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Original Paper

Astragaloside IV Protects Rat Cardiomyocytes from Hypoxia-Induced Injury by Down-Regulation of miR-23a and miR-92a

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Key Words
Astragaloside IV (AS-IV) • Myocardial infarction (MI) • H9c2 cell • miR-23a • miR-92a • Hypoxia

Abstract
Background/Aims: Astragaloside IV (AS-IV), a traditional Chinese medicine isolated from *Astragalus membranaceus*, has been shown to exert cardioprotective effect previously. This study aimed to reveal the effects of AS-IV on hypoxia-injured cardiomyocyte. ***Methods:*** H9c2 cells were treated with various doses of AS-IV for 24 h upon hypoxia. CCK-8 assay, flow cytometry/Western blot, and qRT-PCR were respectively conducted to measure the changes in cell viability, apoptosis, and the expression of miR-23a and miR-92a. Sprague–Dawley rats were received coronary ligation, and were administrated by various doses of AS-IV for 14 days. The infarct volume and outcome of rats followed by ligation were tested by ultrasound, arteriopuncture and nitrotetrazolium blue chloride (NBT) staining. ***Results:*** We found that 10 μg/ml of AS-IV exerted myocardioprotective effects against hypoxia-induced cell damage, as AS-IV significantly increased H9c2 cells viability and decreased apoptosis. Interestingly, the myocardioprotective effects of AS-IV were alleviated by miR-23a and/or miR-92a overexpression. Knockdown of miR-23a and miR-92a activated PI3K/AKT and MAPK/ERK signaling pathways. Bcl-2 was a target gene for miR-23a, and BCL2L2 was a target gene for miR-92a. In the animal model of myocardial infarction (MI), AS-IV significantly reduced the infarct volume, ejection fraction (EF), shortening fraction (FS) and LV systolic pressure (LVSP), and significantly increased left ventricular end-diastolic internal diameter (LVEDd). And also, the elevated expression of miR-23a and miR-92a in MI rat was reduced by AS-IV. ***Conclusion:*** AS-IV protected cardiomyocytes against hypoxia-induced injury possibly via down-regulation of miR-23a and miR-92a, and via activation of PI3K/AKT and MAPK/ERK signaling pathways.

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Introduction

Myocardial infarction (MI) is one of the leading causes of morbidity and mortality worldwide. It is typically caused by the blockage of coronary artery, leading to the chest pain, weakness, diaphoresis, dyspnea, palpitation and even unconsciousness. The incidence of MI is declining in most countries with the development of therapeutic approaches [1]. However, the burden of MI is generally increasing as a result of the aging world population, population growth, and the rising prevalence of long-term survivors of MI [2]. Further deep studies regarding novel therapeutic agents are required for treating this disease.

microRNAs (miRNAs) are a class of single-stranded, non-coding RNAs with approximately 22 nucleotides in length. In cardiovascular system, miRNAs control functions of various cells, such as cardiomyocytes, endothelial cells, smooth muscle cells and fibroblasts [3]. Altered miRNA expression can be found in the blood of patients with acute MI, making them promising candidates for biomarkers [4]. Besides, it has been proposed that miRNA-based therapies might constitute a strategy for protection against cardiomyocyte apoptosis during hypoxia/reoxygenation injury [5, 6]. In brain tissue and blood plasma of rats, miR-23a and miR-92a were up-regulated in response to ischemia [7], indicating these two miRNAs might be new potential markers for ischemic injury. It has been point out that high level of plasma miR-92a might be a promising biomarker to diagnose patients with acute MI from stable coronary heart disease [8]. Besides, the increased plasma miR-23a level may be used to predict the presence and severity of coronary lesions in patients with coronary artery disease [9]. These authors demonstrated miR-23a and miR-92a as MI-related miRNAs.

Astragaloside IV (AS-IV), 3-O-beta-D-xylopyranosyl-6-O-beta-D-glucopyrannosyl-cycloastragenol, is a traditional Chinese medicine isolated from *Astragalus membranaceus* [10]. AS-IV has multiple pharmacologic effects for its potent anti-inflammatory, anti-fibrotic, anti-oxidative stress, anti-asthma, anti-diabetes and immune-regulatory effects [11]. In addition, AS-IV has been shown to exert anti-tumorigenic properties in certain cancers, including breast cancer and colorectal cancer [12, 13]. In terms of MI, the cardioprotective effect of AS-IV has been sparsely reported. It is believed that AS-IV prevents ischemia/reperfusion-induced cardiac malfunction via alleviating myocardial fibrosis, suppressing inflammation, attenuating apoptosis, ameliorating impairment of the myocardial ultrastructural, promoting angiogenesis, and regulating energy metabolism [14-16]. However, the underlying mechanism of which AS-IV exerts cardioprotective effects is still unclear. In the present study, we examined the effect of AS-IV on hypoxia-injured H9c2 cells (a rat cardiomyocyte line), and explored the regulatory correlation between AS-IV and miR-23a/miR-92a in order to decode the molecular mechanisms of which AS-IV attenuated myocardial injury.

Materials and Methods

Cell culture and treatment

Rat cardiomyocyte line H9c2 cells were purchased from the American Type Culture Collection (CRL-1446, ATCC, Manassas, VA, USA), and were cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco). The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. For stimulating hypoxic injury, cells were incubated in hypoxic incubator containing 94% N_2, 5% CO_2, and 1% O_2 for 24 h.

AS-IV with purity greater than 98% was purchased from Sigma-Aldrich (St. Louis, MO, USA). AS-IV was dissolved in DMSO and made up with the medium so that the final concentration of the vehicle was less than 0.1%. AS-IV with concentrations of 2.5, 5, and 10 μg/ml were used to treat cells.

Cell viability assay

H9c2 cells were seeded in 96-well plates with 5000 cells/well for adherence. The cells were treated with AS-IV for 24 h upon hypoxia, and then 10 μl of Cell Counting Kit-8 solution (CCK-8, Dojindo Molecular Technologies, Kyushu, Japan) was added into each well. After incubation at 37°C for 1 h, the absorbance of each well was analyzed using a Microplate Reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

Quantification of apoptotic cells

Cell apoptosis was determined by using the FITC-annexin V/PI detection kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China). In brief, H9c2 cells were treated with AS-IV upon hypoxia for 24 h. Then, 1×10^5 cells were collected from each sample and were resuspended in 200 μL Binding Buffer containing 10 μl FITC-annexin V. Followed by incubation at room temperature for 30 min, 5 μl PI and 300 μl PBS was added, and then the samples were immediately analyzed under a FACS can (Beckman Coulter, Fullerton, CA, USA). Cells in the initial stages of apoptosis characterized by FITC-positive and PI-negative were calculated by using FlowJo software (version 7.2.4, Tree Star Inc, Ashland, OR, USA).

