Iranian Journal of Basic Medical Sciences
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Fisetin attenuates tunicamycin-induced endoplasmic reticulum stress and apoptosis in cardiomyocytes via Nrf2-associated anti-oxidant responses

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ARTICLE INFO

Article type:
Original

Article history:
Received: Jun 30, 2026
Accepted: Nov 7, 2026

Keywords:
Cardiomyocytes
Endoplasmic reticulum-stress
Fisetin
Tunicamycin
Nrf2

ABSTRACT

Objective(s): Fisetin, a natural flavonoid, has anti-oxidant and anti-apoptotic properties; however, its effect on endoplasmic reticulum stress (ERS)-induced cardiomyocyte injury remains unclear. This study aimed to investigate fisetin's protective effect against tunicamycin-induced ERS injury in H9c2 cardiomyocytes and the role of the Nrf2-associated signaling pathway.

Materials and Methods: An ERS model was established in H9c2 cardiomyocytes using tunicamycin. Cell viability, reactive oxygen species (ROS) levels, apoptotic rate, and expression of ERS-related proteins were assessed by CCK-8 assay, DCFH-DA probe, flow cytometry, and Western blotting, respectively. The role of the Nrf2-associated signaling pathway was further investigated using the Nrf2-specific inhibitor ML-385.

Results: Fisetin pretreatment dose-dependently increased the viability of tunicamycin-injured H9c2 cells, reduced intracellular ROS levels, decreased the apoptosis rate, down-regulated GRP78 and CHOP protein expression, and up-regulated Nrf2 and its downstream anti-oxidant proteins HO-1, NQO-1, and catalase. Notably, co-treatment with ML-385 significantly reversed the above protective effects of fisetin.

Conclusion: Fisetin attenuates tunicamycin-induced ERS-mediated cardiomyocyte injury, and the underlying mechanism involves an Nrf2-associated anti-oxidant response. ML-385 reversed this protective effect, supporting a role for Nrf2-related anti-oxidant responses in suppressing oxidative stress and apoptosis.

▶ Please cite this article as:
Chen H, Du M, Chen T, Luo Ch, Cai L, Ning L, Ouyang Ch. Fisetin attenuates tunicamycin-induced endoplasmic reticulum stress and apoptosis in cardiomyocytes via Nrf2-associated anti-oxidant responses. Iran J Basic Med Sci 2027; 30:

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Introduction

The endoplasmic reticulum (ER), the largest membranous organelle in eukaryotic cells, performs essential functions including protein synthesis, folding, modification, and calcium storage (1). Under pathological stimuli such as ischemia, hypoxia, oxidative stress, or impaired glycosylation, unfolded or misfolded proteins accumulate abnormally in the ER lumen, triggering endoplasmic reticulum stress (ERS) and activating the unfolded protein response (UPR). Moderate UPR restores ER homeostasis and exerts cytoprotective effects by up-regulating molecular chaperones (e.g., glucose-regulated protein 78, GRP78), attenuating protein synthesis, and promoting the degradation of misfolded proteins. However, when stress is sustained or excessively severe, the UPR shifts toward pro-apoptotic program, inducing apoptosis through the activation of C/EBP homologous protein (CHOP), c-Jun N-terminal kinase (JNK), caspase-12, and other pathways (2-4).

A substantial body of evidence indicates that ERS-mediated apoptosis is a key pathological mechanism in the pathogenesis and progression of various cardiovascular diseases. In myocardial ischemia/reperfusion (I/R) injury, massive reactive oxygen species (ROS) production during reperfusion disrupts ER redox homeostasis, triggers an excessive UPR, and ultimately leads to cardiomyocyte apoptosis and cardiac dysfunction via CHOP-dependent pathways (5). Furthermore, ERS has been implicated in the pathological processes of atherosclerosis, acute coronary syndrome, myocardial infarction, and heart failure (6). Therefore, identifying novel intervention strategies that can effectively modulate ERS and maintain UPR balance is clinically significant for preventing and treating cardiovascular diseases.

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Fisetin is a natural flavonoid widely present in fruits and vegetables such as strawberries, apples, and onions, and exhibits notable anti-oxidant, anti-inflammatory, and anti-aging activities (7-9). In the context of cardiovascular protection, fisetin acts through multiple mechanisms: it prolongs coagulation time, improves mitochondrial function, reduces oxidative stress, and inhibits glycogen synthase kinase-3 beta (GSK-3β), thereby effectively alleviating myocardial I/R injury (10). Moreover, fisetin suppresses isoproterenol-induced cardiomyocyte apoptosis and ameliorates cardiac hypertrophy by modulating the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway and the expression of GRP78, CHOP, and B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax)(11). Additionally, fisetin alleviates doxorubicin-induced cardiomyopathy by activating the sirtuin 1 (SIRT1)/nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway and inhibiting ferroptosis in cardiomyocytes (12). Collectively, these findings show that fisetin exerts multi-target, multi-pathway cardioprotection. However, whether it protects against ERS-mediated cardiomyocyte injury remains to be systematically investigated.

Nrf2 is a pivotal regulator of intracellular redox homeostasis. It functions by regulating the transcription of downstream anti-oxidant enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase-1 (NQO-1)(13). Nrf2 is widely expressed in the heart, vasculature, brain, kidneys, liver, and muscle (14), and upon activation, it exerts protective effects in cardiovascular diseases through anti-oxidant activity and autophagy regulation (15). Notably, recent studies have revealed close crosstalk between Nrf2 and ERS. Nrf2 activation not only enhances cellular anti-oxidant defense but also protects against multiple disease models by inhibiting CHOP expression and attenuating ERS-mediated apoptosis (16-18). Fisetin has been shown to alleviate liver injury, apoptosis, and oxidative stress induced by hepatic I/R injury via the Nrf2/HO-1 pathway (19), and it markedly ameliorates zinc overload-induced hepatotoxicity in mice through autophagy-dependent Nrf2 activation (20).

Notably, previous studies on the cardioprotective effects of fisetin have largely been based on complex pathological models such as ischemia/reperfusion injury, doxorubicin-induced cardiomyopathy, or cardiac hypertrophy (12, 21). In these models, ERS is not the sole initiating event but rather a common downstream convergence point for multiple injury pathways, including oxidative stress, inflammation, mitochondrial dysfunction, and ferroptosis. Therefore, even when fisetin inhibits CHOP or GRP78 expression, it is difficult to determine whether it regulates ERS directly or indirectly through other pathways. In other words, although previous studies have repeatedly demonstrated that fisetin activates Nrf2 and is accompanied by changes in ERS-related markers, they have not validated its causal protective mechanism in a system where ERS is specifically triggered.

