Salidroside mitigates hypoxia/reoxygenation injury by alleviating endoplasmic reticulum stress-induced apoptosis in H9c2 cardiomyocytes

MENG-YAO SUN¹, DA-SHI MA¹, SONG ZHAO², LEI WANG¹, CHUN-YE MA¹ and YANG BAI¹

Departments of ¹Cardiac Surgery and ²Spine Surgery, The First Hospital of Jilin University, Changchun, Jilin 130021, P.R. China

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Abstract. Endoplasmic reticulum (ER) stress-induced apoptosis serves a crucial role in the development of myocardial ischemia/reperfusion (I/R) injury. Salidroside is a phenylpropanoid glycoside isolated from Rhodiola rosea L., which is a plant often used in traditional Chinese medicine. It possesses multiple pharmacological actions and protects against myocardial I/R injury. Hypoxia/reoxygenation (H/R)-treated H9c2 cardiomyocytes were used in the current study to mimic myocardium I/R injury in vivo. It was hypothesized that salidroside alleviates ER stress and ER stress-induced apoptosis, thereby reducing H/R injury in H9c2 cells. The results demonstrated that salidroside attenuated H/R-induced H9c2 cardiomyocyte injury, as cell viability was increased, lactate dehydrogenase release was decreased, morphological changes in apoptotic cells were ameliorated and the apoptosis ratio was reduced compared with the H/R group. ER stress was reversed, indicated by the downregulation of glucose regulated protein 78 and C/EBP homologous protein following pretreatment with salidroside. In addition, salidroside attenuated ER stress-induced apoptosis, as the expression of cleaved caspase-12 and pro-apoptotic protein Bcl-2 associated X protein and activity of caspase-3 was decreased, while the expression of anti-apoptotic protein Bcl-2 was increased following pretreatment with salidroside. Furthermore, the results indicated that salidroside decreases the activation of the ER stress-associated signaling pathway, as the expression of phosphorylated protein kinase RNA (PKR)-like ER kinase (p-PERK) and phosphorylated inositol-requiring enzyme-1α (p-IRE1α) proteins were decreased following pretreatment with salidroside. These results demonstrate that salidroside protects against H/R injury via regulation of the PERK and IRE1α pathways, resulting in alleviation of ER stress or ER stress-induced apoptosis in H9c2 cardiomyocytes.

Introduction

Myocardial ischemia/reperfusion (I/R) injury remains a major public health problem worldwide, with high rates of morbidity and mortality; 3.8 million men and 3.4 million women succumb to mortality from the disease each year (1). Few investigations have investigated whether perioperative care and surgical techniques may be used stimulate myocardial functional recovery following myocardial ischemia (2). Multiple biological processes and cell signaling pathways that serve a role in myocardial I/R injury have been identified, including apoptosis, inflammation, oxidative stress and mitochondrial dysfunction (2,3). However, these mechanisms are not yet fully understood (4). Thus, it is important to develop novel effective treatments for ischemic damage in cardiac tissue and increase understanding of the potential pathogenesis of myocardial I/R injury.

Endoplasmic reticulum (ER) stress is a cellular process induced by a variety of severe stress conditions, including hypoxia, ischemia, heat shock, gene mutation and oxidative stress, which affects the folding of proteins in the ER and leads to activation of the unfolded protein response (UPR) (5,6). The UPR mediates ER stress and serves a role in the activation of three primary signaling pathways, including protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme-1α (IRE1α) and activating transcription factor 6 (ATF6) (7). Prolonged and/or excessive ER stress may result in apoptosis, which is an essential signaling pathway activated during myocardial damage resulting from I/R injury (8-10). ER stress-mediated apoptosis is associated with the IRE1α-mediated activation of the c-Jun N-terminal kinase cascade (11) and PERK-dependent induction of the pro-apoptotic transcriptional factor C/EBP homologous protein (CHOP) pathways (12). Previous studies have demonstrated that inhibition of ER stress-associated signaling pathways or...
ER stress-mediated apoptosis are potential therapeutic targets for the treatment of myocardial I/R injury (12,13).

