Original Article
Catechin protects rat cardiomyocytes from hypoxia-induced injury by regulating microRNA-92a

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Abstract: *Background:* Myocardial infarction (MI) is a serious condition, caused by acute, persistent ischemia or hypoxia of a coronary artery and responsible for heart failure and sudden death. This study aimed to investigate the effects of catechin, one of the main active components of green tea, on hypoxia-induced MI cell model, as well as the underlying possible mechanism. *Methods:* Cell viability, proliferation, apoptosis, and the expression of microRNA-92a (miR-92a) after hypoxia stimulation and/or catechin treatment were assessed using cell counting kit-8 (CCK-8) assay, western blotting, annexin V-FITC/PI staining and qRT-PCR, respectively. miRNA transfection was performed to change the expression of miR-92a. The effects of miR-92a on hypoxia and catechin-treated H9c2 cell viability, proliferation and apoptosis were evaluated. Finally, western blotting was conducted to measure the expression of core factors in the c-Jun N-terminal kinase (JNK) signaling pathway. *Results:* Hypoxia stimulation significantly inhibited H9c2 cell viability and proliferation, induced cell apoptosis and up-regulated miR-92a expression. Catechin markedly protected H9c2 cells from hypoxia-induced viability loss, proliferation inhibition, and apoptosis enhance, as well as miR-92a expression increase. Furthermore, suppression of miR-92a enhanced the protective effects of catechin on hypoxia-induced H9c2 cells. Overexpression of miR-92a had opposite effects. Catechin activated the JNK pathway in H9c2 cells by down-regulating miR-92a. *Conclusion:* Catechin protected H9c2 cells from hypoxia-induced injury by regulating miR-92a and JNK signaling pathway. Our findings facilitate the understanding of the protective activity of catechin in hypoxia-induced MI cell injury and provide a theoretical basis for further explore treatment of MI by using catechin.

Keywords: Myocardial infarction, hypoxia, catechin, MicroRNA-92a, JNK signaling pathway

Introduction

Myocardial infarction (MI) is a serious condition, caused by acute, persistent ischemia or hypoxia of a coronary artery and responsible for heart failure and sudden death [1, 2]. The main clinical symptoms of MI include severe persistent chest pain, shortness of breath, abnormal heart beating, sweating, nausea, fatigue, and weakness [2, 3]. Increasing numbers of reports provide evidence that multiple factors contribute to the occurrence of MI, including over-fatigue, excitement, overeating, constipation, smoking and drinking [4-6]. Despite the fact that the diagnosis and treatment of MI have been improved in recent years, there are still many people die of this disease all over the world [7]. Searching for novel and more effec-tive therapeutic medicines may be helpful for MI treatment.

Green tea is a well-known healthy drink with a variety of beneficial effects [8]. Catechin is one of major active components found and isolated from green tea, which accounts for approximately 10% of the dry weight of green tea leaves [9]. Researches have demonstrated that catechin possesses multiple activities, such as anti-oxidation [10], anti-obesity [11], anti-cancer [12], antibacterial [13] and cardiovascular-protection [14]. In terms of MI, Othman *et al.* reported that catechin prevented isoproterenol-induced cardiac apoptosis by regulating intrinsic apoptotic pathway in MI [15]. Ortiz-Vilchis *et al.* revealed that co-administration of catechin and doxycycline synergistically sup-
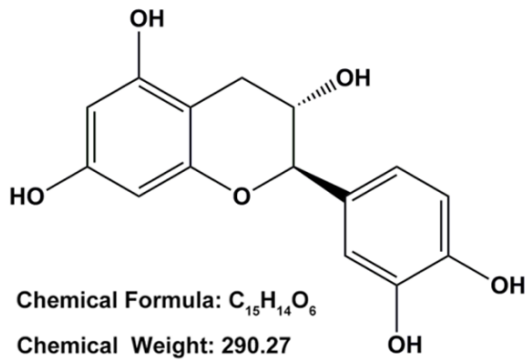
Figure 1. Chemical skeleton structure of catechin.

pressed infarct size in an MI model [16]. More *in vitro* and *in vivo* studies are still needed to further explore the underlying molecular mechanism of the protective effect of catechin on MI.

MicroRNAs (miRNAs) are a class of small, endogenous, and single-stranded RNA transcripts in eukaryotic cells with 20-24 nucleotides (nt) [17]. There are over 2000 miRNAs that have been found in human cells and it is believed that they participate in the regulation of one third of genes in the genome [18]. miRNA-92a (miR-92a) has been demonstrated to up-regulate in the serum of MI patients [19]. Liu *et al.* proved that suppression of miR-92a protected endothelial cells from acute MI in Sprague-Dawley rat models, by modulating kruppel-like factor 2 (KLF2) and KLF4 [20].