Tunicamycin, a classical ERS inducer, directly triggers the UPR by inhibiting protein N-glycosylation, independent of upstream stimuli such as ischemia, oxidative damage, or chemotherapeutic toxicity. This property makes it a key tool for distinguishing between "direct regulation of ERS by fisetin" and "indirect effects of fisetin on ERS markers through other pathways". Previous studies have shown that fisetin can alleviate tunicamycin-induced PC12 cell death, but its mechanism primarily involves ROS scavenging and Nrf2-driven gene expression (22). In cardiomyocytes, a tunicamycin-induced ERS model has been systematically established, in which GRP78 and CHOP are significantly up-regulated within 6-24 hr, providing a reliable platform for evaluating the direct ERS-regulatory effects of candidate drugs (23). However, whether fisetin inhibits the CHOP-mediated pro-apoptotic program through the Nrf2 pathway in this model has not been reported. This study aimed to use this relatively specific ERS model to investigate fisetin's direct regulatory effect on ERS in cardiomyocytes and to determine whether the Nrf2/CHOP axis is a key mechanistic component of its protective effect (Figure 1).

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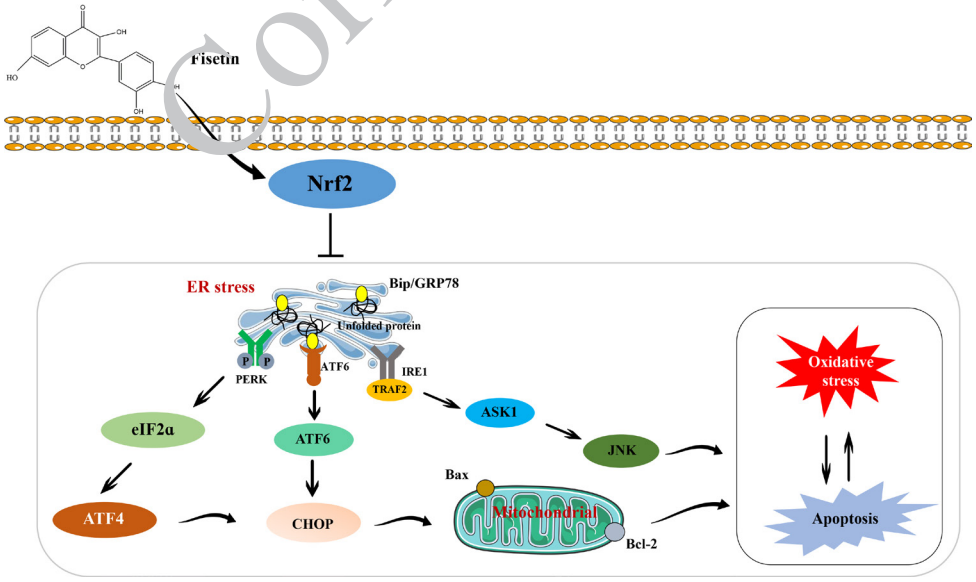


Figure 1. Schematic illustration of the potential mechanism by which fisetin attenuates tunicamycin-induced ERS-injury in H9c2 cardiomyocytes
Nrf2, Nuclear factor erythroid 2-related factor 2; ER stress, Endoplasmic reticulum stress; BiP/GRP78, Binding immunoglobulin protein/Glucose-regulated protein 78; PERK, Protein kinase R-like endoplasmic reticulum kinase; eIF2α, Eukaryotic translation initiation factor 2α; ATF4, Activating transcription factor 4; ATF6, Activating transcription factor 6; IRE1, Inositol-requiring enzyme 1; TRAF2, TNF receptor-associated factor 2; ASK1, Apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; CHOP, C/EBP homologous protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2

Materials and Methods

Materials

Fisetin and ML-385 were purchased from Selleck Chemicals (Texas, USA). Tunicamycin, ammonium persulfate, sodium dodecyl sulfate (SDS), and skimmed milk powder were obtained from Sigma-Aldrich (MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were obtained from Gibco (Uxbridge, UK). RIPA lysis buffer, bicinchoninic acid (BCA) assay kit, Annexin V-FITC apoptosis detection kit, cell counting kit-8 (CCK-8), and BeyoECL Plus kit were purchased from Beyotime Biotechnology. (Jiangsu, China). GAPDH mouse mAb, beta-Actin rabbit mAb, Bcl-2 rabbit mAb, Bax rabbit mAb, CHOP mouse mAb, GRP78 rabbit mAb, Nrf2 rabbit mAb, HO-1 rabbit mAb, NQO-1 rabbit mAb, and Catalase rabbit mAb were purchased from Cell Signaling Technology (MA, USA). Goat Anti-Mouse IgG and goat anti-rabbit IgG were purchased from Bioss (Beijing, China). All other reagents were of analytical grade and were used without further purification.

Cell lines and cultures

H9c2 cells (rat myocardial cell line) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). H9c2 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum at 37 ˚C in a 5% CO_2 atmosphere. Cells were seeded and incubated overnight before experiments.

Tunicamycin-induced ERS injury model in cardiomyocytes

To evaluate the effects of fisetin and ML-385 on basal cell viability, H9c2 cells were divided into the following groups: untreated control, vehicle control (DMSO), fisetin alone (5, 10, 20, 40, or 50 μM), and ML-385 alone (2.5, 5, or 10 μM). Fisetin-alone and ML-385-alone treatments lasted 24 hr.

To determine the optimal dose of fisetin, cells were randomly divided into five groups: Control group, Tm group, Tm+10 μM Fisetin group, Tm+20 μM Fisetin group, and Tm+40 μM Fisetin group. Subsequently, to explore potential mechanisms, cells were assigned to four groups: Control group, Tm group, Tm+Fisetin group, and Fisetin+Tm+ML-385 group. Fisetin and 5 μM ML-385 were added 2 hr before Tm stimulation, respectively. Cells were collected at the end of treatment.

Fisetin, tunicamycin, and ML-385 were dissolved in dimethyl sulfoxide (DMSO). The vehicle control group received the same volume of DMSO, and the final DMSO concentration was 0.1% (v/v) in all treatment groups.

CCK-8 cell viability assay

The CCK-8 stock solution was diluted in complete culture medium at a 1:10 ratio to prepare the CCK-8 assay solution. To the 96-well plate containing the cells (2×10^4 cells per well), the CCK-8 assay solution (10 μL per well) was added. The plate was then incubated at 37 ˚C in the dark for 2 hr. The absorbance at 450 nm (OD450) was measured to determine cell viability. Cell viability (%) was calculated as: (OD_treated−OD_blank)/(OD_Control−OD_blank)×100%.

Measurement of reactive oxygen species (ROS) in H9c2 cells

An ROS detection kit was used to measure the ROS levels. Cells were seeded in 96-well plates, treated with DCFH-DA (10 μM), and then incubated in a humidified environment (5% CO_2, 37 ˚C) in the dark for 20 min. Fluorescence images were acquired using a fluorescence microscope with fixed acquisition parameters. For each group, at least five random fields per well were captured, and three independent experiments were performed. Fluorescence intensity was quantified using ImageJ after background subtraction, and relative ROS levels were normalized to the control group. All images were acquired with identical settings and analyzed using a predefined threshold to minimize subjective bias.