Salidroside is a primary component of *Rhodiola rosea* L., which is used in traditional Chinese medicine (14). Several clinical and experimental studies have identified that salidroside exhibits multiple pharmacological activities, including anti-oxidation, anti-apoptosis, anti-inflammation, anti-stress and enhancement of immune function (15-17). It has also been demonstrated that salidroside has a protective effect on myocardial I/R injury, which is associated with anti-oxidative stress and anti-apoptosis (18,19); however, its exact underlying mechanisms have not yet been determined. Zhu *et al* (20) reported that salidroside protects against homocysteine-induced human umbilical vein endothelial cell injury by inhibiting ER stress via suppression of the ER stress pathway including PERK and IRE1α signaling pathways, suggesting that it may be developed as a promising therapeutic target for atherosclerosis and cardiovascular disease. However, to the best of our knowledge, there have been no reports investigating the role of ER stress or ER pathways in the protective effects of salidroside against myocardium I/R injury.

Therefore, cultured H9c2 cardiomyocytes treated with hypoxia/reoxygenation (H/R) were used in the present study to establish an *in vitro* model of myocardium I/R injury and the underlying mechanisms of salidroside against myocardial I/R injury were subsequently investigated. The results indicated that salidroside alleviates ER stress and ER stress-induced apoptosis, thereby attenuating H/R injury. Therefore, it may serve a role in inhibiting the IRE1α or PERK-mediated ER stress pathways. These results may provide novel insights for the treatment of myocardial I/R injury.

Materials and methods

Reagents. Salidroside (purity >99.7%) was purchased from Shanghai Green Valley Pharmaceutical Co., Ltd. (Shanghai, China), dissolved in PBS to a stock concentration of 10 mM and stored at -20°C. The stock solution was diluted with culture medium immediately prior to treatment. MTT, Hoechst 33258 and enhanced chemiluminescence reagents were obtained from Beyotime Institute of Biotechnology (Haimen, China). Lactate dehydrogenase (LDH) release and caspase-3 activity assay kits were supplied by Nanjing Jiancheng Bioengineering Institute (A020-2, and GO07, respectively, Nanjing, China). The Annexin-V fluorescein isothiocyanate/propidium iodide (FITC/PI) Apoptosis Detection kit was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Antibodies against glucose regulated protein 78 (GRP78) and Bcl-2 were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The primers used in qPCR were: GRP78 forward, 5’-AAA TAA TCA AGG-3’, CHOP forward, 5’-GGA GCT GGA GCC TCA GCG GTT TCT T-3’ and reverse, 5’-TCA AGT TCT GAC TCT CGT TCA AGG-3’. The primers used in qPCR were: GRP78 forward, 5’-AAA TAA TCA AGG-3’, CHOP forward, 5’-GGA GCT GGA GCC TCA GCG GTT TCT T-3’ and reverse, 5’-TCA AGT TCT GAC TCT CGT TCA AGG-3’; CHOP forward, 5’-GGAGCTGGA

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Levels of GRP78 and CHOP mRNA were measured using RT-qPCR. Total RNA was extracted from H9c2 cells using TRIzol reagent (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. An equal amount of RNA (1 µg) was used as a template and was reverse-transcribed into complementary DNA using a QuantiTect Reverse transcription kit (Qiagen GmbH, Hilden, Germany). qPCR was performed using a FastStart Universal SYBR Green Master kit (Roche Applied Science, Madison, WI, USA) on an ABI 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions for qPCR constituted 94°C for 2 min, 40 cycles of 94°C for 20 sec and 60°C for 30 sec. GAPDH was used as an internal control. The primer sequences for GRP78, CHOP and GAPDH were designed as described previously (21-23). primers used in qPCR were: GRP78 forward, 5’-AAATAG GCCCTCAAGGTTTCTT-3’ and reverse, 5’-TCAGTTTCT TGCCGTCAAAGG-3’; CHOP forward, 5’-GGAGCTGGA
AGCTTGGTATG-3' and reverse, 5'-GGGCACGTGACCTC TGTTTC-3' and GAPDH forward, 5'-TGAAGGGTGAG CCAAAG-3' and reverse, 5'-AGTCTTCTGGGTGCGAT GAT-3'. The relative expression of mRNA was calculated using the comparative 2-ΔΔCq method (24) and quantified against GAPDH. All reactions were run in triplicate for each gene.

