

Melatonin protects H9c2 cells against ischemia/reperfusion-induced apoptosis and oxidative stress via activation of the Nrf2 signaling pathway

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Abstract. Melatonin can protect against cardiac ischemia/reperfusion (I/R) injury in models *in vitro* and *in vivo* by regulating oxidative stress and apoptosis; however, the precise molecular mechanisms involved remain unclear. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, which has been associated with the regulation of oxidative stress by translocating to the nucleus. Therefore, the present study investigated whether activation of the Nrf2 signaling pathway may be responsible for the protective effects of melatonin on I/R-injured cardiomyocytes. In the present study, H9c2 cells were subjected to simulated I/R (SIR) injury and pretreated with melatonin and/or Nrf2 small interfering RNA (siRNA). Cell viability was detected via Cell Counting kit-8 assay, apoptosis was examined by caspase-3 cleavage and activity analysis; oxidative stress levels were determined by specific activity analysis assays. In the present study, it was observed that SIR induced significant increases in apoptosis and oxidative stress, and enhanced Nrf2 expression within H9c2 cells. Pretreatment with melatonin partially reversed these alterations and promoted Nrf2 nuclear translocation. Transfection with Nrf2 siRNA inhibited the protective effects of melatonin on SIR-induced H9c2 cells. These results indicated that melatonin may protect H9c2 cells against I/R injury by reducing apoptosis and oxidative stress; this effect may be mediated via activation of the Nrf2 signaling pathway. Collectively, the results of the present study may suggest melatonin as a potential therapeutic agent against cardiac I/R injury.

Introduction

Myocardial ischemia is a major cause of sudden mortality worldwide, which arises from decreased coronary blood flow to the heart due to atherosclerosis or thrombosis (1,2). Restoration of coronary blood flow to the ischemia-injured myocardium in time, which is termed reperfusion, is the most common therapeutic strategy; however, sudden reperfusion to the ischemia-injured myocardium may induce oxidative stress and cell apoptosis, thus further exacerbating myocardial injury, which is known as myocardial ischemia/reperfusion (I/R) injury (3,4). Myocardial I/R injury is a complex pathophysiological process associated with a variety of mechanisms, including oxidative stress, cell apoptosis and inflammatory responses (5-7). The particular mechanisms underlying the development of myocardial I/R injury are unclear; however, it has been reported that excessive production of reactive oxygen species (ROS) is a primary factor that activates a variety of molecular cascades of apoptosis, thereby aggravating myocardial I/R injury (8,9). Therefore, inhibition of ROS production or the scavenging of free radicals may be potential therapeutic strategy to attenuate I/R injury.

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous circadian hormone that is mainly produced by the pineal gland (10). Melatonin and its derivatives possess numerous beneficial effects, including anti-oxidation and anti-apoptotic properties (11,12). Additionally, melatonin has powerful anti-oxidant properties, including scavenging of superfluous free radicals and enhancing the synthesis of intracellular antioxidative enzymes (13). Previous studies have demonstrated that melatonin could protect the myocardium against I/R injury by regulating oxidative stress and apoptosis (14,15); however, the specific mechanism by which melatonin exerts its protective effects on the myocardium against I/R injury requires further investigation.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the basic region-leucine zipper transcription factors (16). Nrf2 is expressed in a variety of organs, and is involved in the regulation of oxidative stress by promoting the transcription and expression of anti-oxidative proteins and phase II detoxifying enzymes, including heme oxygenase 1 (HO-1), sestrin2 and superoxide dismutase (SOD) (17). Under

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normal conditions, Nrf2 is predominantly expressed in the cell cytoplasm and is bound to Kelch-like ECH-associated protein 1 (Keap1), an adaptor protein that facilitates Nrf2 ubiquitination and degradation (18,19). In the presence of oxidative stress, Nrf2 disassociates from Keap1 and translocates to the nucleus; subsequent binding to antioxidant response elements (AREs) regulates the expression of target genes (18,19). Recent studies have suggested that Nrf2 signaling was associated with the cleavage process of myocardial I/R injury (20,21). The Nrf2 signaling pathway was reported to protect against anoxia/reoxygenation-induced apoptosis of H9c2 cells (22); however, whether Nrf2 signaling is involved in the protective effects of melatonin against myocardial I/R injury remains unknown.

Therefore, the present study aimed to investigate whether melatonin exerts protective effects against myocardial I/R injury and whether these protective effects are associated with Nrf2 signaling via a H9c2 cardiomyocyte-simulated I/R injury *in vitro* model.

Materials and methods

Cell culture and treatment. The H9c2 cell line (Tiancheng Technology (Shanghai, China), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% (v/v) penicillin/streptomycin at 37°C with 5% CO₂.

Simulated ischemia reperfusion (SIR) treatment was performed as described (23). The process of SIR treatment was as follows: H9c2 cells (70% confluence) were plated into glucose- and serum-free DMEM in a hypoxia airtight gas chamber containing with 95% N₂ and 5% CO₂ gas mixture at 37°C for 12 h. Subsequently, the medium was replaced with normal DMEM and cells were cultured for 6 h at 37°C with 5% CO₂. H9c2 cells were first treated with different concentrations of melatonin (50, 100 and 200 µmol/l), respectively for 1 h to select the suitable concentration of melatonin.

