Original Article
Protective effects of ginsenoside Rg2 against H$_2$O$_2$-induced injury and apoptosis in H9c2 cells

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Abstract: Ginsenoside Rg2 is one of the major active components of ginseng and has many biological activities. This study aimed to investigate the protective effects of ginsenoside Rg2 against H$_2$O$_2$-induced injury and apoptosis in H9c2 cells. The results showed that pretreatment with ginsenoside Rg2 not only increased cell viability, but also decreased lactate dehydrogenase (LDH) release. Ginsenoside Rg2 inhibited the decrease of SOD, GSH-PX activities and the increase of MDA content induced by H$_2$O$_2$. Meanwhile, the levels of ROS generation and cardiomyocyte apoptosis in ginsenoside Rg2 group significantly reduced when compared with the model group. Western blot analyses demonstrated that ginsenoside Rg2 up-regulate level of Bcl-2 expression and down-regulate levels of Bax, Caspase-3, -9 expression. These findings indicated that ginsenoside Rg2 could protect H9c2 cells against H$_2$O$_2$-induced injury through its actions of anti-oxidant and anti-apoptosis.

Keywords: Ginsenoside Rg2, hydrogen peroxide, oxidative stress, apoptosis

Introduction

Despite significant improvement in treatment strategies in recent years, ischemic heart disease (IHD) remains the leading cause of morbidity and mortality in both the developed and developing countries [1, 2]. Oxidative stress plays a critical role in the pathophysiology of cardiovascular diseases such as atherosclerosis, hypertension, heart failure, and myocardial ischemic reperfusion injury [3-5]. Oxidative stress causes excessive production of reactive oxygen species (ROS), which is an important event in the development of cardiovascular diseases. ROS accumulation may contribute to a number of cardiovascular disorders [6, 7]. Cellular sources of ROS come from the mitochondrial electron transport chain, xanthine oxidase, NADPH oxidase, lipooxygenase/cyclooxygenase, nitric oxide synthase, and autoxidation of various substances particularly catecholamines [8, 9]. If oxidative insult persists, programmed cell death will be initiated partially through the mitochondrial pathway, causing the loss of functional cardiomyocytes. Therefore, treatment strategies to prevent oxidative stress are considered valuable for patients with IHD [10].

Panax ginseng C. A. Mayer (ginseng) has a range of pharmacological and therapeutic uses [11-14]. Ginsenosides are the major active components of ginseng, and they appear to be responsible for the principle pharmacological activities of ginseng, including vasorelaxation and anti-neoplastic, anti-diabetic, anti-inflammatory, and anti-oxidant effects [15-18]. Ginsenoside Rg2, protopanaxatriol-type compound, is one of the major active components in the root and stem leaves of ginseng (Figure 1). Ginsenoside Rg2 has been proposed with a wide range of pharmacological effects. Recently, researchers found that ginsenoside Rg2 blocked calcium channels and displayed anti-free-radical activity [19]. It has been reported that ginsenoside Rg2 inhibited the nicotinic acetylcholine receptors of bovine chromaffin cells from the acetylcholine-stimulated Na$^+$ influx [20, 21]. Then related researches have been shown that ginsenoside Rg2 inhibits the acetylcholine-induced inward current in Xenopus oocytes that express α3β4, α3β2, α4β4, or α4β2 neuronal and αβδε muscle-type nicotinic acetylcholine receptors [22, 23]. But there are few researches about the effect of ginsenoside Rg2 on myocardial injury. This study aimed to investigate the protective
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effects of ginsenoside Rg2 against H2O2-induced injury and apoptosis in H9c2 cells.

