

Ginsenoside Rb1 protects cardiomyocytes from oxygen-glucose deprivation injuries by targeting microRNA-21

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Abstract. Ginsenoside Rb1 (GS-Rb1) is one of the most important active pharmacological extracts of the traditional Chinese medicine, ginseng, and there is extensive evidence of its cardioprotective properties. However, the microRNA (miR) targets of GS-Rb1 and the underlying mechanisms of GS-Rb1 and miR-21 in the progression of cardiomyocyte apoptosis have not been clearly elucidated. The aim of the current study was to investigate the impact of miR-21 and its target gene, programmed cell death protein 4 (PDCD4), on the protective effect of GS-Rb1 in cardiomyocytes injured by oxygen-glucose deprivation (OGD). The miR-21 expression levels were downregulated, and the percentage of the apoptotic cells and reactive oxygen species (ROS) was increased in OGD-cultured neonatal rat cardiomyocytes; however, the effects were reversed by GS-Rb1 treatment. It was demonstrated that GS-Rb1 could reduce intracellular ROS content, and the expression of cytochrome C and the pro-apoptosis protein, apoptosis regulator B-cell lymphoma associated X (Bax) protein while increasing the expression of the anti-apoptosis protein, apoptosis regulator Bcl-2. The target gene, PDCD4, was significantly upregulated in the OGD group; however, the expression of PDCD4 was inhibited by GS-Rb1 treatment. Furthermore, miR-21 inhibitor transfection reduced GS-Rb1-induced miR-21 upregulation compared with the OGD+GS-Rb1 group, indicating that the

miR-21 was involved in the anti-apoptotic effect of GS-Rb1 in cardiomyocytes. The results of the current study highlighted that GS-Rb1 could target miR-21 and its target gene, PDCD4, to protect OGD-injured cardiomyocytes. The results of the current study may provide a novel insight for the treatment of myocardial infarction with Traditional Chinese Medicines, involving miRs as targets.

Introduction

In recent years, the incidence and mortality of cardiovascular disease (CVD) in China have been increasing. It has been estimated that the number of patients with CVD was >290 million in China, 2017. Furthermore, the mortality rate of CVD has increased from ~174 to 298 per 100,000 people from 1990 to 2015. It has therefore become one of the major diseases threatening public health in China and all over the world (1). Myocardial infarctions are a common cause of increased morbidity and mortality in the population, and the onset age is decreasing (2). Early diagnosis and treatment of a myocardial infarction can prevent or reduce myocardial ischemic injury, in addition to preventing ventricular remodeling and heart failure following a myocardial infarction (3).

MicroRNAs (miRs) are short, endogenous, single-stranded, non-coding RNA fragments in animal eukaryotic cells, with a length of 18-26 nucleotides, which can inhibit gene expression at a post-transcriptional level (4). Therefore, miRs are considered to be an important component of the cellular regulatory network (4). miRs that serve an important role in the occurrence and development of cardiovascular diseases have been reported since 2006, when they were first identified by van Rooij *et al* (5). miRs associated with the cardiovascular system are increasingly being identified and studied, thus becoming a research hotspot (4-7). miR-21 is highly expressed in the cardiovascular system and is involved in the pathophysiological mechanisms of various cardiovascular diseases, particularly in myocardial infarctions; high levels of miR-21 expression are associated with cardiovascular diseases (6,7).

Programmed cell death protein 4 (PDCD4) has been demonstrated to be the target protein of miR-21 in tumors and numerous systems, including the circulatory and nervous systems; its role in cellular apoptosis and cellular protection has been increasingly studied (8,9). There are two important α -helical domains at the amino end of PDCD4, through which PDCD4 can bind to eukaryotic initiation factor 4A, the

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Abbreviations: GS-Rb1, ginsenoside Rb1; OGD, oxygen-glucose deprivation; miR-21, microRNA-21; PDCD4, programmed cell death protein 4; Cyt C, cytochrome C; DMEM, Dulbecco's modified Eagle's medium; CCK-8, Cell Counting Kit-8; DCFH, 2,7-dichlorodihydrofluorescein; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN; RECK, reversion-inducing cysteine-rich protein with Kazal motifs

Key words: Ginsenoside Rb1, cardiomyocytes, oxygen-glucose deprivation, apoptosis, microRNA-21, programmed cell death protein 4

initiation factor of eukaryotic translation, and thereby promote cellular apoptosis by inhibiting the formation of ribosome complexes and protein synthesis (10).