Oligonucleotides transfection

The specific mimics, inhibitors and the correspondingly negative controls (NCs) for rno-miR-23a and rno-miR-92a were synthesized by GenePharma Co. (Shanghai, China). The sequences for miR-23a were as follows: mimic sense, AUC ACA UUG CCA GGG AUU UCC, mimic anti-sense, AAA UCC CUG GCA AUG UGA UUU; inhibitor, GGA AAU CCC UGG CAA UGU GAU. The sequences for miR-92a were as follows: mimic sense, UAU UGC ACU UGU CCC GGC CUG, mimic anti-sense, GGA AAU CCC UGG CAA UGU GAU; inhibitor CAG GCC GGG ACA AGU GCA AUA. Transfections were performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h of transfection, cells were collected for use in the following experiments.

Cellular injury assessment

After the indicated treatment, the release of creatine kinase-MB (CK-MB) and cardiac troponin I (cTnI) were respectively assessed by using rat CK-MB ELISA kit (CSB-E14403r, Cusabio, China) and rat cTnI ELISA kit (CSB-E08594r, Cusabio, China) according to the manufacturer's instructions. The viability of mitochondria was measured by Mitochondrial Viability Stain (ab129732, Abcam, Cambridge, UK) [17]. Mitochondrial membrane potential was assessed by JC-1-Mitochondrial Membrane Potential Assay Kit (ab113850, Abcam, Cambridge, UK).

Dual-Luciferase reporter assay

The predicted binding site in the 3'UTR of Bcl-2 and BCL2L2 were respectively amplified by PCR and inserted into the pmirGlO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA), and referred as to Bcl-2 wild type (Bcl-2-WT) and BCL2L2 wild type (BCL2L2-WT). The predicted binding sites were replaced to form the correspondingly negative controls, *i.e.*, Bcl-2 mutated type (Bcl-2-MUT) and BCL2L2 mutated type (BCL2L2-MUT). The vectors were transfected with miR-23a mimic, miR-92a mimic or the mimic NCs by using Lipofectamine 2000 (Invitrogen). At 48 h of transfection, the firefly and renilla luciferase activities were assessed by using the Dual-Luciferase Reporter Assay (Promega).

Animal model of MI

Forty specific pathogen free grade of Sprague–Dawley rats (all male, weighting 280-300 g) were purchased from Vital River Laboratories (Beijing, China), and were routinely cultured as previously described [18]. All the animal experiments of this study were approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University and performed according to the instruction of our institute. The rats were intraperitoneally injected with 350 mg/kg chloral hydrate, and then the proximal left anterior descending branch (LAD) of coronary artery was ligated at 1.0-1.5 mm below the tip of the left auricle with a 5/0 silk. This same procedure except coronary ligation was performed in sham-operated rats.

AS-IV administration in the animal model of MI

The rats were randomly divided into 5 groups (8 rats per group): Sham, MI, AS-IV (20 mg/kg), AS-IV (40 mg/kg), and AS-IV (80 mg/kg). The rats in AS-IV groups were received AS-IV for 14 days after surgery by intragastric route. AS-IV was dissolved in DMSO, and was further diluted with normal saline to concentrations of 20, 40, and 80 mg/kg/day. The rats in the Sham and MI groups were received the same volume of DMSO-saline solution for 14 days.

Echocardiography and hemodynamics

After surgery and AS-IV administration, the rats were intraperitoneally injected with 350 mg/kg chloral hydrate, and ultrasound analyses were performed. Left ventricular end-diastolic internal diameter (LVEDd), ejection fraction (EF) and shortening fraction (FS) were detected. Arteriopuncture was performed to measure the LV pressure. LV systolic pressure (LVSP) was analyzed by PowerLab software (ADInstruments, CO, USA).

Measurement of MI size

Finally, rats were sacrificed by decapitation, and the heart was removed for sampling. The heart samples were washed twice with cold PBS, quickly removed to a -20°C refrigerator, and then sliced into ~1 mm slices. The slices were incubated with 1% nitrotetrazolium blue chloride (NBT) solution (Amresco Company, Solon, Ohio, USA) for 15 min at room temperature, and then were blocked in 4% formaldehyde solution overnight. The infarct (INF) area and the whole heart (WH) area were analyzed by image analysis software (National Institutes of Health, Bethesda, Maryland, USA).

qRT-PCR

Total miRNAs in H9c2 cells and heart samples were extracted by miRNeasy Mini Kit (Qiagen, Shenzhen, China). Reverse transcription was performed with the specific reverse transcription primer for rno-miR-23a (5'-GTC GTA TCC AGT GCG TGT CGT GGA GTC GGC AAT TGC ACT GGA TAC GAC GGA AAU-3') and for rno-miR-92a (5'-GTC GTA TCC AGT GCG TGT CGT GGA GTC GGC AAT TGC ACT GGA TAC GAC CAG GCC-3') under the catalysis of PrimeScript Reverse Transcriptase (Takara, Dalian, China). qRT-PCR was performed by using Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (TaKaRa) with the specific primer for rno-miR-23a (Fw: 5'-GGG ATC ACA TTG CCA GGG-3' and Rv: 5'-CAG TGC GTG TCG TGG AGT-3') and for rno-miR-92a (Fw: 5'-GGG TAT TGC ACT TGT CCC-3' and Rv: 5'-CAG TGC GTG TCG TGG AGT-3'). U6 served as an internal reference for normalizing the expressions of miR-23a and miR-92a. For detection of the mRNA levels of Bcl-2 and BCL2L2, Transcriptor First Strand cDNA Synthesis Kit and FastStart Universal SYBR Green Master both purchased from Hoffmann-La Roche (Basel, Switzerland) were used according to the manufacturer's instructions. The primers used in the procedure are given as follows. Bcl-2, Fw: 5'-AGA GGG GCT ACG AGT GGG AT-3' and Rv: 5'-CTC AGT CAT CCA CAG GGC GA-3'; BCL2L2, Fw: 5'-ATC AGC ACT GGG TCG TAA GAG-3' and Rv: 5'-CCC ACC TGA CCC TAG ACT TTC-3'; β-actin, Fw: 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3' and Rv: 5'-GTG GGC CGC TCT AGG CAC CAA-3'. The expression levels of Bcl-2 and BCL2L2 were normalized to β-actin. Data were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein was isolated from H9c2 cells by using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Protein concentration of the whole-cell extraction was quantified by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Protein (0.1 mg) from each sample was resolved over sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then were probed by primary antibodies: PCNA (sc-9857), cyclin A (sc-53227), Bax (sc-6236), Bcl-2 (sc-56015, Santa Cruz Biotechnology, Santa Cruz, California, USA), p-MAPK (GTX50280, GeneTex, Irvine, California, USA), cyclin E1 (ab52189), CDK1 (ab133327), cyclin D1 (ab134175), CDK4 (ab199728), caspase-3 (ab13586), cytochrome *c* (ab110325), PI3K (ab86714), p-PI3K (ab182651), AKT (ab8805), p-AKT (ab38449), MAPK (ab197348), ERK (ab196883), p-ERK (ab214362), BCL2L2 (ab38629) and β-actin (ab8226, Abcam, Cambridge, UK), overnight at 4°C. The membranes were then incubated with secondary antibodies for 1 h at room temperature. Positive bands were visualized by using enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Little Chalfont, UK) [19]. Intensity of bands was quantified using Image Lab™ Software (Bio-Rad, CA, USA).