Materials and methods

Chemicals and materials

Ginsenoside Rg2 was provided by Professor Yifa Zhou (School of Life Sciences, Northeast Normal University). Analysis by HPLC showed that the purity of ginsenoside Rg2 was >98%. Antibodies against Caspase-3, -9, Bcl-2 and Bax were purchased from Cell Signal Technology (Beverly, MA, USA). Antibody against β-actin was obtained from Tianjing Jingmai. Lactate dehydrogenase (LDH), Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein assay reagent kit, DAPI staining kit, and ROS were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Annexin V-FITC apoptosis detection kit was obtained from Tianjin Sunge Biotech Co. Ltd (Tianjin, China). MTT and all other reagents were purchased from Sigma-Adrich Co. (St. Louis, MO, USA).

Cell culture and treatment

Rat embryonic cardiomyoblast derived H9c2 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academic of Science (Shanghai, China). H9c2 cells were cultured in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (GIBCO, Grand Island, NY, USA) under standard cultured conditions (37°C, 95% humidified air and 5% CO2). Cells were nearly 80% confluent and then treated with different concentrations of ginsenoside Rg2 for 4 h followed by treatment of 150 μM H2O2 for 6 h.

MTT assay

Cell viability was measured by MTT assay as described previously [24]. Cells were dispersed by trypsinization and seeded into 96-well plates overnight before being treated. Subsequently, 10 μL of MTT (Sigma, 5 mg/mL in PBS) solution was added to each well and plates were then incubated another for 4 h. Then 100 μL of dimethyl sulfoxide (DMSO) was added to each well, and the plates were shaken for 10 min. The absorbance was read at 570 nm with a microplate reader (SpectraMax Plus384, Molecular Devices, USA). Percentage of survival was calculated as a fraction of the negative control.

Measurement of lactate dehydrogenase activity

Measurement of Lactate dehydrogenase (LDH) activity was according the method described previously [25]. After treatment, culture medium was collected to measure LDH activity according to the manufacturer’s instruction, which was based on the reaction that LDH can catalyze lactate to form pyruvate. The formed pyruvate can react with 2,4-dinitrophenylhydrazine to form pyruvate-dinitrophenylhydrazone, which can present maroon in alkaline solution. Its absorbance at 440 nm was determined and reflected the LDH activity of the medium.

Determination of malondialdehyde content

After treatment as previous description, H9c2 cells were seeded into 6-well plates. When cells were nearly 80% confluent and then treated with different concentrations of ginsenoside Rg2 for 4 h followed by treatment of 150 μM H2O2 for 6 h. H9c2 cells were adjusted to 1×10^6 cell/mL after trypsinization, washed with phosphate-buffered saline (PBS) twice, and centrifuged at 1000 g for 10 min. The supernatant were removed. The precipitate obtained through centrifugation was crushed by ultrasonic wave, and the cell lysates were resuspended.
The content of cellular malondialdehyde (MDA), which was one product of lipid peroxidation, was determined according to the manufacturer’s instruction. The principle was according to the thiobarbituric acid test as previously described [26, 27]. It is based on the reactivity of MDA with TBA to produce a red adduct which can determine the absorbance at 532 nm with a microplate reader (SpectraMax Plus384, Molecular Devices, USA).

Determination of superoxide dismutase activity, and glutathione peroxidase activity

At the end of the treatment, H9c2 cells were adjusted to 1×10^6 cell/mL after trypsinization, washed with phosphate-buffered saline (PBS) twice, and centrifuged at 1000 g for 10 min. The supernatant were then removed. The precipitate obtained through centrifugation was crushed by ultrasonic wave, and the cell lysates were resuspended. According to the manufac-
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Figure 5. Effects of ginsenoside Rg2 on ROS accumulation in H9c2 cells. H9c2 cells were pretreatment with different concentrations of ginsenoside Rg2 (1, 3, or 10 μg/mL) for 4 h followed by treatment of 150 μM H₂O₂ for 6 h. Model group only treated with 150 μM H₂O₂ for 6 h. A. ROS generation was measured by the DCF fluorescence intensity. B. Quantitative analysis of ROS generation. Data presented are the mean ± SD. **P < 0.01 compared with the control groups; ##P < 0.01 compared with the model group.