Ginseng is one of the most popular herbal medicines that have been used in China for thousands of years (11). Ginsenoside Rb1 (GS-Rb1) is the most active and abundant monomer in ginseng (11). Although a number of studies have demonstrated the protective effects of GS-Rb1 on the heart (11-14) and recent studies have revealed that GS-Rb1 could impact miR expression in hypoxia/ischemia-injured cardiomyocytes (12,13), the miR targets involved and their roles in the heart remain unknown. Therefore, in the current study, the roles and mechanisms of miR-21 and its target gene, which encodes PDCD4, in the protection of cardiomyocytes treated with GS-Rb1 were studied by constructing an oxygen-glucose deprivation (OGD) injury model *in vitro*.

Materials and methods

Culture of neonatal rat cardiomyocytes (NRCMs) from heart tissues. Primary cultures of NRCMs from a total of 180 12-24 h-old male Sprague Dawley rats (weight range, 5-6 g; Laboratory Animal Center, Jilin University, Changchun, China) were prepared through gentle serial trypsinization, as described previously (12-14). All animal protocols were approved by the Animal Care Center and Use Committee of Jilin University (Changchun, China). Briefly, rats were anesthetized without feed and house, and the ventricular myocardium was removed and cut into 1-2-mm³ sections. The obtained ventricles were washed three times in cold phosphate buffered saline (PBS) and digested five times for 5 min each at 37°C with 0.18% (w/v) trypsin and 0.01% EDTA. Digestion was terminated by adding Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; both Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, the cells were collected via centrifugation for 10 min at 1,000 x g at room temperature and resuspended in DMEM with 10% (v/v) FBS for 90 min to facilitate the separation of ventricular myocytes from the more adherent non-myocytes. NRCMs were subsequently collected and plated in collagen-coated 96- or 6-well plates (at a density of 4x10⁴/well and 6x10⁶/well, respectively) and maintained at 37°C in a 5% CO₂/95% air humidified incubator in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin and 100 mg/ml streptomycin.

OGD treatment. Cells were divided into the following groups: A control group, an OGD group and an OGD+GS-Rb1 (4, 8, 16, 32 and 64 µM) group. To generate the OGD group, culture medium was replaced with serum-free DMEM (no glucose; Gibco; Thermo Fisher Scientific, Inc.) and cultured in hypoxic conditions (5% O₂ and 95% N₂) via an automatic injection of N₂ into the incubator at 37°C. The control plates were maintained under normoxic conditions. NRCMs with or without GS-Rb1 (cat. no. 110704; National Institutes for food and drug Control, Beijing, China) (0, 4, 8, 16, 32 and 64 µM) were then subjected to hypoxia at 37°C to the indicated time (0, 6, 12, 18, 24, 36 and 48 h). The control plates were maintained at 37°C in a 5% CO₂/95% air humidified incubator in DMEM containing 10% (v/v) FBS for the same time with the OGD group.

Cell Counting Kit-8 (CCK-8) assay. Following OGD treatment (0, 6, 12, 18, 24, 36 and 48 h), cell viability was determined using CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) in accordance with the manufacturer's protocol. A total of 10 µl CCK-8 solution was added to each well of a 96-well plate (cell density, 4x10⁴/well), followed by an incubation of 3.5 h at 37°C. The optical density of each well was read at a wavelength of 450 nm using a microplate reader (M200 Pro; Tecan Group, Ltd., Mannedorf, Switzerland). The control group was maintained under normoxic conditions for the corresponding times. The results are presented as the fold-change relative to the control group.

Apoptosis assay with annexin V-FITC/propidium iodide (PI) staining. NRCMs were treated with different concentrations of GS-Rb1 (0, 16 and 32 µM) and exposed to 18 h OGD for 18 h and harvested using 0.25% trypsin and 1 mM EDTA for 5 min at 37°C. Samples were then washed with PBS. The percentages of normal nonapoptotic and apoptotic cells were measured via double supravital staining with Annexin V and PI using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (KGA 108; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cells were harvested with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C. Cells were then suspended in 500 µl of binding buffer (provided in the aforementioned Apoptosis Detection kit) and stained with annexin V-FITC and PI at room temperature for 5 min in the dark. Cells were collected via centrifugation at 1,000 x g at room temperature for 3 min and the supernatant containing the unbound annexin V-FITC and PI was aspirated. Then cells were resuspended in 400 µl binding buffer. Flow cytometric analysis was conducted using a Cytomics FC500 flow cytometer with CXP software (both Beckman Coulter, Inc., Brea, CA, USA); the operator was blind to the groups.

Fluorescent measurement of intracellular reactive oxygen species (ROS). The determination of ROS concentrations was based on the oxidation of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In brief, the cells were collected using 0.25% trypsin and 1 mM EDTA for 5 min at 37°C following OGD-injury, washed with serum-free DMEM and incubated with DCFH-DA at 37°C for 20 min. Dichlorofluorescein fluorescence intensity was detected at 488 nm excitation and 525 nm emission using a M200 Pro microplate reader.

