MicroRNA-21 contributes to the puerarin-induced cardioprotection via suppression of apoptosis and oxidative stress in a cell model of ischemia/reperfusion injury

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Abstract. Puerarin, a major bioactive constituent of the *Radix puerariae*, can ameliorate myocardial ischemia/reperfusion (I/R) injury. Emerging evidence supports that microRNA (miR)-21 functions as a protective factor against I/R and/or hypoxia-reperfusion (H/R)-induced myocardial injury. However, the role of miR-21 in the cardioprotective effect of puerarin remains unclear. Therefore, the purpose of the present study was to demonstrate the involvement of miR-21 in the cardioprotective mechanisms of puerarin using a cell model of I/R injury, generated by culturing rat H9c2 cardiomyocytes under H/R conditions. The results demonstrated that pre-treatment with puerarin significantly increased cell viability, decreased lactate dehydrogenase activity and upregulated miR-21 expression in H/R-treated H9c2 cells. Transfection of an miR-21 inhibitor led to an increase in H/R-induced cytotoxicity and reversed the protective effects of puerarin. Additionally, miR-21 inhibition attenuated the puerarin-induced decrease in the rate of apoptosis, caspase-3 activity and the expression of apoptosis regulator Bax, and increased apoptosis regulator Bcl-2 expression, under H/R conditions. Furthermore, puerarin mitigated H/R-induced oxidative stress as evidenced by the decrease in endogenous reactive oxygen species production, malondialdehyde content and NADPH oxidase 2 expression, and enhanced the antioxidative defense system as illustrated by the increase in superoxide dismutase activity, catalase and glutathione peroxidase levels. These effects were all eliminated by miR-21 inhibitor transfection. Furthermore, the miR-21 inhibitor exacerbated the H/R-induced oxidative stress and attenuated the antioxidative defense system in H/R-treated H9c2 cells. Taken together, the results suggested that miR-21 mediated the cardioprotective effects of puerarin against myocardial H/R injury by inhibiting apoptosis and oxidative stress.

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Introduction

Myocardial ischemia/reperfusion (I/R) injury remains a significant clinical problem with a lack of effective therapies, and is a leading cause of morbidity and mortality in patients with cardiovascular disease (1). Previous studies have confirmed that multiple biological processes, including myocardial cell injury, apoptosis, oxidative stress and mitochondrial dysfunction, are involved in the pathophysiology of myocardial I/R injury. However, the underlying molecular and cellular events are complex and remain elusive (2,3). Understanding these mechanisms would be beneficial towards the development of effective interventions and strategies to prevent myocardial I/R injury, and is therefore of great clinical significance.

Puerarin (7,4-dihydroxyisoflavone-8β-glucopyranoside) is a bioactive isoflavone derived from the Kudzu (*Pueraria montana* var. *lobata*) root, a well-known traditional Chinese medicine, which is widely prescribed for patients with cardio- and cerebrovascular diseases in China (4,5). In recent years, a growing body of *in vivo* and *in vitro* evidence has been reported, revealing the value of puerarin in the treatment of cardiovascular diseases, including myocardial I/R injury (6,7). Studies have demonstrated that the therapeutic effect of this compound on I/R-induced myocardial injury is closely associated with its anti-apoptotic role (8) and its regulation of mitochondrial transmembrane pores and/or channels (9). Although puerarin is considered to be a protective agent against myocardial I/R injury, the exact cellular and molecular mechanisms by which it serves this cardioprotective role are not well understood.

Endogenous 18-22 nucleotide microRNAs (miRNAs/miRs) are considered to serve significant roles in cell differentiation, apoptosis and oxidative stress (10). Recently, the function of miR-21 in cardiovascular disease has received increasing attention (11,12). miR-21 has been reported to be highly expressed in many types of cardiovascular cells, including cardiomyocytes, and it functions in myocardial I/R injury protection (13-15). Studies have confirmed that overexpression of miR-21 via plasmid- or adenovirus-mediated gene transfer suppresses cardiomyocyte injury and apoptosis,

providing a novel potential therapeutic target for myocardial I/R injury (16-18). Accordingly, inhibiting miR-21 cancels out the cardioprotective effect of ischemic preconditioning (19). miR-21 has also been reported to be involved in cardioprotection against myocardial I/R and hypoxia/reoxygenation (H/R) injury in cardiomyocytes (20). However, whether miR-21 contributes to puerarin-induced cardioprotection remains unknown.

The present study investigated the role of miR-21 on the effect of puerarin on H/R-induced H9c2 cell injury, an *in vitro* model of myocardial I/R injury. The results revealed that miR-21 mediated the cardioprotective effects of puerarin against H/R injury. Furthermore, apoptosis and oxidative stress was inhibited, and the antioxidative defense system was enhanced. These findings suggested a significant role for miR-21 in the cardioprotective function of puerarin in H/R injury, indicating a potential therapeutic target for treating myocardial I/R injury.

Materials and methods

Cell culture. Embryonic rat myocardium-derived H9c2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin (both Gibco; Thermo Fisher Scientific, Inc., Waltham MA, USA) at 37˚C in a humidified incubator containing 95% air and 5% CO_2.

