Protective Effects of Ginsenoside Rc Against Acute Cold Exposure Induced Myocardial Injury in Rats

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Research

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Abstract

Background: Ginsenoside Rc is one of the cardinal bioactive components of *Panax ginseng* that has been studied for various biological activities. This study aimed to investigate the protective effects of ginsenoside Rc against acute cold exposure induced myocardial injury in rats.

Methods: The rats were intragastrically administrated with ginsenoside Rc (10, 20 mg/kg) or vehicle daily for 7 days. One hour after the seventh-day administration, 3% pentobarbital sodium was used to anesthetize the rats. Rats of the control group were kept in room temperature (22 ± 1 °C) and the other groups of rats were exposed to low ambient temperature (-15 ± 1 °C) in a cold chamber for 6 h. Cardiac function was monitored and recorded as well. To evaluate the protective effects of ginsenoside Rc, we also measured myocardial specific enzymes (lactate dehydrogenase, aspartate aminotransferase and creatine kinase-MB) in plasma, whole blood viscosity (WBV), plasma viscosity (PV), erythrocyte sedimentation rate (ESR) and hematocrit (HCT). The pathological changes of myocardium were observed through histopathological examination. The mRNA expression levels of TNF- α , IL-1 β and IL-6 were by real-time quantitative PCR analysis. And the expressions of silent information regulator1 (SIRT1), B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax) and Procaspase-3 in heart tissues were measured by Western blot method.

Results: Ginsenoside Rc alone had no effect on cardiac function, myocardial enzyme activities and hemorheology in normal rats. Compared with the model group, pretreatment with ginsenoside Rc significantly improved cardiac function, diminished myocardial specific enzymes release and decreased erythrocyte aggregation index. As a result, the pretreatment regulated abnormal hemorheology, attenuated myocardial histological changes and structural abnormalities, reduced the mRNA expression levels of cardiac inflammatory markers. Ginsenoside Rc also resulted in up-regulating SIRT1, Bcl-2 and Procaspase-3 expressions and down-regulating Bax expression.

Conclusion: The current study suggests that ginsenoside Rc alleviates acute cold exposure induced myocardial injury in rats by inhibiting cardiomyocyte apoptosis *via* SIRT1 and reducing inflammatory response.

Background

Exposure to cold weather will have many health effects on human beings, mainly affecting military personnel, homeless people and adventurers (1). In particular, extreme cold temperature condition is a vital risk factor associated with increased cardiovascular morbidity and mortality (2). Many researches have confirmed an association of cold exposure with adverse cardiovascular responses, for example, increased heart rate, blood pressure, systemic vascular resistance and levels of plasma norepinephrine. All of these detrimental reactions can potentially lead to arrhythmias, myocardial ischemia and infarction (2–4). Whereas, the exact mechanisms of these adverse effects in the myocardium under cold exposure remain unclear.

Panax ginseng C. A. Meyer (*P. ginseng*) is an herbal plant that has been used as a traditional medicine in Asian countries for thousands of years. Ginsenosides are the principle active constituent found in *P. ginseng* and exhibit anti-oxidative, anti-inflammatory and anti-cancer activities (5–7). Ginsenoside Rc is one of protopanaxadiol type ginsenosides that has been studied for various biological activities (8–10) (Fig. 1). It is reported that ginsenoside Rc can suppress oxidative stress by influencing FoxO1 activity *via* Akt pathway in human embryo kidney 293T cells (10).

The mammalian sirtuins are a highly conserved family of NAD⁺-dependent enzymes that regulate cellular stress resistance, energy metabolism, genomic stability and tumorigenesis (11). Silent information regulator 1 (SIRT1) is one of the sirtuins family which is implicated in cell survival under stress and longevity in mammals (12–14). Several studies have suggested that SIRT1 plays a critical role in myocardial injury. SIRT1 has cardioprotective effects in the ischemia-reperfusion injury to cardiac muscle (15). Another research elucidated that moderate expression of SIRT1 induces resistance to apoptosis and oxidative stress in the heart (16).