Hoechst 33258 staining. Morphological changes of H9c2 cells treated with salidroside prior to H/R during apoptosis was assessed using a Hoechst 33258 staining kit. Cells at density of 1x10^6 cells/well in 6-well plates were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min at 4°C. Cells were then washed twice with PBS again and incubated with 5 µg/ml Hoechst 33258 for 10 min at 37°C in the dark. Nuclear morphology (magnification, x200) was then observed under a fluorescence microscope (BX51, Olympus Corporation, Tokyo, Japan). The excitation and emission wavelengths were 550 and 460 nm, respectively. Apoptotic cells elicited strong bright turquoise fluorescent signals outlining the chromatin-condensed nuclei, while normal cells were weakly stained with normal nuclei.

Apoptosis detection with Annexin V-FITC/PI staining and flow cytometry. Apoptosis was quantified using an Annexin-V-PI Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. H9c2 cells were seeded in 6-well plates at a density of 1x10^6 cells/well. Treated cells were harvested and rinsed using PBS. Cell suspensions were washed twice with ice-cold PBS prior to further processing. Cells were then gently resuspended in 500 µl Annexin V binding buffer. A total of 5 µl Annexin V-FITC and 5 µl PI were then added to each group and cells were gently vortexed. Following incubation for 10 min at room temperature, apoptosis was analyzed using an FACScan flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) at an excitation wavelength of 488 nm and emittance wavelength of 530 nm. The percentage of cells stained by Annexin V indicated early and late apoptosis. Each assay was performed in triplicate. Analyses were performed with GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

Caspase-3 activity assay. Caspase-3 activity in H9c2 cells was assayed using a Caspase-3 colorimetric assay kit, following the manufacturer's protocols. The caspase-3 enzyme catalyzes the formation of p-nitroaniline (pNA) by the acetyl-Asp-Glu-Val-Asp p-nitroanilide (ACDEVD-pNA) substrate. Following incubation, H9c2 cells were harvested and lysed in lysis buffer included in the kit, and then centrifuged at 1,000 x g for 10 min at room temperature. A total of 10 µl supernatant was co-incubated with 90 µl AC-DEV-pNA substrate solution (0.2 mM) at 37°C for >1 h. Absorbance was then measured at 405 nm using a Varioskan™ LUX multifunctional microplate reader.

Western blot analysis. Following treatment, H9c2 cells were harvested and lysed in radioimmunoprecipitation assay (Beyotime Institute of Biotechnology) buffer supplemented with protease inhibitor (Beyotime Institute of Biotechnology). The cell lysate was centrifuged at 12,000 x g for 10 min at 4°C. Protein concentrations were measured using a BCA protein assay kit. Equal amounts of proteins (50 µg/lane) was separated by 10-12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk in TBST (0.075% Tween in TBS) for 2 h at room temperature, membranes were incubated with primary antibodies against GRP78 (1:2,000; cat. no. 3177), CHOP (1:1,000; ab11419), cleaved caspase-12 (1:2,000; cat. no. 2202), Bax (1:2,000; cat. no. 14796), Bcl-2 (1:2,000; cat. no. 15071), IRE1α (1:2,000; cat. no. 3294), p-IRE1α (1:1,000; ab48187), PERK (1:2,000; cat. no. 3192), p-PERK (1:2,000; cat. no. 3179) and β-actin (1:2,000; cat. no. 3700) antibodies at 4°C overnight. Following incubation, membranes were washed three times with TBST and then probed with horse-radish peroxidase conjugated anti-mouse secondary antibody (1:5,000, cat. no. 14709, Cell Signaling Technology, Inc.) and anti-rabbit secondary antibody (1:5,000, cat. no. 14708, Cell Signaling Technology, Inc.) for 2 h at room temperature. Membranes were visualized using an enhanced chemiluminescence reagent (SignalFire™ Plus ECL Reagent, cat. no. 12757, Cell Signaling Technology, Inc.) with the ChemiDoc™ MP System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were quantified using Image Lab™ Software (version 4.1, Bio-Rad Laboratories, Inc.). The expression of β-actin was used as an internal control. Each experiment was repeated at least three times.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using a one-way analysis of variance followed by post hoc least significant difference tests. P<0.05 was determined to indicate statistically significant difference. Analyses were performed with GraphPad Prism 5.01 (GraphPad Software Inc.).