Flow cytometric analysis of cardiomyocyte apoptosis

The apoptosis rate was measured using an Annexin V-FITC/PI apoptosis detection kit. Following drug treatment as described and an additional 24 hr of culture, cells were digested with 0.25% trypsin, centrifuged, and washed twice with pre-cooled PBS. Cells were resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml, stained with Annexin V-FITC and PI, and analyzed by flow cytometry to assess apoptosis.

Western blotting

Total cellular proteins were extracted from H9c2 cells using RIPA buffer, and protein concentrations were determined with a BCA assay kit. Protein from each sample (25 μg) was subsequently separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The blots were blocked with 5% skimmed milk and incubated with primary antibodies against GAPDH (1:2000), β-actin (1:1000), Bcl-2 (1:1000), Bax (1:1000), CHOP (1:1000), GRP78(1:1000), Nrf2 (1:1000), HO-1 (1:1000), NQO-1 (1:1000), Catalase (1:1000). Next, the blots were incubated with appropriate secondary goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated antibody (both 1:10000) for 1 hr at room temperature. Finally, proteins were visualized using enhanced chemiluminescence reagents, and band densities were quantified with ImageJ analysis software. Target protein abundance was normalized to GAPDH or β-actin. All western blot experiments were performed with at least three independent biological replicates, each using independently prepared cell lysates.

Statistical analysis

Statistical analysis was performed with SPSS 21.0 (IBM, Chicago, IL, USA). All data were expressed as mean±standard deviation (SD). For two-group comparisons, Student's t-test was used. For comparisons involving multiple groups, one-way ANOVA followed by Tukey's post hoc test was used. Differences associated with $P<0.05$ were considered statistically significant.

Results

Tm-induced cytotoxicity in cardiomyocytes in a concentration-dependent manner

The CCK-8 assay revealed that Tm treatment (0.25-2 μg/ml) reduced cardiomyocyte viability in a concentration-dependent manner. As shown in Figure 2, compared with the control group (0 μg/ml), all tested concentrations of Tm (0.25, 0.5, 1, and 2 μg/ml) significantly suppressed cell viability after 24 hr of treatment (Figure 2, $P<0.05$), indicating that Tm induces marked cytotoxicity in cardiomyocytes even at low concentrations.

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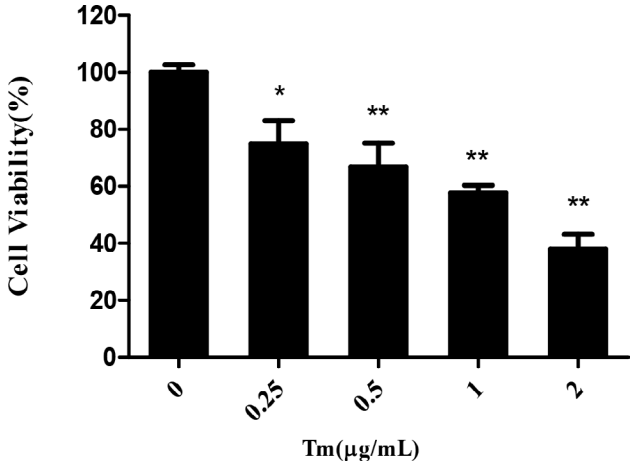


Figure 2. Tunicamycin (Tm) induced cytotoxicity in cardiomyocytes in a concentration-dependent manner
Cell viability was assessed by CCK-8 assay after 24 hr treatment. Data are expressed as mean±SD (n=3, each with four technical replicates), $^{*}P<0.05$, $^{**}P<0.01$ versus Control group. H9c2, rat myocardial cell line

Tm activates the ERS pathway in H9c2 cells

As shown in Figure 3, compared with the control group, treatment with different concentrations of Tm (0.25-2 μg/ml) for 24 hr up-regulated GRP78 and CHOP protein expression in H9c2 cells ($P<0.05$), with the most pronounced increases at 1 μg/ml. Collectively, treatment with 1 μg/ml Tm for 24 hr was therefore selected as the experimental condition for inducing ERS in subsequent studies.

Effects of fisetin and ML-385 on basal and tunicamycin-impaired cell viability

Treatment with fisetin alone at 5, 10, 20, or 40 μM did not significantly alter cell viability measured by the CCK-8 assay compared with the vehicle control (Figure 4A, $P>0.05$). Similarly, treatment with 2.5 or 5 μM ML-385 alone did not significantly affect basal cell viability ($P>0.05$). The vehicle control also showed no significant difference from the untreated control. These findings indicate that the fisetin and ML-385 concentrations used in this study did not cause detectable basal cytotoxicity under the tested conditions. Accordingly, based on the basal cell viability

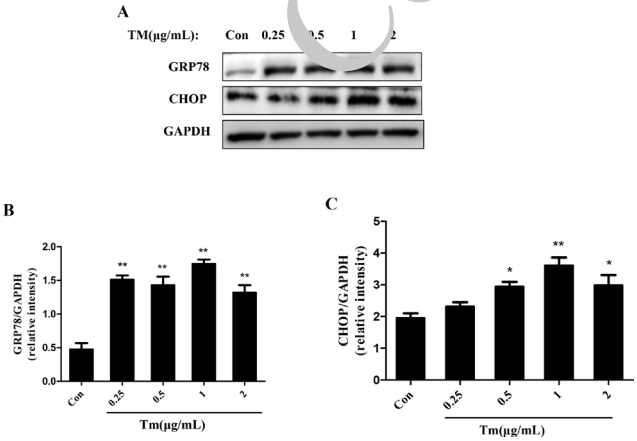


Figure 3. The effects of Tm on the expression of GRP78 and CHOP
(A) Representative Western blotting and (B-C) Quantification of Western blotting data. Data are expressed as mean±SD (n=3), $^{*}P<0.05$, $^{**}P<0.01$ versus Control group
Tm, Tunicamycin; Con, Control; GRP78, Glucose-regulated protein 78; CHOP, C/EBP homologous protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; H9c2, rat myocardial cell line