In vitro H/R model establishment. H9c2 cells were treated with H/R in order to mimic myocardial I/R injury. Following culture to 70-80% confluence under normal conditions as described above, cells were incubated with serum-deficient DMEM in a hypoxic chamber supplied with 95% N_2 and 5% CO_2 at 37˚C for 6 h, and then moved to an incubator with 95% air and 5% CO_2 at 37˚C for 12 h. The serum-deficient DMEM in the hypoxic conditions was replaced by normal medium upon the initiation of reoxygenation. The control cells were kept in normal culture medium with 95% air and 5% CO_2 at 37˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total miRNA was extracted from the cultured H9c2 cells using the mirVana™ miRNA Isolation kit (cat. no. AM1561; Thermo Fisher Scientific, Inc.). cDNA was generated from the total miRNA (1 μg) using a TaqMan™ MicroRNA Reverse Transcription kit (cat. no. 4366597; Thermo Fisher Scientific, Inc.). qPCR amplification was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using TaqMan™ Fast Advanced master mix (cat. no. 4444556; Thermo Fisher Scientific, Inc.). All steps were performed according to the protocols of the kit manufacturers. Following initial incubation at 45˚C for 2 min and 95˚C for 10 min, amplification was performed for 45 cycles of 95˚C for 15 sec, 65˚C for 20 sec and 72˚C for 30 sec. The relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (21). The results were expressed as the ratios of target genes against the small nuclear RNA U6 for miR-21. The primer sequences used in this study are as follows: miR-21 forward, 5'-TGTACCACCTTGTCGGATAG-3'; and reverse, 5'-CTGCTGTTGCCATGAGAT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3'; and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The experiment was performed in triplicate.

Cell transfection. miR-21 inhibitor and an inhibitor negative control (miR-NC;both Thermo Fisher Scientific, Inc.) were transfected into H9c2 cells using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM, according to the manufacturer's protocol. Following transfection for 48 h, the transfection efficiency was determined using RT-qPCR. The sequences were as follows: niR-21 inhibitor, 5'-UCAACAUCAGUCUGAUAAGCUA-3'; miR-NC, 5'-CAGUACUUUUGUGUAGUACAA-3'.

Assessment of cell viability. H9c2 cell viability was assessed using the MTT cell proliferation and cytotoxicity assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. In brief, H9c2 cells were seeded into 96-well plates at $4x10^3$ cells/well. Following incubation with the indicated reagents, MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37˚C. Subsequently, 150 μl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added for 10-15 min to dissolve the precipitate. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The results are represented graphically as a percentage of the control cells.

Measurement of lactate dehydrogenase (LDH) activity in culture supernatants. Following the treatment with miR-21 inhibitors and/or puerarin followed by H/R, cell damage was evaluated by measuring the amount of LDH released into the culture supernatant using a commercial LDH kit (cat. no. C0016; Beyotime Institute of Biotechnology) at 490 nm, using a microplate reader. The results of the LDH activity are expressed as a percentage of the activity in the control group.

Analysis of cell apoptosis by flow cytometry. The measurement of apoptosis was performed using an Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (cat. no. APOAF; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols. Briefly, H9c2 cells were collected, washed twice with cold PBS, and resuspended in 1X binding buffer at $1x10^6$ cells/ml. Subsequently, the cells were stained with 5 μl Annexin V-FITC and 5 μl PI solution for 10 min at room temperature in the dark. Following two washes with PBS, the fluorescence was analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The experiments were performed in triplicate.

Determination of caspase-3 activity. Caspase-3 activity in H9c2 cells was assessed using a caspase-3 colorimetric assay (cat. no. ab39401; Abcam, Cambridge, UK), according to the manufacturer's protocols. The cell lysate was collected and incubated with 150 μM DEVD-p-nitroaniline, a fluorogenic substrate of caspase-3, at 37˚C for 2 h. The fluorescence was