Subsequently, H9c2 cells were randomly divided into the following groups: Control group, H9c2 cells were treated with 1% ethanol in DMEM without SIR treatment for 1 h; Mel group, cells were pretreated with melatonin (100 µmol/l) for 1 h; SIR group, cells were subjected to SIR treatment as aforementioned; SIR + Mel group, cells were pretreated with melatonin (100 µmol/l) for 1 h and then were subjected to SIR treatment and the SIR + Mel + Nrf2 small interfering RNA (siRNA) group, in which cells were transfected with Nrf2 siRNA for 48 h and pretreated with melatonin (100 µmol/l) for 1 h prior to SIR treatment. Additionally, the SIR + Nrf2 siRNA group constituted cells transfected with Nrf2 specific siRNA for 48 h, which were then subject to SIR treatment. Melatonin was dissolved in ethanol and further diluted with DMEM to a concentration of 100 mmol/l (1% ethanol contained in DMEM) prior to application.

Determination of cell viability. Cell viability was detected using Cell Counting kit-8 (CCK-8) assay (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Briefly, H9c2 cells were seeded in 96-well plates at a density of 1×10⁴ cells per well. Following the various treatments described above, 10 µl

CCK-8 reagent was added to 100 µl DMEM medium in each well and then cells were incubated for 2 h at 37°C. The absorbance was measured using a microplate reader at 450 nm and the results were presented as percentages of the control values.

Caspase-3 activity analysis. Caspase-3 activity was measured with a caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were prepared and centrifuged at 12,000 × g for 10 min at 4°C with lysis buffer (included in kit). The protein concentration was determined by Bradford's assay and the caspase-3 activity assay was conducted according to the manufacturer's protocols. The absorbance was measured at 405 nm with a microtiter plate reader.

Quantitative measurement of free radical production. The production of intracellular ROS was measured using a Reactive Oxygen Species Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocols. Following the various treatments, cells (90% confluence) were treated with dichloro-dihydro-fluorescein diacetate (10 µM) at 37°C for 15 min. Following three washes with phosphate-buffered saline (PBS), the fluorescence was measured with an FSX100 microscope (Olympus Corporation, Tokyo, Japan) and the fluorescence intensity was analyzed with ImageJ 1.6.0 software (National Institutes of Health, Bethesda, MD, USA).

Measurement of malondialdehyde (MDA), SOD activity and glutathione (GSH) levels. The MDA content, and the activity levels of SOD and GSH were spectrophotometrically assayed using an MDA Assay, SOD Activity Assay and GSH Activity Assay kits (Jiancheng Bioengineering Institute), respectively according to the manufacturer's protocols. The absorbance of MDA, SOD, and GSH was spectrophotometrically measured at 532, 550 and 420 nm respectively with a SpectraMax M5 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA).

siRNA transient transfection. For Nrf2 silencing experiments, H9c2 cells (50% confluence) were transfected with Nrf2-specific siRNA or scramble siRNA (20 µM) with Micropoly-transfecter™ cell reagent (Micropoly Biotech, Nantong, China) according to the manufacturer's protocols. The particular sequences of Nrf2 siRNA were: Sense, 5'-GAG GAUGGGAAACCUUACUTT-3' and antisense, 5'-AGUAAG GUUCCCAUCCUCTT-3'. The sequences of the scramble siRNA were: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. At 48 h after transfection, the medium was replaced and cells were utilized for further experiments.

Preparation of protein lysates. H9c2 cells were lysed with radioimmunoprecipitation assay buffer (Heart Biological Ltd., Hartlepool, UK) on ice for 30 min, and then centrifuged at 12,000 × g for 10 min at 4°C to remove the insoluble materials. To detect nuclear Nrf2 expression, the nuclear protein fraction was extracted using a Nuclear and Cytoplasmic Protein Extraction kit (Yeasen; Shanghai Shengsheng Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's

protocols. Following the various treatments, H9c2 cells were harvested and centrifuged at 1,000 x g for 5 min at 4°C; the supernatant was subsequently discarded. The precipitate was lysed with the cytoplasmic protein extraction reagent on ice for 30 min and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed and the pellet was further lysed with the nuclear protein extraction reagent on ice for 30 min. The nuclear protein samples and the whole protein samples were mixed with 5X SDS-PAGE loading buffer and then heated to 100°C for 10 min. In total, 30 µg protein lysate was separated by SDS-PAGE (Hart Biological Ltd.) and immunoblotted with specific antibodies.

Western blot analysis. The concentration of the protein samples were measured via a bicinchoninic acid protein assay. A total of 30 µg protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with 10% skimmed milk for 1 h at 37°C and then incubated with specific primary antibodies overnight at 4°C. The next day, the membranes were washed three times with Tris-buffered saline with 1% Tween-20 and incubated with appropriate secondary antibodies for 1 h at room temperature. The bands were detected by chemiluminescence using the FluorChem FC2 system (Alpha Innotech, San Leandro, CA, USA) and the results were analyzed with Quantity One 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Caspase-3 (sc-271759; 1:1,000), cleaved caspase-3 (sc-271759; 1:1,000), Nrf2 (sc-30915; 1:1,000), HO-1 (sc-390991; 1:1,000), lamin A (sc-71481; 1:1,000) and β-actin (sc-70319; 1:1,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Goat anti-rabbit/mouse IgG secondary antibody (BA1054 and BA1051; Wuhan Boster Biological Technology, Ltd., Wuhan, China) were diluted at 1:3,000. Western blot analysis was repeated three times.

