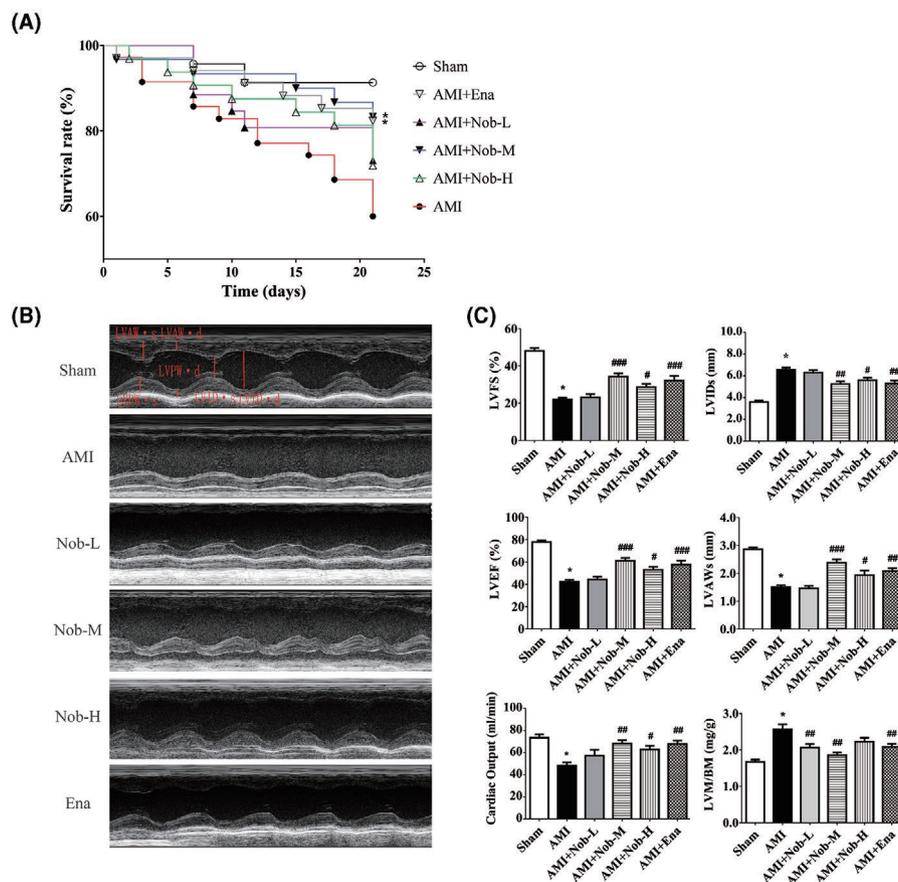


FIGURE 1 Nobiletin's effect on survival rates and cardiac function deterioration in AMI rats. (A) Survival curve for rats 21 day after LAD ligation ($n = 15$ per group). *Kaplan–Meier analysis performed shows p value $<.05$. (B) Representative M-mode echocardiogram recordings of each group 21 days after LAD ligation. (C) Left ventricular mass/body weight (LVM/BM), left ventricular end-systolic anterior wall thickness (LVAWs), left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end-systolic diameter (LVIDs) and cardiac output (CO) were calculated according to M-mode echocardiography in (B). (* $p < .05$ vs. Sham; # $p < .01$ vs. AMI; ### $p < .01$ vs. AMI; ### $p < .001$ vs. AMI; $n = 15$)