Therefore, in this research, the rat embryonic ventricular myocardium-derived H9c2 cells were cultured in hypoxic condition to induce MI cell injury model. The effects of catechin on hypoxia-induced H9c2 cell viability loss, proliferation inhibition, apoptosis enhancement, as well as miR-92a expression increase, were investigated. Our findings will be helpful for further understanding the protective effects of catechin on MI.

Materials and methods

Cell culture and treatment

H9c2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA, catalog number: CRL-1446™) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) supplement-

ed with 10% (v/v) fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA, USA) and 1% (v/v) penicillin-streptomycin-Glutamine (100X) solution (Gibco, Life Technologies, Carlsbad, CA, USA). Cultures were maintained in a humidified incubator (Sanyo, Jencons, Leighton Buzzard, United Kingdom) at 37°C with 95% air and 5% CO_2. H9c2 cells were cultured in hypoxia incubator with 94% N_2, 5% CO_2 and 1% O_2 to stimulate injury [21].

Preparation of catechin solution

Catechin was purchased from Sigma-Aldrich (St Louis, MO, USA, catalog number: 43412) and dissolved in phosphate buffered saline (PBS) to get a storage solution of 200 mM. Then, catechin solution were sterilized through 0.22 µm filter for use in experiments and stored in 4°C. Serum-free DMEM was used to dilute catechin storage solution to 10, 50, 100, 200 and 300 µM before experiments. H9c2 cells were pre-treated by catechin for 30 min before hypoxia stimulation. The chemical skeleton structure of catechin is displayed in **Figure 1**.

Cell viability assay

After hypoxic stimulation and/or catechin treatment or miR-92a inhibitor (mimic) transfection, viability of H9c2 cells was assessed using cell counting kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) in this research. Briefly, transfected or non-transfected H9c2 cells were seeded into a 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) with 1×10^4 per well overnight and exposed to catechin treatment and/or hypoxia stimulation for different times. Then, 10 µl CCK-8 solution was added into the each well of the plate and the plate was incubated for 1 h at 37°C. After that, the absorbance of each well at 450 nm was recorded using a Micro-plate Reader (Bio-Tek Instruments, Winooski, VT, USA). Cell viability (%) was calculated by average absorbance of treatment group/average absorbance of control group × 100%.

Cell apoptosis assay

After hypoxic stimulation and/or catechin treatment or miR-92a inhibitor (mimic) transfection, apoptosis of H9c2 cells was evaluated using the Annexin V-FITC/PI apoptosis detection kit (Invitrogen, Carlsbad, CA, USA). Briefly, trans-

fected or non-transfected H9c2 cells were seeded into 6-well plate (Thermo Fisher Scientific, Waltham, MA, USA) with 1×10^5 per well overnight and exposure to catechin treatment and/or hypoxia stimulation for 16 h. Then, cells in each group were harvested and disposed as following step: washed with PBS for twice, stained using kit solution for 20 min at 37°C in the dark, and subjected to analysis using Guava EasyCyte 8HT flow cytometer (Guava Technologies, Hayward, CA, USA). Data were quantified using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed to measure the expression of miR-92a in H9c2 cells after hypoxia stimulation and/or catechin treatment or miR-92a inhibitor (mimic) transfection. Total RNA in H9c2 cells was isolated using the miRNeasy Mini kit (Qiagen, Germantown, MD, USA). The expression of miR-92a was detected using mir-Vana™ qRT-PCR miRNA Detection kit (Invitrogen, Carlsbad, CA, USA) and the expression of U6 snRNA acted as an endogenous control. Reaction program for cDNA synthesis was 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. Reaction program for PCR was 40 cycles of 15 s at 95°C, 30 s at 55°C and 5 s at 70°C. The data were quantified using $2^{-\Delta\Delta Ct}$ method described previously [22].

miRNA transfection

miRNA transfection was used to up-regulate or down-regulate the expression of miR-92a in H9c2 cells by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). miR-92a mimic, miR-92a inhibitor and negative control (NC) were designed and synthesized by Gene-Pharma Corporation (Shanghai, China). Transfection efficiency was verified using qRT-PCR.