It is still not known, however, whether ginsenoside Rc has protective effects in myocardial injury. In the current study, we aimed to evaluate the effective impacts and latent mechanisms of ginsenoside Rc on protecting myocardial injury in acute cold exposure model rats.

Methods

Animals

Male Wistar rats (240 to 260 g) were from Liaoning Changsheng Biotechnology Co. Ltd. (Benxi, China). Animals were housed and maintained on a 12-h light/dark cycle at a controlled temperature and humidity with unlimited access to food and water. The experiments were approved by the Ethics Committee of Jilin University and were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (publication 86 – 23, revised in 1986). Every effort was made to minimize discomfort and to reduce the number of animals used.

Reagents

Ginsenoside Rc (purity > 98% by HPLC) was provided by Professor YiFa Zhou (School of Life Sciences, Northeast Normal University). Kits for lactate dehydrogenase (LDH), aspartate aminotransferase (AST), creatine kinase-MB (CK-MB) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Hifair® 1st Strand cDNA Synthesis SuperMix and Hieff® qPCR SYBR Green Master Mix (Low Rox Plus) was bought from YEASEN (Shanghai, China). Antibodies against SIRT1, Caspase-3, Bax and Bcl-2 were bought from Affinity Biosciences (OH, USA). Antibodies against β -actin and HRP Goat Anti-Rabbit IgG (H + L) were bought from Beyotime Institute of Biotechnology (Nantong, China).

Experimental protocol

Twenty rats were randomly divided into two groups ($n = 10$ in each group): control and ginsenoside Rc at a dose of 20 mg/kg groups. The rats of control group were intragastrically administrated with 0.5% CMC-Na daily for 7 days. The animals in ginsenoside Rc group were intragastrically treated with ginsenoside Rc daily for 7 days.

Forty rats were randomly divided into four groups ($n = 10$ in each group): control group, model group and ginsenoside Rc (10, 20 mg/kg) groups. The rats of control and model groups were intragastrically administrated with 0.5% CMC-Na daily for 7 days. The animals in ginsenoside Rc groups were intragastrically treated with ginsenoside Rc at doses of 10 or 20 mg/kg, daily for 7 days. One hour after the seventh-day administration, 3% pentobarbital sodium was used to anesthetize the rats. The rats of control group were kept in room temperature (22 ± 1 °C) and the other groups of rats were exposed to low ambient temperature (-15 ± 1 °C) in a cold chamber for 6 h (17).

Cardiac function evaluation

Rats were anesthetized and evaluated for cardiac function immediately after acute cold exposure. A 2 F polyethylene catheter was inserted into the left ventricle by the right common carotid artery. The catheter was connected with a hemodynamic analyzing system (Model RM-6000, Nihon Kohden, Japan). Then, left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), and positive (+dp/dt) and negative (-dp/dt) maximal values of the first derivative of left ventricular pressure were evaluated.

Myocardial specific enzyme and hemorheology assays

Myocardial specific enzymes (LDH, AST and CK-MB) in plasma were examined using a Roche Cobas 8000 automatic biochemical analyzer (Roche Holding Ltd, Basel, Switzerland). Whole blood viscosity (WBV) and plasma viscosity (PV) were measured by LBY-N6 Compact automatic blood rheometer (Beijing Pulisheng Instrument Co., Ltd., Beijing, China). The erythrocyte sedimentation rate (ESR) was recorded by placing 1 mL whole blood in the accretion tube for 1 h. Then, the pipette was centrifuged at 4,000 rpm for 30 min, and the hematocrit (HCT) was recorded.

Histopathological examination

For light microscopic evaluation, tissue sections from the left ventricles were fixed in phosphate buffered 10% formaldehyde buffer at room temperature. The specimens embedded with paraffin were cut into 3–4 μm thick sections and stained with hematoxylin-eosin (HE) (18). The sections were examined by an

experienced observer who was blind to the treatment under light microscope and then photomicrographs were taken.