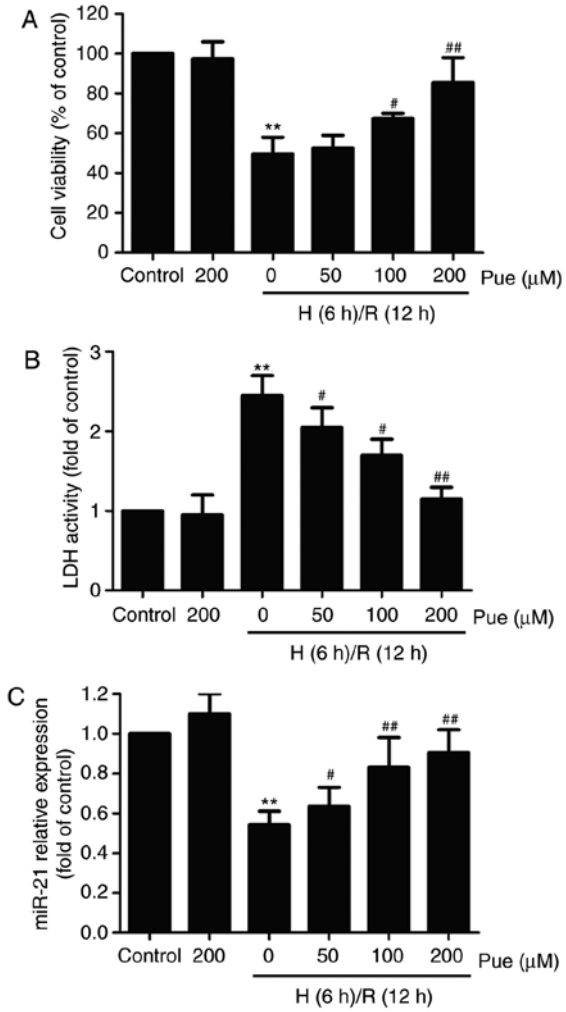

Figure 1. Effects of puerarin on cell viability, cell damage and miR-21 levels in H/R-treated H9c2 cells. Cells were pretreated with 50, 100 or 200 μM puerarin for 1 h prior to incubation under H/R conditions. (A) Cell viability was assessed with MTT assays. The data are expressed as a percentage relative to the control group. (B) Cell damage was assessed by measuring LDH activity in the cell culture supernatant using a commercial kit. The data are expressed relative to the control group. (C) miR-21 expression was determined by reverse transcription-quantitative polymerase chain reaction analysis. The bars represent the mean ± standard deviation from ≥3 independent experiments. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H/R treatment group. LDH, lactate dehydrogenase; miR-NC, miR-21 inhibitor negative control; Pue, puerarin; H/R, hypoxia/reoxygenation.

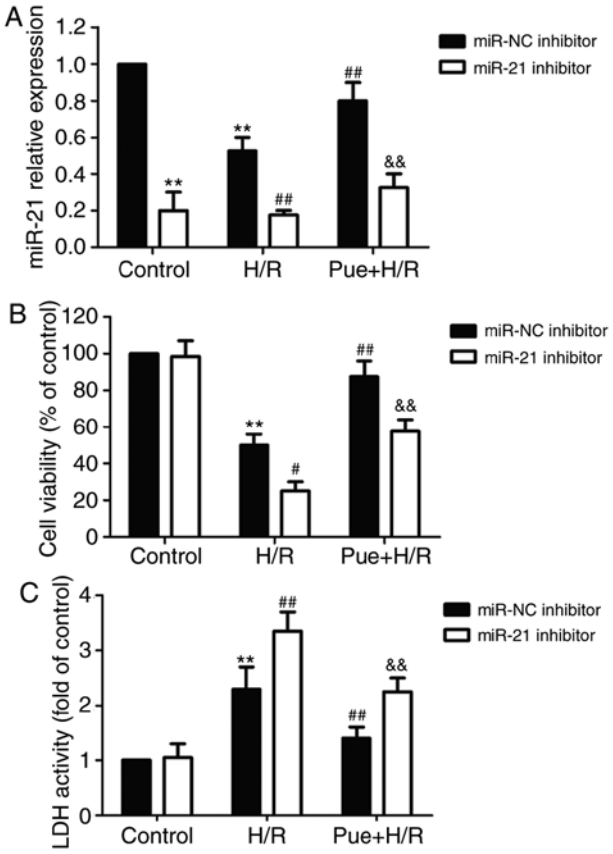

Figure 2. Effects of miR-21 inhibition on the puerarin-induced protection of H9c2 cells against H/R-induced cytotoxicity. Cells were transfected with miR-21 inhibitor or miR-NC followed by treatment with 200 μM puerarin for 1 h prior to incubation under H/R conditions. (A) miR-21 expression was determined by a reverse transcription-quantitative polymerase chain reaction assay. (B) Cell viability was assessed using an MTT assay and the data are expressed as a percentage relative to the control group. (C) LDH activity was measured using a commercial kit and the data are expressed relative to the control group. The bars represent the mean ± standard deviation from ≥3 independent experiments. **P<0.01 vs. control + miR-NC transfection group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. H/R + miR-NC transfection group; $^{\&\&}$P<0.01 vs. Pue + H/R + miR-NC inhibitor transfection group. miR, microRNA; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; Pue, puerarin.

then measured using a Clariostar Monochromator microplate reader (BMG Labtech GmbH, Ortenberg, Germany) with excitation at 360 nm and emission at 460 nm.

Measurement of intracellular reactive oxygen species (ROS) production. To evaluate the intracellular generation of ROS, cells were stained with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich; Merck KGaA). At the final stage of treatment with miR-21 inhibitors and/or puerarin followed by H/R, the H9c2 cells were incubated with 10 μM DCFH-DA at 37˚C for 20 min. Following two washes with PBS, cell images were captured using a fluorescence microscope (Olympus Corporation, Tokyo, Japan) and the DCF fluorescence was analyzed using a FACScanto flow cytometer (BD Biosciences, San Jose, CA, USA) using a 488 nm excitation filter and a 525 nm emission filter. Finally, FlowJo version 7.6 (FlowJo LLC, Ashland, OR, USA) was used to examine the production of intracellular ROS.

Measurement of malondialdehyde (MDA) content, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities. Following treatment with miR-21 inhibitors and/or puerarin followed by H/R, cells were ultrasonicated and centrifuged at 12,000 x g at 4˚C for 10 min. Protein concentration was quantified using a bicinchoninic acid (BCA) protein assay (Beyotime Institute of Biotechnology). MDA (cat. no. CEA597Ge) content and SOD (cat. no. SES134Hu), CAT (cat. no. SEC418Hu) and GSH-Px (cat. no. CEA294Ge) activities in the total cell lysate (10 μl) were determined using commercially available kits (Uscn Life Science, Inc., Wuhan, China), according to the manufacturer's protocols. Optical density was measured using a microplate reader. Each measurement was calculated compared with a standard curve and normalized to the total protein concentration. Experiments were repeated three times.