Measurement of apoptosis. Flow cytometry was performed to determine the levels of apoptosis of cells subject to various treatments using Annexin V-PI Apoptosis Assay kit (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's protocols. Briefly, H9c2 cells were seeded in 6-well plates at 5x10⁴ cells per well. Following the aforementioned treatments, cells were trypsinized and resuspended at 5x10⁵ cells/ml in ice-cold PBS. The cells were washed three times with saline and then were transferred to 1 ml binding buffer (included in kit), and then 5 µl Annexin V-Cy5 and 10 µl propidium iodide (PI) were added. Following thorough mixing, the stained cells were incubated for 15 min in the dark. The samples were measured with a FACScan flow cytometer (BD FACSaria; BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 1x10⁴ cells were analyzed. The data of early apoptosis cells (Annexin-Cy5⁺/PI⁻) were collected for each analysis. The data were analyzed with FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis. All experiment data were expressed as the mean ± standard error (n=8 per group). Comparisons between groups were conducted with using one-way analysis of variance followed by a Bonferroni post-hoc test using GraphPad Prism 6 software (La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Melatonin attenuates SIR-induced apoptosis of H9c2 cells. To investigate the effects of melatonin on cell apoptosis following SIR, cell viability was analyzed. Various concentrations of melatonin (50, 100 and 200 µmol/l) alone exhibited no significant effects on the viability of H9c2 cells, as determined by a CCK-8 assay (Fig. 1A). Conversely, SIR treatment significantly decreased the viability of H9c2 cells compared with in the control; melatonin significantly increased the cell viability of SIR-injured H9c2 compared with SIR treatment alone (Fig. 1B). The pro-survival effects of melatonin did not reveal a significant difference between treatments with 100 and 200 µmol/l. Therefore, 100 µmol/l was selected to investigate the protective effects of melatonin in the present study. To determine the effects of melatonin on cell apoptosis, western blot analysis of cleaved caspase 3 level and flow cytometry were conducted. As presented in Fig. 1C-E, consistent with the results of the cell viability experiments (Fig. 1A and B), SIR treatment significantly promoted cell apoptosis compared with in the control and melatonin significantly reversed this alteration induced by SIR.

Melatonin reverses SIR-induced oxidative stress in H9c2 cells. Additionally, the present study further investigated the effects of melatonin on oxidative stress in H9c2 cells subjected to SIR. The results revealed that SIR treatment induced a significant increase in ROS production and MDA content compared with in the control group (Fig. 2A and B), while the levels of SOD and GSH were significantly decreased following SIR compared with the control group (Fig. 2C and D). Pretreatment with melatonin significantly reduced the effects of SIR on oxidative stress (Fig. 2).

Melatonin attenuates SIR-induced apoptosis and oxidative stress via activation of the Nrf2 signaling pathway. To investigate whether melatonin attenuates SIR-induced apoptosis and oxidative stress via the Nrf2 signaling pathway, the whole cell and nuclear lysates of the cell groups were prepared for immunoblotting. As presented in Fig. 3, SIR induced a significant increase in Nrf2 expression levels within the nuclear and whole H9c2 cell lysates. Compared with in the SIR group, pretreatment with melatonin significantly increased the nuclear and whole cell expression levels of Nrf2. In addition, the present study analyzed the expression levels of HO-1, an important target gene of Nrf2 (24). Consistent with that of Nrf2, the expression levels of HO-1 were significantly increased when subjected to SIR, which further increased in response to pretreatment with melatonin. Interestingly, the results of treatment with melatonin alone also induced a significant increase in the expression levels of nuclear and whole lysate Nrf2, and HO-1 (Fig. 3).

Inhibition of the Nrf2 signaling pathway eliminates the anti-apoptotic effects of melatonin on H9c2 cells following SIR. To further verify the role of Nrf2 in the protective effects of melatonin on SIR treated H9c2 cells, a specific siRNA targeting Nrf2 was employed for the knockdown of its expression. Initially, the knockdown capacity of Nrf2 siRNA was determined. As presented in Fig. 4A, the expression levels of nuclear Nrf2 were markedly reduced in response to Nrf2 siRNA; melatonin was suggested to increase the expression levels of nuclear Nrf2