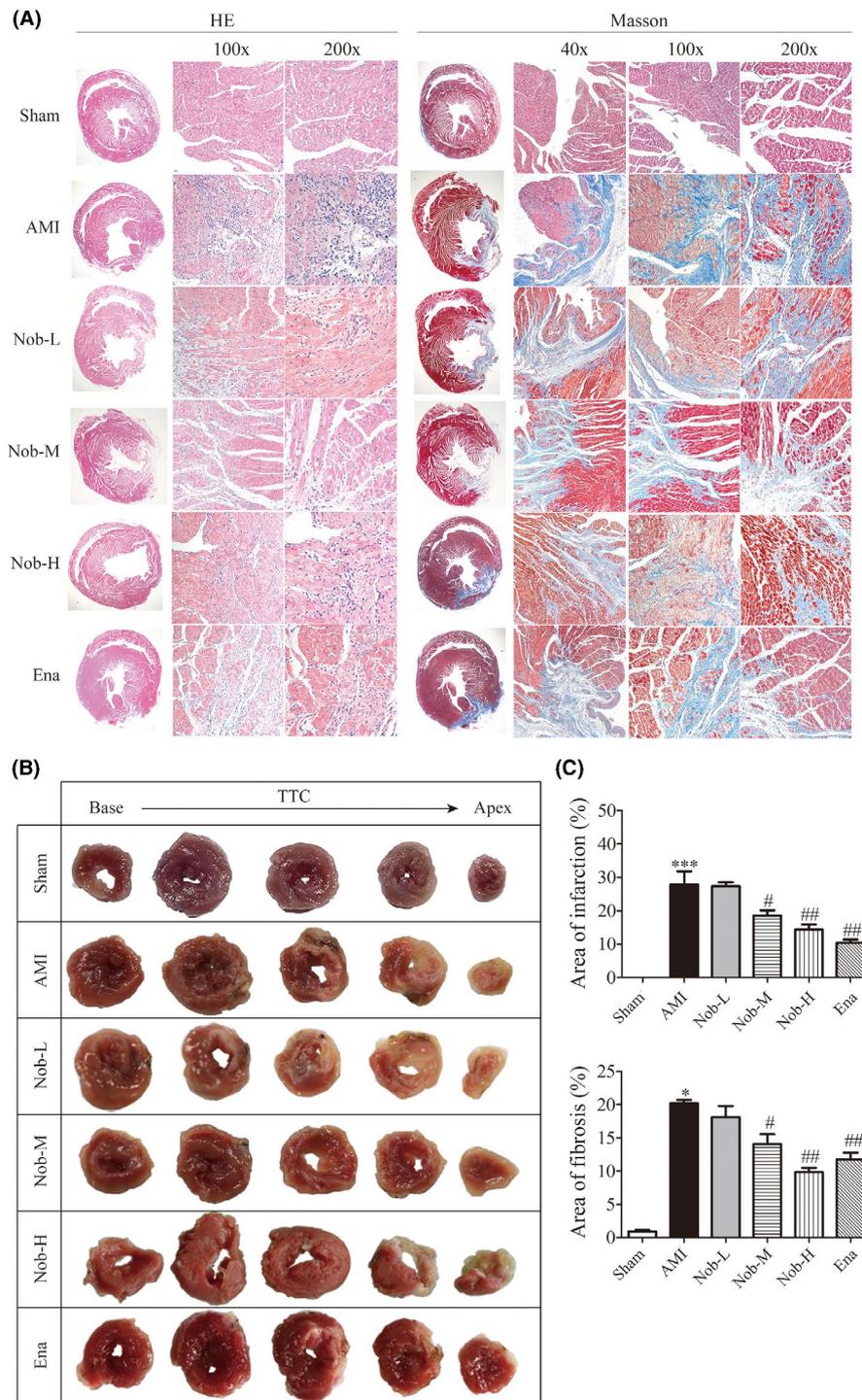


FIGURE 2 Nobiletin's effect on myocardial infarction and fibrosis in AMI rats. (A) Representative images of HE staining and Masson staining with different magnifications. (B) Representative sections of TTC staining. (C) Statistical analysis of area of infarction and fibrosis, from analysis on TTC staining sections and Masson staining sections, respectively. (* $p < .05$ vs. Sham; # $p < .05$ vs. AMI, ## $p < .01$ vs. AMI; $n = 15$)