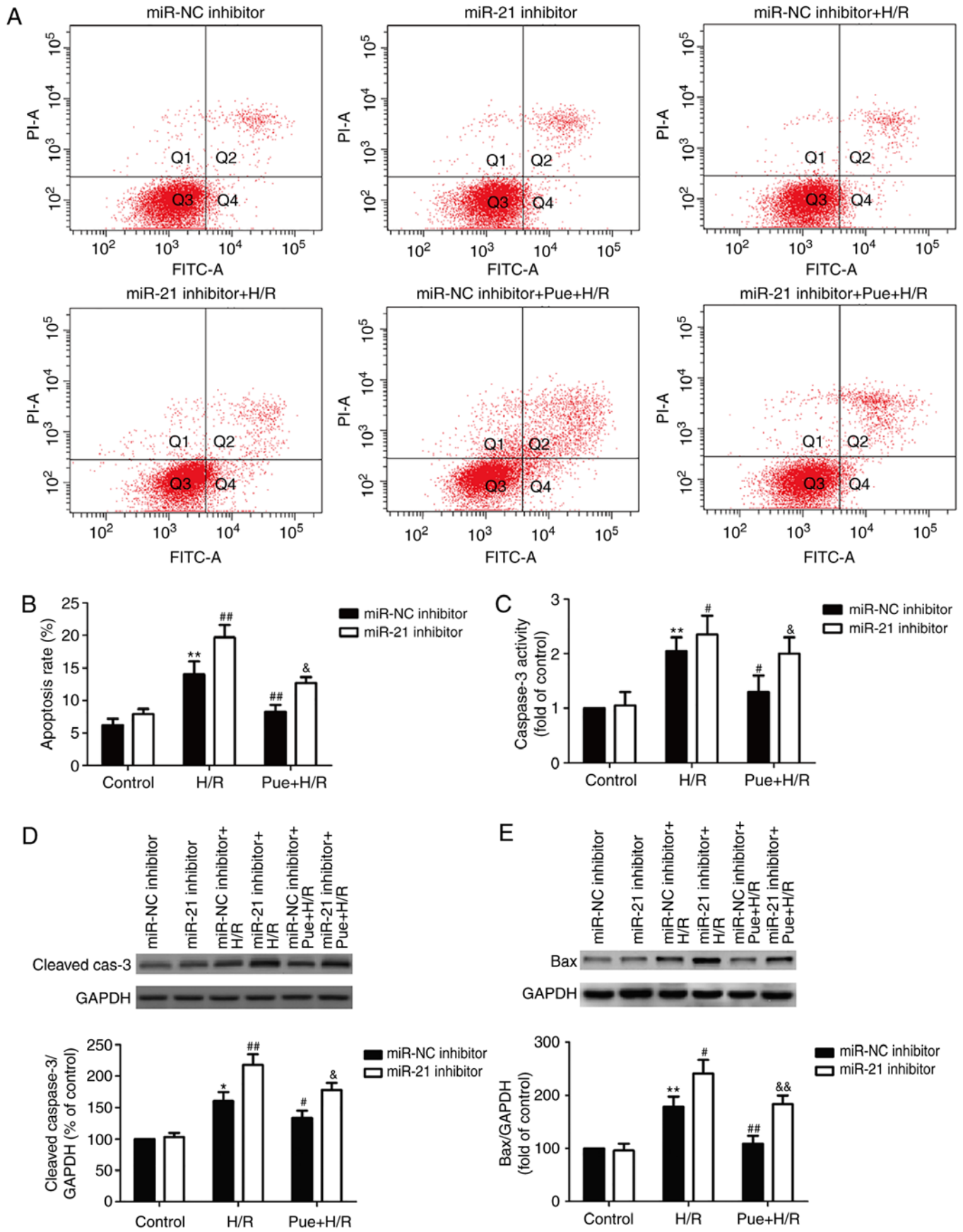

Figure 3. Effects of miR-21 inhibition on apoptosis under H/R conditions in the presence or absence of puerarin in H9c2 cells. Cells were transfected with miR-21 inhibitor or miR-NC followed by treatment with 200 μM puerarin for 1 h prior to incubation under H/R conditions. (A) The apoptotic rate was determined using an Annexin V-FITC/PI apoptosis detection kit. (B) Quantitative analysis was performed following flow cytometry. (C) Caspase-3 activity was detected by a colorimetric assay. The expression of (D) cleaved caspase-3 and (E) Bax were measured by western blot analysis. The bars represent the mean ± standard deviation of ≥3 independent experiments. *P<0.05, **P<0.01 vs. control + miR-NC inhibitor transfection group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. H/R + miR-NC transfection group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. Pue + H/R + miR-NC transfection group.