Western blotting

Protein expression of key factors involved in cell proliferation, apoptosis, and c-Jun N-terminal kinase (JNK) pathways were evaluated using western blotting after relevant treatment or transfection. Total proteins in H9c2 cells were isolated using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing prote-

ase inhibitor (Roche, Basel, Switzerland). The concentrations of total proteins were tested using BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Western blotting was done as previously described [23]. Proteins in equal concentration were electrophoresed in polyacrylamide gels and transfected onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After blocking incubation with 5% bovine serum albumin (BSA, Beyotime Biotechnology, Shanghai, China) for 1 h at room temperature, the membranes were incubated with primary antibodies with a dilution of 1:1000 at 4°C overnight. All primary antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). The information of catalog was as follows: Anti-CDK2 antibody (ab32147), Anti-CDK4 antibody (ab137675), Anti-Cyclin D1 antibody (ab16663), Anti-Bax antibody (ab32503), Anti-Pro-caspase 3 antibody (ab32499), Anti-Cleaved-caspase 3 antibody (ab49822), Anti-Pro-caspase 9 antibody (ab2013), Anti-Cleaved-caspase 9 antibody (ab25758), Anti-t-JNK antibody (ab179461), Anti-p-JNK antibody (ab59-196) and Anti-β-actin antibody (ab8226). After that, the nitrocellulose membranes were incubated with Goat Anti-mouse (Anti-rabbit) IgG H&L (HRP) secondary antibodies (ab205718, ab205719, Abcam Biotechnology, Cambridge, MA, USA) for 1.5 h at room temperature. Followed by adding 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA, USA) to the surfaces of membranes, the signals of proteins were captured using Bio-Rad ChemiDocTM XRS system (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of bands were quantified using Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All experiments were repeated at least three times. The results of multiple experiments were presented as mean ± standard deviation (SD). Statistical analysis was conducted using Graphpad 6.0 statistical software (Graphpad, San Diego, CA, USA). Differences between groups were calculated using one-way analysis of variance (ANOVA) with Sidak post-hot test. $P < 0.05$ was considered to be a significant difference; $P < 0.01$ and $P < 0.001$ were considered extremely significant.
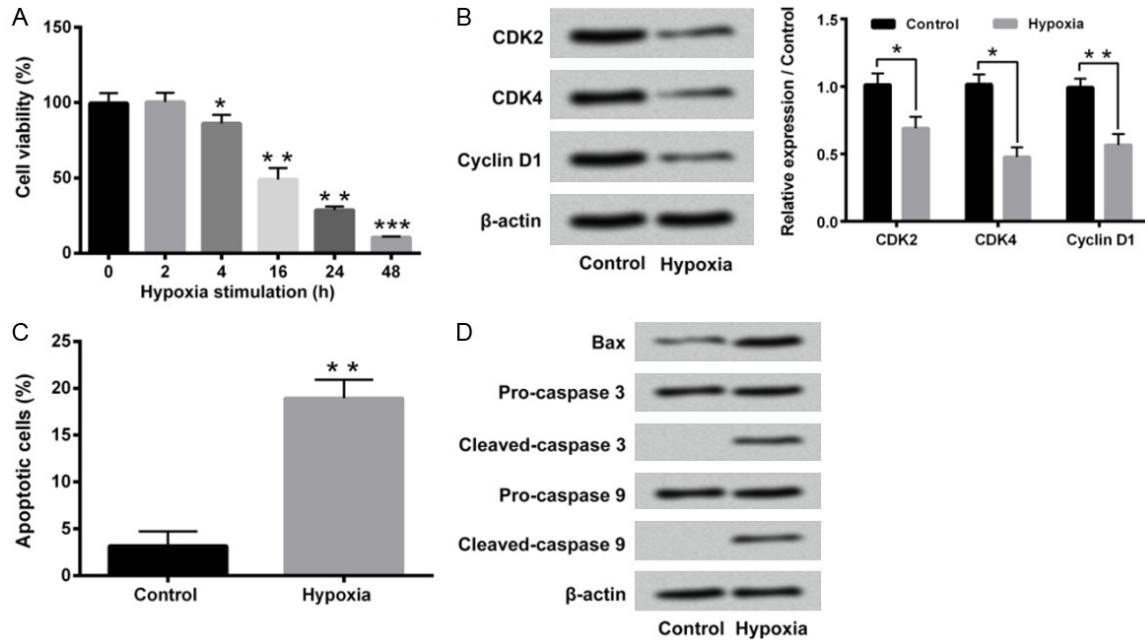
Figure 2. Hypoxia inhibited H9c2 cell viability and proliferation, but induced cell apoptosis. (A) Viability of H9c2 cells after hypoxia stimulation for 0, 2, 4, 16, 24 and 48 h was measured using cell counting kit-8 (CCK-8) assay. After 16 h hypoxia stimulation, (B) protein levels of CDK2, CDK4 and Cyclin D1, (C) apoptosis of H9c2 cells, and (D) protein levels of Bax, Pro-caspase 3, Cleaved-caspase 3, Pro-caspase 9 and Cleaved-caspase 9 were assessed using western blotting and Annexin V-FITC/PI apoptosis detection kit, respectively. CDK: Cyclin-dependent kinase. *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Hypoxia stimulation inhibited H9c2 cell viability and proliferation, but induced cell apoptosis