regularly arranged myocardial fibers in the Sham group, which was replaced by fibrous tissue formation, inflammatory cell infiltration, and evidence of hemorrhage in AMI group. Nobiletin treatment could significantly reduce the disruption of myocardial fiber arrangement, fibrosis formation, and neutrophil infiltration (Figure 2A,

left panel). Masson's trichrome staining could define the degree of fibrogenesis, as the collagen fibers were stained blue and the muscle fibers stained red in the histological sections. The result further confirmed reduced fibrotic area in AMI+Nob-M, AMI+Nob-H, and AMI+Ena groups compared with AMI (Figure 2A, right panel).

TTC-stained sections could delineate the infarct myocardium as the unstained area and the intact myocardium as red area (Figure 2A). Compared to AMI group, treatment of medium- and high-dose Nobiletin and Enalapril could significantly reduce the infarction area (Figure 2B), and intriguingly, the protective effect follows a dose-dependent manner.

These data suggested that Nobiletin-induced cardiac protection against ischemic injury was related to reduction in infarction area and tissue fibrosis. In addition, high Nobiletin concentration (30 mg/kg/day) conferred better cardiac protection on in vivo histological assays.

3.3 | The cytotoxicity and antiapoptotic effect of Nobiletin on H2C9 cardiomyocytes against OGD treatment

The cytotoxic effect of Nobiletin alone was determined by MTT assay and its protective effect on H9C2 cells after 12-h

oxygen-glucose deprivation (OGD) treatment was determined by Western blotting and flow cytometry. In terms of cytotoxicity, at concentrations up to 40 μM , Nobiletin exerted no apparent cytotoxic effect in vitro after 24 h (Figure 3A). As for antiapoptotic protection, cleaved caspase 3, the major effector of apoptosis, increased after 12 h OGD treatment and was alleviated by 2 h Nobiletin pretreatment at 20, 40, and 80 μM (Figure 3B) in a dose-dependent manner. Flow cytometry with FITC-Annexin V/PI double staining confirmed that apoptotic cell population increased after OGD treatment from $7.54 \pm 0.94\%$ to $33.36 \pm 4.13\%$, which was reduced by Nobiletin pretreatment to $16.35 \pm 1.41\%$ at 80 μM (Figure 3C). Moreover, higher dosage (20, 40 and 80 μM) of Nobiletin conferred better protection against apoptosis compared to lower dosages (5 and 10 μM).

These findings confirmed our previous study, that the protective effect of Nobiletin against ischemic injury originates from its modulatory effect on caspase-3 cleavage and cell apoptosis, which follow a dose-dependent manner. However, it could inhibit cell viability at high dosage (80 and 160 μM).