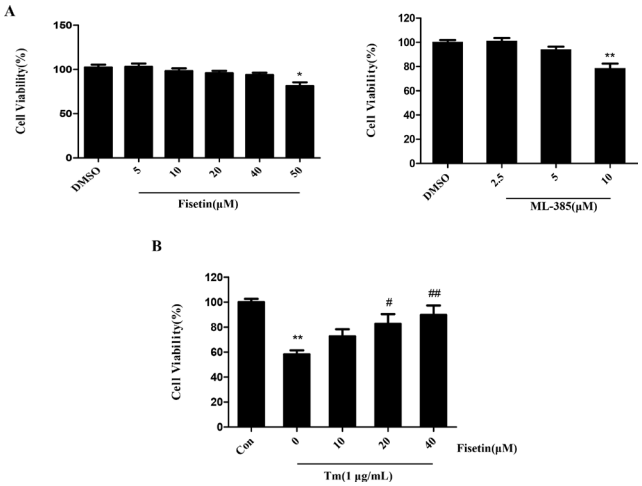


Figure 4. Effects of fisetin and ML-385 on basal cell viability and the protective effect of fisetin against tunicamycin-induced injury
(A) H9c2 cells were treated with vehicle, fisetin alone (5, 10, 20, 40 or 50 μM), or ML-385 alone (2.5, 5, or 10 μM), and cell viability was measured using the CCK-8 assay. Data are expressed as mean±SD (n=3, each with four technical replicates), $^{*}P<0.05$, $^{**}P<0.01$ versus DMSO group. (B) H9c2 cells were pretreated with fisetin for 2 h and subsequently exposed to Tm for 24 h. Data are expressed as mean±SD (n=3, each with four technical replicates), $^{*}P<0.05$, $^{**}P<0.01$ versus Control group; $^{\#}P<0.05$, $^{\#}P<0.01$ versus Tm group
DMSO, Dimethyl sulfoxide; ML-385, Nrf2 inhibitor ML-385; Tm, Tunicamycin; Con, Control; H9c2, rat myocardial cell line

screening and the effective concentrations commonly used in previous cardiomyocyte studies, 5 μM ML-385 was selected for subsequent mechanistic experiments to achieve sufficient pharmacological inhibition of Nrf2 while avoiding detectable basal cytotoxicity (24).

In contrast, 24 hr Tm exposure significantly reduced H9c2 cell viability compared with the vehicle control. Pretreatment with fisetin at 20 μM and 40 μM significantly attenuated the Tm-induced reduction in cell viability ($P<0.05$), whereas 10 μM fisetin pretreatment did not produce a statistically significant difference (Figure 4B, $P>0.05$). These results show that fisetin pretreatment attenuates Tm-induced loss of cardiomyocyte viability in a concentration-dependent manner.

Fisetin reduces ROS levels and up-regulates catalase expression in Tm-injured H9c2 cardiomyocytes

H9c2 cardiomyocytes treated with Tm for 24 hr showed markedly elevated intracellular ROS levels compared with the control group, which fisetin pretreatment prevented (Figure 5A, $P<0.05$). ROS levels decreased as fisetin concentration increased, with the most pronounced effect at 40 μM. We further evaluated catalase expression to assess the anti-oxidant capacity of the cells. Moreover, catalase expression was significantly decreased in the Tm group compared with the control group (Figure 5B). However, fisetin pretreatment up-regulated catalase expression after Tm stimulation, with significant effects observed at 20 and 40 μM ($P<0.05$). These findings indicate that fisetin protects H9c2 cardiomyocytes from Tm-induced injury by reducing oxidative stress.

Fisetin protects H9c2 cardiomyocytes against Tm-induced apoptosis

Compared with the control group, the apoptosis rate was significantly increased in cardiomyocytes after 24 hr of Tm treatment (Figure 6A, $P<0.05$). Fisetin pretreatment

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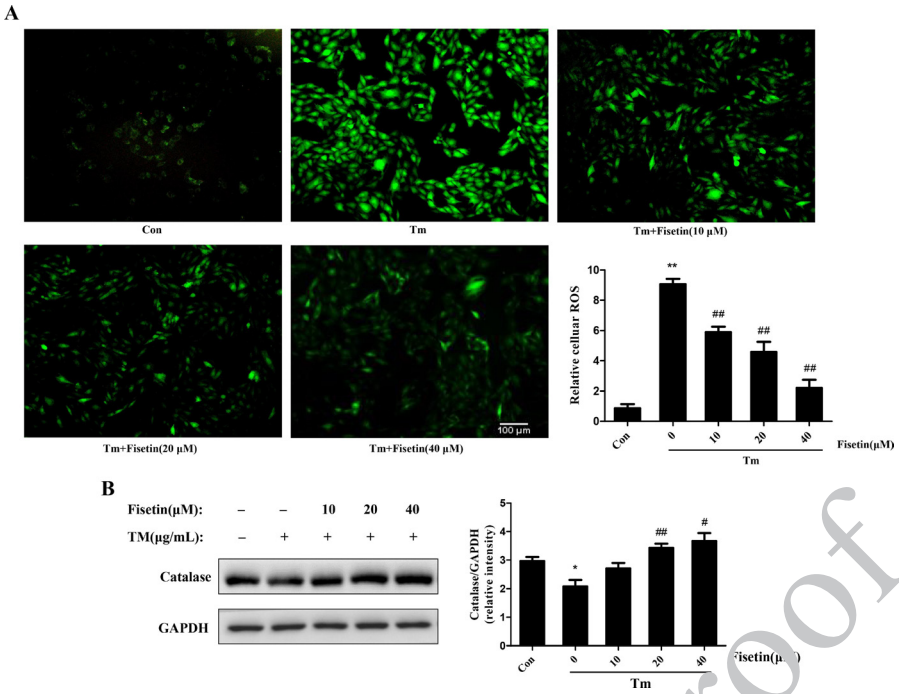


Figure 5. The effects of fisetin on ROS content and antioxidant protein in H9c2 cardiomyocytes injured by Tm
(A) Representative fluorescence microscopy images (magnification: ×100). For each group, five random fields per well were analyzed in three independent experiments (n=3). (B) Representative Western blotting and quantification of Western blotting data. Data are expressed as mean±SD (n=3), $^{*}P<0.05$ versus Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus Tm group
ROS, Reactive oxygen species; Con, Control; Tm, Tunicamycin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; H9c2, rat myocardial cell line

attenuated Tm-induced apoptosis. Similarly, Bax protein expression was elevated, and Bcl-2 expression was decreased in the Tm group compared with the control group (Figure 6B). Fisetin pretreatment down-regulated Bax expression and up-regulated Bcl-2 expression, with statistically significant differences observed at 20 and 40 μM ($P<0.05$). These data further confirm that fisetin inhibits Tm-induced cardiomyocyte apoptosis.

