**Effect of Ginsenoside Rb2 on a Myocardial Cell Model of Coronary Heart Disease through Nrf2/HO-1 Signaling Pathway**

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The ginsenoside Rbs are the primary active compounds of Panax ginseng and ginsenoside Rb2 is a renowned component among the Rbs. This study aimed to investigate the potential effects of ginsenoside Rb2 on coronary heart disease (CHD). H9c2 cells were exposed to H2O2 to establish CHD model in vitro. Gene expression was determined by quantitative realtime PCR (qPCR) and Western blot. Cellular functions were detected by Cell Counting Kit-8 (CCK-8), 5-ethynyl-2’-deoxyuridine (EdU), flow cytometry, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays. We found that Ginsenoside Rb2 promoted cell proliferation while suppressed oxidative stress and apoptosis of H9c2 cells induced by H2O2 exposure. Mechanistically, Ginsenoside Rb2 involves in the regulation of nuclear factor, erythroid 2 like 2 (Nrf2)/heme oxygenase (HO)-1 signaling pathway. Inactivation of Nrf2/HO-1 signaling pathway reversed the effects of ginsenoside Rb2 on H9c2 cells. Taken together, ginsenoside Rb2 exhibited a cardioprotective effect in vitro. The underlying mechanism of ginsenoside Rb2 in H9c2 cells could be standardized to Nrf2/HO-1 signaling pathway, inhibiting cell apoptosis and regaining cell proliferation. The present study has proposed a novel mechanism of ginsenoside Rb2 in the cardioprotective effect.

**Key words** coronary heart disease; H9c2; ginsenoside Rb2; nuclear factor, erythroid 2 like 2 (Nrf2); heme oxygenase (HO)-1

INTRODUCTION

Coronary heart disorder, like myocardial infarction, is a globally leading cause of death.1–3 Primary percutaneous coronary intervention and primary thrombolytic angioplasty are distinct therapeutic strategies that have been evolved to reduce the size of myocardial infarction and improve the expected clinical outcome.4–6 Nevertheless, the myocardial ischemia/reperfusion injury (MIRI) therapy could be reduced and obstructed by reperfusion.7,8 Oxidative stress is a key risk factor for MIRI and its abnormal accumulation induces regulate multiple vascular cell functions, such as cell proliferation, angiogenesis, apoptosis, and genomic stability.9,10 Therefore, exploring novel therapeutics to reduce the MIRI and treat coronary heart disorders becomes a great need.

Panax ginseng C. A. Mayer (ginseng) is a renowned medicinal herb used for over 2000 years in China and has been listed as a first-class article in the Chinese herbal dictionary as it is known to exhibit a vast range of pharmacological and therapeutic applications.11–16 Ginsenosides are major active compounds of ginseng and exhibit distinct pharmacological effects.13–16 Ginsenoside Rb2 is a member of the primary active compounds of Panax ginseng and is known to inhibit angiogenesis and improve wound healing by promoting cell proliferation.17–19 Also, ginsenoside Rb2 has been previously reported to exhibit cardioprotective effects through multiple mechanisms by scavenging oxidative stress products, increasing the concentration of antioxidant defense enzymes, and inhibiting cardiomyocyte apoptosis.20 Besides, ginsenoside Rb2 has been shown to alleviate myocardial ischemia/reperfusion injury in rats through SIRT1 activation.21 Recently, ginsenosides have been shown to prevent cardiac ischemia/reperfusion injury via activating nuclear factor, erythroid 2 like 2 (Nrf2) signaling pathways.22 The activation of Nrf2 signaling exerts cardiac-protective role via suppressing the production of reactive oxygen species (ROS) and the apoptosis of cardiomyocytes.23 For instance, dysregulation of Nrf2 induces oxidative stress, which further promotes chronic heart failure.24 However, activation of Nrf2/heme oxygenase (HO)-1 signaling protects against adverse cardiac remodeling after myocardial infarction.25 Additionally, Nrf2 suppresses inflammatory response and restores heart function.26 Therefore, small molecules that target this signaling pathway may offer the possibility for novel therapeutic strategies in cardiovascular diseases.

Up to the moment, limited studies have been conducted to investigate the cardioprotective effect of ginsenoside Rb2. We aimed in the present study to point out the potential role of ginsenoside Rb2 against H2O2-induced injury in H9c2 cells in vitro and its underlying mechanism concerning Nrf2/HO-1 signaling pathway.