Results

Salidroside alleviates H/R-induced H9c2 cardiomyocyte injuries. H9c2 cells were subjected to hypoxia for 4 h followed by different times of reoxygenation (2, 4, 8, 12, or 16 h). Cell viability was then assessed using an MTT assay. Cell viability was significantly reduced following reoxygenation compared with the group that underwent 4 h hypoxia alone without treatment (Fig. 1A). The cell viability of the group that underwent hypoxia for 4 h and reoxygenation for 12 h was significantly decreased compared with the group that underwent hypoxia alone; therefore it was selected as the optimum treatment condition for subsequent experiments. Salidroside pretreatment significantly increased cell viability compared with the group that underwent H/R alone (Fig. 1B). In addition, H/R treatment significantly increased LDH release from H9c2 cells compared with the control group; however, this effect was reversed by salidroside pretreatment prior to H/R, as LDH release was significantly decreased compared with the H/R group (Fig. 1C). Salidroside treatment alone had no effect on cell viability and LDH release, as there was no significant difference between cell viability and LDH release in the salidroside pretreatment and control groups (Fig. 1B and C). The effect of salidroside on apoptosis under H/R was investigated and the results indicated that salidroside blocked H/R-induced morphological changes in apoptotic cells (Fig. 1D). It also
reversed the increase in the apoptosis ratio; the apoptosis ratio of the H/R group was significantly increased compared with the control but was significantly decreased in the salidroside pretreatment group compared with the H/R group (Fig. 1E and F). These results indicate that salidroside protects H9c2 cardiomyocytes against H/R-inducing cytotoxicity and apoptosis, thereby mitigating myocardial I/R injury.

Salidroside attenuates H/R-induced ER stress in H9c2 cardiomyocytes. Previous studies have established that ER stress serves a pivotal role in myocardial I/R injury (8-10); thus, the effect of salidroside on ER stress under I/R was investigated further. The expression of ER stress-associated mRNA and proteins, including GRP78 and CHOP, was measured using RT-qPCR and western blot analysis, respectively. H/R
significantly increased the expression of GRP78 (Fig. 2A) and CHOP (Fig. 2B) mRNA compared with the control group. These increases were attenuated by salidroside pretreatment, as the expression of the two genes were significantly decreased compared with the H/R group. The results of western blot analysis also indicated that salidroside reversed H/R-induced increases in the expression of GRP78 (Fig. 2C) and CHOP (Fig. 2D) proteins in H9c2 cells. These results suggest that salidroside alleviates H/R-induced ER stress in H9c2 cells.

Salidroside ameliorates ER stress-induced apoptosis in H/R-treated H9c2 cardiomyocytes. It has been confirmed that excessive ER stress in I/R-induced injury induces apoptotic signaling during ER stress (25,26). Thus, the expression and activity of proteins that serve a role in the ER stress-associated apoptotic pathway, including caspase-12, caspase-3 and the Bcl family, were investigated. The results indicated that salidroside significantly reduced the expression of cleaved caspase-12 (Fig. 3A and B) and activity of caspase-3 (Fig. 3C) compared with H/R treatment alone in H9c2 cells. It has been demonstrated that the Bcl-2 family serves a critical role in the pro- and anti-apoptotic system during ER stress (27). The effect of salidroside on the expression of pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 was also investigated using western blot analysis (Fig. 3D-F) and the results indicated that salidroside pretreatment significantly reversed the H/R-induced upregulation of Bax and downregulation of Bcl-2 in H9c2 cells compared with cells that underwent H/R alone (Fig. 3E and F). These results suggest that salidroside inhibits ER stress-induced apoptosis, thereby ameliorating H/R injury.