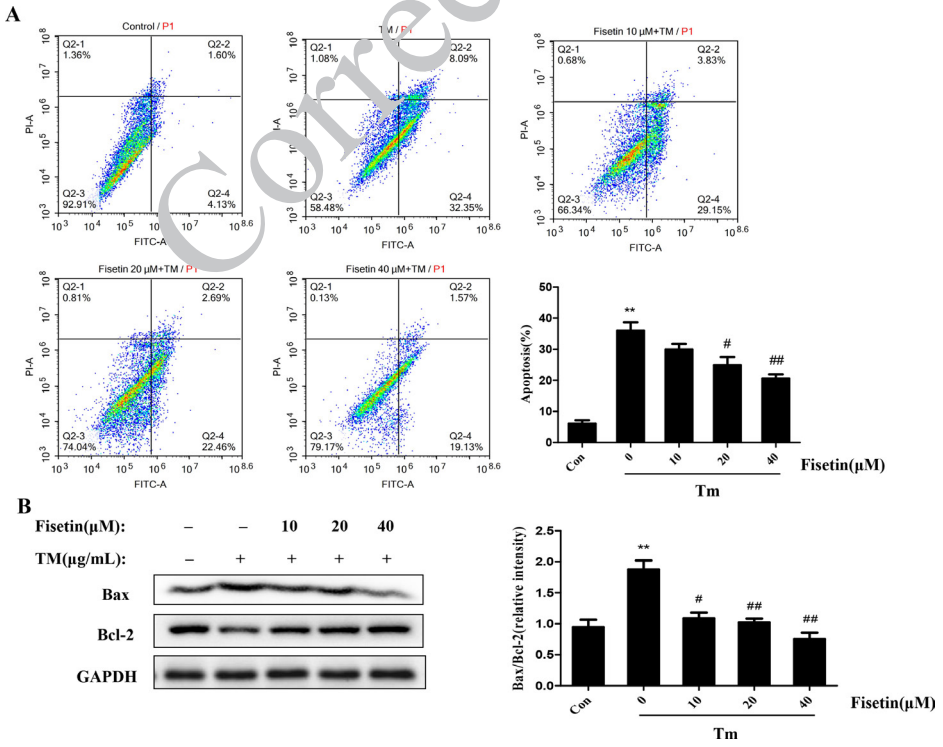


Figure 6. The effects of fisetin pretreatment with different concentrations on apoptosis after TM intervention
(A) Representative flow cytometry plots and quantitative analysis of cell apoptosis rate. (B) Representative Western blotting and quantification of Western blotting data. Data are expressed as mean±SD (n=3), $^{**}P<0.01$ versus Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus Tm group)
Con, Control; TM, Tunicamycin; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; FITC, Fluorescein isothiocyanate; PI, Propidium iodide; H9c2, rat myocardial cell line

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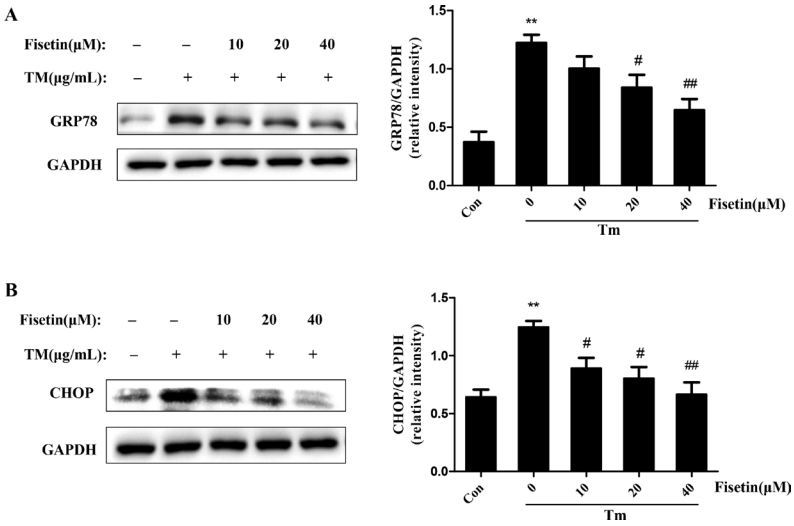


Figure 7. The effects of fisetin pretreatment with different concentrations on the expression of ERS related proteins after Tm intervention in cells
(A)-(B) Representative Western blotting and quantification of Western blotting data. Data are expressed as mean±SD (n=3). $^{**}P$<0.01 versus Control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus Tm group)
Con, Control; Tm, Tunicamycin; GRP78, Glucose-regulated protein 78; CHOP, C/EBP homologous protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ERS, Endoplasmic reticulum stress; H9c2, rat myocardial cell line

Fisetin attenuates Tm-induced ERS in cardiomyocytes

Tm treatment for 24 hr significantly up-regulated GRP78 and CHOP protein expression in cardiomyocytes (P<0.05, Figure 7), indicating successful induction of ERS. Fisetin intervention (10, 20, and 40 μM) down-regulated GRP78 and CHOP expression. Statistically significant differences were observed at 20 and 40 μM versus the Tm group (P<0.05), whereas the reduction in GRP78 expression by 10 μM fisetin did not reach statistical significance (P>0.05). These results indicate that fisetin effectively suppresses Tm-induced ERS in cardiomyocytes.

Fisetin protects against Tm-induced injury in H9c2 cardiomyocytes in association with Nrf2-related anti-oxidant responses

To further verify whether the protective effect of fisetin against tunicamycin-induced cardiomyocyte injury depends on the activation of the Nrf2 signaling pathway, we co-treated H9c2 cells with fisetin (40 μM) and the specific Nrf2 inhibitor ML-385 (5 μM) and assessed the effects on oxidative stress levels, apoptosis, and the expression of ERS-related proteins.

Fisetin up-regulates Nrf2/HO-1/NQO-1-associated anti-oxidant proteins in Tm-injured H9c2 cardiomyocytes

As shown in Figure 8, compared with the control group, Nrf2 and its downstream anti-oxidant proteins HO-1 and NQO-1 were significantly down-regulated in H9c2 cardiomyocytes after Tm treatment (P<0.05). Fisetin intervention significantly up-regulated the expression of these proteins (P<0.05); however, this effect was reversed by ML-385, with the expression levels of Nrf2, HO-1, and

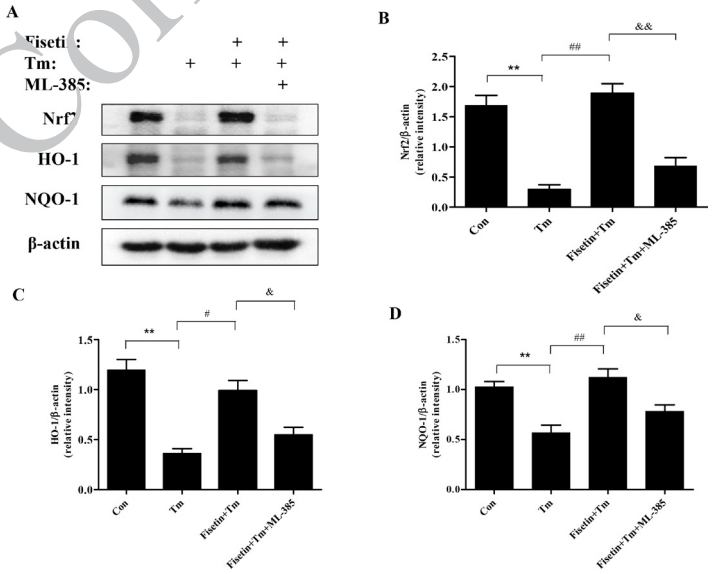


Figure 8. The effects of fisetin on the protein expression of the Nrf2/HO-1/NQO-1-associated antioxidant proteins in Tm-injured H9c2 cardiomyocytes
(A) Representative Western blotting images and (B-D) quantification of Nrf2, HO-1, and NQO-1 protein expression. Data are expressed as mean±SD (n=3). $^{**}P$<0.01 versus Control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus Tm group; $^{\&}P$<0.05, $^{\&\&}P$<0.01 versus Fisetin+Tm+ML-385 group
Con, Control; Tm, Tunicamycin; Nrf2, Nuclear factor erythroid 2-related factor 2; HO-1, Heme oxygenase-1; NQO-1, NAD(P)H quinone dehydrogenase 1; β-actin, Beta-actin; ML-385, Nrf2 inhibitor ML-385; H9c2, rat myocardial cell line

