Journal of Biosciences and Medicines, 2023, 11, 215-231
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Atorvastatin Alleviates Myocardial Ischemia-Reperfusion Injury via miR-26a-5p/FOXO1

Jinlan Duan[1*], Tong Zhang[2*], Ying Zhu[2], Bingtuan Lu[2], Qi Zheng[2], Ninghui Mu[2#]

[1]Department of Geriatric Practice, The First People's Hospital of Yunnan Province, The Affiliated Hospital of Kunming University of Science and Technology, Kunming, China
[2]Department of General Practice, The First People's Hospital of Yunnan Province, The Affiliated Hospital of Kunming University of Science and Technology, Kunming, China
Email: #ninghuimu@163.com

How to cite this paper: Duan, J.L., Zhang, T., Zhu, Y., Lu, B.T., Zheng, Q. and Mu, N.H. (2023) Atorvastatin Alleviates Myocardial Ischemia-Reperfusion Injury via miR-26a-5p/FOXO1. *Journal of Biosciences and Medicines*, **11**, 215-231.
https://doi.org/10.4236/jbm.2023.112017

Received: February 1 2023
Accepted: February 21, 2023
Published: February 24, 2023

Abstract

Purpose: Ischemia-reperfusion (I/R) injury exacerbates myocardial cell death (including apoptosis and necrosis), leading to complications such as arrhythmias, myocardial stenosis, microvascular obstruction and heart failure, and it is particularly important to seek new strategies to mitigate reperfusion injury. In this paper, we will investigate whether atorvastatin can alleviate myocardial ischemia-reperfusion injury and verify its molecular mechanism. **Methods:** We successfully constructed a hypoxia-reperfusion (H/R) H9c2 cell model and transfected miR-26a-5p mimic, miR-26a-5p inhibitor and its negative control NC-mimic or NC-inhibitor into H9c2 cells using a transfection kit. The expression of miR-26a-5p and FOXO1 were detected by RT-qPCR assay, the expression of related proteins by Western blot assay, the cell viability of H9c2 cells by CCK-8 assay, the apoptosis rate of H9c2 cells by flow cytometry, the CK and LDH activity in cells by CK and LDH assay kits. The targeting relationship between miR-26a-5p and FOXO1 was verified by dual luciferase reporter gene assay. **Results:** MiR-26a-5p expression was decreased in H/R-induced cells and FOXO1 expression was increased in H/R-induced cells. Atorvastatin alleviated H/R injury in cardiomyocytes and was most effective at a concentration of 1 μM. Atorvastatin alleviated H/R injury in cardiomyocytes by upregulating miR-26a-5p expression, miR-26a-5p and FOXO1 were negatively regulated by targeting. **Conclusion:** Atorvastatin can alleviate H/R injury in cardiomyocytes by regulating miR-26a-5p/FOXO1.

Keywords

Myocardial Ischemia-Reperfusion Injury, Atorvastatin, miR-26a-5p, FOXO1

*Contributed equally.

1. Introduction

With the deepening of population aging in China, the prevalence of cardiovascular diseases is still in the stage of continuous increase. Among them, ischemic heart disease has become one of the major causes of death in the population [1]. Currently, the treatment of this disease is based on timely restoration of blood perfusion to salvage the ischemic myocardium. Myocardial ischemia-reperfusion injury (MIRI) refers to the progressive aggravation of myocardial depression, arrhythmia, and even irreversible myocardial damage after treatment of ischemic or occluded coronary arteries to restore perfusion [2]. The ischemia-reperfusion (I/R) injury increases myocardial cell death (including apoptosis and necrosis), leading to complications such as arrhythmias, myocardial depression, microvascular obstruction and heart failure [3] [4]. Therefore, it is particularly important to seek new strategies to mitigate reperfusion injury.

MicroRNAs (miRNAs) are small non-coding RNA molecules that mediate gene expression by complementing sites in the 3' UTR (untranslated region) of target mRNAs, resulting in mRNA degradation or translational repression [5]. Studies have shown that miRNAs are involved in a range of processes including embryonic differentiation and development, apoptosis, cell proliferation, and tumor development [6]. miRNAs are involved in a variety of human diseases, and their dysregulation plays a key role in the progression of cerebral ischemia-reperfusion injury [7] [8]. For example: downregulation of miR-146a can prevent cardiac hypertrophy and dysfunction caused by hypertension [9]; miR-532-5p inhibits the progression of cerebral ischemia-reperfusion injury by targeting CXCL1 [10]; miR-30c-5p can effectively reduce myocardial I/R injury Infarct size, cardiac apoptosis, oxidative stress and inflammation [11]. miR-26a-5p acts as a tumor promoter or suppressor in the development and progression of several cancers [12] [13]. In addition, miR-26a-5p has been found to be aberrantly expressed in various cardiovascular diseases such as AMI [14] [15]. As a member of the miRNA family, miR-26a-5p has been found to be involved in pathophysiological processes such as tumor, skeletal system, and sperm apoptosis [16]. The miR-26a-5p is a member of the miRNA family. In recent years, miR-26a-5p has been found to play an important role in myocardial ischemia and ventricular remodeling in various cardiovascular diseases.

Forkhead box protein O1 (FOXO1), a member of the forkhead transcription factor family, is relatively ubiquitously expressed in mammals [17]. As a member of the FOXO family, FOXO1 is considered a tumor suppressor because of its important functions in cell cycle arrest, apoptosis, senescence, differentiation, DNA damage repair, and reactive oxygen species scavenging [18]. Several reports suggest that FOXO1 can regulate the progression of I/R injury. FOXO1 downregulation inhibited the progression of renal ischemia/reperfusion injury [19]. Also, FOXO1 was associated with the development of cerebral ischemia-reperfusion injury [20]. However, whether there is an association between miR-26a-5p and FOXO1 and whether their association is related to MIRI has not been reported.

Atorvastatin is a 3-hydroxy-3-methylglutaryl monoacyl coenzyme A (3-hydroxy-3-meth-yl glutaryl coenzyme A reductase, HMG-CoA) inhibitor, which can inhibit cholesterol synthesis by competitively inhibiting HMG-CoA [21]. At the same time, atorvastatin calcium has a stimulating effect on cell surface LDL receptors, mediating an increase in the number of LDL receptors, reducing LDL levels in the blood, effectively removing intravascular lipids and sclerotic plaques, achieving a lowering effect on blood lipids while reducing whole blood viscosity [22]. The effect of atorvastatin is to lower blood lipids and reduce whole blood viscosity. Clinically, atorvastatin is mainly used for lowering blood lipids, which can reduce blood viscosity and blood blockage, and reduce the risk of ischemic stroke [23]. Atorvastatin is one of the effective drugs widely used in clinical practice to regulate lipids, and as related research continues to progress, many studies have reported that this drug has the ability to improve endothelial function, inhibit inflammatory responses and stabilize atherosclerotic plaques [24]. The study reports have confirmed the effects of this drug on improving endothelial function, inhibiting inflammation and stabilizing atherosclerotic plaque. A number of related studies have reported that statins are effective in improving myocardial ischemia-reperfusion injury [25] [26]. In addition, animal studies suggest that high doses of statins in the pre-MI period may reduce the size of myocardial infarction after reperfusion and help improve cardiac function [27]. The effect of atorvastatin on myocardial infarction is significant. However, the molecular mechanism by which atorvastatin improves myocardial ischemia-reperfusion injury is still unclear and needs to be further investigated.

In this paper, we will investigate whether the molecular mechanism of myocardial ischemia-reperfusion injury ameliorated by atorvastatin is related to the regulation of miR-26a-5p/FOXO1.