Detection of miR-21 expression using poly(A) tailing SYBR green reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cell in the control group, OGD group (12-48 h), OGD 18 h + 32 µM GS-Rb1 group, miR-21 vehicle control group, miR-21 scramble control group, miR-21 inhibitor group and the miR-21 inhibitor + 32 µM GS-Rb1 group were lysed with 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The reaction mixture was then extracted with phenol/chloroform, precipitated with isopropanol and resuspended in 25 µl diethylpyrocarbonate-treated water. Total RNA (5 µg) was subsequently treated with DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 22°C and poly(A) polymerase (provided in the miRNA cDNA kit) at 37°C for 20 min. The poly(A)-tailed RNA (6 µl) was

reverse-transcribed into first-strand cDNA using an miRNA cDNA kit (cat. no. cw2141; Beijing ComWin Biotech, Co., Ltd., Beijing, China) according to the manufacturer's protocol. For qPCR analysis, 30 ng cDNA was employed as a template in each reaction using the miRNA qPCR Assay kit (cat. no. cw2142; Beijing ComWin Biotech, Co., Ltd.). The following primers for miR-21 (mirBase no. MIMAT0000790) were utilized: Forward, 5'-GCTAGCTTATCAGACTGAT GTTGAAA-3' and reverse, provided in the qPCR Assay kit (cat. no. cw2142; Beijing ComWin Biotech, Co., Ltd.). U6 small noncoding RNA was used as an internal control using the following primers: 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse). The thermocycling conditions were as follows: Denaturation at 94°C for 20 sec, annealing at 60°C for 45 sec followed by an extension at 72°C for 30 sec for 40 cycles. Gene expression was normalized to that of U6 and relative fold changes were calculated using the $2^{-\Delta\Delta C_q}$ method (15).

Western blotting. NRCMs subjected to different conditions and treatments were harvested and lysed using the Cell lysis buffer for western and immunoprecipitation IP kit (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China). For cytochrome *c* (Cyt C) western blotting, preparation of the mitochondrial and cytosolic protein fractions was conducted with a Cell Mitochondria Isolation kit (Beyotime Institute of Biotechnology). Protein concentrations were measured using a BCA protein assay kit. Equal amounts of the sample lysate (30 μ g) were separated via 12% SDS-PAGE and then transferred through electroblotting to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) overnight at 40°C. The following primary antibodies were utilized: B-cell lymphoma (Bcl-2; 1:1,000; cat. no. 2872), Bcl-2-associated X protein (Bax; 1:1,000; cat. no. 2772), cytochrome *c* (1:1,000; cat. no. 4272), PDCD4 (1:1,000; cat. no. 9535) and GAPDH (1:1,000; cat. no. 2118; all, Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was subsequently incubated with the aforementioned primary antibodies for 2 h at 37°C and an immunoglobulin G horseradish peroxidase-conjugated anti-rabbit antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at room temperature. The resultant signals were visualized using the SuperSignal West Femto Trial kit (cat. no. 34095; Pierce; Thermo Fisher Scientific, Inc.) on a Syngene G: BOX Chemi gel documentation system (Syngene Europe, Cambridge, UK). The densitometric values were normalized using GAPDH as an internal control. ImageJ software was also used for densitometry (version 1.6; National Institutes of Health, Bethesda, MD, USA).

In vitro caspase-3 activity assay. Caspase-3 activity was measured with a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, NRCM lysates were prepared following 18 h OGD treatment with or without different doses of GS-Rb1 (4, 8, 16 and 32 μ M). The assays were performed in 96-well micro plates by incubating 10 μ l protein cell lysate per sample in 80 μ l reaction buffer (provided by the Caspase-3 Activity Assay kit) containing

10 μ l substrate (Asp-Glu-Val-Asp-p-nitroaniline). The lysates were incubated at 37°C for 4-6 h. Samples were subsequently measured with a M200 Pro microplate reader at an absorbance of 405 nm. Caspase-3 activity was expressed as the percentage relative to the control group.

NRCMs transfection. A microOFF™ rno-miR-21 inhibitor (cat. no. miR20000790-1-5; 10 nM) or the microOFF™ inhibitor Negative Control (cat. no. miR02201-1-5; 5 nM; each, RiboBio, Guangzhou, China) was transfected into NRCMs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Subsequent GS-Rb1 treatment, and total RNA and protein extraction were performed 48 h post-transfection for qPCR and western blot analyses, respectively.

Statistical analysis. The data were expressed as the mean \pm standard deviation of at least three independent experiments. The group results were analyzed for variance using one-way analysis of variance followed by a post-hoc Bonferroni test. GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was employed for all the analyses. $P < 0.05$ indicated that the difference between groups was statistically significant.