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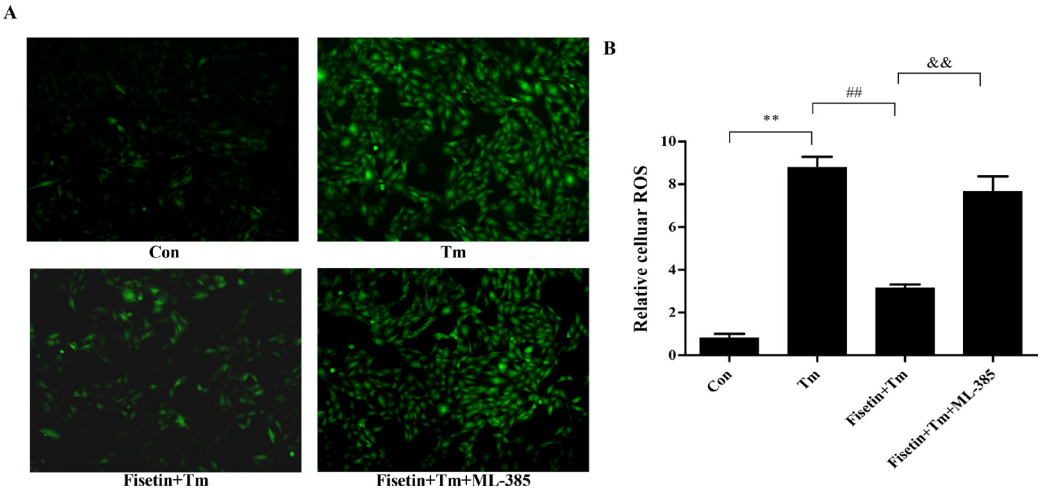


Figure 9. ML-385 reverses the protective effect of fisetin against oxidative stress in Tm-injured H9c2 cardiomyocytes
(A) Representative fluorescence microscopy images (magnification: ×100). For each group, five random fields per well were analyzed in three independent experiments (n=3). (B) quantitative analysis of cell ROS. Data are expressed as mean±SD. $^{*}P<0.01$ versus Control group; $^{\#\#}P<0.01$ versus Tm group; $^{\&\&}P<0.01$ versus Fisetin+Tm+ML-385 group.
Con, Control; Tm, Tunicamycin; ROS, Reactive oxygen species; ML-385, Nrf2 inhibitor ML-385; H9c2, rat myocardial cell line

NQO-1 falling back to levels close to those in the Tm group ($P<0.05$), confirming that ML-385 effectively blocked the activation of Nrf2 signaling. These results indicate that fisetin's cardioprotective effect against Tm-induced injury involves activation of the Nrf2 pathway.

ML-385 reverses the ROS-scavenging effect of Fisetin in Tm-injured cardiomyocytes

After 24 hr of Tm treatment, intracellular ROS levels were significantly elevated in H9c2 cardiomyocytes (Figure 9, $P<0.01$). Fisetin intervention significantly reduced Tm-induced ROS accumulation ($P<0.01$). However, cotreatment with ML-385 again significantly increased intracellular ROS levels ($P<0.01$), indicating that fisetin's anti-oxidant effect depends on Nrf2 pathway activation.

ML-385 reverses the inhibitory effects of fisetin on Tm-induced ERS and apoptosis

Tm treatment significantly up-regulated GRP78 and CHOP expression in H9c2 cardiomyocytes (Figure 10, $P<0.01$). Fisetin intervention effectively suppressed these changes ($P<0.01$); however, cotreatment with ML-385 again increased GRP78 and CHOP expression ($P<0.05$), indicating that Nrf2 inhibition impaired fisetin-mediated alleviation of ERS. Regarding apoptosis-related proteins, fisetin significantly down-regulated the pro-apoptotic protein Bax and up-regulated the anti-apoptotic protein Bcl-2 ($P<0.05$). After combined treatment with ML-385, Bax expression increased significantly, and Bcl-2 expression decreased significantly compared with the fisetin-alone group ($P<0.05$), suggesting that Nrf2 inhibition partially reversed fisetin's regulation of apoptotic signaling. Collectively, these results indicate that fisetin-mediated suppression of Tm-induced ERS and apoptosis is dependent on Nrf2 pathway activation.

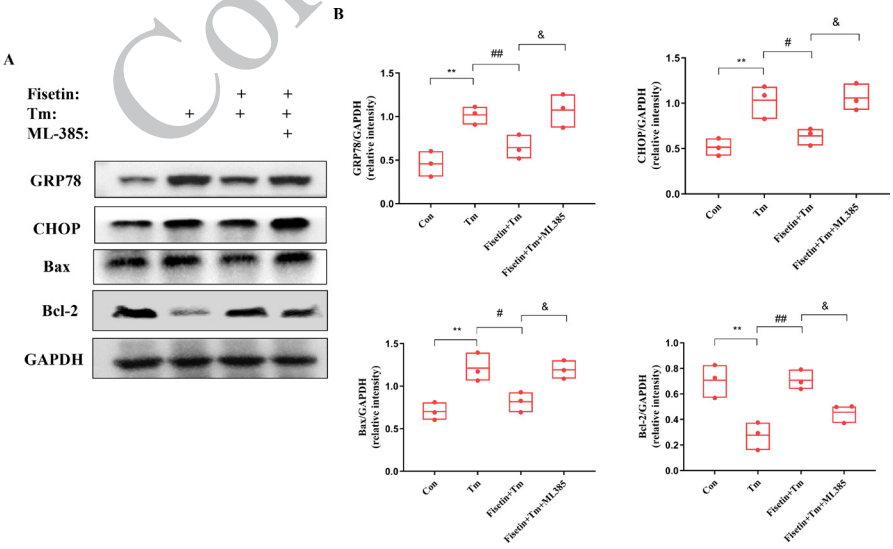


Figure 10. ML-385 reverses the effects of fisetin on the expression of ERS markers and apoptosis-related proteins in Tm-injured H9c2 cardiomyocytes
(A) Representative Western blotting and (B) Quantification of Western blotting data. Data are expressed as mean±SD (n=3). $^{**}P<0.01$ versus Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus Tm group; $^{\&}P<0.05$ versus Fisetin+Tm+ML-385 group
Con, Control; Tm, Tunicamycin; GRP78, Glucose-regulated protein 78; CHOP, C/EBP homologous protein; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ML-385, Nrf2 inhibitor ML-385; H9c2, rat myocardial cell line