2. Methods and Materials

2.1. Cell Culture and Transfection

H9c2 cells (Shanghai Cell Bank, Chinese Academy of Sciences, stock no. GNR5) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in an incubator (ESCO CelMate,CLM-170B-8-NF) at 37˚C and 5% CO_2. To establish the hypoxia-reperfusion (H/R) model, cells were first cultured under anaerobic conditions (94% N_2, 5% CO_2 and 1% O_2) for 4 h, and then transferred to a conventional oxygen incubator for 4 h for reoxygenation, with cells cultured under conventional oxygen conditions serving as controls. After successful modeling, cells were treated with 0.5, 1, 2, 4, 8 and 16 μM concentrations of atorvastatin (purchased from Tocris, UK) to screen the best concentration of atorvastatin for subsequent experiments.

The miR-26a-5p mimic, miR-26a-5p inhibitor or its negative control NC-mimic (both purchased from GeneChem, Shanghai, China) were transfected into H9c2

cells using the Lipofectamine™ 2000 transfection kit (Invitrogen, USA) according to the kit instructions. The transfection efficiency was verified after incubation at 37˚C and 5% CO_2 for 6 hours. The transfected cells were collected for subsequent experiments. The FOXO1 sequence was subcloned into pcDNA3.1 vector (Invitrogen, USA) to construct pcDNA3.1-FOXO1-GFP (pcDNA-FOXO1) and transfected with empty pcDNA3.1 (pcDNA-NC) as a control. pcDNA-FOXO1 and pcDNA-NC vectors were both synthesized in Shanghai, China. GenePharma synthesized.

2.2. Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from cardiomyocytes using Trizol reagent (Invitrogen, USA). 2 μg of RNA was reverse transcribed into cDNA using cDNA synthesis kit (TaKaRa, Japan) based on cDNA sequences obtained from GenBank. The quantitative PCR (qPCR) experiments were performed using the MiniOpticon qPCR detection system (Bio-Rad Laboratories). Using GAPDH or U6 as reference control. Relative quantification results were calculated according to the $2^{-\Delta\Delta CT}$ formula($2^{-\Delta\Delta CT} = 2^{-(\text{experimental group } \Delta Ct - \text{control group } \Delta Ct)}$, ΔCt = Ct target gene -Ct internal reference gene). Primer Premier 5 and miRNA Design V1.01 software were used to design primers. The primer sequence is as follows (see Table 1):

2.3. Western Blot Experiment

Frozen RIPA lysis buffer (Shanghai Biyuntian Biotechnology, China) was added to H9c2 cardiomyocytes. Cells were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes and lysed with FOXO1 (1:1000, ab179450, Abcam), Bax (1:1000, ab32503, Abcam), Bcl-2 (1:1000, ab32124, Abcam), caspase-3 (1:5000, ab32351, Abcam), Cytochrome C (1:5000, ab133504, Abcam), and β-actin (1:1000, ab8226, Abcam) overnight at 4˚C. After washing, membranes were treated with the corresponding secondary antibodies and incubated at 25˚C for 1 h. β-actin was used as an internal control. Protein blot imaging was viewed using the Bio-Rad VersaDocTM Imaging System (Bio-Rad Laboratories,

Table 1. Primer sequences.

Gene	Primer	Sequence (5'-3')
miR-26a-5p	Forward	CGCGTTCAAGTAATCCAGGA
	Reverse	AGTGCAGGGTCCGAGGTATT
FOXO1	Forward	AGCAAATCAAGTTATGGAGGAT
	Reverse	CTGGCATGACTGAGTTAGGG
U6	Forward	TGGCATTGGCAGTACATA
	Reverse	AGGTCACTCTTGCACAGG
GAPDH	Forward	CACCATCTTCCAGGAGCGAG
	Reverse	CTCGTGGTTCACACCCATCA

Freiburg, Germany).

2.4. CCK-8 Experiment

H9c2 cells were cultured in 96-well plates at 1×10^3 cells/well. Cell counting kit-8 (MedChem Express, USA) was used for analysis according to the kit instructions. After 48 h of cell culture, 10 μL CCK-8 reagent was added and the plates were incubated at 37˚C for another 2 h. The absorbance at 450 nm was then measured using an enzyme marker (BIO-TEK, ELX800), and cell proliferation viability was calculated.

2.5. Flow Cytometry

Apoptosis was assessed using a flow cytometer (Beckman Coulter, USA) and Annexin V-FITC Apoptosis Detection Kit (Procell Life Science & Technology, China) to determine the percentage of apoptotic cells. A total of approximately 1×10^6 cells were suspended in 200 μL binding buffer, 10 μL Annexin V-Fluorescein isothiocyanate and 5 μL propidium iodide (BD Biosciences, Germany) were added to the cells, followed by incubation for 30 min at room temperature and protected from light. Apoptosis was assessed using flowjo V10 software (BD Biosciences).

2.6. Measurement of LDH (Lactate Dehydrogenase) and CK (Creatine Kinase) Activities

Cell supernatants were collected and CK and LDH activities were measured using the CK (Catalog No. A032) and (Catalog No. A020-1) LDH assay kits (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China) according to the kit instructions.

2.7. Dual Luciferase Reporter Gene Assay

The binding site of miR-26a-5p and FOXO1 gene was predicted utilizing (https://starbase.sysu.edu.cn/). After construction of the FOXO1-WT and FOXO1-MUT vectors, Lipofectamine2000 (Invitrogen) was used to transfect the vectors and miR-26a-5p mimic into cells. Cells were harvested 48 hours after transfection and lysed using Lysis Buffer (Beyotime, China), followed by measurement of luciferase activity with a dual-luciferase reporter assay system (Promega). Two parallel holes were set in each group, and the experiment was repeated three times.

2.8. Statistical Analysis

All experimental data were processed and analyzed using GraphPad Prism 7 software, and the results were expressed as mean ± standard deviation (SD). The t-test was used for comparison between two groups, and one-way analysis of variance (ANOVA) was used for comparison between multiple groups. Differences were statistically significant when $P < 0.05$.

3. Results

3.1. Expression of miR-26a-5p and FOXO1 in H/R-Induced Cardiomyocytes

To verify the expression of miR-26a-5p and FOXO1 in H/R-induced cardiomyocytes, the expression of miR-26a-5p in H/R-induced cardiomyocytes was significantly decreased ($P < 0.001$) as detected by RT-qPCR assay, see Figure 1(A). Western blot assay showed that FOXO1 in H/R-induced cardiomyocytes was significantly increased ($P < 0.001$), see Figure 1(B). As seen, the induction of H/R resulted the abnormal expression of miR-26a-5p and FOXO1 in cardiomyocytes.