Real-time quantitative PCR analysis

The total RNA from cardiac tissue was prepared using TRIzol (Thermo Fisher, USA) following the manufacturer's protocols for RT-qPCR. Synthesis of cDNA and qPCR were determined using Hifair® 1st Strand cDNA Synthesis kit and SYBR Green Master Mix kit (YEASEN, China). The amount of TNF- α , IL-1 β and IL-6 mRNA was normalized to the expression of β -actin. All of the primers were synthesized by Beijing Dingguo Changsheng Biotechnology Co. Ltd. The following primers were used: TNF- α forward 5'-GTCGTAGCAAACCAAGC-3', reverse 5'-TGTGGGTGAGGAGCACGTAG-3'; IL-1 β forward 5'-GCAATGGTCGGGACATAGTT-3', reverse 5'-AGACCTGACTTGGCAGAGG-3'; IL-6 forward 5'-AGTGCATCATCGCTGTTCATACA-3', reverse 5'-ATATGTTCTCAGGAGATCTTGGAA-3'; β -actin forward 5'-GATCAAGATCATTGCTCCTCCTG-3', reverse 5'-AGGGTGTAACGCAGCTCA-3'. Fold changes in the target gene expression were calculated by the relative quantification method ($2^{-\Delta\Delta CT}$).

Western blot

The proteins of heart tissue samples were loaded (50 μ g) and subjected to 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes (EMD Millipore Corporation, Billerica, USA) for 1.5 h at 100 V. The membranes were blocked with 5% nonfat milk for 1 h and incubated with the primary antibodies: rabbit anti-SIRT1 (1:2,000, Affinity Biosciences, OH, USA), rabbit anti-caspase-3 (1:1,000, Affinity Biosciences, OH, USA), rabbit anti-Bax (1:1,000, Affinity Biosciences, OH, USA), rabbit anti-Bcl-2 (1:1,000, Affinity Biosciences, OH, USA) or rabbit anti- β -Actin (1:1,500, Beyotime Institute of Biotechnology, Nantong, China) overnight at 4 °C. The membranes were processed with the horseradish peroxidase-labeled secondary antibody (1:5,000, Beyotime Institute of Biotechnology, Nantong, China). The bands were visualized using ECL plus enhanced chemiluminescence kit (Affinity Biosciences, OH, USA). The intensity of protein bands was measured with the NIH Image J software (version 1.62; National Institutes of Health, Bethesda, MD, USA). The ratio of the density of bands of the detected protein to that of β -actin was used for statistical analysis.

Statistical analysis

Experiments were implemented in duplicate/triplicate at least thrice. All values are expressed the mean value $\pm$ standard deviation (SD). Analysis was conducted using SPSS 22.0. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test. The *P* less than 0.05 was considered as statistically significant.

Results

Effects of ginsenoside Rc on cardiac function, myocardial enzyme activities and hemorheology in normal rats

Firstly, we evaluated the effect of ginsenoside Rc treatment on normal rats. Under experimental dosages, pretreatment with ginsenoside Rc had no significant effect on the LVSP, LVEDP, +dp/dt and -dp/dt ($P > 0.05$, compared with the control group, Fig. 2). In addition, the activities of LDH, AST and CK-MB were not significantly changed ($P > 0.05$, compared with the control group, Fig. 3). Then, we also examined WBV, including 20, 60, and 120 s^{-1} high and low shear rates, PV, ESR and HCT. There were no significant differences among the 2 groups ($P > 0.05$, Fig. 4). These findings indicated that ginsenoside Rc alone had no effect on cardiac function, myocardial enzyme activities and hemorheology in normal rats.

Effects of ginsenoside Rc on cardiac function in acute cold exposure rats

The cardiac function of rats was evaluated with a hemodynamic analyzing system. The LVEDP of the rats in the model group was significantly increased, as compared with the control group ($P < 0.01$). The LVSP, the + dp/dt, and the -dp/dt in the model group were markedly decreased ($P < 0.01$). Compared with the model group, LVEDP of the rats in the ginsenoside Rc groups was significantly decreased, whereas the LVSP, the + dp/dt, and the -dp/dt of the ginsenoside Rc groups were increased ($P < 0.05$ or $P < 0.01$). These results demonstrated that ginsenoside Rc has a property improving cardiac function in acute cold exposure rats (Fig. 5).