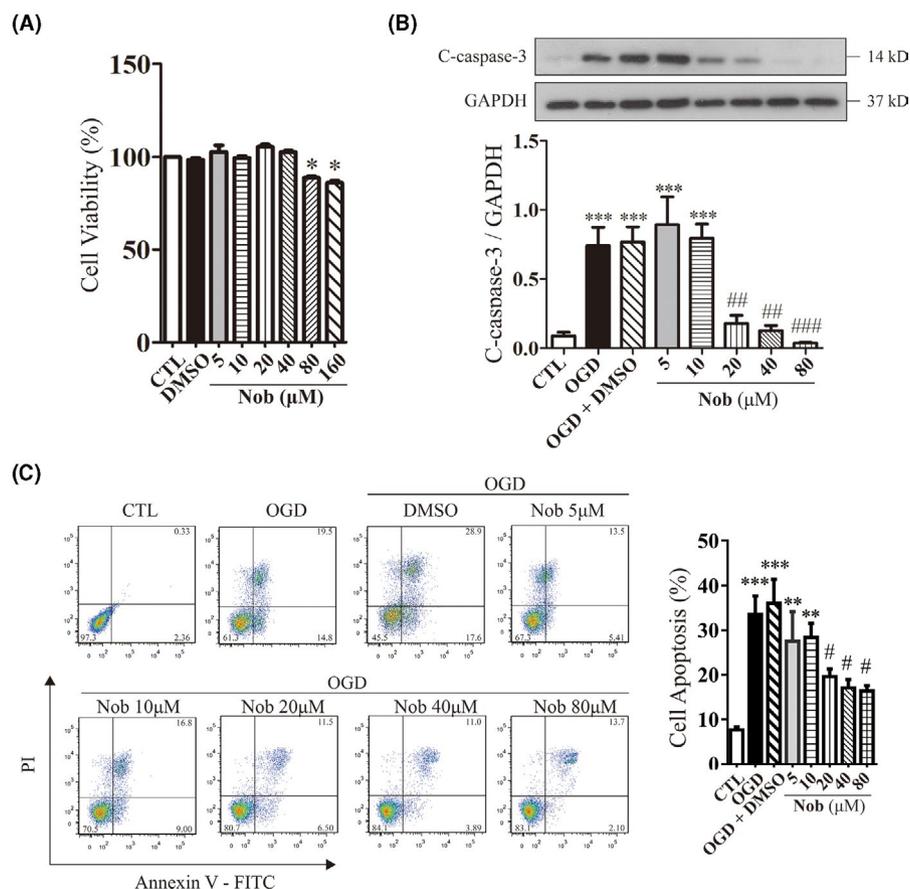


FIGURE 3 The cytotoxicity and antiapoptotic effect of Nobiletin on H2C9 cardiomyocytes against OGD treatment. (A) Cell viability was analyzed by MTT assay on H9C2 cardiomyocytes pretreated with various concentrations of Nobiletin for 24 h. (* $p < .05$ vs. CTL; $n = 6$). (B) Representative immunoblots and analysis of cleaved caspase-3 levels in H9C2 cardiomyocytes after 12 h OGD treatment in the presence or absence of 2 h Nobiletin pretreatments. The data were represented as the mean \pm SEM. (** $p < .05$ vs. CTL, # $p < .05$ vs. OGD; $n = 6$). (C) Representative images and analysis of FITC-Annexin V/PI double stain of H9c2 cardiomyocytes by flow cytometry. (** $p < .01$ vs. CTL, *** $p < .001$ vs. CTL, # $p < .05$ vs. OGD; $n = 6$)

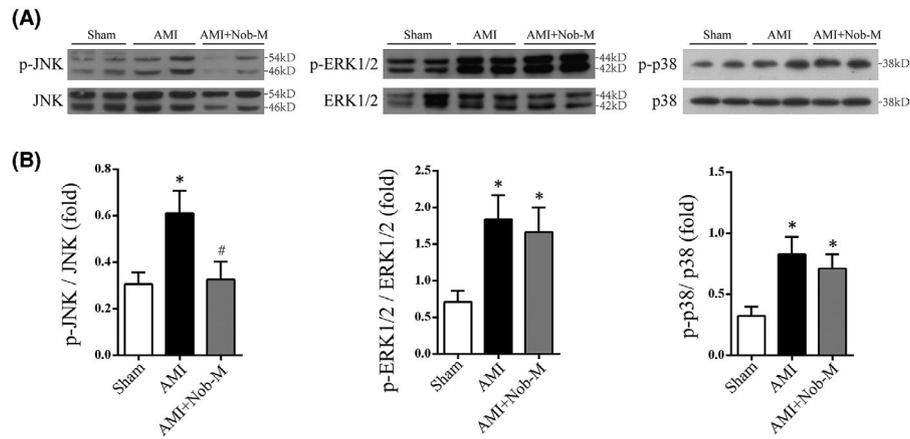


FIGURE 4 Nobiletin exerts antiapoptotic effect via modulation of JNK pathway in AMI rat model. (A) Representative immunoblots of p-JNK, JNK, p-ERK1/2, ERK1/2, p-p38, and p38 levels in tissue lysate from Sham, AMI, and AMI+Nob-M. (B) Statistical analysis of Western blot in (A). Data were represented as mean ± SEM. (**p* < .05 vs. Sham, #*p* < 0.05 vs. AMI; *n* = 5)