3.2. Effectiveness of Atorvastatin in the Treatment of Myocardial Ischemia-Reperfusion Injury

To investigate the effect of atorvastatin in the treatment of myocardial ischemia-reperfusion injury, the effect of atorvastatin on cell viability was examined using CCK-8. The results showed that treatment with different concentrations of atorvastatin revealed no significant effect on the viability of H9c2 cells when the concentration of atorvastatin was less than 2 μM, and cell viability decreased significantly with increasing concentrations when the concentration of atorvastatin was higher than 2 μM (Figure 2(A)). In addition, the therapeutic effects of concentrations of 0.5 μM, 1 μM, and 2 μM atorvastatin on myocardial ischemia-reperfusion injury were examined (Figure 2(B)). CCK-8 results showed that cell viability was significantly inhibited in the H/R group compared with the NC group, and cell viability was significantly increased after treatment with 1 μM atorvastatin.The results of CK and LDH activity assays showed that after induction of H/R, the cellular CK and LDH activities were significantly higher than those in the NC group ($P < 0.001$), and the activities of CK and LDH were significantly decreased after the addition of 1 μM atorvastatin ($P < 0.001$), as shown in Figure 2(C) and Figure 2(D). Western blot experiments showed that the protein expression of Bax, caspase-3 and cyto-c were significantly increased

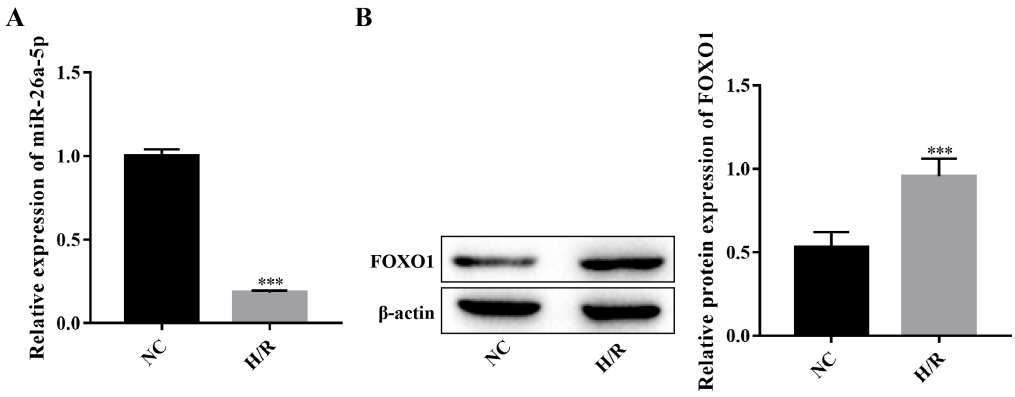

Figure 1. Expression of miR-26a-5p and FOXO1 in H/R-induced cardiomyocytes. (A) RT-qPCR for miR-26a-5p expression; (B) Western blot for FOXO1 protein expression. Compared with NC group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

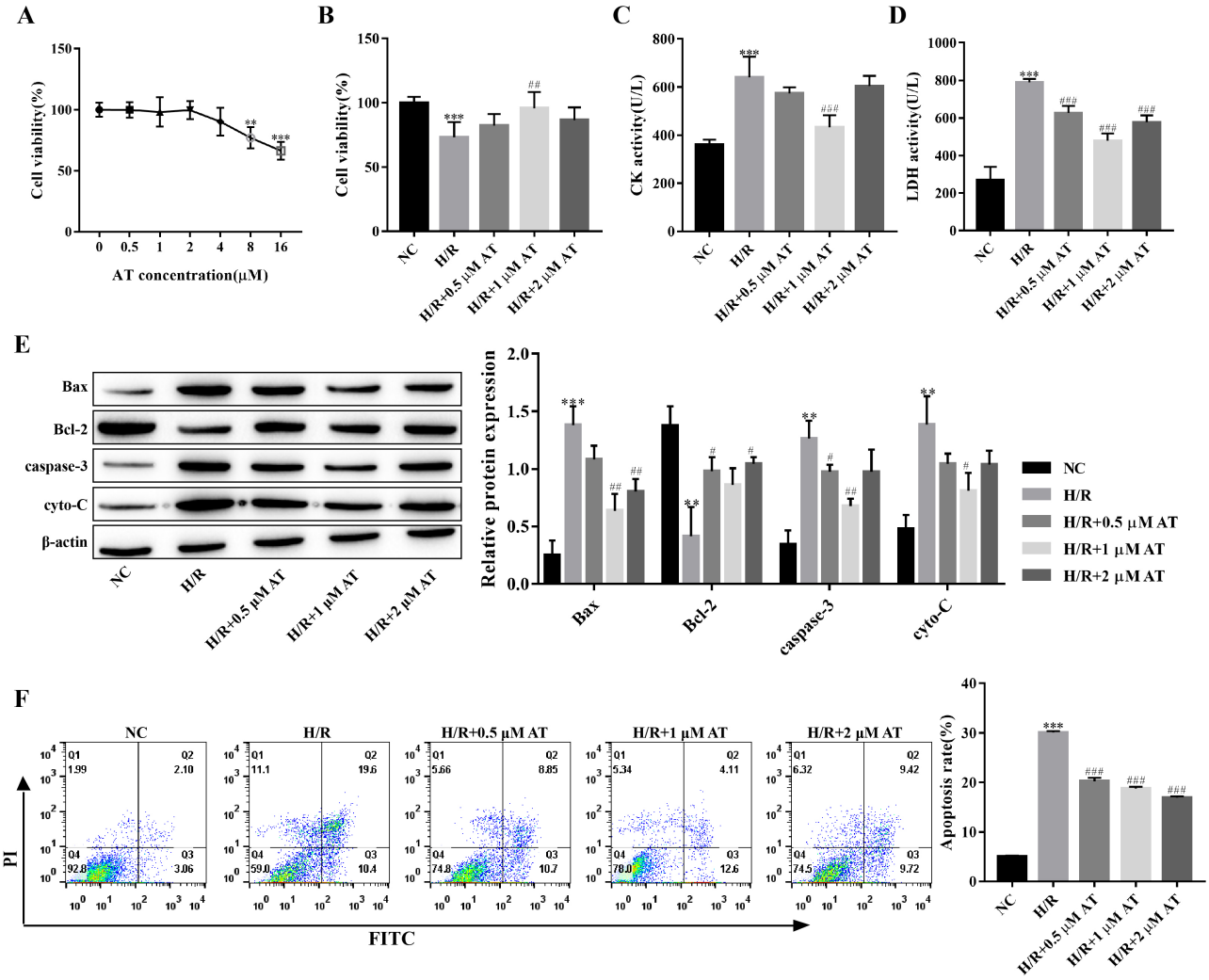

Figure 2. Effect of atorvastatin in the treatment of myocardial ischemia-reperfusion injury. ((A), (B)): CCK-8 assay for H9c2 cell viability; ((C), (D)): Kits for CK and LDH activity; E: Western blot for related protein expression; F: Flow cytometry for H9c2 cell apoptosis rate. Compared with NC group, *P < 0.05, **P < 0.01, ***P < 0.001; Compared with H/R group, #P < 0.05, ##P < 0.01, ###P < 0.001.

after the induction of H/R (P < 0.01) and the protein expression of Bcl-2 was significantly decreased (P < 0.01); while the protein expression of Bax, caspase-3 and cyto-c was least elevated and the protein expression of Bcl-2 was least decreased after treatment with 1 μM atorvastatin (Figure 2(E)). By detecting the apoptosis rate using flow cytometry, it was found that the apoptosis rate was significantly increased after H/R induction (P < 0.01), while the apoptosis rate was significantly decreased after the addition of atorvastatin treatment (P < 0.01), as shown in Figure 2(F). It is thus clear that atorvastatin can alleviate the H/R injury in cardiomyocytes, with 1 μM atorvastatin being the most effective, so subsequent experiments were performed using 1 μM atorvastatin.