**MATERIALS AND METHODS**

**Chemicals and Reagents** Ginsenoside Rb2 was purchased from MedChemExpress (U.S.A.). Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) Nick End Labeling (TUNEL) Apoptosis Detection Kit was purchased from Roche (Switzerland). Antibodies against Nrf2 and HO-1 were purchased from Abcam (England). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), BMI-111, and all other chemicals were purchased from Beyo-
time (Shanghai, China).

Human and Animal Rights The study protocol was approved by the 921st Hospital of Joint Logistic Support Force of PLA. All patients provided the informed consent.

Cell Culture H9c2 cell was routinely cultivated in Dulbecco’s modified eagle medium (ThermoFisher, U.S.A.) supplemented with 10% fetal bovine serum (ThermoFisher), 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidiﬁed atmosphere at 37 °C with 5% CO₂. For the establishment of cell injury model, 200 µM H₂O₂ was used to treat the H9c2 cells for 12 h.

Western Blotting The cells were collected and lysed in radio immunoprecipitation assay (RIPA) buffer supplied with protease inhibitors. An equal amount of extracted proteins were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene difluoride (PVDF) membrane membranes (Millipore, U.S.A.). The membranes were blocked with 10% non-fat milk at room temperature for one hour and then incubated with the primary antibody at four °C overnight. The next day, we incubated the membrane with a horseradish peroxidase-coupled secondary antibody. The protein expression was detected using a chemiluminescent substrate system (Beyotime). We measured the band intensity using Image J software.

Determination of Creatine Kinase-MB (CK-MB), Troponin I (cTn-I) and Lactate Dehydrogenase (LDH) Levels

The CK-MB, cTn-I and LDH levels of the H9c2 cells were determined by corresponding commercial kits purchased from the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). All operations were carried out in strict accordance with the instructions of the kits.

5-Ethynyl-2′-deoxyuridine (EdU) Assay The H9c2 were seeded in an 8-wells plate (200 µL) on a glass chamber slide at the desired density. The cells were allowed to recover overnight (until they reached 80% confluence). Next, a 10× working solution of the EdU staining was prepared following the manufacturer’s protocol. The cells were incubated with the EdU staining for the indicated time course followed by immediate fixation and permeabilization. Briefly, the cells were first fixed using 3.7% formaldehyde diluted in phosphate buffered saline (PBS), followed by a 0.5% Triton X-100 permeabilization step. After permeabilization, the cells were washed twice with 200 µL of 3% bovine serum albumin (BSA) in PBS and then proceeded into the fluorescent EdU staining using a kit (Abcam, England).

Flow Cytometry The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was purchased from Solebo Company (Beijing, China). The cells of each group were seeded in 6-well plates at a density of 2 × 10⁵ cells/well. Next, the cells were digested with trypsin, and centrifuged at 1500 r/min for 5 min. Four hundred microliters binding buffer was added to resuspend the cells. Then 4 µL annexin V-FITC and 4 µL PI were added. The cells were cultured in dark for 15 min. Cell apoptosis was detected by flow cytometry.

TUNEL Assay Apoptosis of H9c2 cells were examined by TUNEL assay. Cells were ﬁxed with 4% formaldehyde for 15 min and dehydrated with 50, 75, 95, and 100% ethanol for 5 min. Then the cells washed twice with phosphate buffer solution and treated with 0.5% Triton X-100 for 20 min. At last, TUNEL working solution was added and the cells were incubated at 37 °C for 1 h. The apoptotic cells were detected by ﬂuorescence microscope.

Statistical Analysis Each independent was conducted in triplicated (n = 3). All data were analyzed using SPSS 19.0 and expressed as mean ± standard deviation (S.D.). The difference among multiple groups were analyzed using one-way ANOVA. p < 0.05 was deemed as statistical significance.

RESULTS

Ginsenoside Rb2 Promoted Cell Proliferation in H9c2 Cells Previous studies reveal that Ginsenoside Rb2 play a crucial role CVD. To test the effects of ginsenoside Rb2 on the myocardial cells, we ﬁrst tried to ﬁnd out the suitable concentration of ginsenoside Rb2. The results indicated that 100 µM of Rb2 inhibited the proliferation of H9c2 cells. Thus, we chose 10 µM as the low concentration of Rb2 while 50 µM as the high concentration (Fig. 1, n = 3).