First, we assessed the effects of hypoxia on H9c2 cell viability, proliferation, and apoptosis by using CCK-8 assay, western blotting, and Annexin V-FITC/PI apoptosis detection kit, respectively. **Figure 2A** presented that hypoxic stimulation remarkably inhibited the viability of H9c2 cells in a time-dependent manner (P < 0.05, P < 0.01 or P < 0.001). Considering that 16 h of hypoxia stimulation inhibited viability at a rate of 51.02 ± 5.22%, 16 h hypoxic stimulation was chosen for subsequent experiments. **Figure 2B** displayed that 16 h hypoxic stimulation significantly suppressed the protein expressions of CDK2, CDK4 and Cyclin D1 in H9c2 cells (P < 0.05 or P < 0.01), which indicated that hypoxic stimulation inhibited the proliferation of H9c2 cells. The result in **Figure 2C** showed that hypoxic stimulation drastically induced H9c2 cell apoptosis (P < 0.01). Moreover, **Figure 2D** illustrated that the protein expressions of Bax, Cleaved-caspase 3 and

Cleaved-caspase 9 in H9c2 cells were all increased after hypoxia stimulation. These above results suggested that hypoxia stimulation notably inhibited H9c2 cell viability and proliferation, but induced cell apoptosis.

Catechin protected H9c2 cells from hypoxia stimulation-induced viability and proliferation inhibition, as well as apoptosis enhancement

Then, we evaluated the protective effects of catechin on hypoxia-induced H9c2 cell viability and proliferation inhibition, as well as apoptosis enhancement, in our research. As displayed in **Figure 3A**, 10, 50, 100 and 200 μM catechin treatment had no significant effects on H9c2 cell viability and 300 μM catechin treatment decreased the viability of H9c2 cells (P < 0.05). The catechin concentrations of 100 and 200 μM were selected for subsequent experiments. **Figure 3B** shows that 100 and 200 μM catechin treatments remarkably alleviated the hypoxia-induced H9c2 cell viability inhibition (P < 0.05). **Figure 3C** shows that 100 and 200 μM catechin treatments obviously attenuated the hypoxia-induced CDK2, CDK4 and Cyclin D1
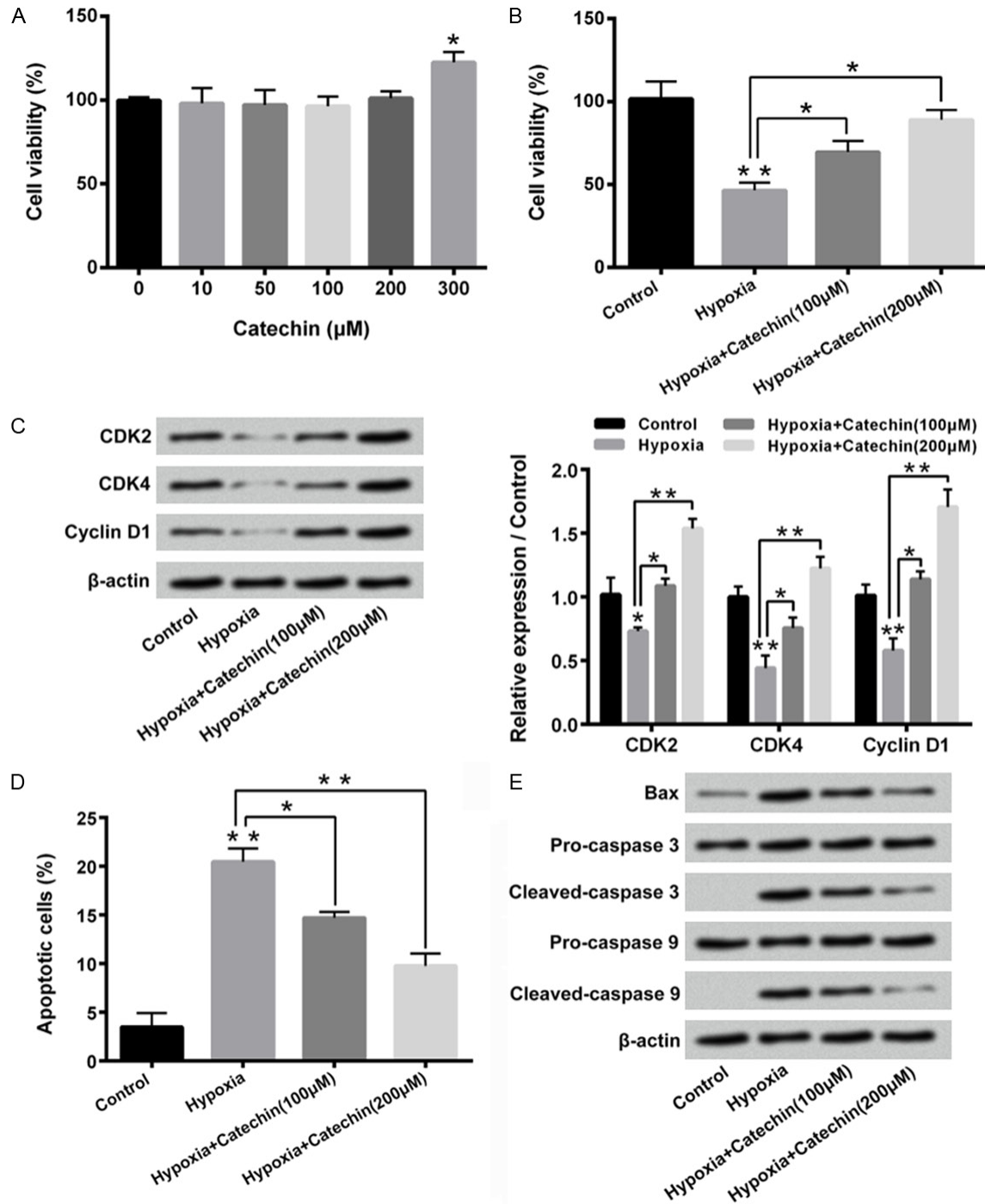
Figure 3. Catechin protected H9c2 cells from hypoxia stimulation-induced viability and proliferation inhibition, as well as apoptosis inducement. (A) Viability of H9c2 cells after 0, 10, 50, 100, 200 and 300 µM catechin treatment was detected using cell counting kit-8 (CCK-8) assay. After 16 h hypoxic stimulation and/or 100 (or 200) µM catechin treatment, (B) viability of H9c2 cells, (C) protein levels of CDK2, CDK4 and Cyclin D1, (D) apoptosis of H9c2 cells, and (E) protein levels of Bax, Pro-caspase 3, Cleaved-caspase 3, Pro-caspase 9 and Cleaved-caspase 9 were tested using CCK-8 assay, western blotting and Annexin V-FITC/PI apoptosis detection kit, respectively. CDK: Cyclin-dependent kinase. *$P < 0.05$, **$P < 0.01$.