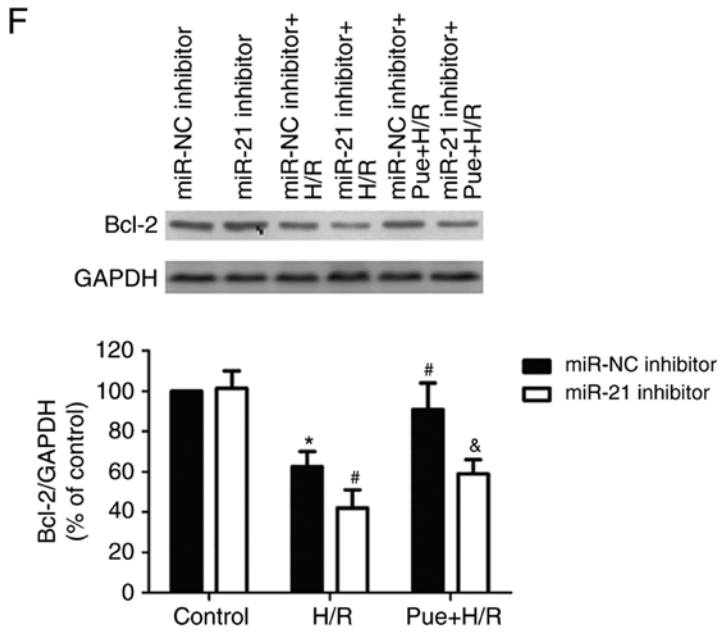

Figure 3. Continued. Effects of miR-21 inhibition on apoptosis under H/R conditions in the presence or absence of puerarin in H9c2 cells. The expression of (F) Bcl-2 was measured by western blot analysis. The bars represent the mean ± standard deviation of ≥3 independent experiments. *P<0.05 vs. control + miR-NC inhibitor transfection group; #P<0.05 vs. H/R + miR-NC transfection group; &P<0.05 vs. Pue + H/R + miR-NC transfection group. miR, microRNA; miR-NC, miR-21 inhibitor negative control; H/R, hypoxia/reoxygenation; Bax, apoptosis regulator Bax; Bcl-2, apoptosis regulator Bcl-2; Pue, puerarin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Western blot analysis. Following treatment with miR-21 inhibitors and/or puerarin followed by H/R, the H9c2 cells were collected and lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) supplemented with a protease inhibitor cocktail (cat. no. 78425; Thermo Fisher Scientific, Inc.) on ice for 30 min. The protein concentration was measured using the BCA protein assay. Equal amounts of protein (30 μg/lane) were separated by SDS-PAGE (12% gel; Beyotime Institute of Biotechnology) and transferred to a polyvinylidene fluoride membrane membranes (Merck KGaA). Non-specific binding was blocked with 5% non-fat milk for 2 h at room temperature, and then the membranes were incubated overnight at 4˚C with the following specific primary antibodies: Anti-apoptosis regulator Bcl-2 (1:1,000, cat. no. 15071; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-apoptosis regulator Bax (1:1,000, cat. no. 2774; Cell Signaling Technology, Inc.), cleaved caspase-3 (Asp175; 1:1,000, cat. no. 9661; Cell Signaling Technology, Inc.), anti-NADPH oxidase 2 (NOX2; 1:1,000; cat. no. ab129068; Abcam) and anti-GAPDH (1:2,000; cat. no. 5174; Cell Signaling Technology, Inc.). Following three washes with Tris-buffered saline with 0.1% (v/v) Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, 1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature. Finally, the protein bands were detected with a Super ECL Detection kit (Beyotime Institute of Biotechnology). The band intensities were quantified using Quantity One software version 4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to GAPDH.

Statistical analysis. Data were expressed as the mean ± standard deviation. Data were analyzed in SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) using one-way analysis of variance followed by Bonferroni's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Puerarin attenuates cytotoxicity and increases miR-21 levels in H/R treated H9c2 cells. Firstly, to investigate the cardioprotective effect of puerarin in H/R-induced injury, H9c2 cells were pre-treated with various doses of puerarin (50, 100 or 200 μM) for 1 h, followed by H/R conditions. The results from the MTT and LDH activity assays revealed that the H/R treatment alone significantly decreased the H9c2 cell viability (Fig. 1A) and increased LDH activity in the culture supernatant (Fig. 1B), whereas these effects were blocked by the puerarin pretreatment in a dose-dependent manner. Subsequently, the effect of puerarin on the expression of miR-21 under H/R conditions was investigated, and the results demonstrated that the H/R conditions led to a decrease in the endogenous miR-21 levels in the H9c2 cells; this was also prevented by the puerarin pretreatment in a concentration-dependent manner (Fig. 1C). The maximum protective effect was achieved with 200 μM puerarin, which was the concentration selected for subsequent experiments. These results suggested that miR-21 upregulation may be associated with the cardioprotective function of puerarin against H/R injury.

Inhibition of miR-21 eliminates the puerarin-induced cardioprotection against H/R injury. To further confirm the role of miR-21 in puerarin-induced protection against H/R injury, H9c2 cells were transfected with miR-21 inhibitor to block miR-21 expression. As demonstrated in Fig. 2A, cells transfected with the miR-21 inhibitor exhibited a notable decrease