3.3. Atorvastatin Mitigates Myocardial Ischemia-Reperfusion Injury via miR-26a-5p

To explore the molecular mechanism of atorvastatin to alleviate H/R injury in

cardiomyocytes, we transfected miR-26a-5p inhibitor into H9c2 cells. As detected by RT-qPCR assay, the expression of miR-26a-5p in cells was significantly decreased after transfection of miR-26a-5p inhibitor ($P < 0.01$) and increased after addition of atorvastatin ($P < 0.01$), as shown in Figure 3(A). The results of CCK-8 assay showed that after transfection of miR 26a-5p inhibitor resulted in a significant decrease in cell viability ($P < 0.05$) and an increase in cell viability after treatment with atorvastatin ($P < 0.001$), see Figure 3(B). CK and LDH activities were detected using CK and LDH kits, and the results showed that after

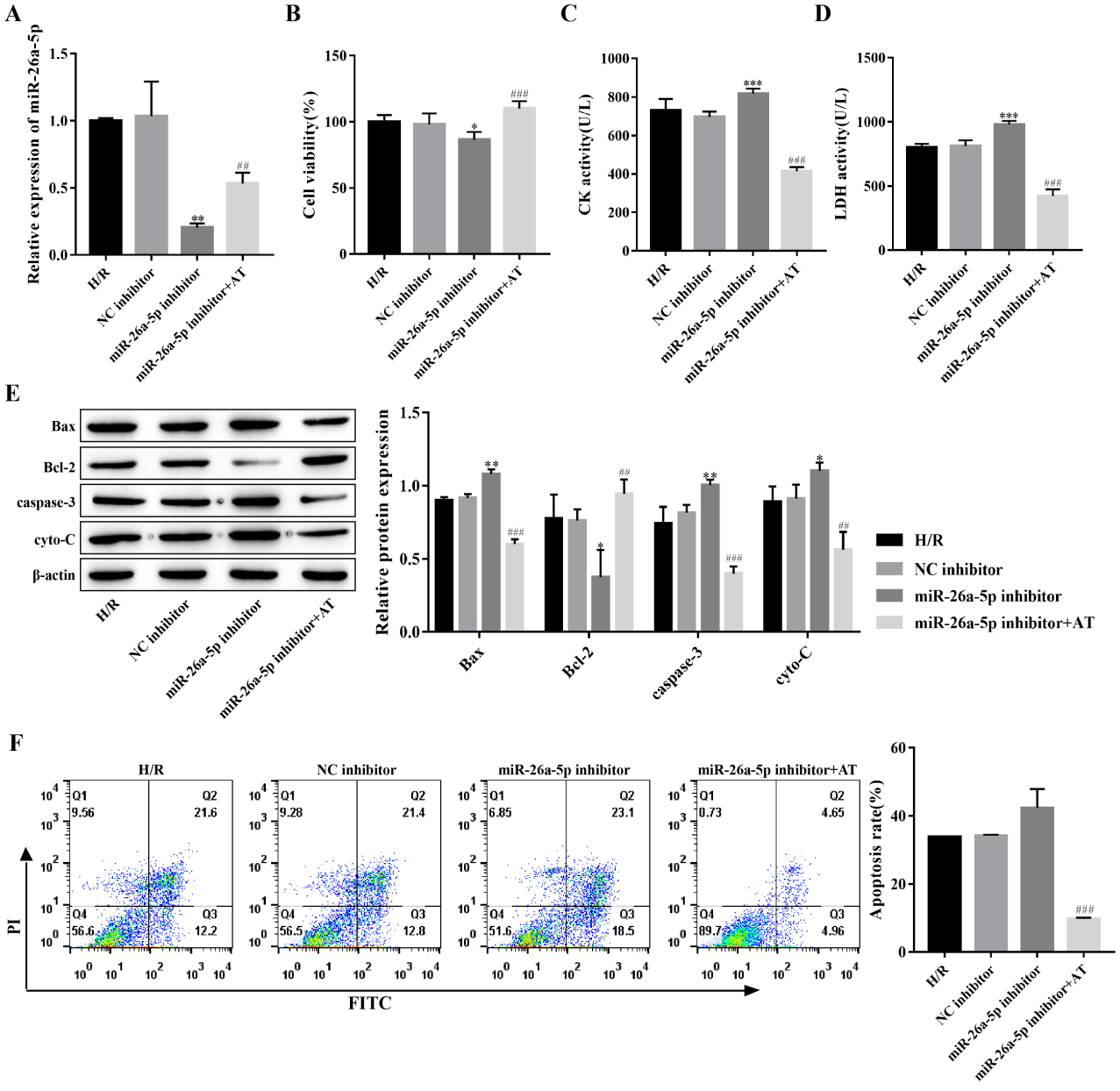

Figure 3. Atorvastatin alleviates myocardial ischemia-reperfusion injury via miR-26a-5p. (A) RT-qPCR for miR-26a-5p expression; (B) CCK-8 for H9c2 cell viability; ((C), (D)): Kits for CK and LDH activity; E: Western blot for related protein expression; F: Flow cytometry for H9c2 cell apoptosis rate. Compared with NC inhibitor group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Compared with miR-26a-5p inhibitor group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

transfection with miR-26a-5p inhibitor, CK and LDH activities were significantly increased ($P < 0.001$), and the addition of atorvastatin significantly decreased CK and LDH activities ($P < 0.001$), see Figure 3(C), 3 Figure 3(D). To detect the effect of atorvastatin on apoptosis of H9c2 cells, the apoptosis-related proteins and apoptosis rate were detected using western blot and flow cytometry, respectively. The results of western blot experiment showed that the protein expression of Bax, caspase-3 and cyto-c was significantly increased ($P < 0.01$) and that of Bcl-2 was significantly decreased ($P < 0.05$) after the transfection of miR-26a-5p inhibitor; The protein expression of Bax, caspase-3 and cyto-c was decreased after the addition of atorvastatin ($P < 0.01$) and protein expression of Bcl-2 was increased ($P < 0.01$), see Figure 3(E). Flow cytometry results revealed that apoptosis rate was increased after transfection with miR-26a-5p inhibitor, while apoptosis rate was significantly decreased after the addition of atorvastatin treatment ($P < 0.001$), see Figure 3(F). It is evident that atorvastatin can alleviate H/R injury in cardiomyocytes through upregulation of miR-26a-5p expression.

3.4. miR-26a-5p Affects Myocardial Ischemia-Reperfusion Injury by Targeting FOXO1

To verify the targeting relationship between miR-26a-5p and FOXO1, we used the StarBase website to predict the binding site of miR-26a-5p to FOXO1 (Figure 4(A)). As detected by dual luciferase reporter gene assay, miR-26a-5p reduced the luciferase activity of wild-type FOXO1 ($P < 0.01$) and had almost no effect on the luciferase activity of mutant FOXO1 (Figure 4(B)). The transfection efficiency of pc-DNA3.1-FOXO1 was examined by RT-qPCR assay, and the results showed that transfection of pc-DNA3.1-FOXO1 significantly increased the expression of FOXO1 ($P < 0.001$) (Figure 4(C)). The mRNA and protein expression levels of FOXO1 were detected by RT-qPCR and Western blot assays, respectively, and both results showed that the mRNA and protein expression of FOXO1 were significantly decreased ($P < 0.001$) after transfection with miR-26a-5p mimic, and after transfection with miR-26a-5p inhibitor, FOXO1 mRNA and protein expression were elevated (Figure 4(D), Figure 4(F)). CCK-8 results showed that cell viability was significantly higher after transfection with miR-26a-5p mimic compared to NC group ($P < 0.01$). Cell viability was reduced after transfection with miR-26a-5p mimic + pc-DNA3.1-FOXO1 compared with the miR-26a-5p mimic group (Figure 4(E)). Western blot detection of apoptosis-related proteins showed that compared with the NC group, the expression of Bax, caspase-3 and cyto-c proteins were significantly reduced ($P < 0.01$) and the expression of Bcl-2 protein was significantly increased ($P < 0.01$) after miR-26a-5p mimic transfection, while after transfection with miR-26a-5p mimic + pc-DNA3.1-FOXO1, Bax, caspase-3 and cyto-c protein expression was increased and Bcl-2 protein expression was decreased, indicating that transfection with miR-26a-5p inhibited apoptosis, while FOXO1 decreased this inhibition (Figure 4(G)). In addition, the same results were obtained for apoptosis detection by flow cytometry (Figure 4(H)). CK and LDH activity assays showed that CK