Results

GS-Rb1 protects NRCMs from OGD impairment in a dose-dependent manner. As indicated in Fig. 1, cell activity was detected after 6, 12, 18, 24, 36 and 48 h of cultivation, and it was determined that cell viability gradually decreased as the duration of OGD treatment was prolonged (Fig. 1A). OGD for 18 h reduced cell activity by ~50%; therefore, 18 h OGD was used to induce cell damage in subsequent experiments (Fig. 1A and B). When GS-Rb1 was applied at different concentrations (4-64 μ M) and the protective effect of GS-Rb1 was examined using a cell viability assay, the effect of GS-Rb1 at 32 μ M was revealed to be the most significant in OGD-damaged cells compared with the OGD group (Fig. 1C). Annexin V-FITC/PI double staining was used to detect apoptosis and the results demonstrated that the cell percentage of living cells in the OGD 18 h-damaged group was ~40.2%, while the apoptosis rate, which included early and late apoptosis, reached about 58.8% compared with the control group (Fig. 1D and E). When GS-Rb1 was applied at 32 μ M in the model group, the percentage of living cells significantly increased compared with the OGD group (Fig. 1E), whereas the percentage of apoptotic cells were decreased.

Anti-apoptotic signaling pathway activities in OGD-injured NRCMs are increased by GS-Rb1 treatment. Furthermore, the apoptotic signaling pathways in OGD-injured NRCMs were evaluated following GS-Rb1 treatment. OGD treatment significantly increased the intracellular level of ROS, with an >10-fold increase in the fluorescence intensity of DCF. Co-treatment with GS-Rb1 significantly inhibited the increase in the intracellular concentration of ROS induced by OGD (Fig. 2A). Western blotting revealed the cytosolic Cyt C and Bcl-2/Bax expression levels (Fig. 2B). GS-Rb1 significantly reduced Bax (a pro-apoptosis protein) expression and the release of Cyt C from