Ginsenoside Rb2 Inhibited H9c2 Cells Apoptosis To investigate whether Ginsenoside Rb2 play a role in the H₂O₂ induced cell injury model. We ﬁrst detected the level of several cell injury related biomarkers. The results indicated that H₂O₂ treatment signiﬁcantly elevated the level of CK-MB, cTn-I and LDH, while Ginsenoside Rb2 administration decreased the level of them compared to the model group (Figs. 2A–C, n = 3). CCK-8 and EdU staining were used to detect the cell proliferation. We found that H₂O₂ treatment inhibited the proliferation of H9c2 cells while Ginsenoside Rb2 inhibited it (Figs. 2D, E, n = 3). Apoptosis is known to be critically engaged in coronary heart disease. 25,26 We used ﬂow-cytometric analysis to test the ginsenoside Rb2 effect on H9c2 cells apo-

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**Fig. 1. The Effect of Ginsenoside Rb2 on the Proliferation of H9c2 Cells**

(A) The Chemical structure of Ginsenoside Rb2. (B) CCK-8 was used to evaluate the proliferation of H9c2 cells. n = 3. **p < 0.01.**
ptosis. Treating H9c2 cells with ginsenoside Rb2 has markedly reduced apoptotic cell presence compared to control group in a dose-dependent manner (Fig. 2F, \(n = 3\)).

**Ginsenoside Rb2 Exhibited a Cardioprotective Effect in H9c2 Cells through Nrf2/HO-1 Signaling Pathway**

We utilized Western blotting to elucidate the underlying mechanism of the cardioprotective effect of ginsenoside Rb2 in H9c2 cells. Compared to the control group, \(\mathrm{H}_2\mathrm{O}_2\) treatment attenuated the expression of Nrf2 and HO-1. Ginsenoside Rb2 has been shown to promote the expression of Nrf2 and HO-1 protein (Figs. 3A, B, \(n = 3\)).

**BML-111 Obstructed the Cardioprotective Effect of Ginsenoside Rb2**

Following our previous finding where ginsenoside Rb2 exhibited its cardioprotective effect through
the Nrf2/HO-1 signaling pathway. We used the Nrf2 inhibitor BML-111 to confirm whether the ginsenoside Rb2 exerts its effect through the Nrf2/HO-1 signaling pathway or differently. Compared to the control group, the co-treatment of H9c2 cells with ginsenoside Rb2 and BML-111 has reversed the cardioprotective effect of ginsenoside Rb2 and inhibited the H9c2 cell injury (Figs. 4A–C, n = 3). Cell proliferation and apoptosis was further analyzed. The results indicated that BML-111

Fig. 4. BML-111 Obstructed the Cardioprotective Effect of Ginsenoside Rb2

(A–D) The level of cell injury related biomarker including CK-MB, cTn-I and LDH was detected by ELISA. Cell proliferation was detected by (D) CCK-8 and (E) EdU staining. (F) Cell apoptosis was evaluated by flow cytometry. *p < 0.05, **p < 0.01, n = 3.

![Figure 4](image_url)

Fig. 5. BML-111 Obstructed the Effect of Ginsenoside Rb2 on Nrf2/HO-1 Signaling Pathway

(A, B) Western blot was used to evaluate the expression level of Nrf2 and HO-1. **p < 0.01 vs. CON, *p < 0.05 vs. Model, **p < 0.01 vs. Model. n = 3.

![Figure 5](image_url)
reversed the effect of ginsenoside Rb2 on the proliferation and apoptosis of H9c2 cells. (Figs. 4D–F, n = 3).

BML-111 Attenuated Ginsenoside Rb2 Effect on the Nrf2/HO-1 Signaling Pathway We used Western blotting to confirm the inhibitory effect of BML-111 on ginsenoside Rb2 regulation of Nrf2/HO-1 signaling pathway. Compared to the control group, the co-treatment of BML-111 and ginsenoside Rb2 has reversed the effect of ginsenoside Rb2 effect on the Nrf2/HO-1 signaling pathway and significantly inhibited the expression of Nrf2 and HO-1 (Fig. 5, n = 3).