expressions decreases in H9c2 cells ($P < 0.05$ or $P < 0.01$). In addition, the results of **Figure**

3D shows that 100 and 200 µM catechin treatments significantly protected H9c2 cells from
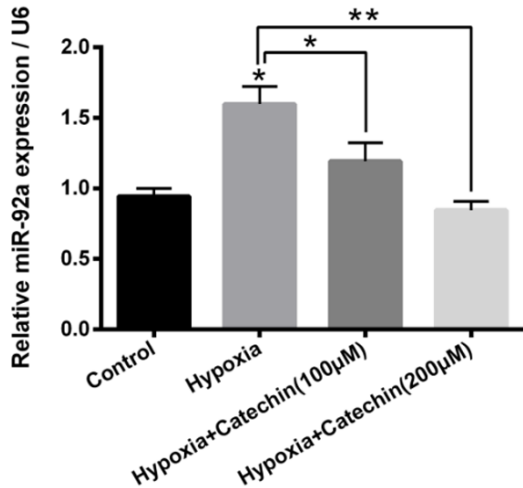
Figure 4. Catechin reversed hypoxia-induced miR-92a expression increase in H9c2 cells. The expressions of miR-92a in H9c2 cells after 16 h hypoxia and/or 100 (or 200) μM catechin treatment were assessed using quantitative reverse transcription PCR (qRT-PCR). miR-92a: MicroRNA-92a. *P < 0.05, **P < 0.01.

hypoxia-induced apoptosis (P < 0.05 or P < 0.01). The results of **Figure 3E** show that hypoxia-induced Bax, Cleaved-caspase 3, and Cleaved-caspase 9 increases in H9c2 cells were inhibited by 100 and 200 μM catechin treatments. These above findings revealed that catechin treatment protected H9c2 cells from hypoxia-induced cell viability and proliferation inhibition, as well as increased apoptosis.