Figure 6. Effects of ginsenoside Rg2 on (A) MDA, (B) SOD and (C) GSH-PX. H9c2 cells were pretreatment with different concentrations of ginsenoside Rg2 (1, 3, or 10 μg/mL) for 4 h followed by treatment of 150 μM H₂O₂ for 6 h. Model group only treated with 150 μM H₂O₂ for 6 h. Data presented are the mean ± SD. **P < 0.01 compared with the control group; *P < 0.05, **P < 0.01 compared with the model group.

Figure 7. Effects of ginsenoside Rg2 on apoptosis. H9c2 cells were pretreatment with different concentrations of ginsenoside Rg2 (1, 3, or 10 μg/mL) for 4 h followed by treatment of 150 μM H₂O₂ for 6 h. Model group only treated with 150 μM H₂O₂ for 6 h. Cell apoptosis was evaluated by DAPI staining. Magnification: ×200.

turer’s instruction, superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were determined with a microplate reader (SpectraMax Plus384, Molecular Devices, USA). Protein content was measured with the BCA Bradford protein assay (Jiangsu, China).
Measurement of ROS

The production of the reactive oxygen species (ROS) was monitored by nonfluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) as previously described [28]. DCFH-DA readily diffuses into the cell and is deacetylated by nonspecific esterases to yield nonfluorescent 2',7'-dichlorofluorescein (DCFH). In the presence of cellular oxidizing agent, DCFH is oxidized to the highly fluorescent compound dichlorofluorescein (DCF), which is trapped inside the cells [29]. Thus, the fluorescence intensity is proportional to the amount of ROS produced in the cells. After treatment, H9c2 cells were collected, washed twice with PBS and then incubated with DCFH-DA (10 μM) at 37°C for 20 min in the dark. H9c2 cells after incubation with H2O2 were washed three times with medium. The fluorescence of DCFH was detected by flow cytometry.

DAPI staining

The DAPI staining was performed as previously described [30]. Briefly, H9c2 cells were collected by trypsin and seeded in the coverslips. After treatment, the coverslips were washed twice with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with 2 μg/mL DAPI for 10 min. Finally, the cells were observed under the fluorescence microscope (Nikon TE-2000U, Nikon Corporation, Tokyo, Japan).

Annexin V-FITC/PI assay

Annexin V-FITC/PI staining was done by flow cytometry as previously described [24]. After treatment, H9c2 cells were harvested with 0.25% trypsin and washed twice in ice cold PBS and resuspended in 300 μL of binding buffer containing 1 μg/mL PI and 0.05 μg/mL Annexin V-FITC. The samples were incubated for 15 min at room temperature in dark and were analyzed by flow cytometry (Becton-Dickinson, USA).

Western blot analysis

Western blot was performed for detection of Caspase-3,-9, Bax and Bcl-2 proteins. After treatment, H9c2 cells were harvested and lysed in RIPA buffer for 30 min on ice. The protein concentration was determined using the BCA protein assay kit. The cell extract (20 μg) were loaded onto 12% polyacrylamide-SDS gel. After electrophoresis, the gel was blotted onto a PVDF membrane, blocked with 5% (w/v) non-fat milk for 1 h. The transferred membrane was incubated with appropriate primary antibodies at 4°C overnight. Primary antibody binding was detected with secondary antibody conjugated to HRP, and visualized using ECL chemiluminescence.

Statistical analysis

The results are expressed as mean ± SD for three independent experiments. Statistical differences were evaluated using Student’s test or one-way analysis of variance (ANOVA). \( P < 0.05 \) was considered to be significant.