Statistical analysis

The results are expressed as mean ± SD from three or eight independent experiments. Statistical analyses between groups were analyzed by the one-way analysis of variance (ANOVA) with Duncan procedure of the SPSS version 19.0 program (SPSS Inc., Chicago, IL, USA). A *P*-value of <0.05 was considered to indicate a statistically significant result.

Results

Hypoxia induced H9c2 cells damage

To start with, H9c2 cells were subjected to hypoxic condition to stimulate an *in vitro* model of MI. As a result, the viability of H9c2 cells was significantly reduced, while the apoptotic cell rate was significantly increased in response to hypoxia when compared to the control group ($P < 0.01$, Fig. 1A and 1B). These data suggested that apoptosis was induced by hypoxia in H9c2 cells.

AS-IV protected H9c2 cells against hypoxia-induced cell damage

To explore the impacts of AS-IV on hypoxic-injured H9c2 cells, various doses of AS-IV (2.5, 5, and 10 μg/ml) were used to treat cells. By performing CCK-8 assay and flow cytometer, we found that 5 and 10 μg/ml of AS-IV significantly alleviated hypoxia-induced cell damage by increasing cell viability and decreasing apoptotic cell rate ($P < 0.05$ or $P < 0.01$, Fig. 2A and 2B). AS-IV with dose of 2.5 μg/ml slightly increased cell viability and decreased apoptotic cell rate upon hypoxia, but not reach the significant difference compared to the control group with hypoxia only. Thus, 10 μg/ml of AS-IV was selected for use the following investigations. Next, western blot analysis was performed to detect the expression changes of proliferation- and apoptosis-related proteins. As results shown in Fig. 2C and 2D, down-regulations of PCNA ($P < 0.001$), cyclin E1 ($P < 0.001$), CDK1 ($P < 0.001$), cyclin D1 ($P < 0.001$), CDK4 ($P < 0.001$), Bcl-2 ($P < 0.01$), up-regulations of Bax ($P < 0.01$) and cytochrome *c* ($P < 0.001$), and the cleavage of caspase-3 ($P < 0.001$) were observed in hypoxic cells, while these protein alteration induced by hypoxia were recovered by AS-IV ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Cyclin A and pro form of caspase-3 were unaffected.

AS-IV reduced the expression of miR-23a and miR-92a

The expression levels of miR-23a and miR-92a in H9c2 cells treated with AS-IV were monitored by qRT-PCR. Results indicated that the RNA levels of miR-23a and miR-92a were significantly reduced by AS-IV treatment ($P < 0.01$, Fig. 3A and 3B). And also, the expression changes of miR-23a and miR-92a in AS-IV-treated cells upon hypoxia were detected. qRT-PCR data in Fig. 3C and 3D showed that, miR-23a and miR-92a were significantly up-regulated in response to hypoxia exposure ($P < 0.001$). However, AS-IV treatment significantly reduced miR-23a and miR-92a expression upon hypoxia ($P < 0.01$ and $P < 0.001$).

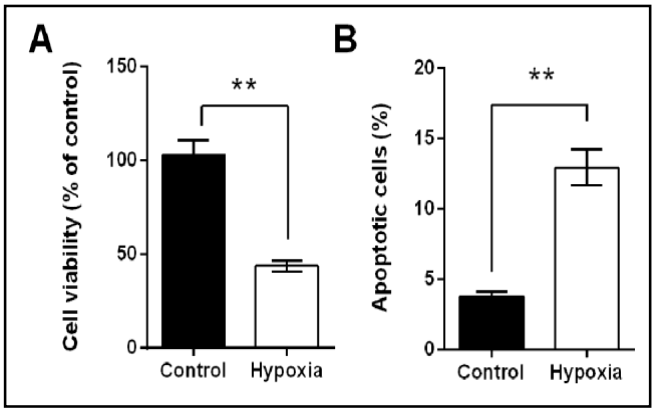

Fig. 1. Hypoxia induced H9c2 cells damage. H9c2 cells were subjected to hypoxic conditions (94% N_2, 5% CO_2, and 1% O_2) for 24, then (A) the viability of cells, and (B) apoptotic cell rate were detected. Data expressed as mean ± SD (n = 3 per group). ** P<0.01.