Effects of ginsenoside Rc on myocardial enzyme activities in acute cold exposure rats

The elevations of cardiac markers enzymes (such as LDH, AST and CK-MB) are important bases for the diagnosis of myocardial injury. As shown in Fig. 6, acute cold exposure resulted in a significant increase in the activities of LDH, AST and CK-MB ($P < 0.01$, compared with the control group). However, pretreatment with ginsenoside Rc (10 and 20 mg/kg) remarkably alleviated these conditions ($P < 0.01$, compared with the model group).

Effects of ginsenoside Rc on hemorheology in acute cold exposure rats

In this study, the results of WBV, including 20, 60, and 120 s^{-1} high and low shear rates, PV, ESR and HCT are shown in Fig. 7. Compared with the control group, WBV, PV, ESR, and HCT in the model group were

significantly increased ($P < 0.01$). Compared with the model group, pretreatment with ginsenoside Rc (10 and 20 mg/kg) notably reduced WBV at high and low shear rates (120, 60 and 20 s^{-1}), PV, ESR and HCT ($P < 0.05$ or $P < 0.01$). These results indicated that ginsenoside Rc could improve acute cold exposure rats by decreasing erythrocyte aggregation index and regulating abnormal hemorheology.

Effects of ginsenoside Rc on histopathological examination of cardiac tissues in acute cold exposure rats

As shown in Fig. 8, HE staining of heart tissues in the model group showed myocardial histological changes and structural abnormalities in acute cold exposure rats, including myocardial cell loss, widespread myocardial structure disorder, myocardium fragment and a high number of inflammatory cell infiltration. Pretreatment with ginsenoside Rc (10 and 20 mg/kg) significantly attenuated the pathophysiological changes in the cardiac muscle fiber.

Effects of ginsenoside Rc on inflammation response in acute cold exposure rats

Acute cold exposure induced large amounts of inflammatory factors in myocardium, and then aggravated myocardial damage. The pro-inflammatory factor levels were elevated noticeably in the model group ($P < 0.01$, compared with the control group, Fig. 9). Pretreatment with ginsenoside Rc decreased in the mRNA expression levels of TNF- α , IL-1 β and IL-6 ($P < 0.01$, compared with the model group, Fig. 9). These results showed that ginsenoside Rc reduced inflammation response in acute cold exposure induced injury.

Effects of ginsenoside Rc on expression of SIRT1, Bcl-2, Bax and Procaspase-3 in acute cold exposure rats

We further evaluated the expression of SIRT1 to explore the molecular mechanisms underlying the protective effects of ginsenoside Rc. We found that SIRT1 expression was markedly decreased in the model group ($P < 0.01$, compared with the control group, Fig. 10). However, pretreatment of the rats with ginsenoside Rc increased SIRT1 expression ($P < 0.01$, compared with the model group, Fig. 10). We next evaluated the apoptotic-related protein expressions. As shown in Fig. 10, acute cold exposure notably decreased the expression of procaspase-3 and Bcl-2, but increased the expression of Bax ($P < 0.01$, compared with the control group, Fig. 10). In contrast, pretreatment with ginsenoside Rc (10 and 20 mg/kg) decreased the expression of Bax and increased the expression of procaspase-3 and Bcl-2 ($P < 0.05$ or $P < 0.01$, compared with the model group). These results indicated that pretreatment with ginsenoside Rc may activate the SIRT1 expression, thus inhibiting the apoptotic signaling pathway.

Discussion

The results show that ginsenoside Rc protects the heart from inflammation and apoptosis. Stimulating endogenous SIRT1 appears to be beneficial to reduce the level of cold exposure induced myocardial injury.