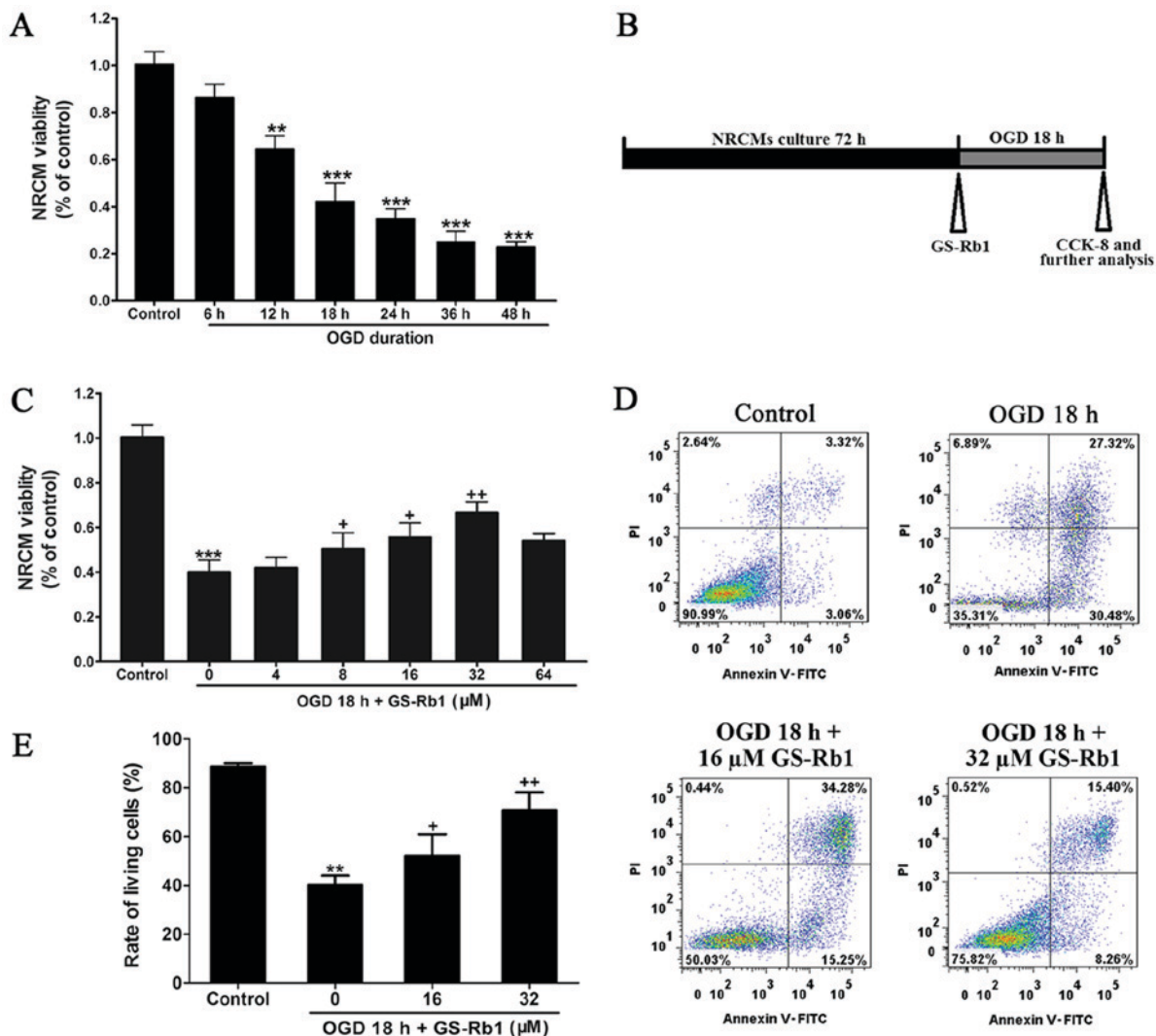


Figure 1. GS-Rb1 protects NRCMs from OGD impairment in a dose-dependent manner. (A) Cell viability of NRCMs after 6, 12, 18, 24, 36 and 48 h of OGD cultivation. (B) For the experimental procedure, NRCMs were cultured for 72 h and subjected to the hypoxia conditions with or without GS-Rb1 for 18 h (95% N₂ and 5% O₂), then cells were collected for further analysis. (C) Cell viability of NRCMs following OGD and GS-Rb1 treatment at different concentrations. (D) Effect of GS-Rb1 on OGD-damaged NRCMs detected via flow cytometry. (E) Quantification of the percentage of apoptotic and living cells in each group. Data is represented as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. the control group; +P<0.05 and **P<0.01 vs. the OGD group (n=3). GS-Rb1, ginsenoside Rb1; NRCMs, neonatal rat cardiomyocytes; OGD, oxygen-glucose deprivation; PI, propidium iodide; FITC, fluorescein isothiocyanate; CCK-8, Cell Counting Kit-8.

the nucleus to cytosol, while increasing Bcl-2 (an anti-apoptosis protein) expression, compared with the OGD group (Fig. 2C). In addition, OGD significantly increased the activity of caspase-3 to 216.24% compared with the control group (Fig. 2D). When co-treated with different doses of GS-Rb1, the activity of caspase-3 was attenuated from 207.3 to 121.4%. These results indicated that GS-Rb1 inhibited OGD-induced apoptosis by regulating apoptotic signaling pathways.

GS-Rb1 treatment increases miR-21 expression and cell viability in OGD-injured NRCMs. In the current study, RT-qPCR analysis was used to detect the expression of miR-21 in various groups. Compared with the control group, as the OGD duration increased, the expression of miR-21 continuously declined (Fig. 3A). When the duration of OGD treatment reached 18 h, miR-21 expression in cardiac muscle cells was only ~45% of the control group. Following treatment with 32 μM GS-Rb1, the expression of miR-21 significantly

increased compared with the OGD 18 h group (Fig. 3B). Following the transfection of NRCMs with a miR-21 inhibitor, the expression of miR-21 was significantly reduced compared to the vehicle control group and the increase in miR-21 expression caused by GS-Rb1 was inhibited by the miR-21 inhibitor (Fig. 3C). Further analysis of cell viability demonstrated that the inhibition of miR-21 weakened the protective effect of GS-Rb1 on NRCMs damaged by OGD, which demonstrated that miR-21 is likely to act as a target of GS-Rb1 in the protection of cardiac muscle cells (Fig. 3D).

GS-Rb1 decreases PDCD4 in NRCMs damaged by OGD. The PDCD4 downstream target protein of miR-21 was evaluated using western blot analysis (Fig. 4A). In the control group, PDCD4 exhibited almost no expression; however, PDCD4 expression was higher in cardiac muscle cells damaged by OGD (Fig. 4B). Increasing GS-Rb1 concentration gradually decreased the expression of PDCD4. When the expression