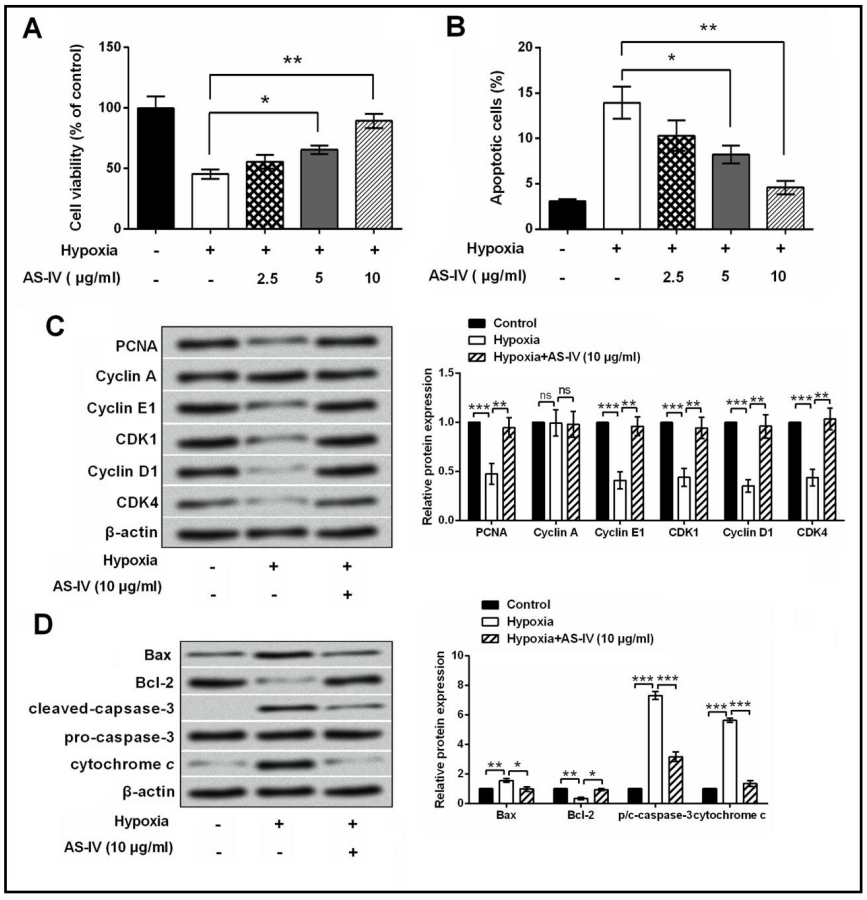

Fig. 2. AS-IV protected H9c2 cells against hypoxia-induced cell damage. H9c2 cells were treated with AS-IV (2.5, 5, and 10 μg/ml) for 24 h upon hypoxia, and then (A) cell viability and (B) apoptotic cell rate were detected. H9c2 cells were treated with 10 μg/ml AS-IV for 24 h upon hypoxia, and then protein levels of (C) proliferation- and (D) apoptosis-related factors were assessed. β-actin acted as an internal reference. Data expressed as mean ± SD (n = 3 per group). ns, no significance, * P<0.05, ** P<0.01, *** P<0.001.

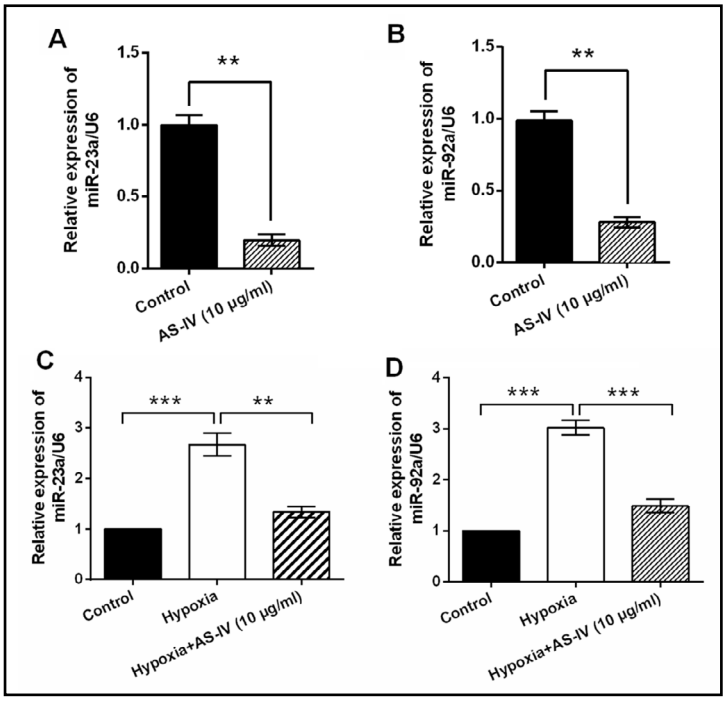

Fig. 3. AS-IV reduced the expression of miR-23a and miR-92a. H9c2 cells were treated with 10 μg/ml AS-IV for 24, RNA levels of (A) miR-23a, and (B) miR-92a were determined. H9c2 cells were treated with 10 μg/ml AS-IV for 24 h upon hypoxia, RNA levels of (C) miR-23a, and (D) miR-92a were determined. Data expressed as mean ± SD (n = 3 per group). U6 acted as an internal reference. ** P<0.01, *** P<0.001.