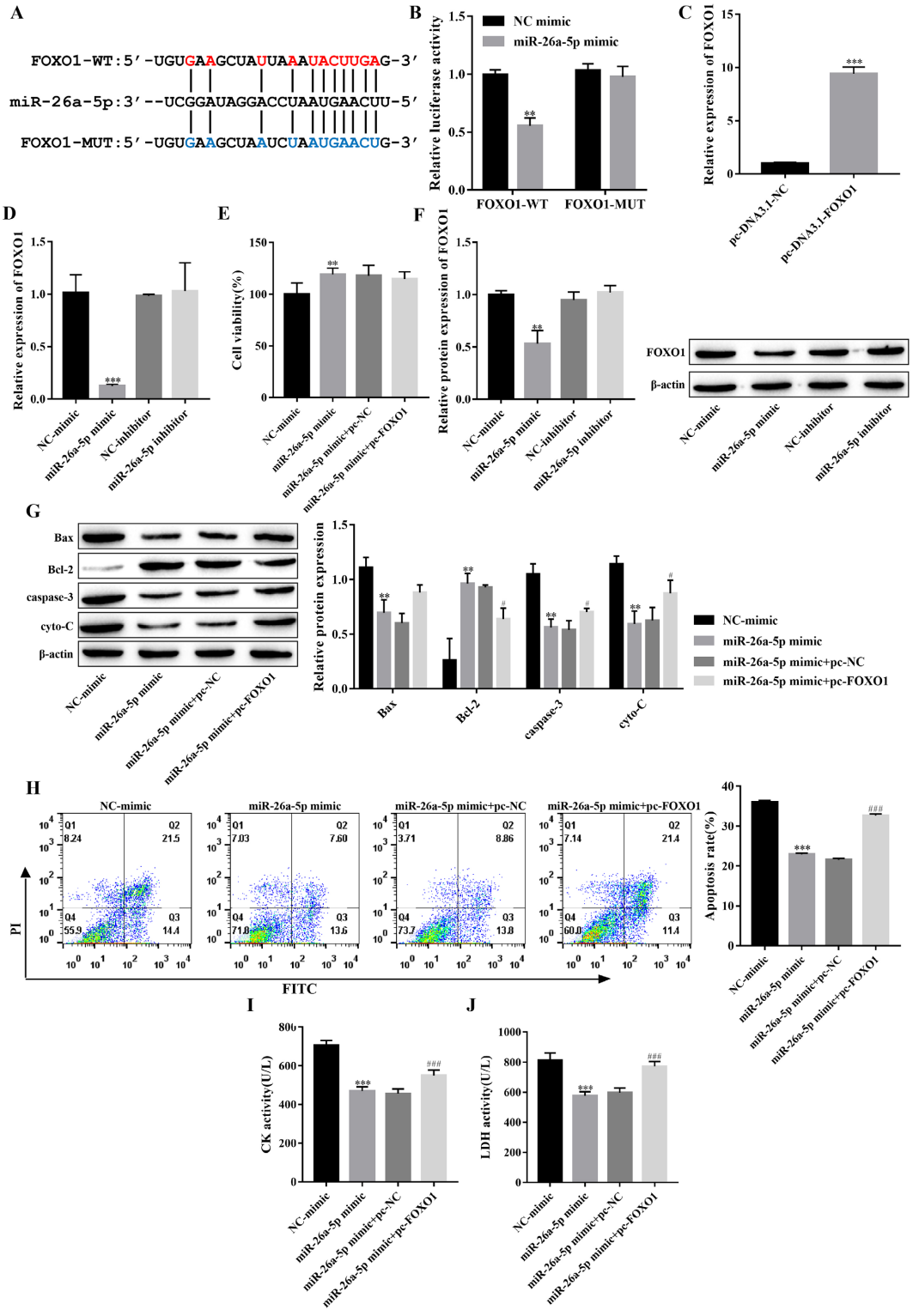

Figure 4. miR-26a-5p affects myocardial ischemia-reperfusion injury by targeting FOXO1. (A) Targeted binding site of miR-26a-5p to FOXO1; (B) Dual luciferase gene reporter assay to verify the targeting relationship between miR-26a-5p and FOXO1; (C) RT-qPCR to detect the transfection efficiency of pc-DNA3.1-FOXO1; (D) RT-qPCR for FOXO1 expression; (E) CCK-8 assay for cell viability; (F) Western blot for FOXO1 protein expression; (G) Western blot for related protein expression; (H): Flow cytometry for H9c2 apoptosis rate; (I) and (J): Kits for CK and LDH activity. Compared with NC mimic group, *P < 0.05, **P < 0.01, ***P < 0.001; Compared with miR-26a-5p mimic + pc-NC group, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001.

and LDH activities were significantly reduced after transfection with miR-26a-5p mimic compared with the NC group ($P < 0.001$). In contrast, CK and LDH activities were significantly increased after transfection with miR-26a-5p mimic+pc-DNA3.1-FOXO1 compared with the miR-26a-5p mimic group ($P < 0.001$), as shown in **Figure 4(I)** and **Figure 4(J)**. It is evident that miR-26a-5p alleviates H/R injury in cardiomyocytes by targeting negative regulation of FOXO1.

3.5. Atorvastatin Mitigation of Myocardial Ischemia-Reperfusion Injury via miR-26a-5p/FOXO1

To verify whether atorvastatin could alleviate myocardial ischemia-reperfusion injury through miR-26a-5p/FOXO1, we transfected miR-26a-5p inhibitor into cells and detected the expression of FOXO1 by RT-qPCR and Western blot assay, and the results showed that the expression of FOXO1 in the cells decreased after adding atorvastatin ($P < 0.01$) (**Figure 5(A)**, **Figure 5(B)**). And the expression of FOXO1 was significantly increased ($P < 0.01$) after the addition of atorvastatin in cells transfected with miR-26a-5p inhibitor compared with AT+NC inhibitor group, see **Figure 5(A)**. CCK-8 results showed that the cell viability was significantly increased ($P < 0.001$) after the addition of atorvastatin. In contrast, cell viability was decreased in cells transfected with miR-26a-5p inhibitor by addition of atorvastatin compared with the AT + NC inhibitor group (**Figure 5(E)**). CK and LDH activity assay results showed that the addition of atorvastatin significantly decreased CK and LDH activity ($P < 0.001$), while compared with the AT + NC inhibitor group, CK and LDH activities were increased after the addition of atorvastatin treatment in cells transfected with miR-26a-5p inhibitor ($P < 0.001$), as shown in **Figure 5(C)** and **Figure 5(D)**. Western blot assay results showed that the protein expression of Bax, caspase-3, cyto-c and FOXO1 were significantly decreased ($P < 0.01$) and the protein expression of Bcl-2 increased significantly ($P < 0.001$); after transfection with miR-26a-5p inhibitor and addition of atorvastatin, the protein expression of Bax, caspase-3, cyto-c and FOXO1 increased and the protein expression of Bcl-2 decreased (**Figure 5(F)**). The results of flow cytometry apoptosis assay showed that the apoptosis rate was decreased after the addition of atorvastatin ($P < 0.001$) and increased after the addition of atorvastatin after transfection with miR-26a-5p inhibitor ($P < 0.001$), as shown in **Figure 5(G)**. Thus, it is clear that atorvastatin can reply to the effect of transfection with miR-26a-5p inhibitor induced of H/R injury in cardiomyocytes, and atorvastatin alleviated H/R injury in cardiomyocytes through miR-26a-5p/FOXO1.