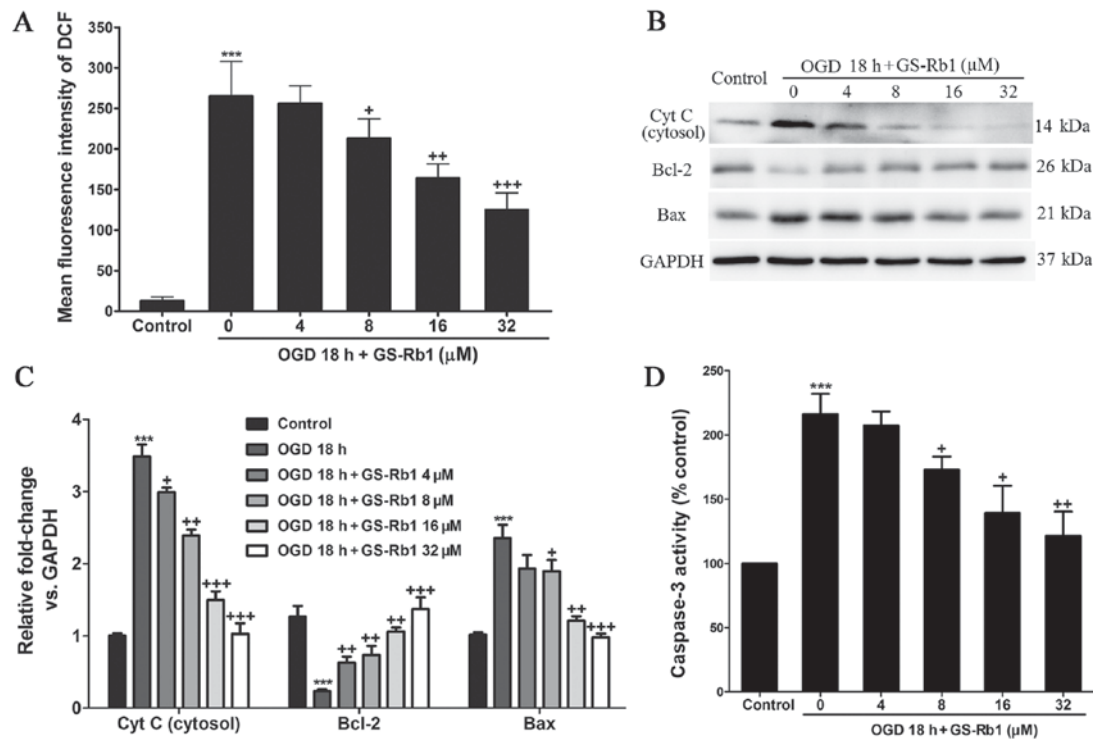


Figure 2. Anti-apoptotic signaling pathway activities in OGD-injured neonatal rat cardiomyocytes are increased by GS-Rb1 treatment. Cells with or without GS-Rb1 treatment were harvested after 18 h of OGD. (A) Analysis of the mean fluorescence intensity of DCF, an indicator of reactive oxygen species. (B) Western blot analysis and (C) quantification of western blot analysis for cytosolic Cyt C, Bcl-2 and Bax. (D) Caspase-3 activity. Data is represented as the mean \pm standard deviation *** P <0.001 vs. the control group; * P <0.05, ** P <0.01 and *** P <0.001 vs. the OGD group (n =3). GS-Rb1, ginsenoside Rb1; OGD, oxygen-glucose deprivation; DCF, dichlorofluorescein; Cyt C, cytochrome c; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein.