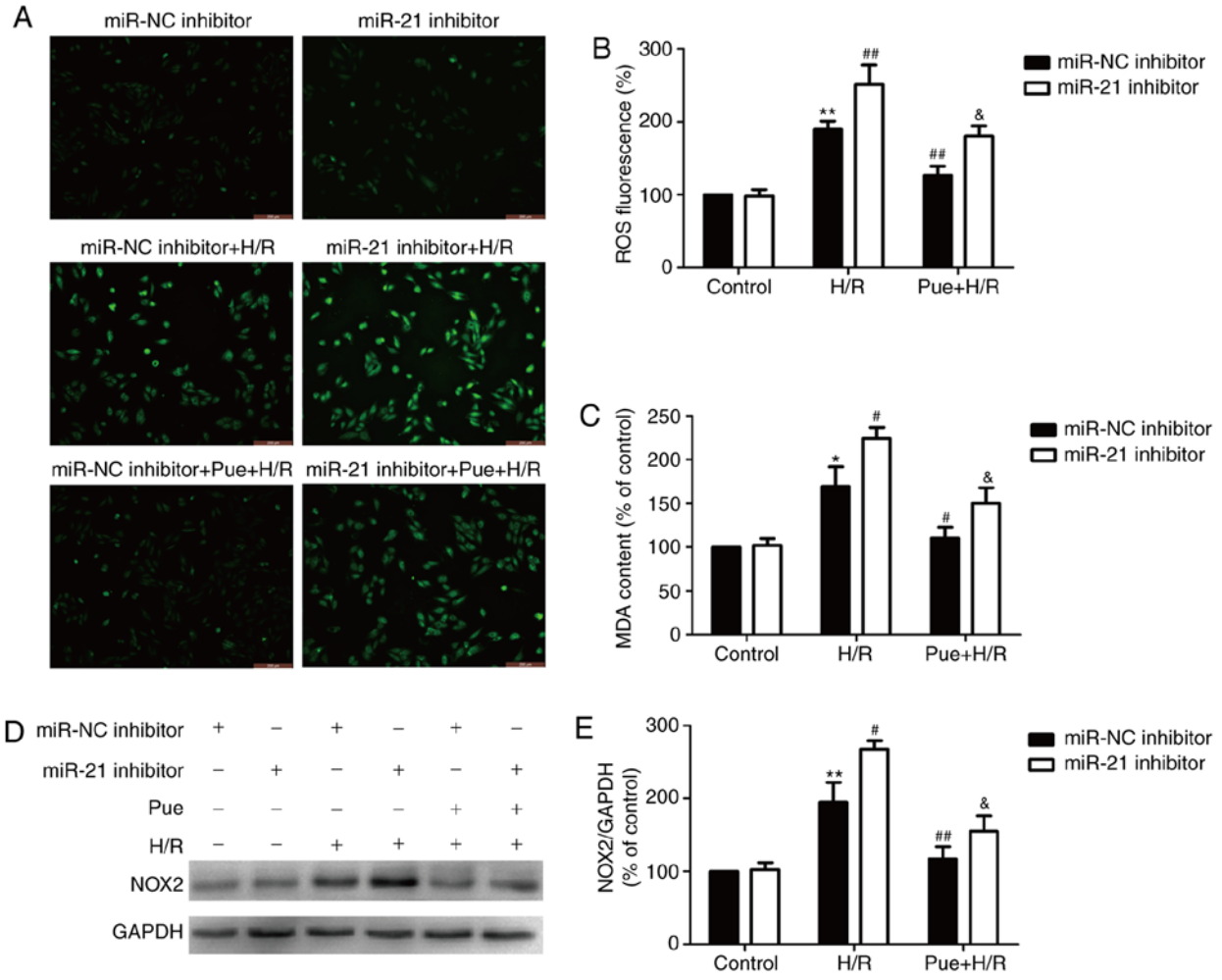

Figure 4. Effects of miR-21 inhibition on oxidative stress in the presence or absence of puerarin in H/R-treated H9c2 cells. Cells were transfected with miR-21 inhibitor or miR-NC followed by treatment with 200 μM puerarin for 1 h prior to incubation under H/R conditions. (A) Endogenous ROS production was determined by DCFH-DA staining. Scale bar, 100 μm. (B) Quantitative analysis of ROS production was processed by flow cytometry analysis. (C) MDA content was measured using a commercially available kit, calculated based on a standard curve and normalized to the total protein concentration. (D) NOX2 expression was detected by western blot analysis. (E) Quantitative analysis of NOX2 expression. Bars represent the mean ± standard deviation from ≥3 independent experiments. *P<0.05, **P<0.01 vs. control + miR-NC transfection group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. H/R + miR-NC transfection group; $^{\&}$P<0.05 vs. Pue + H/R + miR-NC transfection group. miR, microRNA; H/R, hypoxia/reoxygenation; miR-NC, miR-21 inhibitor negative control; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; MDA, malondialdehyde; NOX2, NADPH oxidase 2; Pue, puerarin.

in miR-21 expression compared with those transfected with the miR-NC. In the miR-21 inhibitor transfected group, no significant difference in miR-21 expression was observed between the control and H/R groups (Fig. 2A). In addition, the inhibition of miR-21 further intensified the H/R-induced decrease in cell viability (Fig. 2B) and increase in LDH activity (Fig. 2C), and markedly reduced the puerarin-induced protection against H/R-induced cytotoxicity in H9c2 cells. These results indicated that miR-21 mediated the cardioprotective function of puerarin against H/R injury.

Inhibition of miR-21 reverses the puerarin-induced reduction of apoptosis in H/R-treated H9c2 cells. The effect of puerarin on apoptosis, and the role of miR-21 in this process, was examined. The results revealed that the H/R-induced apoptosis in H9c2 cells was enhanced following the inhibition of miR-21 expression, but reduced following puerarin treatment (Fig. 3A and B). However, this effect of puerarin was blocked by miR-21 inhibition. Furthermore, in H/R-treated cells, miR-21 inhibition increased the activity of caspase-3 (Fig. 3C) and the expression of cleaved caspase-3 (Fig. 3D), an apoptosis mediator in intrinsic and extrinsic pathways (22), and reversed the puerarin-induced repression. The western blot analysis results revealed that puerarin alleviated the H/R-induced upregulation of the expression of pro-apoptotic protein Bax, and downregulated that of anti-apoptotic protein Bcl-2 (Fig. 3E and 3F). These effects were blocked by miR-21 inhibition. In addition, transfection with miR-21 inhibitor intensified the H/R-induced changes in the expression of Bax and Bcl-2. These results suggested that miR-21 contributed to the protective function of puerarin against H/R-induced apoptosis, likely in a mitochondrial-dependent manner.