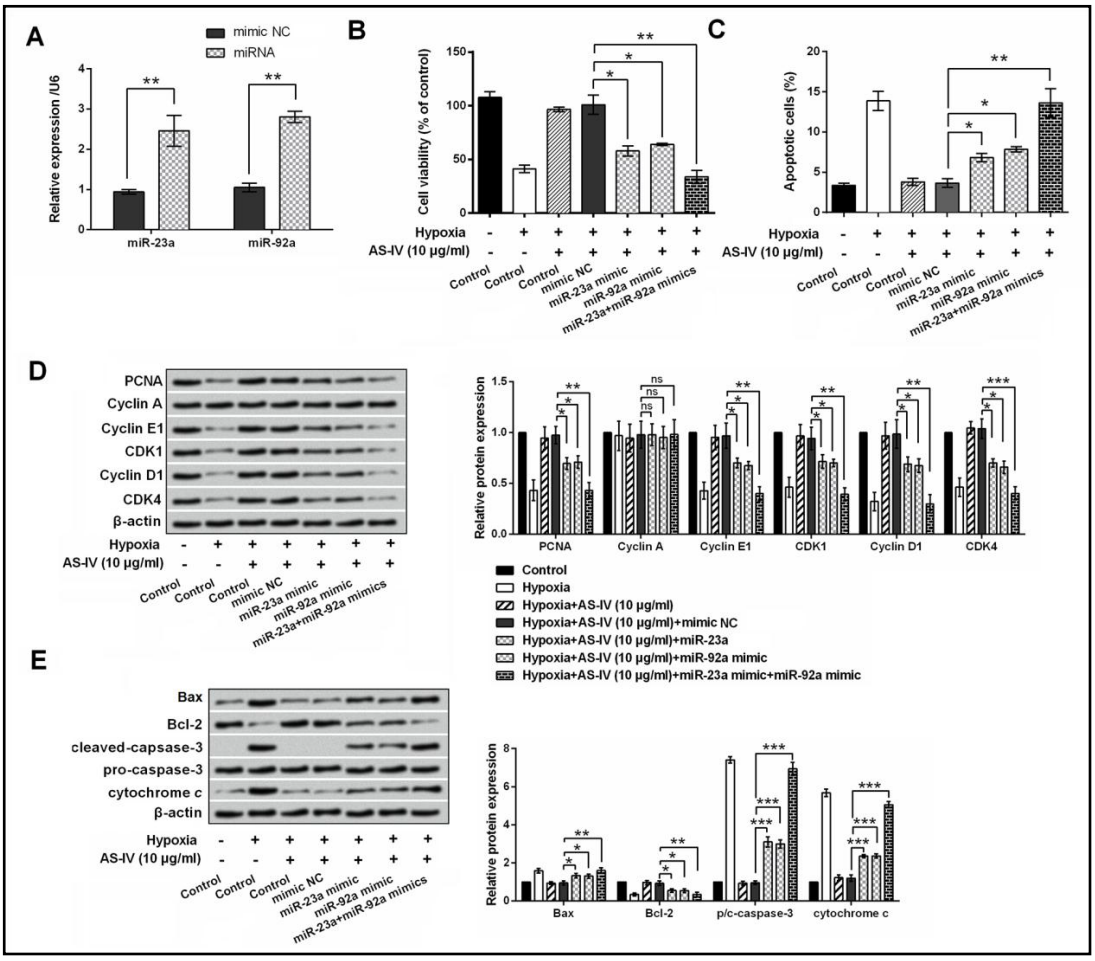

Fig. 4. AS-IV protected H9c2 cells against hypoxia-induced apoptosis and viability impairment via down-regulation of miR-23a and miR-92a. (A) The RNA levels of miR-23a and miR-92a in H9c2 cells after transfection with mimics specific for miR-23a and miR-92a. U6 acted as an internal reference. Cells transfected with scrambled oligonucleotides served as controls (mimic NC). (B) Viability and (C) apoptotic rate of cells transfected with miR-23a and/or miR-92a mimic, and then treated with 10 μg/ml AS-IV for 24 h upon hypoxia. Protein expression levels of (D) proliferation- and (E) apoptosis-related factors in cell after the indicated treatment. β-actin acted as an internal reference. Data expressed as mean ± SD (n = 3 per group). ns, no significance, * P<0.05, ** P<0.01, *** P<0.001.

AS-IV protected H9c2 cells against hypoxia-induced cell damage via down-regulation of miR-23a and miR-92a

To test whether miR-23a and miR-92a were involved in the protective functions of AS-IV on hypoxia-injured cardiomyocyte, the expression levels of miR-23a and miR-92a in H9c2 cells were respectively overexpressed by mimic transfection. As shown in Fig. 4A, the RNA levels of miR-23a and miR-92a were highly expressed in mimic-transfected cells compared with the cells transfected with mimic NC ($P < 0.01$). More surprisingly, we found that the protective effects of AS-IV on hypoxia-induced cell damage were alleviated by miR-23a and/or miR-92a overexpression, as evidenced by the decrease of cell viability ($P < 0.05$ or $P < 0.01$, Fig. 4B), the increase of apoptotic cell rate ($P < 0.05$ or $P < 0.01$, Fig. 4C), the down-regulations of PCNA ($P < 0.05$ or $P < 0.01$), cyclin E1 ($P < 0.05$ or $P < 0.01$), CDK1 ($P < 0.05$ or $P < 0.01$), cyclin D1 ($P < 0.05$ or $P < 0.01$), CDK4 ($P < 0.05$ or $P < 0.001$) and Bcl-2 ($P < 0.05$ or $P < 0.01$), the up-regulations of Bax ($P < 0.05$ or $P < 0.01$) and cytochrome *c* ($P < 0.001$), and the cleavage of caspase-3 ($P < 0.001$) (Fig. 4D and 4E). Cyclin A and pro form of caspase-3

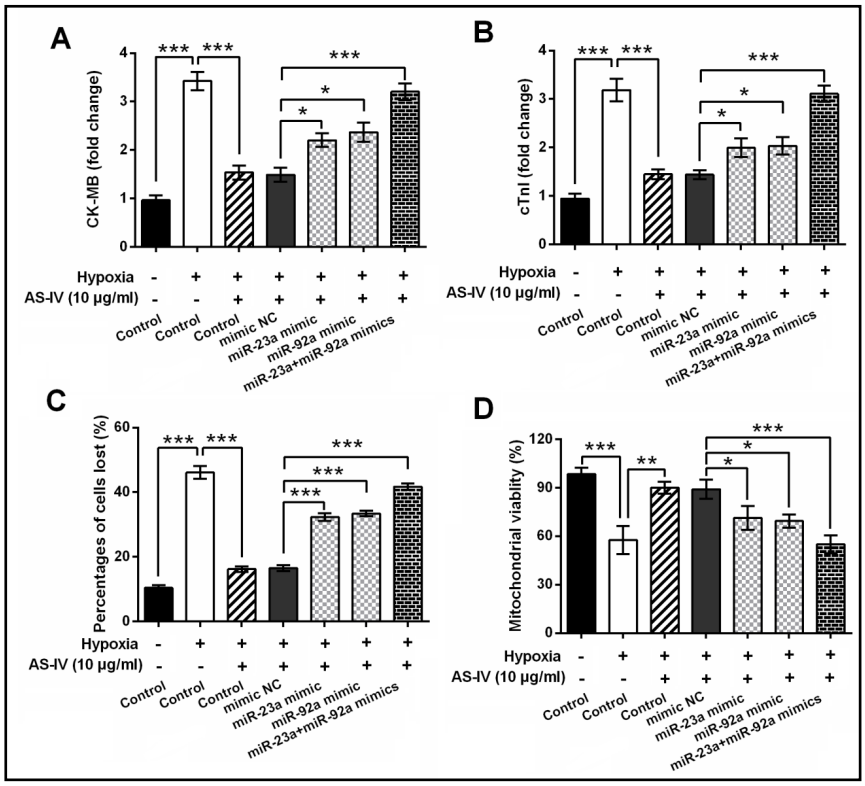

Fig. 5. AS-IV protected H9c2 cells against hypoxia-mediated cellular injury via down-regulation of miR-23a and miR-92a. H9c2 cells were transfected with miR-23a and/or miR-92a mimic, and then treated with 10 μg/ml AS-IV for 24 h upon hypoxia. The release of (A) creatine kinase-MB (CK-MB) and (B) cardiac troponin I (cTnI), (C) the mitochondrial membrane potential, and (D) the viability of mitochondria were respectively assessed. Data expressed as mean ± SD (n = 3 per group). * P<0.05, ** P<0.01, *** P<0.001.

were unaffected.

Next, the effects of AS-IV on hypoxia-mediated cellular injury were tested by measuring the release of CK-MB and cTnI, two biomarkers of myocardial cellular injury. Results in Fig. 5A and 5B showed that, both CK-MB and cTnI were significantly increased in response to hypoxia ($P < 0.001$). AS-IV treatment significantly reduced the levels of CK-MB and cTnI in hypoxia-injured cells ($P < 0.001$). And the effects of AS-IV treatment on the release of CK-MB and cTnI were attenuated by miR-23a and/or miR-92a overexpression ($P < 0.05$ or $P < 0.001$). Same trend was observed in the lost mitochondrial membrane potential (Fig. 5C), the decrease of which marks the early apoptosis. Besides, we found that hypoxia significantly reduced the viability of mitochondria ($P < 0.001$), and AS-IV treatment increased it ($P < 0.01$, Fig. 5D). As expected, AS-IV treatment induced the increase of mitochondrial viability was attenuated by miR-23a and/or miR-92a overexpression ($P < 0.05$ or $P < 0.001$).