DISCUSSION

There is an increase in the research on ginsenosides in the treatment of cardiovascular disease. Researchers have investigated different ginsenosides compounds and their underlying mechanisms in coronary heart disorders.27,28 Recently, there is little research about the ginsenoside Rb2 cardioprotective effects. It has been previously reported that the ginsenoside Rb2 could exhibit a cardioprotective effect by scavenging oxidative stress products29, it can also alleviate the myocardial ischemia/reperfusion injury in rats through SIRT1 activation.30 In the present research, we studied the cardioprotective effect of ginsenoside Rb2 on H9c2 cells in vitro. Our analysis has found that compared to the control group (H2O2-induced stress in H9c2 cells), ginsenoside Rb2 has significantly promoted H9c2 cell proliferation. Several studies have elucidated that cardiomyocyte apoptosis is a pathophysiological hallmark in injury induced by oxidative stress and MI/R.31 Furthermore, ginsenoside Rb2 exerted its anti-apoptotic effects via activating Nrf2/HO-1 signaling pathways. Our results have rigor confirmed that ginsenoside Rb2 exhibits an anti-apoptotic effect where it diminished the apoptotic cell presence in H9c2 cells compared to the control group.

Nrf2/HO-1 signaling pathway has been long known as a major regulatory pathway in the oxidative stress. Shanmugam et al. reveal that cardiac-specific Nrf2 induces redox homeostasis and protect against oxidative stress and pathologic remodeling.32 The degradation of Nrf2 contributes to heart failure after myocardial infarction.33 Overexpression of Nrf2 suppresses the ferroptosis of cardiomyocytes as well as cardiomyopathy.34 Thence, to activate Nrf2 signaling pathways may be a primising therapy for oxidative stress as well as oxidative stress induced cell death in CVD. In this study, ginsenoside Rb2 suppressed the release of MDA, and increased the level of glutathione (GSH). Additionally, ginsenoside Rb2 suppressed the apoptosis of cardiomyocytes. These results suggested that possessed anti-oxidant and anti-apoptotic properties, which is in line with Choi et al. study.35 Moreover, ginsenoside Rb2 stimulated Nrf2/HO-1 signaling pathways. Allopurinol alleviates diabetic cardiomyopathy in vivo via activating Nrf2 signaling.36 Myricetin suppresses pathological cardiac hypertrophy via activating TRAF6/TAK1/mitogen-activated protein kinase (MAPK) and Nrf2 signaling pathway.37 In this study, Nrf2 was downregulated after CHD in vitro. Knockdown of Nrf2 alleviated the effects of ginsenoside Rb2 and promoted the apoptosis of cardiomyocytes. These results suggested that ginsenoside Rb2 may restore heart function via activating Nrf2 signaling.

However, previous study mainly focus on the roles of Nrf2 in renal disease, skeletal muscle abnormalities, and multiple sclerosis.38 However, the potentials of Nrf2 signaling in CHD has not been fully elucidated. Recently, its anti-oxidant and hyper-proliferative properties attaches increasing attention in CHD. The activation of Nrf2 suppresses oxidative stress and restores heart function.4,39 Hence Nrf2 may play a heart protective role in CHD. However, after BML-111 treatment, the effect of Rb2 was not completely reversed to the level of the model group. We suspected there may be other signaling pathway involved in the CHD development, such as phosphatidylidyinositol 3-kinase (PI3K)/AKT40 or NF-KappaB41 signaling pathways. Further study will be carried out to investigate the underlying mechanisms.

To our knowledge, the results and cogitations in this manuscript of authors’ is the first to investigate the effects of ginsenoside Rb2 on oxidative stress in CHD. Ginsenoside Rb2 exerts a cardioprotective effect in vitro through the Nrf2/HO-1 signaling pathway in H9c2 cells. The underlying mechanism involved in this process may be attributed to the scavenging of the apoptotic presence by increasing the protein levels of Nrf2 and HO-1.

CONCLUSION

Ginsenoside Rb2 exerts its cardioprotective effect through Nrf2/HO-1 pathway and inhibiting cellular apoptosis. These findings highlighted a novel mode of action of the ginsenoside Rb2 and its possible use in treating coronary heart disorders.

Conflict of Interest The authors declare no conflict of interest.

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