Results

Effects of ginsenoside Rg2 on cell viability

Cell viability was measured by MTT assay. H9c2 cells were treated with ginsenoside Rg2 (0, 1, 3, 10, 30, or 300 μg/mL) for 10 h. None of these ginsenoside Rg2 caused damage to H9c2 cells compared with the control group \( (P > 0.05) \). Treatment with H2O2 (150 μM, 6 h) significantly decreased the viability of H9c2 cells compared with the control group \( (P < 0.01) \). Pretreatment with ginsenoside Rg2 (1, 3, 10 μg/mL) for 4 h increased cell viability compared with the model group \( (P < 0.01) \). The results showed that 1, 3, 10 μg/mL of ginsenoside Rg2 protected H9c2 cells from oxidative damage (Figure 2B).

Effects of ginsenoside Rg2 on morphologic changes

The morphologies of H9c2 cells treated with H2O2 (150 μM) in the presence of ginsenoside...
Rg2 (1, 3, 10 μg/mL) were observed with an inverted phase-contrast microscope (Nikon TE-2000U, Nikon Corporation, Tokyo, Japan). H9c2 cells in the control group were normal with long fusiform shapes. H2O2 treatment induced distinctive morphological changes, such as cell shrinkage, irregular shape, and a wider intercellular gap. However, the proportion of abnormal cells in 1, 3, 10 μg/mL of ginsenoside Rg2-pretreated groups decreased significantly (Figure 3).

**Effects of ginsenoside Rg2 on LDH activity**

To confirm the protective effects of ginsenoside Rg2 against oxidative stress-induced cell injury,
we measured LDH release in each group. LDH activity reflects the cell membrane damage caused by oxidative stress. LDH activity in the culture medium of the model group was significantly higher than that in the control group ($P < 0.01$, Figure 4), suggesting that H$_2$O$_2$ exposure led to severe cell injury. Pretreatment with ginsenoside Rg2 significantly reduced the levels of LDH activity in a concentration-dependent manner compared with the model group ($P < 0.01$, Figure 4).

**Effects of ginsenoside Rg2 on oxidative stress**

To determine whether the protective effects of ginsenoside Rg2 on H9c2 cells were related to reduce oxidative stress, we evaluated the effects of ginsenoside Rg2 on intracellular ROS in H9c2 cells. H$_2$O$_2$ exposure increased cellular ROS production significantly, reflected by the higher DCFH-DA fluorescence intensity in the model group compared with the control group ($P < 0.01$, Figure 5). However, ROS generation was significantly reduced by ginsenoside Rg2 pretreatment in a dose-dependent manner compared with the model group ($P < 0.01$, Figure 5).

It was found that ginsenoside Rg2 pretreatment reduced intracellular ROS, suggesting that ginsenoside Rg2 may reduce the ROS production induced by H$_2$O$_2$ exposure. An excessive accumulation of ROS may lead to severe cellular membrane damage through peroxidative lipid injury. As shown in Figure 6, the content of MDA, an index of lipid peroxidation was significantly increased, while the activities of SOD and GSH-PX were decreased significantly in the model group compared with the control group ($P < 0.01$). Ginsenoside Rg2 pretreatment significantly decreased the MDA content and increased SOD and GSH-PX activities compared with the model group ($P < 0.05$ or $P < 0.01$, Figure 6A-C).

**Effects of ginsenoside Rg2 on apoptosis**

To clarify whether ginsenoside Rg2 protects H9c2 cells by anti-apoptosis, we detected apoptotic nuclei and DNA fragmentation in H$_2$O$_2$-treated cells with or without ginsenoside Rg2. Most nuclei in either control or ginsenoside Rg2 group displayed uniform blue chromatin with organized structure. In contrast, intense DAPI stained nuclei that indicate cells undergoing apoptosis were frequently observed in H$_2$O$_2$-stimulated cells compared to control cells. However, pretreatment with ginsenoside Rg2 decreased apoptotic nuclei induced by H$_2$O$_2$. These data proposed that ginsenoside Rg2 has a protective role in H$_2$O$_2$-induced apoptotic cell death of H9c2 cells (Figure 7).