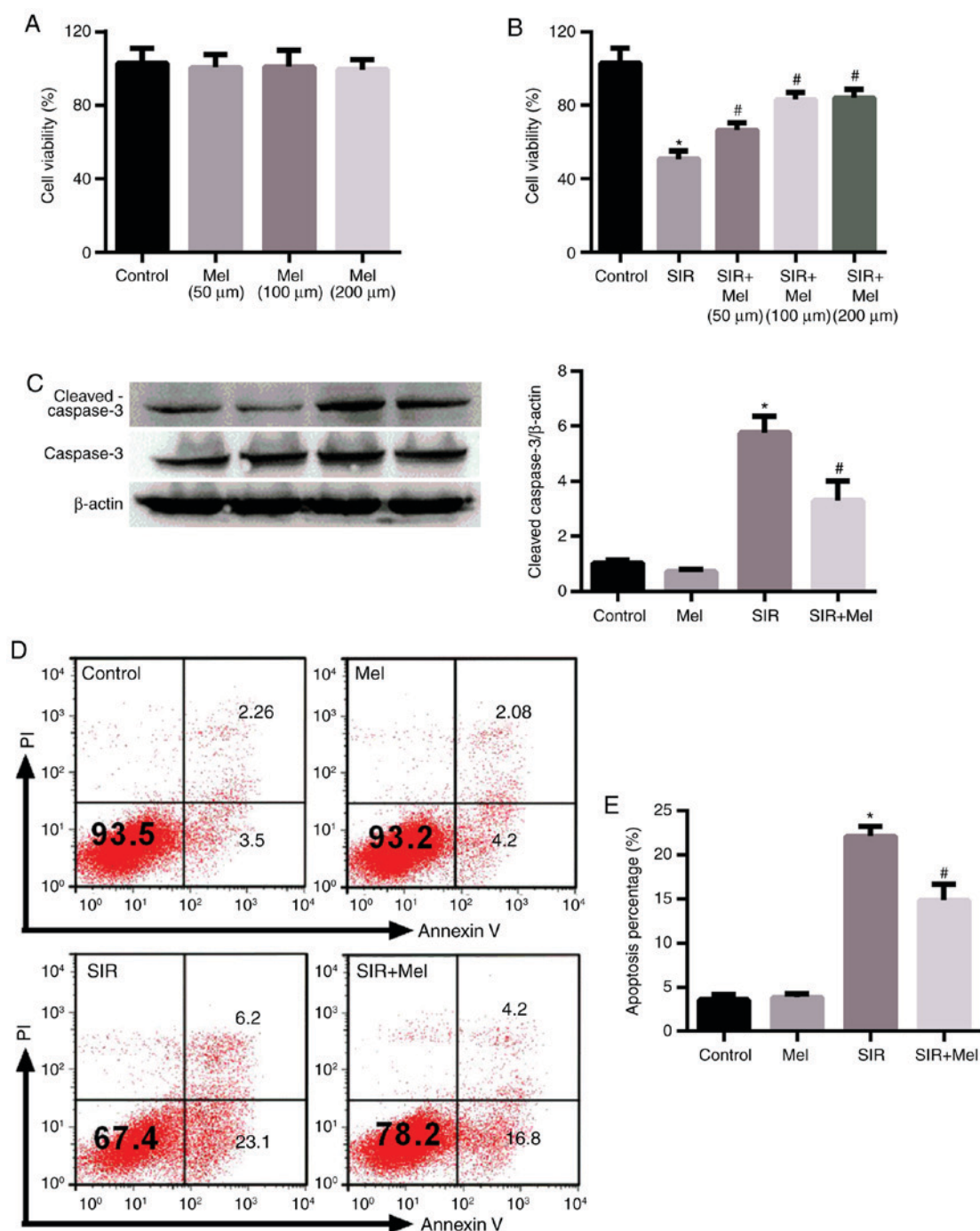


Figure 1. Mel attenuates SIR-induced apoptosis in H9c2 cells. (A) H9c2 cells were treated with various concentrations of Mel (50, 100 or 200 $\mu\text{mol/l}$); cell viability was detected by a Cell Counting kit-8 assay. (B) H9c2 cells were pretreated with Mel for 1 h and then subjected to SIR; cell viability was detected. (C) Cleaved caspase-3 expression was examined by western blotting. Cell apoptosis was measured by (D) flow cytometry and (E) the percentage of apoptosis of cells labeled as Annexin V (+) PI (-) was determined. The results were reported as the mean \pm standard error of the mean ($n=8$ per group). * $P<0.05$ compared with the control group; # $P<0.05$ compared with the SIR group. Mel, melatonin; SIR, simulated ischemia reperfusion; PI, propidium iodide.

and HO-1 (Fig. 3). However, the effects of melatonin on the expression levels of nuclear Nrf2, and its target gene HO-1, were also significantly reduced by Nrf2 siRNA (Fig. 4B). In addition, when the Nrf2 signaling pathway was inhibited by Nrf2 siRNA, cell viability significantly decreased and the caspase-3 activity of H9c2 cells was significantly elevated compared with in the SIR + Mel group (Fig. 4C and D). Cell apoptosis was also analyzed by flow cytometry (Fig. 4E). This indicated that the effects of melatonin on cell viability and apoptosis were abolished when Nrf2 signaling was inhibited. The data from the

Control and Melatonin groups were not analyzed to avoid any overlap as described previously (25). These results confirmed that Nrf2 signaling may serve a crucial role in the protective effects of melatonin on SIR-induced apoptosis in H9c2 cells.