Inhibition of miR-21 reverses the puerarin-induced inhibition of oxidative stress in H/R-treated H9c2 cells. Oxidative stress serves a fundamental role in the physiological process of myocardial I/R injury (23). Therefore, to examine the effects of miR-21 in puerarin-induced cardioprotection against this

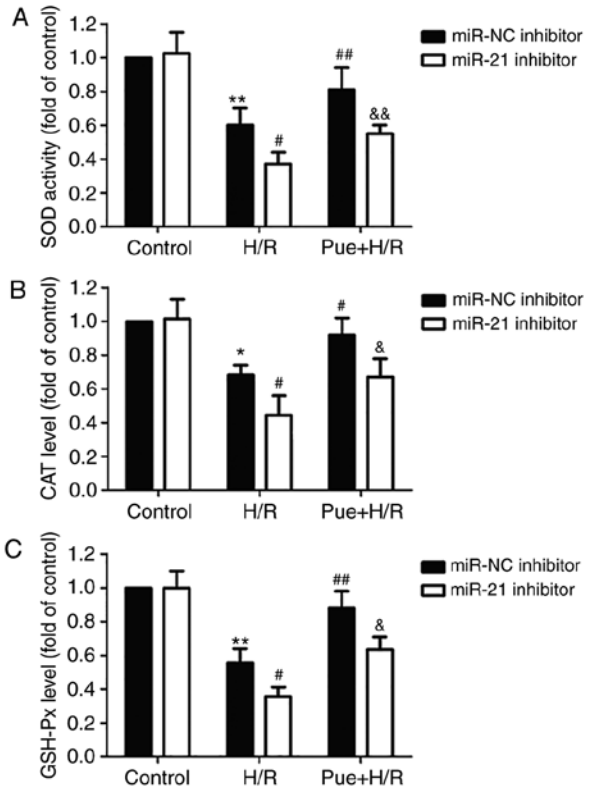

Figure 5. Effects of miR-21 inhibition on SOD activity, as well as levels of CAT and GSH-Px in the presence or absence of puerarin in H/R-treated H9c2 cells. Cells were transfected with miR-21 inhibitor or miR-NC followed by treatment with 200 μM puerarin for 1 h prior to incubation under H/R conditions. (A) SOD activity, (B) CAT levels and (C) GHS-Px levels were determined using commercially available kits. Data are presented relative to the miR-NC transfection group. Bars represent the mean ± standard deviation from ≥3 independent experiments. *P<0.05, **P<0.01 vs. control + miR-NC transfection group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. H/R + miR-NC transfection group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. Pue + H/R + miR-NC transfection group. miR, microRNA; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; H/R, hypoxia/reoxygenation; miR-NC, miR-21 inhibitor negative control; Pue, puerarin.

factor, oxidative stress parameters, including ROS production, MDA content and NOX2 expression, were measured. As shown in Fig. 4, puerarin treatment led to a marked decrease in endogenous ROS production induced by H/R, whereas this effect was reversed by the miR-21 inhibitor (Fig. 4A and B). In the H/R-treated group, the miR-21 inhibitor led to the intensification of the H/R-induced increase in ROS. In addition, the miR-21 inhibition led to the inhibition of the puerarin-induced decrease in MDA content in the H/R-treated group, and aggravated the H/R-induced increase (Fig. 4C). Furthermore, H/R treatment resulted in an increase in NOX2 expression, which was mitigated by puerarin pretreatment, and enhanced by miR-21 inhibition (Fig. 4D and E). Notably, miR-21 inhibition eradicated the effects of puerarin. These results indicated that puerarin inhibits H/R-induced oxidative stress by enhancing miR-21 expression.

Inhibition of miR-21 halts the puerarin-induced improvement of the antioxidant defense system in H/R-treated H9c2 cells. The endogenous antioxidant system provides defense against oxidative stress-induced cytotoxicity, through enzymatic and/or non-enzymatic mechanisms (24). In the present study, the effects of puerarin on the antioxidant enzymes SOD, CAT and GSH-Px in H9c2 cells were evaluated. The results revealed that H/R leads to a decrease in SOD activity and CAT and GSH-Px levels (Fig. 5A-C). These effects were enhanced by miR-21 inhibition and abated by puerarin pretreatment. Furthermore, miR-21 inhibition reversed the puerarin-induced upregulation of SOD activity and CAT and GSH-Px levels in the H/R-treated group (Fig. 5A-C). These results demonstrated that miR-21 caused the puerarin-induced enhancement of the antioxidant defense system under H/R conditions.

Discussion

The present study was the first to demonstrate that miR-21 mediated the cardioprotective effect of puerarin against H/R-induced injury in H9c2 cells by inhibiting apoptosis and oxidative stress.