3.4 | Nobiletin exerts antiapoptotic effect via modulation of JNK pathway in AMI rat model

MAPK is a family of kinases that regulate cell proliferation, differentiation and survival through a sequential three-tiered kinase phosphorylation,^{34,35} which was shown to be modulated by Nobiletin in cerebral ischemia injury.⁹ Western blotting was conducted to identify the effect of Nobiletin on JNK, p38 MAPK, and ERK1/2, three important MAPK members that transduce respective signals to downstream pathways in AMI tissue. JNK and p38 MAPK, two pro-death MAPK responsive to stress stimuli were significantly activated, as shown by increased phosphorylation. Similarly, ERK1/2, a prosurvival MAPK molecule was also activated in AMI model (Figure 4A and B). Pretreatment with Nobiletin (15 mg/kg/day) exerted differential modulatory effects on MAPK molecules, as the activation of JNK in AMI group was

significantly reversed (from 0.61 ± 0.09 fold to 0.32 ± 0.08 fold, *p* < .01), whereas the phosphorylation of ERK1/2 and p38 MAPK remained unchanged (Figure 4C).

4 | DISCUSSION

Loss of cardiomyocyte after myocardial infarction is a critical event that involves necrosis, apoptosis and autophagic cell death, which leads to impairment in left ventricular function, pathological cardiac remodeling, and heart failure.^{36,37} The regulated cell death mechanisms such as apoptosis and autophagic cell death have been under investigation as important therapeutic targets in the treatment of AMI.³⁸⁻⁴⁰

Consistent with our previous research, Nobiletin can significantly reduce the myocardial infarction area, improve cardiac function, and reduce left ventricular remodeling in vivo (Figure 1). In

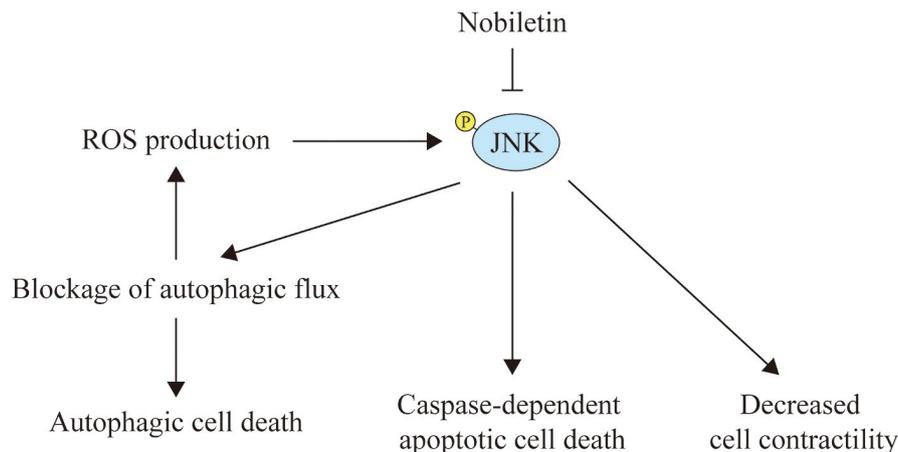


FIGURE 5 Schematic representation of JNK interaction with autophagic flux and its biological functions in Nobiletin-modulated AMI protection. The activated JNK by ROS accumulation could mediate the blockage of autophagic flux, which could exacerbate the production of ROS, leading to a feedforward loop of JNK activation. Nobiletin treatment could alleviate the activation of JNK, which help to curb the ensuing autophagic cell death and caspase-dependent apoptosis. A recent study also suggest a possible role of increasing cardiomyocyte contractility by JNK inhibition⁵⁴