Knockdown of miR-23a and miR-92a activated PI3K/AKT and MAPK/ERK signaling pathways

Further, we focused on PI3K/AKT and MAPK/ERK signaling pathways, in order to reveal the underlying mechanisms of which miR-23a and miR-92a mediated hypoxia-induced cell damage. Western blot analytical results showed that, both PI3K/AKT and MAPK/ERK signaling pathways were blocked by hypoxia exposure, as the phosphorylated levels of PI3K, AKT, MAPK, and ERK were all down-regulated ($P < 0.001$). Hypoxia-induced down-regulations of these phosphorylated proteins were alleviated by miR-23a or miR-92a knockdown ($P < 0.05$ or $P < 0.01$). Moreover, the down-regulations induced by hypoxia were recovered to the normal levels by simultaneous silence of miR-23a and miR-92a ($P < 0.001$, Fig. 6A and 6B). Total levels of PI3K, AKT, MAPK, and ERK were unaffected neither by hypoxia nor by miRNA transfection.

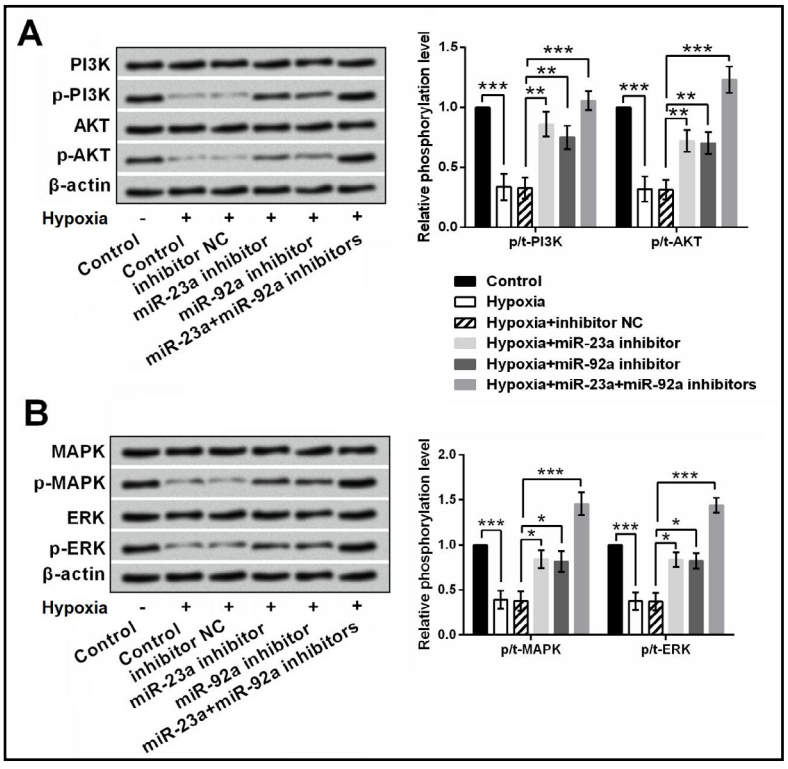

Fig. 6. Knockdown of miR-23a and miR-92a activated PI3K/AKT and MAPK/ERK signaling pathways. H9c2 cells were transfected with the inhibitors specific for miR-23a and miR-92a, followed by hypoxia exposure for 24 h. The expression levels of core proteins in (A) PI3K/AKT and (B) MAPK/ERK signaling pathways were detected. β-actin acted as an internal reference. Data expressed as mean ± SD (n = 3 per group). ns, no significance, * P<0.05, ** P<0.01, *** P<0.001.

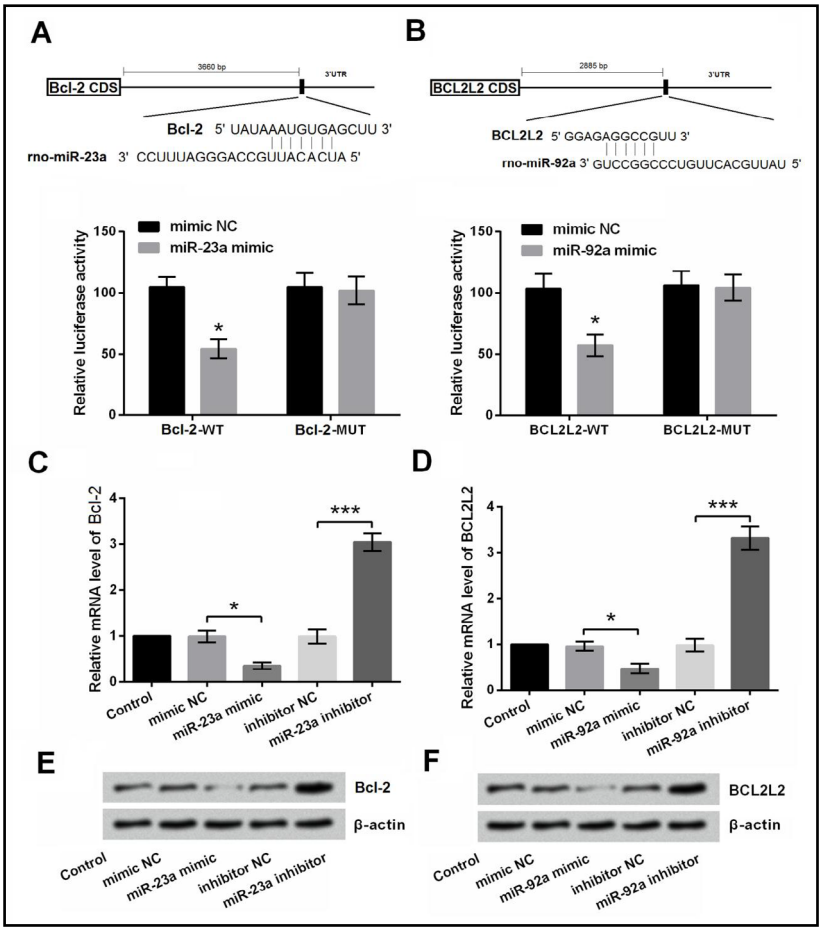

Fig. 7. Bcl-2 was a target gene for miR-23a and BCL2L2 was a target gene for miR-92a. Dual-Luciferase reporter assay was performed to assess whether (A) Bcl-2 was a target for miR-23a, and whether (B) BCL2L2 was a target for miR-92a. The mRNA levels of (C) Bcl-2 and (D) BCL2L2, as well as the protein levels of (E) Bcl-2 and (F) BCL2L2 were tested in H9c2 cells after transfection with the inhibitors specific for miR-23a and miR-92a. Data expressed as mean ± SD (n = 3 per group). * P<0.05, *** P<0.001.