4. Discussion and Conclusion

Statins prevent coronary artery disease and stroke by lowering serum LDL cholesterol and inhibiting hepatic cholesterol biosynthesis [28]. Atorvastatin is cardioprotective against cardiac I/R injury by reducing myocardial infarct size and cardiomyocyte apoptosis [29] [30] [31]. In this study, atorvastatin was selected

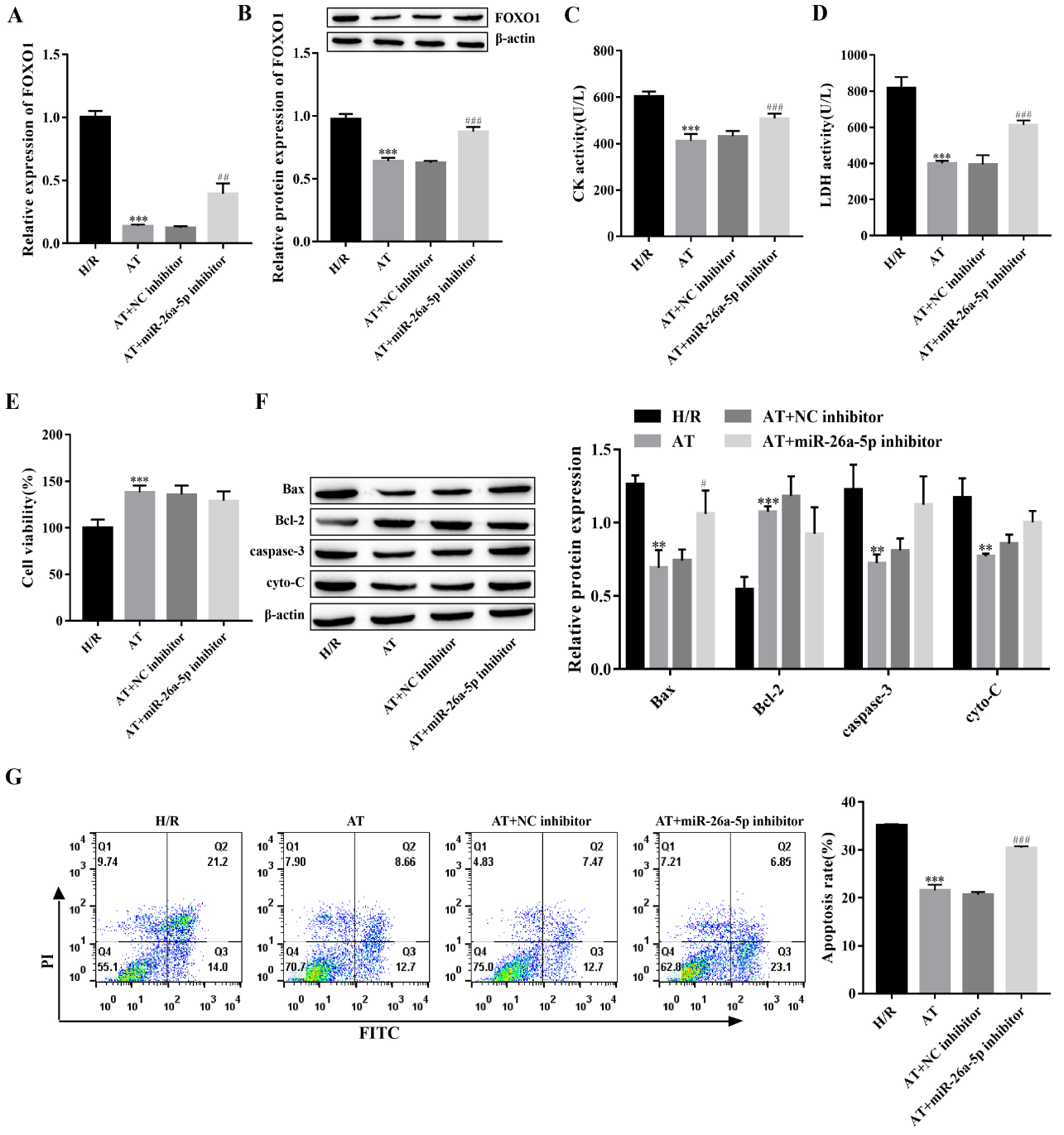

Figure 5. Atorvastatin alleviates myocardial ischemia-reperfusion injury via miR-26a-5p/FOXO1. (A) RT-qPCR for FOXO1 expression; (B) Western blot for FOXO1 expression ((C), (D)): Kit for CK and LDH activity; (E) CCK-8 assay for cell viability; (F) Western blot for related protein expression; (G) Flow cytometry for H9c2 cell apoptosis rate. Compared with H/R group, *P < 0.05, **P < 0.01, ***P < 0.001; Compared with AT + NC inhibitor group, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001.

as the experimental drug and different concentrations of atorvastatin were set as the control group, and atorvastatin was added to the medium of H9c2 cells while the experiment was in progress, and the results showed that treatment with atorvastatin improved cell viability, decreased CK and LDH activities of cells, altered the expression of ischemia-reperfusion injury-related proteins, and re-

duced the hypoxia-reperfusion injury after apoptosis rate of cardiomyocytes, with the best effect of 1 μM atorvastatin.

When the organism is subjected to sustained or strong external stimuli, the activation of apoptotic signals within the cell induces molecular conformational changes in Bax and Bcl-2 to promote disruption of mitochondrial membrane integrity and increase membrane permeability, leading to changes in mitochondrial membrane potential, which in turn activates Caspase-3 to mediate apoptosis [32]. Cytochrome C (cyto-C) is a pro-apoptotic factor, and when mitochondria are damaged, mitochondrial membrane potential decreases, membrane permeability increases, and pro-apoptotic factors such as cyto-C enter the cell and cause apoptosis in the mitochondrial pathway. In this study, we examined the Caspase cascade proteases Bax, Bcl-2, Caspase-3 and cyto-C that mediate the apoptotic pathway, and found that the protein expression of Bax, Caspase-3 and cyto-C were significantly elevated and that of Bcl-2 was decreased in cardiomyocytes in the H/R model, and that administration of atorvastatin treatment was able to revert. The protein expression of Bax, Bcl-2, Caspase-3 and cyto-C, which were altered due to H/R induction, was restored after administration of atorvastatin. The results of the apoptosis rate using flow cytometry were also consistent, with a significant increase in the apoptosis rate induced by H/R and a decrease in the apoptosis rate after administration of atorvastatin treatment.

Previous studies only elucidated the involvement of miR-26a-5p in the regulation of cardiomyocyte apoptosis in vivo,a study showed that targeted inhibition of miR-26a rapidly increased angiogenesis, decreased infarct size, and improved cardiac function after myocardial infarction in a mouse model [33]. Another study showed that high level of circulating miR-26a-5p was likely to have elevated risk for MI [34]. However, there were no reports on miR-26a-5p associated with myocardial ischemia-reperfusion injury until 2019, when Wei *et al.* showed that LncRNA AK038897 exacerbated cerebral ischemia/reperfusion by acting as a ceRNA for miR-26a-5p targeting DAPK1 injury [35]; Xing *et al.* [36] reported that miR-26a-5p can protect against myocardial ischemia/reperfusion injury by regulating PTEN/PI3K/AKT signaling pathway. Yang *et al.* [37] found that melatonin exerted protective effects by regulating miR-26a-5p-NRSF and JAK2-STAT3 pathways to improve autophagy, inflammation and oxidative stress in cerebral ischemia/reperfusion injury. We found that downregulation of miR-26a-5p expression in H9c2 cells by transfection of miR-26a-5p inhibitor resulted in decreased cell viability, increased intracellular CK and LDH activity, increased protein expression of Bax, Caspase-3 and cyto-C, decreased protein expression of Bcl-2 expression, and increased apoptosis, whereas atorvastatin replied to the effects brought by downregulation of miR-26a-5p on cells, while upregulation of miR-26a-5p resulted in the opposite. Therefore, this study demonstrated that miR-26a-5p was associated with myocardial ischemia-reperfusion injury by positive and negative validation. In addition, we also found a targeted regulatory relationship between miR-26a-5p and FOXO1.