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Discussion

This study investigated the protective effect of fisetin against tunicamycin-induced ERS injury in H9c2 cardiomyocytes and the underlying molecular mechanisms. The results demonstrated that fisetin pretreatment alleviated tunicamycin-induced cardiomyocyte injury in a dose-dependent manner, as evidenced by increased cell viability, increased protein abundance of Nrf2, HO-1, NQO-1, and catalase, and concurrent reductions in ROS accumulation, GRP78 and CHOP expression, and apoptotic injury. In addition, ML-385 attenuated several of these protective effects. These observations extend previous work by linking an Nrf2-associated anti-oxidant response to the suppression of maladaptive ERS and CHOP-associated apoptosis in a cardiac-derived cellular context. Nevertheless, because Nrf2 nuclear translocation, anti-oxidant response elements (ARE) transcriptional activity, and genetic loss-of-function were not examined, the present results support the involvement of an ML-385-sensitive, Nrf2-associated component rather than definitively establishing direct Nrf2 activation or an exclusively Nrf2-dependent mechanism.

To establish a relevant model for this investigation, we employed tunicamycin, a commonly used ERS inducer that inhibits protein N-glycosylation, leading to the accumulation of unfolded or misfolded proteins in the ER lumen and thereby triggering the UPR (25). Unlike ischemia/reperfusion or doxorubicin-induced cardiomyopathy, which simultaneously involve oxidative stress, inflammation, mitochondrial dysfunction, ferroptosis, and other forms of cellular injury, tunicamycin provides a relatively defined trigger of the UPR. Previous animal studies have shown that tunicamycin-induced ERS is associated with impaired cardiac function and altered mitochondrial function in cardiomyocytes (26). In the present study, tunicamycin (1 μg/ml) treatment significantly reduced H9c2 cell viability, increased the apoptosis rate, and up-regulated GRP78 and CHOP expression, confirming the successful establishment of an ERS-induced cardiomyocyte injury model. GRP78 is a canonical ERS chaperone whose up-regulation reflects disrupted ER homeostasis, while CHOP is a key transcription factor mediating ERS-induced apoptosis; its activation promotes the expression of the pro-apoptotic protein Bax and concurrently inhibits the anti-apoptotic protein Bcl-2.

Having validated the ERS injury model, we next examined whether fisetin could counteract this damage. Numerous studies have established that fisetin possesses anti-oxidant, anti-inflammatory, and anti-apoptotic properties. Adeli *et al.* (21) systematically reviewed the protective role of fisetin in I/R injury, demonstrating that fisetin reduces ROS production and up-regulates anti-oxidant enzymes including superoxide dismutase, glutathione, catalase, and glutathione peroxidase. Consistent with these reports, we observed that fisetin pretreatment dose-dependently increased cell viability, decreased the apoptosis rate, reduced the Bax/Bcl-2 ratio, and down-regulated GRP78 and CHOP expression in tunicamycin-treated cells, collectively indicating that fisetin effectively attenuates tunicamycin-induced ERS and apoptosis.

A closer examination of the dose–response relationship revealed an important nuance: fisetin's cytoprotective effects showed clear concentration dependence, with 10 μM failing to reach statistical significance for GRP78 suppression and cell viability improvement. This pattern may be attributable to two non-mutually exclusive factors. First, at lower concentrations, fisetin may primarily act as a direct ROS scavenger-as demonstrated by Yen *et al.* (22) in PC12 cells-without sufficiently triggering the nuclear translocation of Nrf2 required for transcriptional activation of its full target gene repertoire. Second, a threshold concentration of approximately 20 μM may be necessary to overcome the strong ERS stimulus imposed by tunicamycin in the present model. Notably, similar biphasic or threshold-dependent effects have been documented for other natural polyphenols, including resveratrol and curcumin, wherein low concentrations predominantly engage direct anti-oxidant activity. In contrast, higher concentrations are required to initiate transcriptional reprogramming via Nrf2 and other signaling cascades. This functional switch from "direct anti-oxidant" to "signaling modulator" may represent a general pharmacological feature of dietary polyphenols and warrants careful consideration in dose selection for both experimental and translational studies (27-29).

The concentration-dependent up-regulation of anti-oxidant proteins by fisetin prompted us to investigate the role of Nrf2, a key transcription factor governing the intracellular anti-oxidant defense system. Under physiological conditions, Nrf2 is sequestered in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (Keap1). Upon oxidative stress stimulation, Nrf2 dissociates from Keap1 and translocates to the nucleus, where it binds to ARE and initiates transcription of downstream anti-oxidant and phase II detoxifying enzymes, including HO-1 and NQO-1 (13). In the present study, tunicamycin treatment significantly decreased Nrf2 expression and the expression of its downstream targets HO-1 and NQO-1, as well as the anti-oxidant protein catalase, suggesting that the anti-oxidant defense system in cardiomyocytes is impaired under ERS conditions. Fisetin pretreatment significantly up-regulated the expression of these proteins, enhanced cellular anti-oxidant capacity, and consequently reduced tunicamycin-induced ROS accumulation. Previous studies have established that fisetin exerts cardioprotective effects through multiple mechanisms. In myocardial ischemia/reperfusion injury, fisetin induces Nrf1/2 expression, scavenges ROS, reduces mitochondrial oxidative stress, improves mitochondrial function, and inhibits GSK3β activity. In contrast, its anti-hypertrophic effects were primarily associated with suppression of oxidative stress and downstream mitogen-activated protein kinase (MAPK)/mechanistic target of rapamycin (mTOR) signaling (8, 21, 30). More directly, fisetin was reported to promote Nrf2 nuclear translocation and inhibit ferroptosis through SIRT1/Nrf2 signaling in doxorubicin-induced cardiomyopathy (12). Fisetin also protected tunicamycin-treated PC12 cells by reducing ROS production and modulating Nrf2-regulated anti-oxidant genes, MAPK signaling, and SIRT1 (22). Collectively, these studies underscore the central role of the Nrf2 signaling pathway in fisetin's cardioprotective effects.

To determine whether Nrf2-related transcriptional activity causally contributes to the observed cardioprotection, we used ML-385. This small-molecule inhibitor directly binds the Nrf2-ECH homology 1 (Neh1) domain and inhibits its binding to ARE sequences, thereby suppressing Nrf2 transcriptional activity (31). Our results

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showed that ML-385 significantly attenuated fisetin's protective effects against tunicamycin-induced injury, as evidenced by decreased cell viability, increased apoptosis, and elevated ROS levels. Moreover, ML-385 blocked fisetin-induced up-regulation of Nrf2, HO-1, and NQO-1 and reversed fisetin's regulatory effects on GRP78, CHOP, and the Bax/Bcl-2 ratio. These findings are consistent with those of Zhang *et al.* (32), who showed that ML-385 counteracted ferulic acid-mediated activation of the Nrf2/HO-1/NQO-1 pathway in isoproterenol-induced heart failure. Similarly, Sun *et al.* (33) reported that ML-385 reversed the activation of the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK)/Nrf2/heme oxygenase (decycling) 1 (HMOX1) pathway and its anti-oxidant effects in a study of ginsenoside Rb3-mediated cardiomyocyte protection. Together, these independent lines of evidence support Nrf2 as a convergent target through which diverse natural products confer cardioprotection.