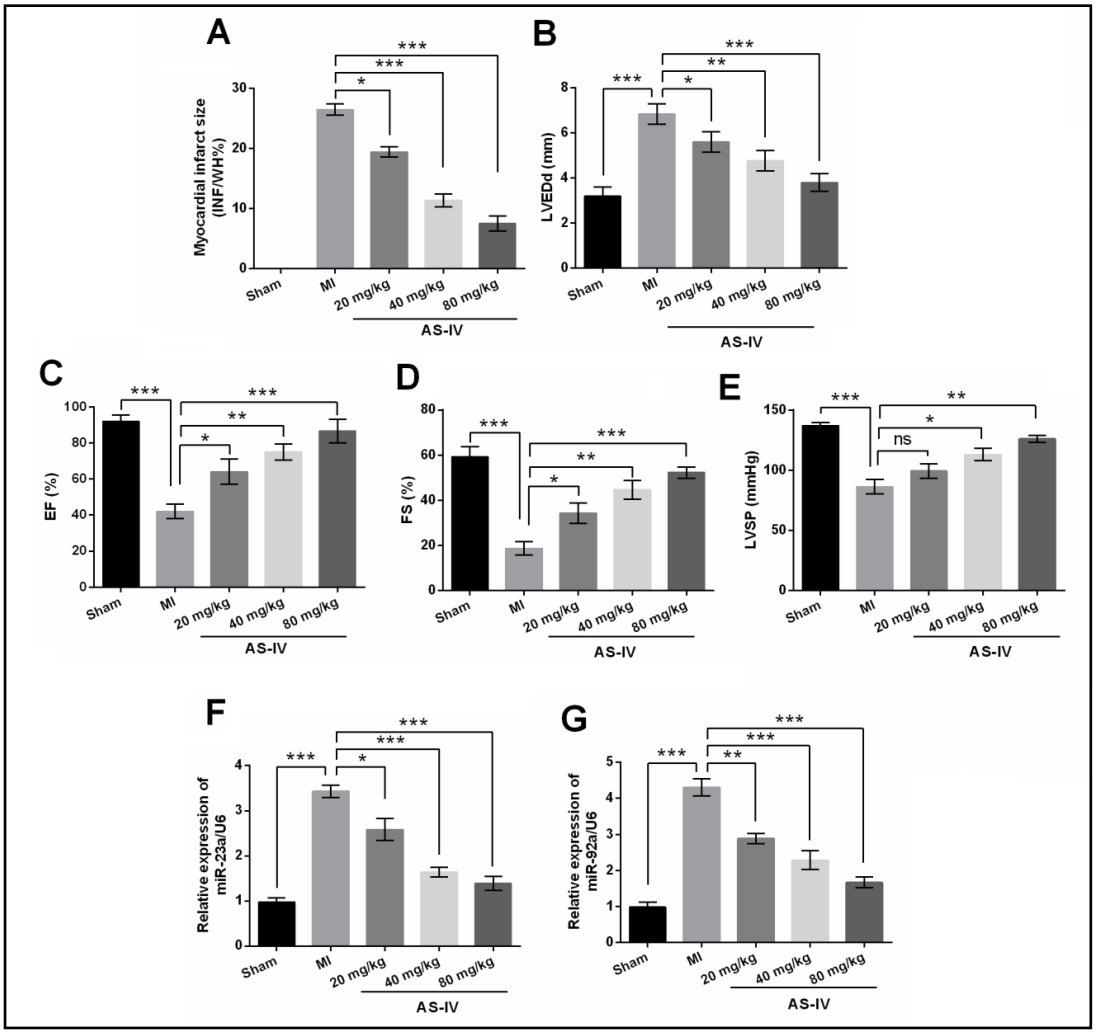

Fig. 8. AS-IV administration reduced infarct volume and improved outcome of MI rats. Sprague–Dawley rats were received coronary ligation or the sham operation, after which the rats were injected with 20, 40, and 80 mg/kg/day of AS-IV for 14 days by intragastric route. (A) The infarct volume of rat heart, (B) The left ventricular end-diastolic internal diameter (LVEDd), (C) ejection fraction (EF), (D) shortening fraction (FS), (E) LV systolic pressure (LVSP), as well as the expression levels of (F) miR-23a and (G) miR-92a were detected. Data expressed as mean ± SD (n = 8 per group). ns, no significance, * P<0.05, ** P<0.01, *** P<0.001.

Bcl-2 was a target gene for miR-23a and BCL2L2 was a target gene for miR-92a

By using the online databases (TargetScan and microrna), Bcl-2 was predicted as a target gene for miR-23a and BCL2L2 was predicted as a target gene for miR-92a. These predictions were confirmed by Dual-Luciferase reporter assay, as the luciferase activities were significantly reduced by co-transfection with Bcl-2-WT and miR-23a mimic ($P < 0.05$, Fig. 7A), and by co-transfection with BCL2L2-WT and miR-92a mimic ($P < 0.05$, Fig. 7B). The expression changes of Bcl-2 and BCL2L2 in miR-23a or miR-92a dysregulated cells were detected by performing qRT-PCR and western blot. Fig. 7C-7F showed that, both mRNA and protein levels of Bcl-2 were negatively regulated miR-23a, and both mRNA and protein levels of BCL2L2 were negatively regulated by miR-92a.

AS-IV administration reduced infarct volume and improved outcome of MI rats

Finally, the *in vivo* effect of AS-IV on animal model of MI was tested. NBT staining results showed that AS-IV administration significantly reduced the infarct volume in a dose-dependent fashion ($P < 0.05$ or $P < 0.001$, Fig. 8A). Fig. 8B-8E showed that, compared to the Sham group, rats in the MI group exhibited significant left ventricular dilation, and congestion and systolic dysfunction, as LVEDd was significantly increased ($P < 0.001$), and EF, FS and LVSP were significantly reduced (all $P < 0.001$). Of note, AS-IV administration attenuated the left ventricular dilation, and congestion and systolic dysfunction in MI rat ($P < 0.05$, $P < 0.01$ or $P < 0.001$).