One of mechanisms for cold exposure induced myocardial injury is ischemic. Microcirculatory perfusion depends not only on vascular contraction and perfusion pressure, but also on rheologic characteristics of the blood. Cold exposure can directly cause cardiovascular stress due to vasoconstriction and variations in blood pressure (19). The effect of hypothermia includes reductions in leukocyte mobility, elevations in plasma viscosity and red cell deformability which may lead to a reduction of blood flow through capillaries (20–22). It further slows oxygen delivery and reduces the elimination of toxic metabolites. Therefore, hypothermia will lead to myocardial dysfunction in clinic (23). However, there are few experiments on cold exposure induced heart injury in rats, and our results can play a complementary role. According to our experiments, ginsenoside Rc can effectively reduce the damage of acute cold exposure to myocardial function in rats.

Cytokines are known as signaling molecules to regulate cellular function and specifically involved in inflammation and immune response as well as in heart disease. Acute cardiac injury is associated with increased tissue level of IL-1 β (24). Additionally, IL-6 also plays a negative role in cardiac injury in most experimental and clinical studies (25, 26). Another class of cytokines, the tumor necrosis factor (TNF), is a ubiquitous cell signaling protein produced by various cells. Sustained inflammatory signaling by cardiac overexpression of TNF- α leads to proteotoxicity and cell death in the heart (27). In the present study, the high expression of IL-1 β , IL-6 and TNF- α in the myocardium may aggravate myocardial damage. The pretreatment with ginsenoside Rc exhibits anti-inflammatory effect by inhibiting expression of these cytokines.

Our further study shows that acute cold exposure induced cardiomyocyte apoptosis. Previously, several studies have demonstrated that apoptotic death of cardiomyocytes appears after exposure various damaging stimuli both in vitro and in vivo (28–31). SIRT1, Bcl-2 family and caspases are associated with apoptotic cell death in cardiomyocytes (32–34). Bcl-2 is a key mediator in the regulation of cardiac myocyte apoptosis which has a protective effect on mitochondria permeability (35). By contrast, Bcl-2-associated X (Bax), a proapoptotic member of the Bcl-2 family, can neutralize the activation of Bcl-2 by forming Bcl-2/Bax heterodimers (36). Bax also directly activates caspase-3 which results in cleavage of cytoskeletal and nuclear proteins (37). SIRT1 was observed in regulating expression of anti-/pro-apoptotic molecules. It was reported that SIRT1 upregulates Bcl-2 and downregulates Bax during ischemia-reperfusion in SIRT1 transgenic mice (38). Thus, SIRT1 is a potential target in the therapy of cardiac injury. In different models of cardiac injury, resveratrol as a SIRT1 activator has a protective effect (39, 40). Our previous study has showed ginsenoside Rg2 alleviates ischemia-reperfusion injury by activating SIRT1 signaling (41). Currently, our results show that ginsenoside Rc inhibits expression of Bax in cardiomyocytes after acute cold exposure. The levels of SIRT1, Bcl-2 and procaspase-3 are upregulated

by pretreatment of ginsenoside Rc. Thus, our results suggest that ginsenoside Rc inhibits cardiomyocyte apoptosis by activating SIRT1 in rats.

Conclusions

The current study suggests that ginsenoside Rc alleviates acute cold exposure induced myocardial injury in rats. The mechanisms of ginsenoside Rc involve suppression of inflammatory cytokine production and inhibition of cardiomyocyte apoptosis by activating SIRT1. Our findings indicate that ginsenoside Rc is a key cardioprotective component of *P. ginseng* which may be potentially valuable as a monomeric drug. However, acute cold exposure clinically is mostly unpredictable. The effect of ginsenoside Rc after the occurrence of cold exposure needs to be further studied. Furthermore, the cellular targets of SIRT1 during cardiac injury should be elucidated. Therefore, it is indeed necessary to develop more effective methods to protect the heart from acute cold exposure.