Catechin reversed hypoxia-induced miR-92a expression increase in H9c2 cells

The expression of miR-92a in H9c2 cells after hypoxic stimulation and/or catechin treatment were measured using qRT-PCR. As presented in **Figure 4**, hypoxic stimulation significantly up-regulated the expression of miR-92a in H9c2 cells (P < 0.05). In addition, the hypoxia-induced miR-92a expression increase in H9c2 cells was obviously alleviated by 100 and 200 μM catechin treatments (P < 0.05 or P < 0.01). This finding implied that miR-92a might participate in the protective effects of catechin on hypoxia-stimulated H9c2 cells.

miR-92a participated in the protective effects of catechin on hypoxia-stimulated H9c2 cells

To verify the roles of miR-92a in the protective activity of catechin, miR-92a inhibitor and miR-

92a mimic were transfected into H9c2 cells, respectively. **Figure 5A** displayed that the expression of miR-92a in H9c2 cells was markedly decreased by miR-92a inhibitor transfection and obviously increased by miR-92a mimic transfection (P < 0.01). **Figure 5B** showed that compared to hypoxia+catechin+NC group, the viability of H9c2 cells was markedly increased in hypoxia+catechin+miR-92a inhibitor group (P <0.05) and decreased in hypoxia+catechin+miR-92a mimic group (P < 0.05). Similar results were found in **Figure 5C**, which displayed that compared to hypoxia+catechin+NC group, the expressions of CDK2, CDK4 and Cyclin D1 in H9c2 cells were significantly enhanced in hypoxia+catechin+miR-92a inhibitor group (P < 0.05) and markedly reduced in hypoxia+catechin+miR-92a mimic group (P < 0.05 or P < 0.01). Furthermore, **Figure 5D** and **5E** showed that miR-92a inhibitor transfection enhanced the protective effects of catechin on hypoxia-stimulated H9c2 cells by further reducing cell apoptosis (P < 0.05) and expressions of Bax, Cleaved-caspase 3, and Cleaved-caspase 9. miR-92a mimic transfection had the opposite effects. These results suggested that miR-92a participates in the protective effects of catechin on hypoxia-stimulated H9c2 cells and implyd that catechin protects H9c2 cells from hypoxia-induced injury by down-regulating miR-92a.

Catechin activated JNK pathway in H9c2 cells by down-regulating miR-92a

Finally, we assessed the effects of catechin and miR-92a on the JNK pathway in hypoxia-stimulated H9c2 cells. As displayed in **Figure 6**, hypoxia dramatically reduced the expression rate of p/t-JNK in H9c2 cells (P < 0.01), which indicated that hypoxia inactivated the JNK pathway in H9c2 cells. Catechin treatment significantly attenuated the hypoxia-induced inactivation of the JNK pathway (P < 0.05). Moreover, compared to the hypoxia+catechin+NC group, the activation of the JNK pathway in H9c2 cells was notably increased in hypoxia+catechin+miR-92a inhibitor group (P < 0.01) and distinctly decreased in hypoxia+catechin+miR-92a mimic group (P < 0.01). This finding revealed that catechin attenuated the hypoxia-induced inactivation of the JNK pathway in H9c2 cells by down-regulating miR-92a.
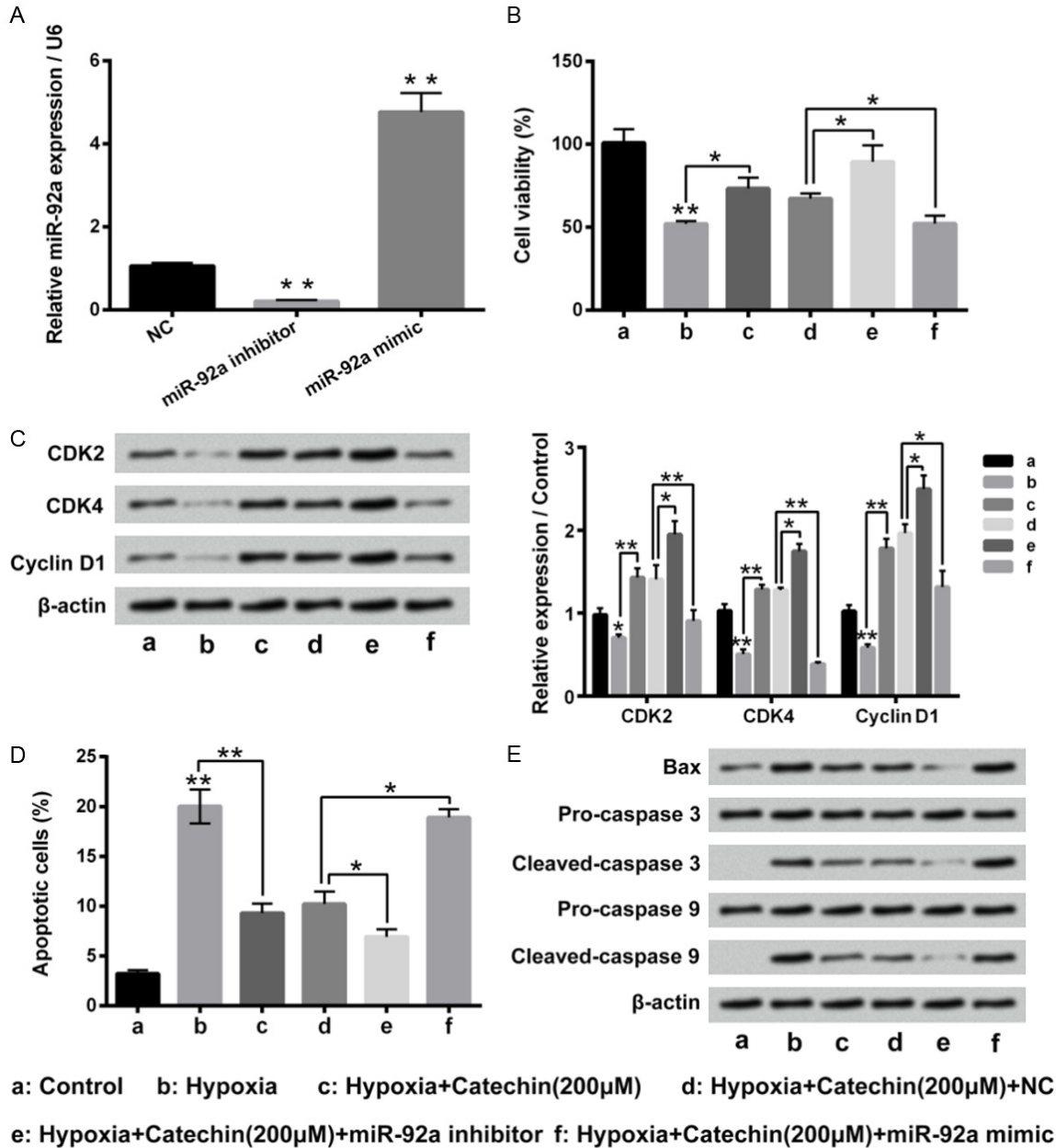
Figure 5. miR-92a participated in the protective effects of catechin on hypoxia-stimulated H9c2 cells. (A) Quantitative reverse transcription PCR (qRT-PCR) was performed to detect the expression of miR-92a in H9c2 cells after miR-92a inhibitor transfection or miR-92a mimic transfection. After 16 h hypoxia and/or 100 (or 200) μM catechin treatment or miR-92a inhibitor (mimic) transfection, (B) viability of of H9c2 cells, (C) protein levels of CDK2, CDK4 and Cyclin D1, (D) apoptosis of H9c2 cells, and (E) protein levels of Bax, Pro-caspase 3, Cleaved-caspase 3, Pro-caspase 9 and Cleaved-caspase 9 were evaluated using cell counting kit-8 (CCK-8) assay, western blotting and Annexin V-FITC/PI apoptosis detection kit, respectively. miR-92a: MicroRNA-92a; NC: Negative control; CDK: Cyclin-dependent kinase. *P < 0.05, **P < 0.01.