Furthermore, we also detected the apoptotic rate by Annexin V-FITC/PI staining. As shown in Table 1, the apoptotic rate was increased significantly in the model group compared with the control group ($P < 0.01$). Pretreatment with ginsenoside Rg2 treatment significantly reduced the percentage of Annexin V-FITC positive cells compared with the model group ($P < 0.01$). The apoptotic rate was 14.43%, 9.42%, and 8.01% in H9c2 cells treated with 1, 3, 10 μg/mL of ginsenoside Rg2, respectively.

**Effects of ginsenoside Rg2 on expression of Bcl-2, Bax, and Caspase-3, -9**

Ginsenoside Rg2 pretreatment restored protein levels of the anti-apoptotic molecule Bcl-2 and reduced expression of the apoptotic molecule Bax, Caspase-3, -9 in H$_2$O$_2$-subjected H9c2 cells (Figure 8).

**Discussion**

We chose a model of H$_2$O$_2$-induced oxidative stress in H9c2 cells and found that H$_2$O$_2$ exposure led to significant activated oxidative stress in the cells, which was characterized by reduced cell viability, increased intracellular ROS and lipid peroxidation, and reduced intracellular antioxidant activity. These pathophysiological processes led to apoptosis. More importantly, pretreatment with ginsenoside Rg2 significantly increased cell viability and decreased LDH release. In addition, ginsenoside Rg2 reduced ROS generation, cardiomyocyte apoptosis and enhanced antioxidant abilities, such as increasing SOD, GSH-PX activities and decreased MDA content. The present study indicated that ginsenoside Rg2 may protect H9c2 cells from H$_2$O$_2$-induced injury through its actions of anti-oxidant and anti-apoptosis.

Oxidative stress plays an important role in the pathogenesis of many cardiovascular diseases [31]. The balance between ROS production and clearance of endogenous antioxidants is destroyed during oxidative stress, resulting in
up-regulation of endogenous antioxidants. A previous study reported that ginsenoside Rg2 was capable of blocking calcium channels and exerting anti-free radical actions in cultured myocardiocytes [19]. It was also reported that ginsenoside Rg2 could decrease the elevated level of intracellular Ca\(^{2+}\) and reduce the lipid peroxidation (the excessive production of MDA and NO) induced by glutamate in PC12 cells [32]. In this study, pretreatment with ginsenoside Rg2 significantly not only increased SOD, GSH-PX activities, but also decreased ROS, MDA content. Our results were in agreement with previous results [19, 32], which results indicated that ginsenoside Rg2 has anti-oxidant effect.

Apoptotic process regulatory proteins, including Bcl-2 and Bax, are located in the mitochondrial membrane. Imbalance of these regulatory proteins plays a key role on apoptosis [33]. The caspase proteases are also believed to play a critical role in mediating apoptosis. Two different caspase pathways (extrinsic and intrinsic pathways) are involved in mediating the response. Both extrinsic and intrinsic pathways lead to activation executioner Caspase (Caspase-3). Caspase-3 induces cell shrinkage, nuclear condensation and DNA fragmentation [34]. Previous report indicated that ginsenoside Rg2 has a beneficial effect on the rat memory impairment in vivo induced by ischemia-reperfusion, possibly through prevention of the development of apoptosis. Ginsenoside Rg2 might mediate this effect by down-regulating the expression of pro-apoptotic factors Bax and P53 and up-regulating Bcl-2 and HSP70 [35]. Our data indicated that ginsenoside Rg2 could attenuate the apoptosis induced by H\(_2\)O\(_2\) in H9c2 cells through restored protein levels of Bcl-2 and reduced expression of Bax and Caspase-3, -9. Therefore, the underlying mechanism of protective effects of ginsenoside Rg2 was associated with its anti-apoptosis potency.

In conclusion, our study indicated that ginsenoside Rg2 may protect cardiomyocytes from H\(_2\)O\(_2\)-induced injury by inhibiting ROS production, increasing intracellular antioxidants, and attenuating apoptosis.

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Disclosure of conflict of interest

None.

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