Abbreviations

ANOVA: analysis of variance; AST: aspartate aminotransferase; Bax: Bcl-2-associated X; Bcl-2: B-cell lymphoma-2; CK-MB: creatine kinase-MB; +dp/dt: positive maximal values of the first derivative of left ventricular pressure; -dp/dt: negative maximal values of the first derivative of left ventricular pressure; ESR: erythrocyte sedimentation rate; HCT: hematocrit; HE: hematoxylin-eosin; IL-1 β : interleukin-1 β ; IL-6: interleukin-6; LDH: lactate dehydrogenase; LVEDP: left ventricular end diastolic pressure; LVSP: left ventricular systolic pressure; NAD⁺: nicotinamide adenine dinucleotide; *P. ginseng*: *Panax ginseng* C. A. Meyer; PV: plasma viscosity; SD: standard deviation; SIRT1: silent information regulator 1; TNF: tumor necrosis factor; TNF- α : tumor necrosis factor- α ; WBV: whole blood viscosity.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Jilin University. All animals were treated in accordance with the Animal Care and Use Committee of Jilin University (Changchun, China).

Consent for publication

Not applicable.

Availability of data and materials

Available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SDY, YJA and FWW conceived and designed the study. XY, YXF, YP, LYG and FWW performed the experiments. XY wrote the paper. XY, FWW and ZXH were responsible critical revision of the manuscript. All authors read and approved the final version manuscript for publication.

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Figures

Figure 1

Molecular structure of ginsenoside Rc.

Figure 2

Effects of ginsenoside Rc on cardiac function in normal rats. Data were presented as the mean $\pm$ SD. n=10 per group. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test.

Figure 3

Effects of ginsenoside Rc on myocardial enzyme activities in normal rats. Data were presented as the mean $\pm$ SD. n=10 per group. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test.

Figure 4

Effects of ginsenoside Rc on hemorheology in normal rats. Data were presented as the mean $\pm$ SD. n=10 per group. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test.

Figure 5

Effects of ginsenoside Rc on cardiac function in acute cold exposure rats. Data were presented as the mean $\pm$ SD. n=10 per group. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test. ^{##}P< 0.01, compared with the control group; ^{*}P< 0.05, ^{**}P<0.01, compared with the Model group.

Figure 6

Effects of ginsenoside Rc on myocardial enzyme activities in acute cold exposure rats. Data were presented as the mean $\pm$ SD. n=10 per group. Statistical analysis among various groups was conducted

by one-way analysis of variance (ANOVA) with Tukey's post hoc test. ##P< 0.01, compared with the control group; **P<0.01, compared with the Model group.

Figure 7

Effects of ginsenoside Rc on hemorheology in acute cold exposure rats. Data were presented as the mean ± SD. n=10 per group. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey's post hoc test. ##P< 0.01, compared with the control group; *P< 0.05, **P<0.01, compared with the Model group.

Figure 8

Effects of ginsenoside Rc on histopathological examination of cardiac tissues in acute cold exposure rats. Representative hematoxylin-eosin (H&E) pathological photomicrographs of left ventricular tissue. n=4 per group. The cardiac apex was excised and fixed with 10% formaldehyde buffer for subsequent H&E staining. The sections were examined under light microscope, and then photomicrographs were taken. The top line: magnification× 200; the bottom line: magnification× 400.

Figure 9

Effects of ginsenoside Rc on inflammation response in acute cold exposure rats. The mRNA expression levels of TNF- α , IL-1 β and IL-6 in myocardium were measured. Data were presented as the mean $\pm$ SD. n=10 per group. Statistical analysis among various groups was conducted by one-way analysis of variance (ANOVA) with Tukey’s post hoc test. ##P< 0.01, compared with the control group; **P<0.01, compared with the Model group.

Figure 10

Effects of ginsenoside Rc on expression of SIRT1, Bcl-2, Bax and Procaspase-3 in acute cold exposure rats. Representative photograph and bar graph of SIRT1, Bcl-2, Bax and Procaspase-3. Data were presented as the mean $\pm$ SD. n=3 per group. Statistical analysis among various groups was conducted by

one-way analysis of variance (ANOVA) with Tukey's post hoc test. $##P < 0.01$, compared with the control group; $*P < 0.05$, $**P < 0.01$, compared with the Model group.