Discussion

Plant-derived medicines have made their own niche in the treatment of multiple diseases all over the world [24, 25]. In this study, we revealed that catechin, a phenolic compound isolated from green tea, significantly alleviated hypoxic stimulation-induced H9c2 cell viability loss, cell proliferation inhibition, and cell apoptosis enhancement. Mechanistically, catechin remarkably attenuated hypoxia stimulation-induced miR-92a expression increase in H9c2
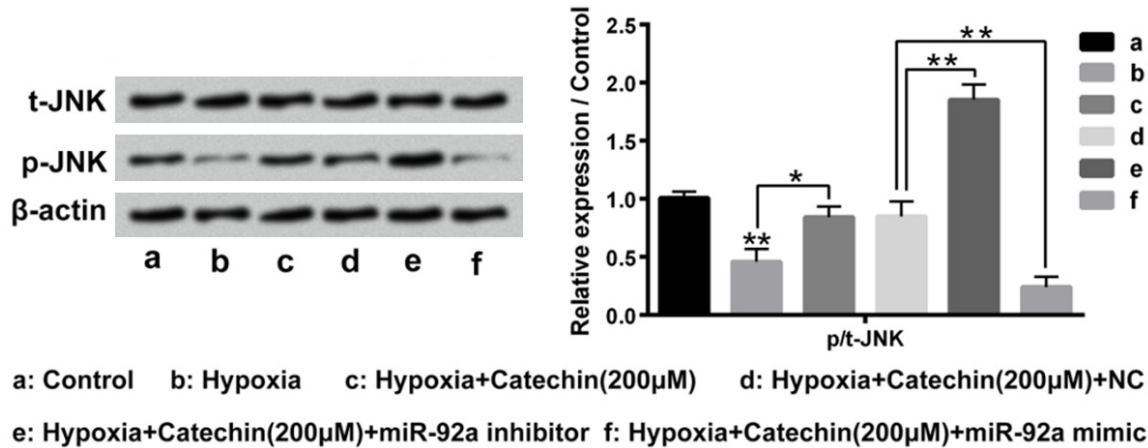
a: Control   b: Hypoxia   c: Hypoxia+Catechin(200µM)   d: Hypoxia+Catechin(200µM)+NC
e: Hypoxia+Catechin(200µM)+miR-92a inhibitor   f: Hypoxia+Catechin(200µM)+miR-92a mimic