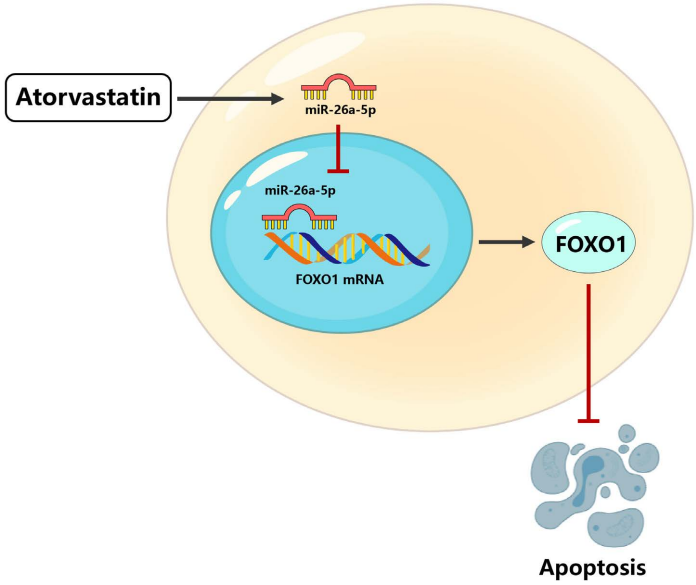

Figure 6. Mechanism diagram.

In summary, atorvastatin alleviates H/R-induced cardiomyocyte injury, and the molecular mechanism associated with this effect may be the negative regulation of FOXO1 targeting by miR-26a-5p. This study will provide a theoretical basis for the clinical treatment of atorvastatin on myocardial ischemia-reperfusion injury.

5. Conclusion

In conclusion, atorvastatin can promote the expression of miR-26a-5p and inhibit the expression of FOXO1, inhibit myocardial cell apoptosis caused by myocardial ischemia-reperfusion, and alleviate myocardial ischemia-reperfusion injury (**Figure 6**). This study will provide a theoretical basis for the clinical treatment of atorvastatin in the treatment of myocardial ischemia-reperfusion injury.

Funding

This work was supported by the Applied Basic Research Project of Yunnan provincial Science and Technology Department and Kunming Medical University, Grant Number: 2019FE001(-295).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Zhou, M., *et al.* (2020) Mortality, Morbidity, and Risk Factors in China and Its Provinces, 1990-2017: A Systematic Analysis for the Global Burden of Disease Study 2017. *The Lancet* (*London*, *England*), **394**, 1145-1158. https://doi.org/10.1016/S0140-6736(19)30427-1

[2] Li, T., Wc, B., Rya, B., *et al.* (2019) Pretreatment with Tilianin Improves Mitochon-

drial Energy Metabolism and Oxidative Stress in Rats with Myocardial Ischemia/Reperfusion Injury via AMPK/SIRT1/PGC-1 Alpha Signaling Pathway. *Journal of Pharmacological Sciences*, **139**, 352-360. https://doi.org/10.1016/j.jphs.2019.02.008

[3] Plantinga, T.S., Joosten, L.A., *et al.* (2012) Modulation of Inflammation by Autophagy: Consequences for Crohn's Disease. *Current Opinion in Pharmacology*, **12**, 497-502. https://doi.org/10.1016/j.coph.2012.01.017

[4] Yu, Y., Sun, G., Luo, Y., *et al.* (2016) Cardioprotective Effects of Notoginsenoside R1 against Ischemia/Reperfusion Injuries by Regulating Oxidative Stress- and Endoplasmic Reticulum Stress-Related Signaling Pathways. *Scientific Reports*, **6**, Article No. 21730. https://doi.org/10.1038/srep21730

[5] Fabris, L., Ceder, Y., Chinnaiyan, A.M., *et al.* (2016) The Potential of MicroRNAs as Prostate Cancer Biomarkers. *European Urology*, **70**, 312-322. https://doi.org/10.1016/j.eururo.2015.12.054

[6] Kozomara, A., *et al.* (2018) miRBase: From microRNA Sequences to Function. *Nucleic Acids Research*, **47**, D155-D162. https://doi.org/10.1093/nar/gky1141

[7] Liu, W., Miao, Y., Zhang, L., *et al.* (2020) MiR-211 Protects Cerebral Ischemia/Reperfusion Injury by Inhibiting Cell Apoptosis. *Bioengineered*, **11**, 189-200. https://doi.org/10.1080/21655979.2020.1729322

[8] Chen, X., Li, C., Li, J., *et al.* (2019) Upregulation of miR-1306-5p Decreases Cerebral Ischemia/Reperfusion Injury *in Vitro* by Targeting BIK. *Bioscience Biotechnology and Biochemistry*, **83**, 2230-2237. https://doi.org/10.1080/09168451.2019.1654846

[9] Heggermont, W.A., Papageorgiou, A.P., Quaegebeur, A., *et al.* (2017) Inhibition of MicroRNA-146a and Overexpression of Its Target Dihydrolipoyl Succinyltransferase Protect against Pressure Overload-Induced Cardiac Hypertrophy and Dysfunction. *Circulation*, **136**, 747-761. https://doi.org/10.1161/CIRCULATIONAHA.116.024171

[10] Shi, Y., Yi, Z., Zhao, P., *et al.* (2021) MicroRNA-532-5p Protects against Cerebral Ischemia-Reperfusion Injury by Directly Targeting CXCL1. *Aging* (*Albany NY*), **13**, 11528-11541. https://doi.org/10.18632/aging.202846

[11] Sun, M., Guo, M., Ma, G., *et al.* (2021) MicroRNA-30c-5p Protects against Myocardial Ischemia/Reperfusion Injury via Regulation of Bach1/Nrf2. *Toxicology and Applied Pharmacology*, **40**, Article ID: 115637. https://doi.org/10.1016/j.taap.2021.115637

[12] Heggermont, W.A., Papageorgiou, A.P., Quaegebeur, A., *et al.* (2017) Inhibition of MicroRNA-146a and Overexpression of Its Target Dihydrolipoyl Succinyltransferase Protect against Pressure Overload-Induced Cardiac Hypertrophy and Dysfunction. *Circulation*, **14**, 4223-4228.