While our inhibitor experiments supported an association between fisetin-mediated protection and Nrf2-related anti-oxidant responses, it is important to consider the broader context of ERS-oxidative stress interplay. Protein folding during ERS generates ROS as byproducts, leading to redox imbalance; conversely, oxidative stress can interfere with proper protein folding in the ER, thereby exacerbating ERS and creating a self-amplifying pathological loop. In the present study, fisetin not only enhanced cellular anti-oxidant capacity by activating the Nrf2 pathway but also alleviated ERS itself, which may be attributable to Nrf2-mediated anti-oxidant defense breaking this vicious cycle. However, the residual protection observed after ML-385 treatment suggests that Nrf2-independent mechanisms also contribute to fisetin's overall cardioprotective effect. Indeed, Yen *et al.* (22) reported that fisetin modulates multiple signaling pathways in tunicamycin-treated PC12 cells-including MAPKs (extracellular signal-regulated kinase (ERK), JNK, p38 mitogen-activated protein kinase (p38) and SIRT1-indicating that the protective mechanisms of fisetin are likely more complex and involve coordinated multi-pathway regulation.

Beyond this indirect redox-based interplay, emerging evidence has revealed that Nrf2 and ERS engage in direct molecular interactions at multiple levels, providing a deeper mechanistic framework for our observations. First, the ERS sensor PERK has been shown to directly phosphorylate Nrf2 at threonine residues within its Nrf2-ECH homology 2 (Neh2) domain, promoting its dissociation from Keap1 and subsequent nuclear translocation. This positions Nrf2 not merely as an oxidative stress responder but as a direct downstream effector of the UPR (34, 35). Second, activating transcription factor 4 (ATF4), a transcription factor downstream of the PERK-eIF2α axis, can heterodimerize with Nrf2 and cooperatively drive the transcription of anti-oxidant genes including HO-1 and NQO-1, revealing a synergistic interplay between the UPR and anti-oxidant programs (36, 37). Third, CHOP-the key pro-apoptotic effector of the PERK-ATF4 cascade-is transcriptionally antagonized by Nrf2 through competitive binding to ARE elements, providing a direct mechanistic basis for how Nrf2 activation suppresses ERS-mediated apoptosis beyond ROS scavenging alone. These multi-level interactions offer a plausible molecular explanation for our observation that fisetin-mediated Nrf2 activation simultaneously alleviated oxidative stress, ERS, and apoptosis (38).

Despite these mechanistic insights, several limitations remain. First, this study was limited to *in vitro* experiments using the H9c2 cardiomyocyte cell line, which cannot fully recapitulate the complex *in vivo* myocardial microenvironment. Second, while we focused primarily on the Nrf2 pathway, whether fisetin also participates in regulating ERS through other signaling routes-such as PI3K/AKT, MAPKs, or SIRT1-remains to be systematically explored. Third, the direct interaction mechanisms between Nrf2 and key ERS molecules, including PERK, ATF4, and CHOP, were inferred from existing literature and were not directly interrogated in this study.

Looking forward, several specific avenues should be prioritized to advance these findings toward *in vivo* validation and eventual clinical translation. All findings in this study were obtained exclusively from H9c2 cells. As embryonic rat cardiomyoblasts that retain some skeletal muscle characteristics, H9c2 cells cannot fully recapitulate the phenotype of mature cardiomyocytes or the complex myocardial microenvironment *in vivo*. Therefore, future studies should validate fisetin's protective effects in primary cardiomyocytes and further confirm its translational potential *in vivo*. Second, the pharmacokinetic limitation of fisetin must be addressed; recent advances in nanoformulation strategies, including PLGA-based nanoparticles and liposomal encapsulation, have demonstrated promising improvements in fisetin solubility, half-life, and tissue distribution (39, 40). Third, the choice of animal model is critical: tunicamycin administered via intraperitoneal injection provides a well-characterized *in vivo* ERS model that would allow direct extrapolation of the present *in vitro* findings. In contrast, myocardial ischemia/reperfusion or pressure-overload models would assess the translational relevance of fisetin-mediated cardioprotection in clinically relevant disease settings. Fourth, generating and using cardiomyocyte-specific Nrf2 conditional knockout mice would provide the most rigorous causal evidence at the gene-function level for the Nrf2 dependence of fisetin-mediated cardioprotection. In parallel, subsequent experiments should directly examine Nrf2 nuclear translocation, ARE luciferase reporter activity, and Keap1-Nrf2 dissociation to determine whether fisetin activates the canonical Nrf2 pathway by promoting Nrf2 nuclear accumulation, enhancing ARE-mediated transcriptional activity, and facilitating Keap1 dissociation from Nrf2. Such mechanistic assays would complement the proposed conditional knockout strategy and help distinguish direct Nrf2 activation from indirect or Nrf2-associated anti-oxidant effects. In addition, the present study relied on the pharmacological inhibitor ML-385 to probe Nrf2 involvement. Because ML-385 may have off-target effects, genetic loss-of-function approaches, such as Nrf2 siRNA or shRNA knockdown, would provide stronger evidence for Nrf2's causal role. Therefore, future studies should incorporate these genetic tools to complement the ML-385 findings and more definitively establish whether Nrf2 is required for fisetin's protective effects. Finally, given the broad expression of Nrf2 across multiple organs-including the kidney, liver, and brain-future *in vivo* studies should systematically evaluate the multi-organ effects of fisetin administration to assess both therapeutic benefits beyond the heart and potential off-target toxicities. Addressing

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these translational gaps will be essential to determine whether fisetin can progress from a promising experimental cardioprotectant to a clinically viable therapeutic agent for ERS-associated cardiovascular diseases.

Conclusion

In conclusion, this study shows that fisetin protects H9c2 cardiomyocytes against tunicamycin-induced ERS injury. The underlying mechanism may involve an Nrf2-related anti-oxidant mechanism, including up-regulation of the anti-oxidant enzymes HO-1, NQO-1, and catalase, attenuation of oxidative stress, and inhibition of ERS-mediated apoptosis. These findings provide a novel theoretical basis for using fisetin to prevent and treat cardiovascular diseases.

Acknowledgment

The National Natural Science Foundation of China (62372088) financially supported this work.

Authors' Contributions

H C and C O conceived and designed the experiments. H C and M D conducted the experiments. T C, C L, and L C contributed to data collection and analysis. H C and L N contributed to project development and manuscript writing. L N and C O edited and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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