addition, the study on dose–effect relationship showed medium-dose Nobiletin treatment (15 mg/kg/day) showed better protective effect than high-dose treatment (30 mg/kg/day), although the difference is statistically insignificant. Interestingly, *in vivo* and *in vitro* assays (HE, TTC and Masson staining; and cell viability assay) suggested better protection by high-dose Nobiletin (Figures 2 and 3) against histological changes and cell apoptosis induced by AMI. The antiapoptotic effect also seemed to be dose-dependent by Nobiletin treatment on both *in vitro* and *in vivo* levels, as apoptotic population and caspase 3 cleavage were reduced by Nobiletin preconditioning in OGD-treated cells (Figure 3B and C). On the other hand, Nobiletin at a higher dosage (80 μ M) showed an inhibitory effect on cell viability (Figure 3A) while maintaining its antiapoptotic effect (Figure 3B and C). This may help to explain the discrepancy in the dose–effect relationship in functional study result (Figure 1).

Our previous study showed that by restoring autophagic flux, Nobiletin could partially alleviate AMI-induced apoptosis, and classical autophagy signaling pathways were unaffected. Our current research took a step further to investigate the roles of major MAPK pathway effectors in the protective effect of Nobiletin against AMI. All three MAPK effectors, including proapoptotic JNK and p38 MAPK and antiapoptotic ERK1/2 were activated in ischemia injury (Figure 4). Our study showed that only JNK was suppressed by Nobiletin treatment.

The results suggested that inhibition of JNK is the key mechanism of action. JNK activation by ROS production was found to mediate autophagy initiation,^{41,42} which leads to caspase-dependent apoptosis.^{25,43–45} In addition, a previous study suggested that JNK mediates autophagic cell death in Bax/Bak double-knockout cells, a pathway independent of apoptosis,⁴⁶ which is in line with the other studies suggesting caspase-independent autophagic cell death mediated by JNK.^{26,47,48} On the other hand, the activation of JNK could be induced by blockage of autophagic flux, which leads to increased ER stress,^{49,50} activation of PERK and IRE1, and ROS production.^{18,51} Studies also found enhanced autophagic flux could reverse JNK activation, leading to reduced apoptotic cell death.^{52,53} Therefore, we hypothesize a feedforward loop of ROS production, JNK activation, and autophagic flux blockage, which is interrupted by Nobiletin treatment (Figure 5). As autophagic cell death and apoptosis are two separate downstream death pathways in this process, this is in agreement with our previous results, that inhibition of autophagy initiation (by 3-MA) and more importantly the inhibition of autophagic flux (by CQ) could partially reverse the antiapoptotic effect of Nobiletin.¹⁴

In addition, JNK inhibition was reported to play a protective effect on cardiomyocyte contractility,⁵⁴ thus inhibition of JNK activation by Nobiletin might also help to explain the discrepancy that medium dose (15 mg/kg/d) exhibited better cardiac function and overall animal survival, whereas high dose showed better protective effect for cell survival and structural preservation. However, this warrants further study for confirmation.

Collectively, our study confirmed that natural product Nobiletin exerts protection against AMI by inhibiting myocardial cell death

both *in vitro* and *in vivo*, whereas the dose–effect relationship remains differential between functional and histological studies. We also proposed that inhibition of JNK activation by Nobiletin could disrupt the vicious feedforward loop of ROS production, autophagic flux blockage, and JNK activation, leading to the rescue of both non-canonical autophagic death and caspase-dependent apoptosis. This finding could tie up the loose ends in our previous study and explain the findings in our study. Since the modulation of JNK is increasingly investigated in the treatment of myocardial ischemic injury, Nobiletin could be a potential therapeutic option in the treatment of AMI patients.

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CONFLICT OF INTEREST

We declared that the authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated and analyzed in the study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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