Inhibition of the Nrf2 signaling pathway eliminates the anti-oxidative effects of melatonin on H9c2 cells following SIR. The present study investigated alterations in oxidative stress within H9c2 cells expressing Nrf2 siRNA. The results revealed that Nrf2 siRNA significantly attenuated the

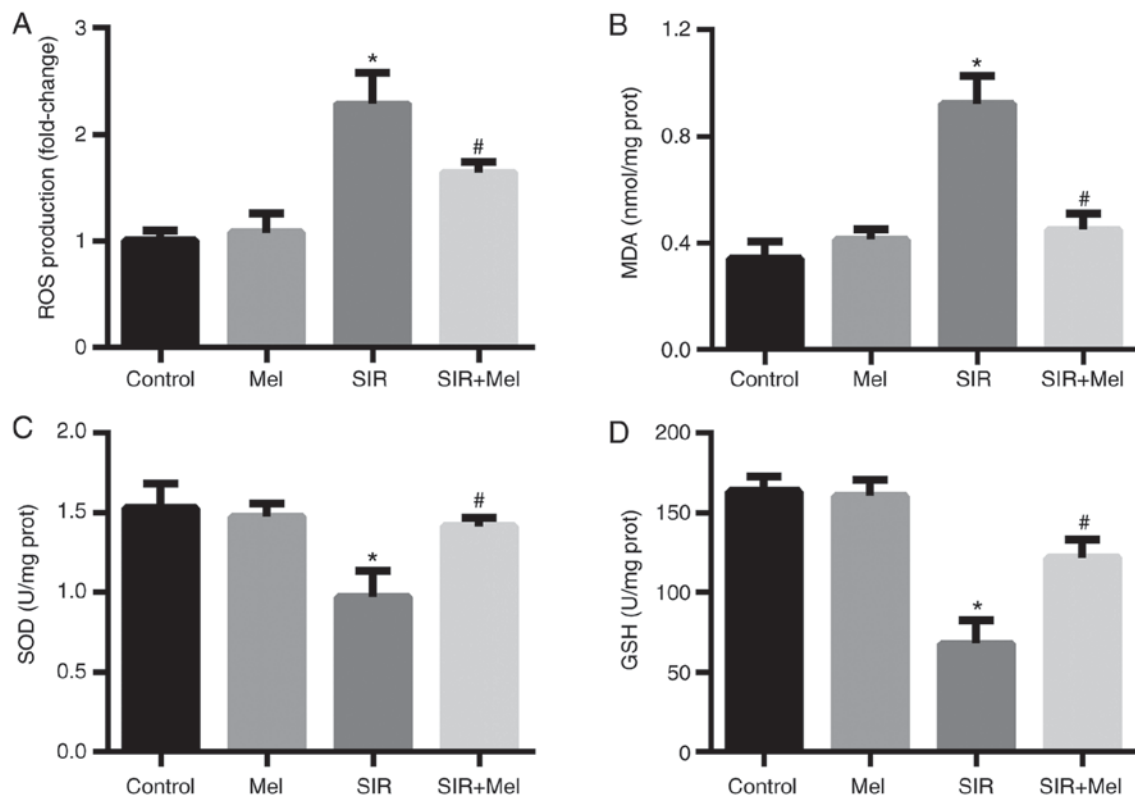


Figure 2. Mel reverses SIR-induced oxidative stress in H9c2 cells. H9c2 cells were pretreated with Mel for 1 h and then subjected to SIR. (A) ROS production was measured using a ROS Assay kit. (B) MDA content, (C) SOD activity levels and (D) GSH expression were assayed using appropriate kits. The results were reported as the mean ± standard error (n=8 per group). *P<0.05 vs. control group; #P<0.05 compared with the SIR group. ROS, reactive oxygen species; Mel, melatonin; SIR, simulated ischemia reperfusion; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione.