Salidroside alleviates H/R-induced ER stress-associated signaling pathway activation in H9c2 cardiomyocytes. The underlying mechanism of the protective effect of salidroside against H/R-induced injury was investigated by determining the effects of salidroside on the ER stress-associated signaling proteins IRE1α and PERK. H/R treatment significantly increased the expression of p-IRE1α and p-PERK proteins in H9c2 cells compared with the control, while these changes were reversed by salidroside pretreatment, as the expression of these proteins was significantly decreased in the salidroside pretreatment group compared with the H/R group (Fig. 4A and B). Salidroside treatment alone had no effect on the expression of these proteins, as the difference between their expression in the salidroside pretreatment and control groups was not significant. These results suggest that salidroside may...
inhibit the IRE1α or PERK pathways, thereby attenuating ER stress-mediated apoptosis and eliciting cardioprotection in myocardial I/R injury.

**Discussion**

It has been demonstrated that ER stress contributes to cardiomyocyte apoptosis, which results in myocardial I/R and suppressing ER stress-associated apoptosis may be a critical therapeutic approach for treating myocardial I/R injury (19,28,29). Salidroside is an active ingredient extracted from *Rhodiola rosea L.*, which has various pharmacological functions, including anti-apoptosis, anti-oxidation and cardioprotection (30). However, the underlying protective mechanisms of salidroside in myocardial I/R-injury remain unclear. The results of the current study demonstrate that salidroside protects against myocardial H/R injury by inhibiting the IRE1α or PERK pathways, thereby reducing ER stress or ER stress-mediated apoptosis.

To the best of our knowledge, the present study is the first to indicate that salidroside protects against H/R-treated H9c2 cardiomyocyte injury, which is a frequently used *in vitro* model of myocardial I/R injury (31). The protective effects of salidroside on hypoxia-induced cardiac injuries via inhibition of apoptotic pathways have previously been demonstrated (32). Zhu et al (19) determined that salidroside exhibits a cardioprotective function in myocardial I/R injury and is associated with the inhibition of cell apoptosis. Similarly, the results of the current study indicated that salidroside significantly increased H9c2 cardiomyocyte viability and decreased apoptosis, which was identified by decreases in caspase-3 activity, morphological changes of apoptotic cells and changes in the apoptotic

Figure 3. Effects of SAL on endoplasmic reticulum stress-mediated apoptosis under H/R in H9c2 cardiomyocytes. H9c2 cells were pre-incubated with or without 10 µM SAL for 30 min prior to H/R. (A) Expression of cleaved caspase-12 measured using western blot analysis and (B) quantification of western blotting results. (C) Caspase-3 activity was measured using a caspase-3 colorimetric assay kit. (D) The expression of Bax and Bcl-2 were determined using western blot analysis. β-actin was used as a loading control. Quantitative analysis of the expression of (E) Bax and (F) Bcl-2. Values are expressed as the mean ± standard deviation from three independent experiments. *P<0.01 vs. control; **P<0.05 and ***P<0.01 vs. H/R group. SAL, salidroside; H/R, hypoxia for 4 h/reoxygenation for 12 h; cle cas-12, cleaved caspase-12; Bax, Bcl-2 associated X protein.
rate following H/R, suggesting that it exhibits cardioprotection against myocardial I/R injury.

It has been demonstrated that prolonged and/or excess ER stress may eventually lead to significant apoptosis and be a major contributor to myocardium IR injury (33). GRP78 is a primary ER molecular chaperone, which is widely used as a marker for ER stress under pathological conditions (34). CHOP is another ER stress index, which is a major stress-induced pro-apoptotic gene in ER stress-induced apoptosis (35). In the current study, H/R treatment significantly increased the mRNA and protein expression of GRP78 and CHOP in H9c2 cells, indicating that it induces ER stress. In addition, it has been demonstrated that salidroside may exert its protective effect by suppressing the ER stress pathway in homocysteine-induced injury in human umbilical vein endothelial cells (20) and 6-Hydroxydopamine-induced cytotoxicity (36). The results of the current study are consistent with these studies, as it was identified that salidroside significantly decreased the levels of GRP78 and CHOP, thereby reducing ER stress. This suggests that the inhibition of ER stress may contribute to the cardioprotective role of salidroside against myocardial I/R injury.