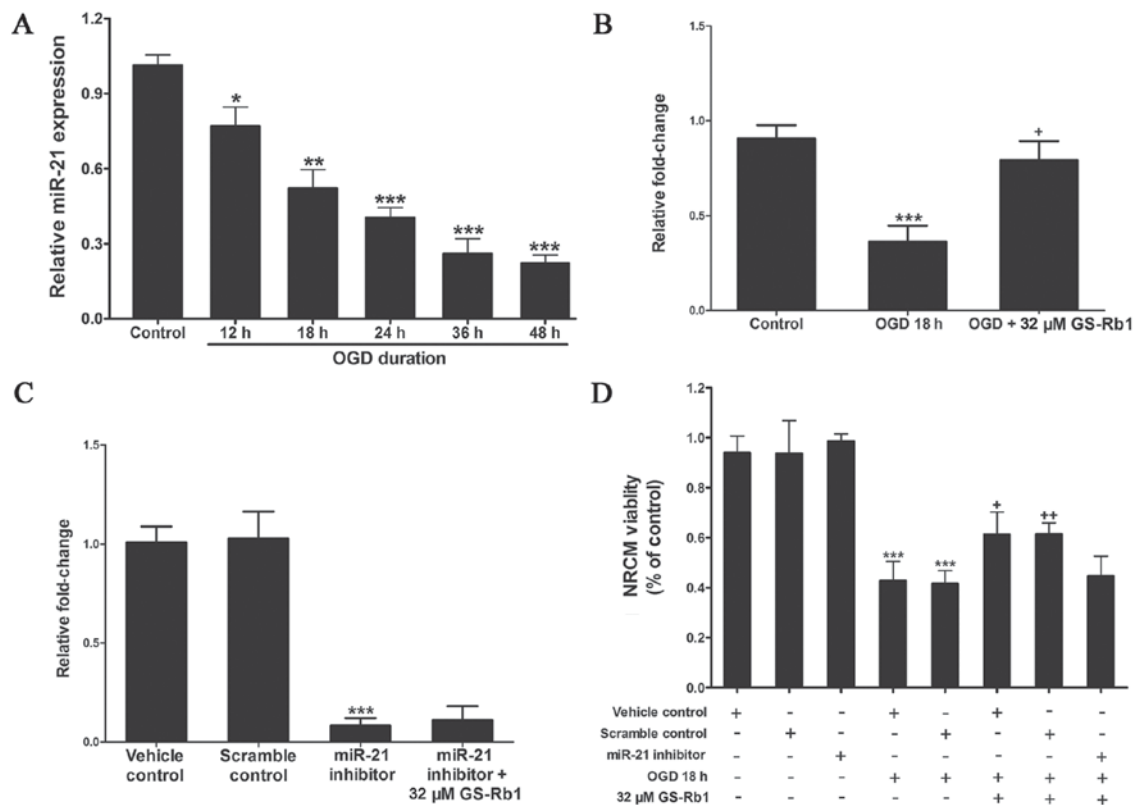


Figure 3. GS-Rb1 treatment increases miR-21 expression and cell viability in OGD-injured NRCMs. (A) Expression of miR-21 at different durations of OGD culturing. The relative fold change of miR-21 expression in (B) untreated NRCMs, NRCMs cultured under OGD with or without 32 μ M GS-Rb1 and (C) NRCMs treated with a vehicle, scramble miR or a miR-21 inhibitor with or without OGD. (D) Cell viability of NRCMs with different treatments and conditions. * P <0.05, ** P <0.01 and *** P <0.001 vs. the control group; * P <0.05, ** P <0.01 vs. the OGD group (n =3). GS-Rb1, ginsenoside Rb1; NRCMs, neonatal rat cardiomyocytes; OGD, oxygen-glucose deprivation; miR, microRNA.

of miR-21 was inhibited by the miR-21 inhibitor, the expression of PDCD4 was significantly increased compared with the control group, while OGD and GS-Rb1 treatment had no significant effect on the increased PDCD4 expression induced by miR-21 expression inhibition.

Discussion

Previous studies have only identified differentially expressed miRNAs in OGD-injured NRCMs with GS-Rb1 treatment (12,13). The present study is the first to verify the miRNA target of GS-Rb1 in the OGD induced cardiomyocytes apoptosis. Through inhibition of cardiac muscle cell apoptosis, GS-Rb1 may greatly alleviate cardiac muscle cell reduction and relieve myocardial damage (16).

In the current study, cardiac muscle cell damage following a myocardial infarction was simulated by establishing a model of OGD damage in NRCMs. GS-Rb1 was then added to the cells at different concentrations to determine the optimum dose of GS-Rb1 for the protection of cardiac muscle cells, which appeared to be dose dependent. Through the detection of apoptosis-associated proteins, it was determined that GS-Rb1 may reduce OGD-induced intracellular ROS contents, and decrease the expression of Cyt C in the cytoplasm and Bax. Simultaneously, GS-Rb1 may increase the expression of Bcl-2 and inhibit the activity of caspase-3, ultimately inhibiting the activation of the apoptosis signaling pathway and contributing to myocardial preservation. Further experiments revealed that GS-Rb1 may increase miR-21 expression following OGD damage, thereby reducing the expression of PDCD4 and limiting the activation of the apoptosis signaling pathway, which ultimately decreased apoptosis caused by OGD.

Studies on the regulatory effect of miR-21 on myocardial infarctions and apoptosis have been increasing. Numerous studies have verified the overexpression of miR-21 protects the heart (17,18). The investigation of a model of ischemia-reperfusion injury in mice and rats demonstrated that the expression of miR-21 in ischemic regions of the myocardium was lower when compared with the control group, miR-21 overexpression may reduce myocardial ischemia-reperfusion injury and a miR-21 inhibitor can eliminate the protective effect of a miR-21 stimulant (19,20). Studies on ischemic cardiomyopathy revealed that the expression levels of miR-21 in the heart ischemia border zone and non-ischemic regions were higher, while miR-21 expression in ischemic regions was lower compared with the normal tissues (7,21). Furthermore, it was demonstrated that the overexpression of miR-21 through a virus transfection method could reduce the ischemic area and relieve congestive heart failure after 2 weeks (21).