[13] Chang, L., Li, K. and Guo, T. (2017) miR-26a-5p Suppresses Tumor Metastasis by Regulating EMT and Is Associated with Prognosis in HCC. *Clinical and Translational Oncology*, **19**, 695-703. https://doi.org/10.1007/s12094-016-1582-1

[14] Kong, B., Qin, Z., Ye, Z., *et al.* (2019) microRNA-26a-5p Affects Myocardial Injury Induced by Coronary Microembolization by Modulating HMGA1. *Journal of Cellular Biochemistry*, **120**, 10756-10766. https://doi.org/10.1002/jcb.28367

[15] Zheng, L., Lin, S. and Lv, C. (2018) MiR-26a-5p Regulates Cardiac Fibroblasts Collagen Expression by Targeting ULK1. *Scientific Reports*, **8**, Article No. 2104. https://doi.org/10.1038/s41598-018-20561-4

[16] Shi, D., Wang, H., Ding, M., *et al.* (2019) MicroRNA-26a-5p Inhibits Proliferation, Invasion and Metastasis by Repressing the Expression of Wnt5a in Papillary Thy-

roid Carcinoma. *OncoTargets and Therapy*, **12**, 6605-6616. https://doi.org/10.2147/OTT.S205994

[17] Bernardi, H., *et al.* (2014) FoxO Transcription Factors: Their Roles in the Maintenance of Skeletal Muscle Homeostasis. *Cellular & Molecular Life Sciences*, **71**, 1657-1671. https://doi.org/10.1007/s00018-013-1513-z

[18] Jiramongkol, Y. and Lam, E.W.-F. (2020) FOXO Transcription Factor Family in Cancer and Metastasis. *Cancer and Metastasis Reviews*, **39**, 681-709. https://doi.org/10.1007/s10555-020-09883-w

[19] Wang, D., Wang, Y., Zou, X., *et al.* (2020) FOXO1 Inhibition Prevents Renal Ischemia-Reperfusion Injury via cAMP-Response Element Binding Protein/PPAR-γ Coactivator-1α-Mediated Mitochondrial Biogenesis. *British Journal of Pharmacology*, **177**, 432-448. https://doi.org/10.1111/bph.14878

[20] Wen, Y., Zhang, X., Liu, X., *et al.* (2020) Suppression of lncRNA SNHG15 Protects against Cerebral Ischemia-Reperfusion Injury by Targeting miR-183-5p/FOXO1 Axis. *American Journal of Translational Research*, **12**, 6250-6263.

[21] Nawrocki, J.W., Weiss, S.R., Davidson, M.H., *et al.* (1995) Reduction of LDL Cholesterol by 25% to 60% in Patients with Primary Hypercholesterolemia by Atorvastatin, a New HMG-CoA Reductase Inhibitor. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **15**, 678-682. https://doi.org/10.1161/01.ATV.15.5.678

[22] Zhou, H., Xie, Y.Q., Baloch, Z., Shi, Q.P., *et al.* (2017) The Effect of Atorvastatin, 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Inhibitor (HMG-CoA), on the Prevention of Osteoporosis in Ovariectomized Rabbits. *Journal of Bone & Mineral Metabolism*, **35**, 245-254. https://doi.org/10.1007/s00774-016-0750-2

[23] Cheng, Z.-J., *et al.* (2018) Atorvastatin Pretreatment Attenuates Ischemic Brain Edema by Suppressing Aquaporin 4. *Journal of Stroke & Cerebrovascular Diseases*, **27**, 3247-3255. https://doi.org/10.1016/j.jstrokecerebrovasdis.2018.07.011

[24] Pei, W., *et al.* (2018) C-reactive Protein Aggravates Myocardial Ischemia/Reperfusion Injury through Activation of Extracellular-Signal-Regulated Kinase 1/2. *Journal of Geriatric Cardiology*, **15**, 492-503.

[25] Reichert, K., Carmo, H., Torina, A.G., *et al.* (2017) Correction: Atorvastatin Improves Ventricular Remodeling after Myocardial Infarction by Interfering with Collagen Metabolism. *PLOS ONE*, **12**, 172453-172455. https://doi.org/10.1371/journal.pone.0172453

[26] Lu, J., Jie, H., Hong, D., *et al.* (2016) Amlodipine and Atorvastatin Improved Hypertensive Cardiac Remodeling through Regulation of MMPs/TIMPs in SHR Rats. *Cellular Physiology & Biochemistry*, **39**, 47-60. https://doi.org/10.1159/000445604

[27] Lu, J., Hao, J., Du, H., Xiao, B., Li, Y., Yang, X. and Cui, W. (2016) Amlodipine and Atorvastatin Improved Hypertensive Cardiac Hypertrophy through Regulation of Receptor Activator of Nuclear Factor Kappa B Ligand/Receptor Activator of Nuclear Factor Kappa B/Osteoprotegerin System in Spontaneous Hypertension Rats. *Experimental Biology & Medicine*, **241**, 1237-1249. https://doi.org/10.1177/1535370216630180

[28] Sirtori, C.R. (2014) The Pharmacology of Statins. *Pharmacological Research*, **88**, 3-11. https://doi.org/10.1016/j.phrs.2014.03.002

[29] Tao, S., Zhang, H.J., Krittanawong, C., *et al.* (2017) Acute Atorvastatin Treatment Restores the Cardioprotective Effects of Ischemic Postconditioning in Hyperlipidemic Rats. *Oncotarget*, **8**, 55187-55193. https://doi.org/10.18632/oncotarget.19232

[30] Desoky, E.E., Hassan, A., Salem, S.Y., *et al.* (2016) Cardioprotective Effect of Ator-

vastatin Alone or in Combination with Remote Ischemic Preconditioning on the Biochemical Changes Induced by Ischemic/Reperfusion Injury in a Mutual Prospective Study with a Clinical and Experimental Animal Arm. *International Journal of Cardiology*, **222**, 866-873. https://doi.org/10.1016/j.ijcard.2016.07.178

[31] Rohilla, A., Ahmad, A., Khan, M.U., *et al.* (2015) A Comparative Study on the Cardioprotective Potential of Atorvastatin and Simvastatin in Hyperhomocysteinemic Rat Hearts. *European Journal of Pharmacology*, **764**, 48-54. https://doi.org/10.1016/j.ejphar.2015.06.045

[32] Luna-Vargas, P.A.M., *et al.* (2016) The Deadly Landscape of Pro-Apoptotic BCL-2 Proteins in the Outer Mitochondrial Membrane. *FEBS Journal*, **283**, 2676-2689. https://doi.org/10.1111/febs.13624

[33] Icli, B., Wara, A., Moslehi, J., *et al.* (2013) MicroRNA-26a Regulates Pathological and Physiological Angiogenesis by Targeting BMP/SMAD1 Signaling. *Circulation Research*, **113**, 1231-1241. https://doi.org/10.1161/CIRCRESAHA.113.301780

[34] Velle-Forbord, T., Debik, M., Saether, J., *et al.* (2019) Circulating microRNAs as Predictive Biomarkers of Myocardial Infarction: Evidence from the HUNT Study. *Atherosclerosis*, **289**, 1-7. https://doi.org/10.1016/j.atherosclerosis.2019.07.024

[35] Wei, R., Zhang, L., Hu, W., *et al.* (2019) Long Non-Coding RNA AK038897 Aggravates Cerebral Ischemia/Reperfusion Injury via Acting as a ceRNA for miR-26a-5p to Target DAPK1. *Experimental Neurology*, **314**, 100-110. https://doi.org/10.1016/j.expneurol.2019.01.009

[36] Xing, X., Guo, S., Zhang, G., *et al.* (2020) miR-26a-5p Protects against Myocardial Ischemia/Reperfusion Injury by Regulating the PTEN/PI3K/AKT Signaling Pathway. *Brazilian Journal of Medical and Biological Research*, **53**, e9106. https://doi.org/10.1590/1414-431x20199106

[37] Yang, B., Zang, L.-E., Cui, J.-W., *et al.* (2020) Melatonin Plays a Protective Role by Regulating miR-26a-5p-NRSF and JAK2-STAT3 Pathway to Improve Autophagy, Inflammation and Oxidative Stress of Cerebral Ischemia-Reperfusion Injury. *Drug Design, Development and Therapy*, **14**, 3177-3188. https://doi.org/10.2147/DDDT.S262121