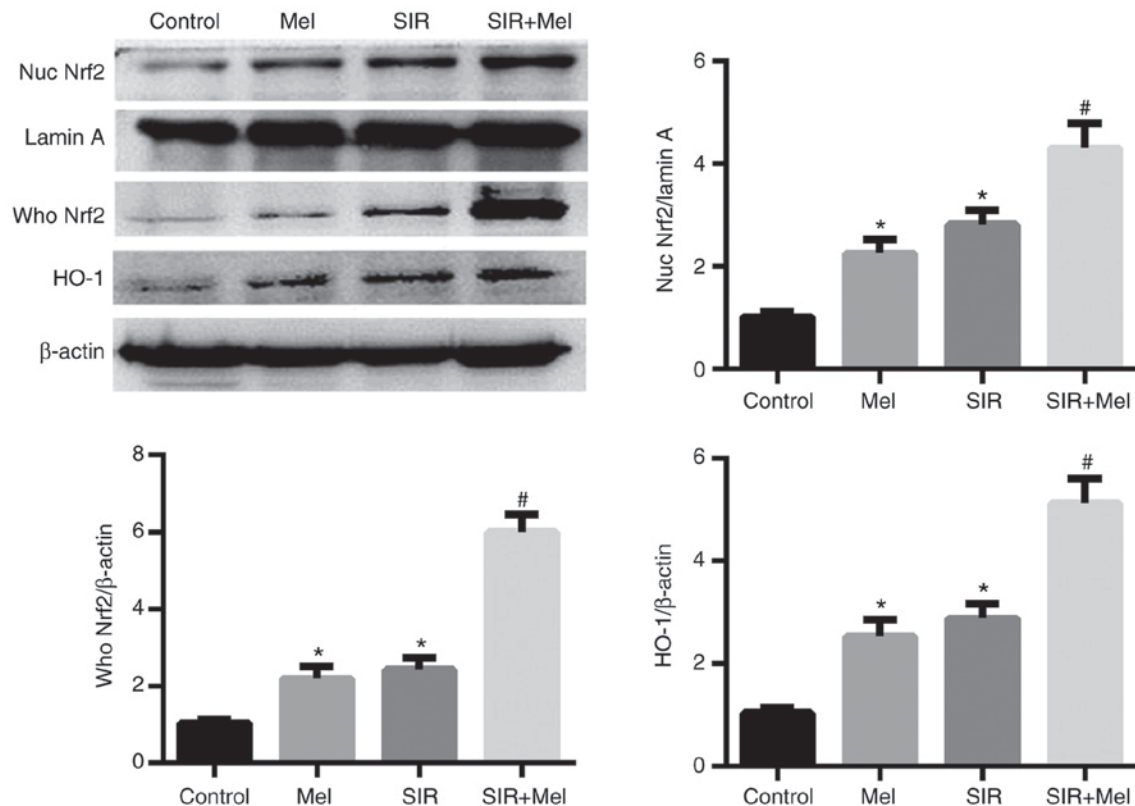


Figure 3. Mel attenuates SIR-induced apoptosis and oxidative stress via the activation of the Nrf2 signaling pathway. H9c2 cells were pretreated with Mel for 1 h and then subjected to SIR. Protein expression levels of Nrf2 in the nucleus and the whole cell lysate, and HO-1 were examined by western blotting. The results were reported as the mean ± standard error (n=8 per group). *P<0.05 vs. control group; #P<0.05 vs. SIR group. Mel, melatonin; SIR, simulated ischemia reperfusion; Nuc, nuclear; Nrf2, nuclear factor erythroid 2-related factor 2; Who, whole cell lysate; HO-1, heme oxygenase 1.