Figure 6. Catechin activated the JNK pathway in H9c2 cells by down-regulating miR-92a. Western blotting was conducted to measure the protein level of t-JNK and p-JNK in H9c2 cells after 16 h hypoxia stimulation and/or 100 (or 200) µM catechin treatment or miR-92a inhibitor (mimic) transfection. JNK: c-Jun N-terminal kinase; miR-92a: MicroRNA-92a; NC: Negative control. *$P < 0.05$, **$P < 0.01$.

cells. miR-92a participated in the protective effects of catechin on hypoxia-stimulated H9c2 cells by influencing the JNK pathway.

Appropriate oxygen supply is critical for maintaining function of cardiomyocytes [26]. Hypoxic stimulation will disrupt the balance of the intracellular microenvironment, leading to irreparable functional injury of cardiomyocytes and resulting in MI occurrence [27]. Gong *et al.* pointed out that hypoxia reduced the viability, migration and invasion of H9c2 cells, and increased the apoptosis of H9c2 cells [28]. Consistent with the previous study, we proved that hypoxic stimulation suppressed the viability and proliferation of H9c2 cells and notably induced H9c2 cell apoptosis in the present study, which suggested that the MI cell injury model induced by hypoxia was established successfully. As one of the major active components of green tea, catechin has been demonstrated to reduce cardiac apoptosis and infarct size in MI [15, 16]. In this study, we found that catechin significantly attenuated hypoxia -induced H9c2 cell viability loss, proliferation inhibition, and apoptosis enhancement, which suggested that catechin exerts protective roles in MI by increasing cardiomyocyte viability and proliferation, as well as decreasing cardiomyocyte apoptosis.

One of the most important findings in this study was that miR-92a was involved in the protective effects of catechin on hypoxia-induced H9c2 cell injury. miRNAs do not encode proteins, but regulate intracellular gene expression at the post-transcriptional level [29]. Many reports provided evidence that abnormal expressions of miRNAs were associated with the pathogenesis of multiple human diseases, including MI [18, 30, 31]. Zhang *et al.* [32] and Wang *et al.* [19] indicated that miR-92a had high expression in the serum of MI patients. We pointed out that hypoxia significantly up-regulated the expression of miR-92a in H9c2 cells and catechin co-treatment alleviated the hypoxia-induced miR-92a expression increase in H9c2 cells. More importantly, miR-92a suppression remarkably enhanced the protective effects of catechin in hypoxia-induced H9c2 cells, as evidenced by the increases of cell viability and proliferation, as well as decrease of cell apoptosis in the hypoxia+catechin+miR-92a inhibitor group, compared to hypoxia+catechin+NC group. On the contrary, overexpression of miR-92a had opposite effects, which notably reversed the protective effects of catechin on hypoxia-induced H9c2 cell injury. The above results suggested that catechin exerted protective roles in hypoxia-stimulated H9c2 cells at least in part by down-regulating miR-92a. Moreover, these results were consistent with the previous study, which revealed that suppression of miR-92a also protected endothelial cells from acute MI in Sprague-Dawley rat models by modulating KLF2 and KLF4 [20].

The inactivation of the JNK pathway in cardiomyocytes has been demonstrated to contribute to the occurrence of MI [33, 34]. In our research,

we found that hypoxia dramatically inactivated the JNK pathway in H9c2 cells. Catechin treatment notably alleviated the hypoxia-induced inactivation of JNK pathway in H9c2 cells. In addition, the activation of JNK pathway induced by catechin was further enhanced by miR-92a suppression and reduced by miR-92a overexpression. Considering that the JNK pathway played critical regulatory roles in multiple myocardial cell functions, such as cell proliferation, cell differentiation, cell autophagy, and cell apoptosis [35, 36], the findings in our research suggested that the JNK pathway might be involved in the protective effects of catechin on MI and be regulated by miR-92a.

To sum up, our research verified that catechin protected H9c2 from hypoxia-induced injury by regulating miR-92a and JNK pathway. This study will be helpful for further understanding the protective effects of catechin on MI and provides a theoretical basis for deeply exploring the treatment of MI by using catechin.

Acknowledgements

Disclosure of conflict of interest

None.

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