The expression changes of miR-23a and miR-92a in the heart tissues of MI rats were tested by qRT-PCR. Data in Fig. 8F and 8G indicated that, miR-23a and miR-92a was highly expressed in MI group ($P < 0.001$), and the elevated expression of miR-23a and miR-92a was reduced by AS-IV administration in a dose-dependent fashion ($P < 0.05$, $P < 0.01$ or $P < 0.001$).

Discussion

MI has been defined as myocardial cell death due to prolonged ischaemia [20]. Hypoxia, a condition of insufficient O_2 to support metabolism, is one of the major risks of MI. Under hypoxia, energy metabolism shifts from mitochondrial respiration to anaerobic glycolysis, and simultaneously causes large numbers of physiological and pathological responses, resulting in acidosis and cell necrosis [21]. Currently, protection of cardiomyocyte from hypoxia-induced injury has been regarded as a promising therapeutic strategy for MI. In the current study, rat cardiomyocyte line H9c2 was subjected to hypoxic condition for 24 h. In consistence with previous studies [22, 23], 24 h of hypoxic exposure significantly induced apoptosis and impaired viability of H9c2 cells, indicating cell damage occurred in cardiomyocytes by hypoxia *in vitro*. Then, we found that 5 and 10 μg/ml of AS-IV protected H9c2 cells against hypoxia-induced injury by promoting cell proliferation and inhibiting apoptosis.

AS-IV, a popular traditional Chinese medicine, is commonly utilized for the prevention and treatment of cardiovascular and cerebrovascular ischemia [24]. Zhang *et al.*, revealed that AS-IV alleviated hypoxia/reoxygenation-induced neonatal rat cardiomyocyte injury possibly through up-regulation of PKA and Ser (16)-PLN, thereby restoring SERCA2a function [25]. Tu *et al.*, revealed that AS-IV prevented ischemia/reperfusion-induced cardiac malfunction, maintained the integrity of myocardial structure through regulating energy metabolism [16]. In addition, the cardioprotective effects of AS-IV against LPS [26], isoproterenol [27] and doxorubicin [28] via diverse effects upon a variety of intracellular signaling pathways have been previously described, including the activation of TLR4/NF-κB and PI3K/AKT pathways. Consistent with the findings of these previous studies, our study showed that AS-IV significantly increased H9c2 cells viability, and decreased apoptotic cell rate *in vitro*, and significantly reduced infarct volume and improved outcome *in vivo*. These data confirmed the cardioprotective effects of AS-IV against hypoxia.

PCNA is an essential protein for cell replication. It is specially expressed in proliferating cells and tumor cells. In this study, the protein levels of PCNA, cyclins, and CDKs were detected to reveal the effects of AS-IV on the proliferation of hypoxic cardiomyocyte. We found that PCNA was highly expressed after AS-IV treatment, implying cell proliferation was improved by AS-IV. Cyclin E1, cyclin D1, CDK1 and CDK4 were all up-regulated, while cyclin A was unaffected by AS-IV, suggesting AS-IV led to a cyclin/CDK-dependent and checkpoint-type arrest. Besides, our western blot data showed the anti-apoptotic effect of AS-IV was associated with mitochondria-dependent pathway, as AS-IV increased the ratio of Bcl-2 to Bax, repressing the release of cytochrome *c*, and sequential deactivation of caspase-3, which is consistent with the findings of Xu *et al.* and Jia *et al* [28, 29].

It is well-known that CK-MB and cTnI are two biomarkers of myocardial cellular injury. Herein, we found that AS-IV treatment significantly reduced the levels of CK-MB and cTnI in hypoxia-injured cells, further suggesting AS-IV treatment attenuated myocardial cellular injury induced by hypoxia. Besides, we found that AS-IV could prevent energy metabolism shifts from mitochondrial respiration to anaerobic glycolysis, as the lost mitochondrial membrane potential and mitochondrial viability were reduced by AS-IV administration in MI rats.

Following that, the mechanisms by which AS-IV protected H9c2 cells from hypoxia-induced injury were detected. Interestingly, we observed that miR-23a and miR-92a (two myocardial ischemia-related miRNAs) levels were significantly reduced by AS-IV treatment. Moreover, AS-IV could not alleviate hypoxia-induced cell damage when miR-23a and/or miR-29a were overexpressed. Collectively, the *in vitro* and *in vivo* data suggested that AS-IV exerted cardioprotective effects might be via down-regulation of miR-23a and miR-92a. We additionally found that Bcl-2 was a target gene for miR-23a, and BCL2L2 was a target gene for miR-92a. Bcl-2 and BCL2L2 are two inhibitors of apoptosis, and the expression of Bcl-2 and BCL2L2 plays pathophysiological role in the protection or acceleration of the apoptosis of cardiomyocytes after ischemia [30, 31]. Thus, it can be inferred that AS-IV treatment prevented myocardial cellular injury via down-regulation of miR-23a and miR-92a, and avoiding Bcl-2 and BCL2L2 from degradation by miR-23a and miR-92a. Presently, miRNAs studies have obtained an in-depth understanding of the pathological mechanisms of MI and pharmacological mechanisms of natural product medicine at the genomic level [32, 33], whereas only few reports explored the role of miRNAs in the pharmacological mechanism of AS-IV [13, 34]. Our findings highlight down-regulation of miR-32a and miR-92a may mediate cardioprotective mechanisms of AS-IV in MI for the first time.

In the setting of ischaemia-reperfusion injury, several kinases signaling have been implicated through their effects on cell death and survival, including PI3K/AKT, MAPK/ERK, and JAK/STAT [35, 36]. It has been demonstrated in the myocardium that the activation of PI3K/AKT and MAPK/ERK pathways by procedures such as ischaemic pre- or post-conditioning or by the administration of pharmacological agents is crucial for the salvage of the ischaemic/reperfused myocardium [37-39]. Herein, we demonstrated that silence of miR-23a and/or miR-92a activated PI3K/AKT and MAPK/ERK signaling pathways, since the phosphorylated levels of PI3K, AKT, MAPK, and ERK proteins were remarkably up-regulated by miR-23a and/or miR-92a overexpression.

Conclusion

AS-IV has a certain role in protecting cardiomyocyte against hypoxia-induced injury. Its mechanism of action involves the expression of miR-23a and miR-92a, as well as involves the activation of PI3K/AKT and MAPK/ERK signaling pathways. The findings of this study suggest AS-IV has potential for use in the clinical treatment of MI, and provide a novel viewpoint regarding the underlying mechanism of AS-IV's cardioprotective effect.

Disclosure Statement

The authors declare to have no competing interests.

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