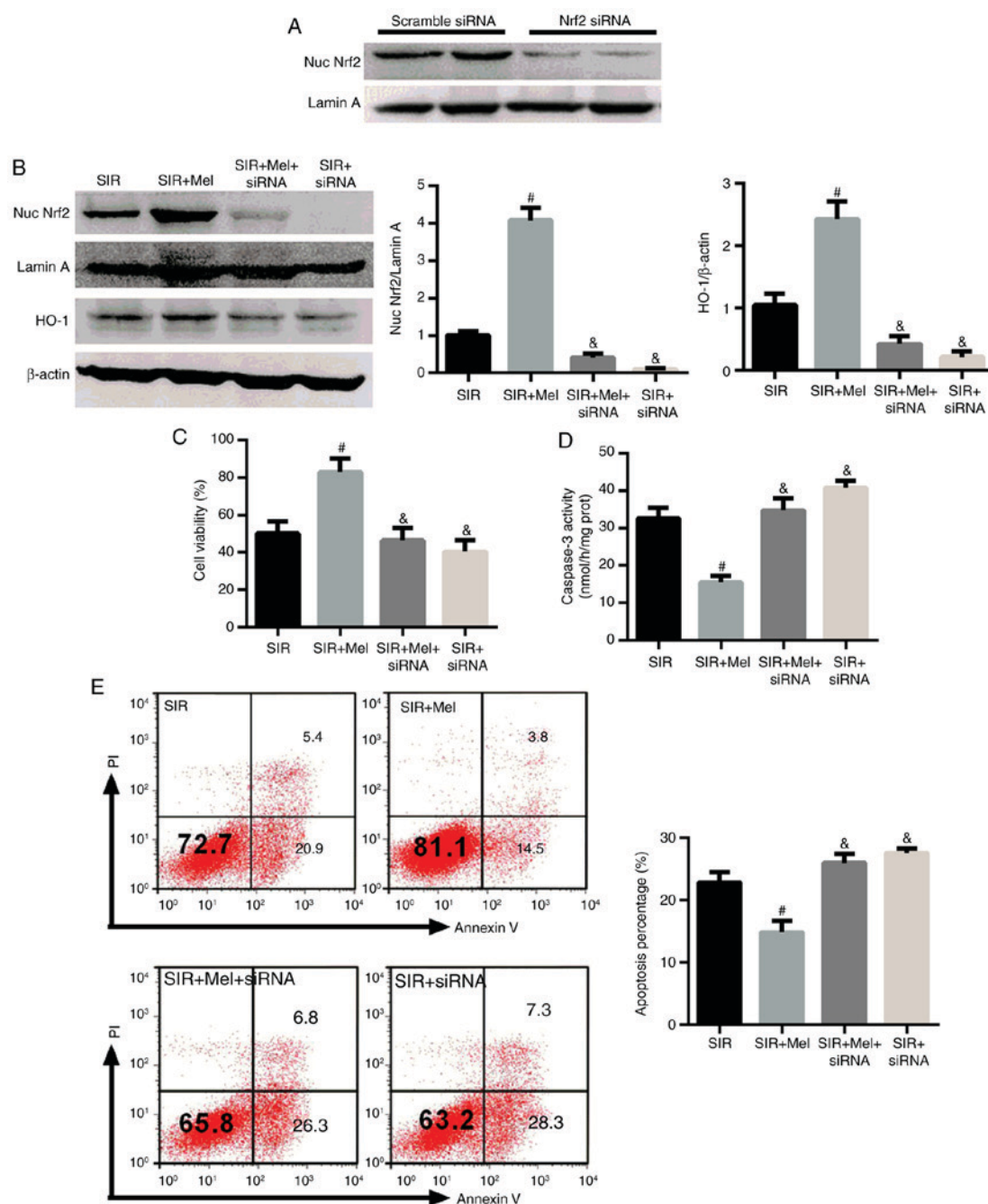


Figure 4. Inhibition of the Nrf2 signaling pathway eliminated the antiapoptotic effects of Mel on H9c2 cells following SIR. H9c2 cells were transfected with scramble or Nrf2 siRNA for 48 h and then subjected to SIR. The expression of (A) nuclear Nrf2 expression was examined by western blotting. H9c2 cells were transfected with scramble siRNA or Nrf2 siRNA for 48 h and then subjected to SIR with or without Mel pretreatment. (B) Protein expression levels of nuclear Nrf2 and HO-1 were examined by western blotting. (C) Cell viability was detected and (D) caspase-3 activity was examined using a particular kit. (E) Cell apoptosis was analyzed by flow cytometry and the percentage of apoptotic cells labeled as Annexin V (+) PI (-) were collected. The results were reported as the mean \pm standard error ($n=8$ per group). $^{\#}P<0.05$ vs. SIR group; $^{\&}P<0.05$ vs. SIR+Mel group. siRNA, small interfering RNA; Nrf2, nuclear factor erythroid 2-related factor 2; Nuc, nuclear; SIR, simulated ischemia reperfusion; Mel, melatonin; HO-1, heme oxygenase 1; PI, propidium iodide.

anti-oxidative effects of melatonin on SIR-treated H9c2 cells as demonstrated by elevated ROS production and MDA content, decreased SOD activity and GSH expression (Fig. 5). These results suggested that Nrf2 signaling may serve a crucial role in mitigating SIR-induced oxidative stress in H9c2 cells.

Discussion

Melatonin is a multifunctional and ubiquitous hormone that exhibits potent cardioprotective effects due to its notable

antioxidant ability (14). Studies have demonstrated that the administration of melatonin may alleviate several cardiovascular diseases, including myocardial I/R injury (26), atherosclerosis (27) and hypertension (28); however, the specific mechanism by which melatonin protects the myocardium against I/R injury requires further investigation. In the present study, H9c2 cardiomyocytes subjected to SIR treatment were used to mimic *in vivo* myocardial I/R injury. Consistent with a previous study (4), SIR induced significant apoptosis and oxidative stress; melatonin pretreatment may