Puerarin is a major bioactive ingredient of the *Pueraria* lobata (Willd.) Ohwi. root, a traditional Chinese medicine widely used in the treatment of cardiovascular diseases, including myocardial I/R injury (4,8,25). miR-21 has been reported to be involved in the regulation of I/R injury and the associated processes (14,15,26). However, its role in the cardioprotective effect of puerarin has not been reported to date. To the best of our knowledge, the present study was the first to demonstrate that puerarin reduced the H/R-induced downregulation of miR-21 in H9c2 cells. Notably, the inhibition of miR-21 in H/R-treated H9c2 cells blocked the puerarin-induced increase in cell viability and decrease in LDH activity. These results indicated that miR-21 mediated the puerarin-induced cardioprotection against H/R injury.

Apoptosis is a form of cardiomyocyte damage in the early stages of myocardial I/R injury, and is crucial in its pathophysiological processes (27,28). Studies have demonstrated that puerarin exhibits protective effects against I/R injury in cardiac tissues (29-31). The potential underlying mechanisms involve its anti-apoptotic functions. Guo *et al* (8) reported that puerarin decreases I/R-induced myocardial injury in diabetic rats via the suppression of apoptosis. In line with these studies, the present study demonstrated that puerarin attenuated the H/R-induced increase in apoptosis, as evidenced by the increase in apoptotic rate and caspase-3 activity. Notably, miR-21 is pro-survival and anti-apoptotic, and a number of studies have confirmed the protective function of miR-21 in ischemic heart diseases (16,32,33). The present results further revealed that the inhibition of miR-21 in H/R-treated H9c2 cells enhanced the apoptotic rate, and reversed the puerarin-induced downregulation of apoptosis and caspase-3 activity, indicating that miR-21 mediated the protective effect of puerarin against H/R-induced apoptosis.

The Bcl-2 family proteins are also major regulators of the apoptotic process, and one of the major processes regulated by these factors is mitochondria-dependent apoptosis (34). The present results revealed that the inhibition of miR-21 in H/R-treated H9c2 cells mitigated the puerarin-induced decrease in pro-apoptotic protein Bax expression and increase in anti-apoptotic protein Bcl-2 expression. Existing studies have confirmed that puerarin inhibits apoptosis via modulation of caspase-3 activity and Bcl-2/Bax expression, resulting in the restoration of mitochondrial function, and that miR-21

eliminates mitochondrial apoptosis (35-37). The present results further demonstrated that miR-21 inhibition in H/R-treated H9c2 cells enhanced the H/R-induced changes in Bax and Bcl-2 expression, and reversed the puerarin-induced downregulation of Bax and upregulation of Bcl-2. These results suggested that miR-21 mediated the protection of puerarin against apoptosis, and this process may be mitochondria-dependent.

Oxidative stress is a major contributor to myocardial I/R injury (23). *In vivo* and *in vitro* experiments have demonstrated that puerarin exhibits an anti-oxidative function (38-40). Gang *et al* (41) demonstrated that puerarin suppresses angiotensin II-induced cardiac hypertrophy by inhibiting NOX2 activation and oxidative stress. However, the effect of puerarin on oxidative stress in myocardial I/R injury remains unclear. In the present study, puerarin significantly attenuated H/R-induced oxidative stress in H9c2 cells, as evidenced by the decrease in endogenous ROS production, MDA content and NOX2 expression. Furthermore, it has been reported that miR-21 overexpression alleviates cardiomyocyte apoptosis induced by oxidative stress (42). The results of the study by Wu *et al* (37) revealed that miR-21 mimic transfection inhibits ROS-activated mitochondrial apoptosis. In line with these findings, the present study demonstrated that miR-21 inhibition exacerbated H/R-induced oxidative stress and reduced the protective effect of puerarin in H9c2 cells. These results suggested that miR-21 contributed to the protective effects of puerarin against H/R-induced cell damage and apoptosis by inhibiting oxidative stress processes.

Oxidative stress is caused by the imbalance between ROS production and their neutralization and removal by the antioxidant defense system (43). Studies have suggested that myocardial I/R injury can lead to a decrease in the levels of endogenous antioxidants, including SOD, CAT and GSH-Px, and abolish antioxidant defenses, resulting in cellular oxidative stress (43,44). In addition, puerarin attenuates anoxia/reoxygenation-induced ROS production, and SOD and GSH-Px inhibition in rat primary cardiomyocytes (45). The present findings confirmed that puerarin pre-treatment notably increased SOD activity, and CAT and GSH-Px levels in H/R treated cells, indicating the effects of puerarin on the promotion of the anti-oxidative defense system in H/R injury. Furthermore, miR-21 inhibition worsened the H/R-induced loss of the anti-oxidative system and reversed the puerarin-induced enhancement of antioxidant levels under H/R conditions. These results showed that puerarin protected against H/R-induced oxidative stress by enhancing the anti-oxidative defense system, in a miR-21-dependent manner.

In summary, the results of the present study indicated that miR-21 served an important role in the puerarin-induced protection against myocardial H/R injury. Enhancement of endogenous miR-21 expression, induced by puerarin, alleviated H/R-induced cardiomyocyte injury, and the underlying mechanism potentially involved the inhibition of apoptosis and oxidative stress. The present study demonstrated that the use of puerarin may be promising in the management of ischemic heart diseases, which was involved in regulating miR-21.

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Availability of data and materials

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

Authors' contributions

HXX analyzed the data and wrote the manuscript. WP and JFQ performed the experiments and analyzed of data. FL and HQD made substantial contributions to the acquisition and analysis of the data. QJL designed the study and was involved in drafting the manuscript. All authors read and approved the final 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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