The results of the current study demonstrated that after 18 h of OGD, the miR-21 expression level was decreased, while apoptosis was increased. Additionally, GS-Rb1 enhanced miR-21 expression while reducing NRCM apoptosis, demonstrating that miR-21 may be a miR target through which GS-Rb1 protects the myocardium. Although the effect of miR-21 mimics on OGD-injured NRCMs was evaluated with and without GS-Rb1, and miR-21 mimics upregulated miR-21 expression, NRCM viability did not change with culturing under OGD conditions or GS-Rb1 treatment (data not shown). It was hypothesized that the stagnation of cell viability may

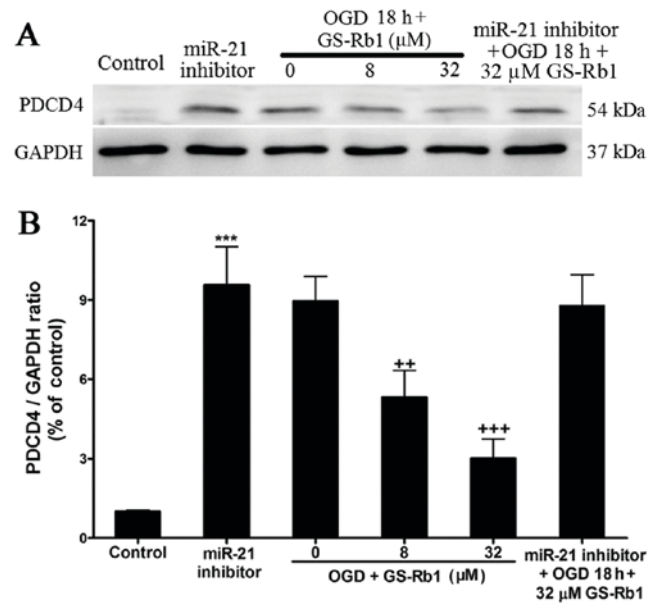


Figure 4. GS-Rb1 decreases PDCD4 in NRCMs damaged by OGD. NRCMs were untreated, treated with a miR-21 inhibitor, treated with GS-Rb1 under OGD for 18 h or co-treated with 32 μM GS-Rb1 and a miR-21 inhibitor under OGD for 18 h. (A) Western blot analysis and (B) quantification of western blot analysis for PDCD4. Data is represented as the mean ± standard deviation. ***P<0.001 vs. the control group; **P<0.01 and ***P<0.001 vs. the OGD group (n=3). GS-Rb1, ginsenoside Rb1; NRCMs, neonatal rat cardiomyocytes; OGD, oxygen-glucose deprivation; miR, microRNA; PDCD4, programmed cell death protein 4.

be associated with the NRCM injury model (e.g., OGD/reoxygenation model or the oxidative stress injury model). Previous studies revealed that miR-21 overexpression could protect against ischemia-reperfusion- and doxorubicin-induced cardiac cell death (18,22). However, the results of miR-21 overexpression in the OGD model require further study. In addition, future study should focus on other signaling pathways and miRs involved in the process.

miRs can regulate gene expression at the post-transcription level. The major mechanism of action of miRs involves the binding of the 'seed sequence' of the miR to the 3'-untranslated region of the target mRNA to inhibit the translation of the target mRNA or promote its degradation, thereby restricting gene and protein expression (23). Previous studies have highlighted that miR-21-specific targets include phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (PTEN) (24), reversion-inducing cysteine-rich protein with Kazal motifs (RECK) (25) and PDCD4 (26-28). However, positive results were not obtained in western blotting for PTEN and RECK in the present study (data not shown). The PDCD4 gene is a cancer suppressor gene that may directly regulate the apoptosis of cells (29). A number of studies have verified that apoptosis in a tumor can be regulated by PDCD4, a target gene of miR-21 (30,31). In the cardiovascular system, it has been demonstrated that the upregulation of miR-21 expression can inhibit the expression of PDCD4 to protect cardiomyocytes (26-28). The present study also verified that treatment with GS-Rb1 *in vitro* can protect OGD-damaged NRCMs through the upregulation of miR-21 and the inhibition of PDCD4 expression.

Myocardial apoptosis caused by OGD is one of the key characteristics of congestive heart failure following a myocardial infarction (32). The identification of a method for reducing the apoptosis of cardiac muscle cells under OGD conditions is an important research direction in regard to myocardial infarction treatment.

Although the cause-effect association between GS-Rb1 and miR-21/PDCD4 requires further clarification, to the best of our knowledge, the *in vitro* experiments in the current study revealed for the first time that the protective effect of GS-Rb1 may act directly via miR-21 and its target gene, PDCD4. Future *in vivo* studies to investigate the effects of GS-Rb1 treatment and role of miR-21 in other ischemic cardiovascular diseases will be performed. To conclude, the current study not only provides new options and concepts for cardiovascular disease-associated medicines involving miRs as targets, but also provides direct laboratory data for the clinical application of GS-Rb1 in the treatment of myocardial infarction.

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Availability of data and materials

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

Authors' contributions

CY performed the western blot analysis and analyzed the data. BL performed the cell biology experiments. YSL performed the transfections into the neonatal rat cardiomyocytes and reverse transcription-quantitative polymerase chain reaction analysis. YX designed the current study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal protocols in current research were approved by the Animal Care Center and Use Committee of Jilin University.

Patient consent to publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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