ER stress leads to cardiomyocyte apoptosis following myocardial I/R by modulating the CHOP and caspase-12 signaling pathways (37). CHOP sensitizes cells to ER stress-induced apoptosis by causing an imbalance of Bcl-2 family members and then promoting cytochrome c release from the mitochondria to activate the apoptotic cascade (26). The results of the present study demonstrated that salidroside treatment reversed the H/R-induced upregulation of Bax expression and downregulation of Bcl-2 expression in H9c2 cells, which is consistent with the results of previous studies (29,38). These results indicate that salidroside may inhibit myocardial I/R injury by attenuating ER stress or ER stress-induced apoptosis by mitigating the mitochondria-dependent apoptotic pathway. ER stress-specific caspase-12 normally exists in an inactive pro-caspase form and serves a pivotal role in initiating ER stress-mediated apoptosis by activating caspase-3 and caspase-9 (39). It has been demonstrated that during reperfusion of the ischemic myocardium, caspase-12 and caspase-3 activities are increased (40). Furthermore, salidroside significantly reduces cleaved caspase-12 levels under 6-OHDA-induced neurotoxicity (36) and inhibits caspase-3 activity during chronic intermittent hypoxia (32). The results of the current study are consistent with these results, as they indicate that salidroside significantly decreases the expression of cleaved caspase-12 and caspase-3 activity under H/R conditions in H9c2 cardiomyocytes. These results therefore suggest that the caspase-12 pathway serves a role in the protection of salidroside against H/R injury.

The PERK, IRE1α and ATF6 pathways are well characterized as the three primary ER stress associated pathways in mammals (7). In the presence of ER stress, PERK and IRE1 are activated by trans-autophosphorylation and oligomerization (41,42). The PERK-activated CHOP pathway contributes to apoptosis (43) and activated IRE1 forms a complex with other molecules, which also leads to apoptosis (44,45). Yu et al (46) determined that pre-treatment with melatonin attenuates PERK-eukaryotic initiation factor 2 α-activating transcription factor-4-mediated ER stress and apoptosis during myocardial I/R injury. In addition, Wang et al (47) demonstrated that the ER stress-PERK signaling pathway may be a novel therapeutic target for attenuating myocardial I/R injury. Zhu et al (20) also demonstrated that salidroside attenuates the ER stress induced by homocysteine by inhibiting the phosphorylation of PERK or IRE1α. Similarly, the results of the current study indicated that following salidroside treatment, levels of PERK or IRE1α phosphorylation were reduced compared with H/R treatment in H9c2 cells, indicating that inhibition of the PERK or IRE1α pathways may contribute to the protection of salidroside against myocardial I/R injury. Although the results of the present study identified that salidroside has a protective effect against myocardial I/R injury and determined its underlying mechanism of action, there were several limitations. Further investigations of the explicit effects of the PERK or IRE1α...
pathways on ER stress or ER stress-mediated apoptosis in the cardioprotection of salidroside under H/R are required. In addition, many other signaling pathways or molecules, including the adenosine monophosphate-activated protein kinase α pathway, insulin receptor A and sirtuin 1 contribute to the beneficial effects of salidroside during hypoxia; therefore further studies are required to verify the mechanism by which salidroside protects against H/R injury (48,49).

In conclusion, the current study investigated the cardioprotection of salidroside in a cell model of myocardial I/R injury and the results indicated that the PERK or IRE1α pathways, or ER stress-mediated apoptosis contribute to this process. Furthermore, the results suggest that the inhibition of PERK or IRE1α pathways may contribute to the inhibition of salidroside on ER stress or ER stress-induced apoptosis during myocardial I/R injury. Thus, the results of the present study provide an insight into the protective effect of salidroside and ER stress-induced apoptosis in salidroside-elicted cardioprotection.

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Availability of data and materials

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

Authors' contributions

MYS analyzed and wrote the manuscript. SZ and DSM participated in the experiments and analysis of data. LW and CYM made substantial contributions to the acquisition of statistical analysis data. YB conceived the study and was involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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