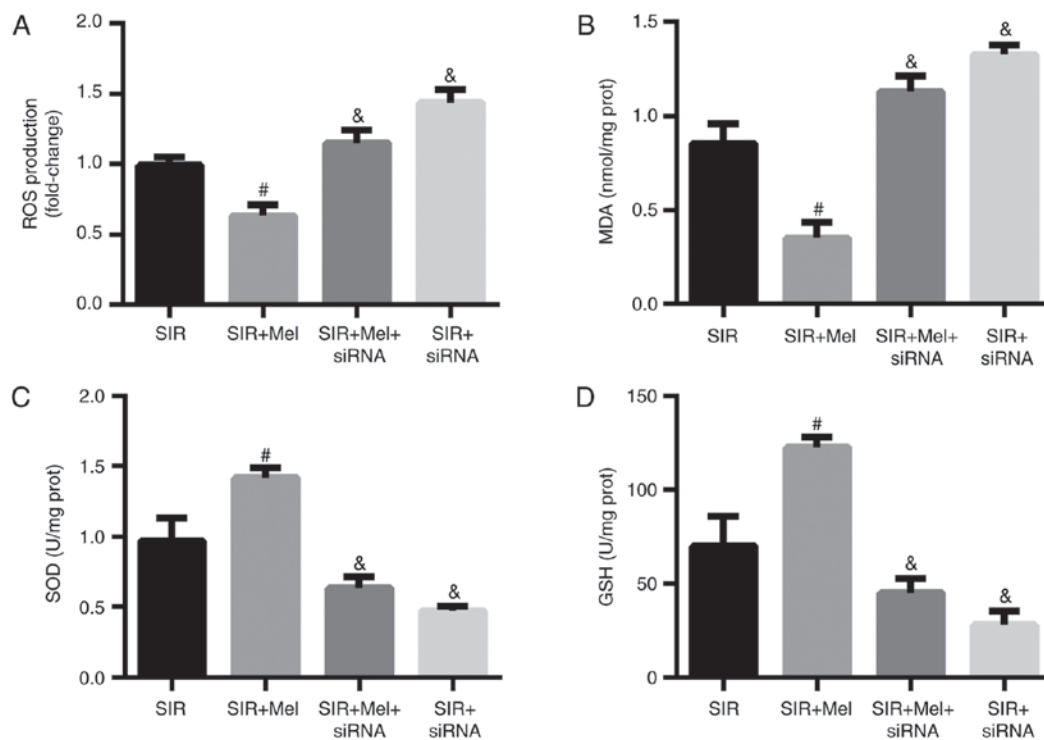


Figure 5. Inhibition of the Nrf2 signaling pathway abolishes the antioxidative effects of Mel on H9c2 cells following SIR. H9c2 cells were transfected with scramble siRNA or Nrf2 siRNA for 48 h and then subjected to SIR with or without Mel pretreatment. (A) ROS production was measured using a ROS assay kit. (B) MDA content, (C) SOD activity and (D) GSH expression were assayed using appropriate kits. The results are presented as the mean \pm standard error ($n=8$ per group). [#] $P<0.05$ vs. SIR group; [&] $P<0.05$ vs. SIR+Mel group. Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SIR, simulated ischemia reperfusion; Mel, melatonin; siRNA, small interfering RNA; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione.

have protected H9c2 cells against SIR-induced apoptosis and oxidative stress as reported in the present study. To the best of our knowledge, the present study is the first to demonstrate that the protective effects of melatonin on SIR-induced H9c2 cells may occur via the activation of the Nrf2 signaling pathway.

It has been reported that myocardial I/R injury resulted in cellular oxidative stress responses, which further led to lipid peroxidation, Ca^{2+} overload, protein oxidation and myocardial apoptosis (29). Additionally, it has been demonstrated that melatonin has notable myocardial protective effects due to its potent anti-oxidant and anti-apoptotic properties (15). In accordance with these studies, the present study revealed that SIR treatment significantly reduced the cell viability and enhanced the extent of apoptosis of H9c2 cells. Furthermore, oxidative stress was investigated in the present study, which indicated that SIR induced significant increases in ROS and MDA production, but decreased SOD activity and GSH expression; however, pretreatment with melatonin revealed that the effects of SIR treatment in H9c2 cells were all reversed. These results indicated melatonin may protect H9c2 cells from I/R injury via inhibition of oxidative stress and apoptosis.

The Nrf2 signaling pathway serves a crucial role in reducing oxidative stress-induced injury (30,31). Under normal conditions, Nrf2 is mainly located in the cytoplasm; under conditions of oxidative stress, Nrf2 translocates to the nucleus, binds to AREs and then initiates the activation of the endogenous anti-oxidative response to inhibit oxidative stress (32,33). Activation of Nrf2 signaling has been reported to increase cell anti-oxidative ability (34). HO-1 is a target gene of Nrf2,

which is involved in regulating cellular ROS production and scavenging (35,36). Activation of Nrf2-HO-1 signaling may alleviate myocardial I/R injury (37). In the present study, SIR treatment was observed to enhance the expression of Nrf2 in the nucleus and the whole cell, which may suggest increased nuclear translocation. As the expression of Nrf2 in whole lysate and nucleus was not directly compared, the increased nuclear translocation of Nrf2 may be likely; however, further investigation is required. However, pretreatment with melatonin further promoted Nrf2 translocation, as well as the expression of the downstream gene, HO-1, which encodes an enzyme involved in heme metabolism. In the present study, only the expression levels of HO-1 were analyzed; however, analysis of the activity of HO-1 may improve understanding of its role in Nrf2 signaling. In addition, the present study observed that pretreatment with melatonin alone without SIR also promoted Nrf2 nuclear transport, which further demonstrated the protective effect of melatonin. These results indicated that the protective effects of melatonin on SIR-induced H9c2 cells may occur via activation of the Nrf2 signaling pathway.

To further verify the effects of Nrf2 on SIR-induced H9c2 cells pretreated with melatonin, a specific siRNA targeting Nrf2 was employed in the present study. The inhibition of Nrf2 signaling eliminated the anti-apoptotic and anti-oxidative effects of melatonin on H9c2 cells in the present study. These results further suggested that the protective effects of melatonin on SIR-induced H9c2 cells may be dependent on the Nrf2 signaling pathway.

However, there were limitations of the present study as whether melatonin exhibited its protective effects on

myocardial I/R injury via activation of the Nrf2 signaling pathway was not investigated *in vivo*. Thus, the mechanism underlying the effects of melatonin in activating the Nrf2 signaling pathway requires further study.

In conclusion, the present study proposed that melatonin may protect H9c2 cells against I/R injury by reducing apoptosis and oxidative stress, and this effect may be mediated via the activation of the Nrf2 signaling pathway, suggesting that melatonin may be considered as a potential therapeutic target for the prevention of myocardial I/R injury.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and HZ designed the study. YZ and HW conducted the experiments. BQ and FG performed the statistical analysis. HW and SM contributed to the interpretation of the experimental results, produced the figures and wrote the manuscript. HZ revised and edited the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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