Pharmacogn. Mag.
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ORIGINAL ARTICLE

Sugemule-3 Protects against Isoprenaline-induced Cardiotoxicity *In vitro*

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Submitted: 27-03-2016 Revised: 19-05-2016 Published: 19-07-2017

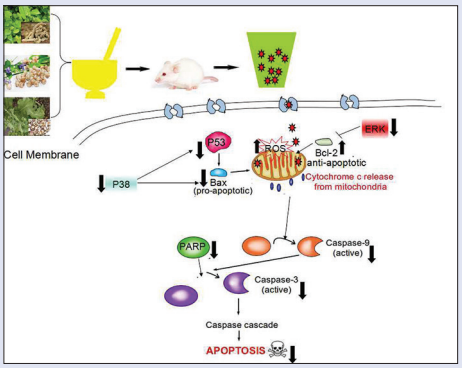
ABSTRACT

Background: Sugemule-3 (SD) is a traditional Chinese medicine with protective effect of myocardium. However, the underlying mechanisms of the effect had not been elucidated. **Materials and Methods**: In the present study, the serum of SD was prepared. A model of β-adrenergic agonist isoprenaline (ISO)-induced H9c2 cardiomyocytes injury was established *in vitro*. The changes in cell viability were examined to determine the available concentration of ISO and serum of SD. ELISA, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, and flow cytometry were used to detect the effect of serum of SD on oxidative stress and apoptosis. The expression levels of the mitochondria-dependent apoptotic pathway and mitogen-activated protein kinase signalling-related proteins were analyzed. **Results:** Incubation with different dose of ISO (0.015, 0.01, 0.005, and 0.0025 mol/L) for 24 h caused dose-dependent loss of cell viability and 0.01 mol/L of ISO approximately reduced the cell viability to 50%. Pretreatment with 50 μ mol/L serum of SD effectively decreased the levels of ISO-induced cell toxicity. Serum of SD relived ISO-induced oxidative stress and apoptosis in H9c2 cardiomyocytes. A further mechanism study indicated that serum of SD inhibited the mitochondria-dependent apoptotic pathways and regulated the expression levels of Bcl-2 family. ISO activated ERK and P38, whereas serum of SD inhibited their activation. **Conclusion:** Serum of SD inhibits the ISO-induced activation of the mitochondria-dependent apoptotic pathway, oxidative stress, and ERK, P38 inactivation. Serum of SD is used for the treatment of ISO-induced cardiomyopathy.

Key words: Cardiomyopathy, mitochondria-dependent apoptotic pathway, MAPK, Sugemule-3

SUMMARY

- The serum of SD pretreatment significantly ameliorated ISO-induced H9c2 cardiomyocytes injuries.
- The protective effect related with apoptosis and oxidative stress
- Inhibition of MAPK pathway was involed in serum of SD induced cardioprotection.
- The serum of SD is used for the treatment of ISO-induced cardiomyopathy.



Abbreviations used: ELISA: Enzyme-linked Immunosorbent Assay; TUNEL: TdT-mediated dUTP nick end labeling; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, DMSO: dimethyl sulfoxide; MDA: Malondialdehyde; SOD: Superoxide Dismutase; GSH-Px: Glutathione peroxidase.

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DOI: 10.4103/0973-1296.211018

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INTRODUCTION

Heart failure (HF) is the culmination of diverse cardiovascular diseases, such as ischaemic disease, myocarditis, and is uniformly characterized by a progressive loss of contractile function and reserve.[1] The number of patients with HF has reached epidemic proportions in industrialized countries and affects more than 20 million people worldwide.[2] In spite of the development of device therapies and effective medical for HF, the disease ultimately progresses, leading to recurrent hospitalizations and death. It is worth noting that 30%-40 % of patients die within 1 year of diagnosis of HF and 60%-70 % die within 5 years.[3]

The pathogenesis of HF is difficult to elaborate. Some studies remain in favor of the view, among the proposed hypotheses, that the upregulated reactive oxygen species (ROS) and subsequent apoptosis are major adverse factors in the pathogenesis of HF.[4] A number of cardiomyopathies were associated with mitochondrial DNA damage which leads to increasing of ROS also have been reported.[5] The ratio of Bax/Bcl-2 is postulated as an important factor in the increased rated of apoptosis in cardiac myocytes.[6] The left ventricular hypertrophy and left ventricular dysfunction in rat model of chronic pressure overload were accompanied by increased the ratio of Bax/Bcl-2 which leaded to cardiomyocyte apoptosis.[7] Kang *et al.*[8] indicated that apoptosis predominated in cardiomyocytes after reoxygenation by a mitochondrial apoptotic pathway and the protein Bcl-2 prevented reoxygenation-induced apoptosis by inhibiting the release of cytochrome c from mitochondria.

Cite this article as: Wang Y, Gong GH, Xu YN, Yu LJ, Wei CX. Sugemule-3 protects against isoprenaline-induced cardiotoxicity *In vitro*. Phcog Mag 2017;13:517-22.

Then, these proteins regulated activation of caspase-9 and caspase-3 which is the key executioner of apoptosis.[9] The activation of caspase-3 has been reported in the myocardium of end-stage HF patients.[10,11] Several transgenic animal models have demonstrated that apoptotic myocyte death is a determining factor involved in the transition to failure.[12] Some reporters have proved that NF-κB and mitogen-activated protein kinase (MAPK) family related with Bcl-2 family mediated apoptosis.[13]

As an infarct-like myocardial necrosis-inducing drug, isoproterenol (ISO) was used in research since 1959.[14] Prolonged ISO exposure causes myocardial hypertrophy in animals, even in subhypertensive dosed.[15] Some reports have demonstrated that the histopathological lesions produced due to ISO resemble myocardial infarcts seen after acute myocardial infarction in humans.[16] Now, ISO is employed at submaximal dose as a noninvasive method to induce myocardial lesions in rodents.[17] Some mechanisms proposed for understanding ISO-induced myocardial injury, production of highly cytotoxic free radicals is accepted.[18]

Sugemule-3 (SD) is a traditional Mongolian medicine that has been used for many years in China. It contains the fruit of *Amonum Kravanh Pierre ex Cagnep*, *Piper Longum L* and the fruit of *Cuminum cyminum L*, and is used to treat heiyi disease (such as insominia), especially heart disease.[19] However, little is known about the possible protective effect of SD against ISO-induced myocardial injury model and the underlying mechanism. On the basis of these reasons, we first used an ISO-induced myocardial injury model to test the protect function of SD in cardiac injury in H9c2 cardiomyocytes and investigated the possible reasons of *in vitro* experiment. Then, we clarified the related mechanisms through investigating the mitochondria-dependent apoptotic pathway and MAPK signaling pathway.

MATERIALS AND METHODS

Instruments and chemicals

ISO was obtained from Sigma Biotechnology (Sigma, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay kit was purchased from Beyotime Biotechnology (Shang Hai, China). AnnexinV-FITC/PI assay kit was obtained from Invitrogen (Eugene, USA). Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA) assay kit were purchased from Naning Jiancheng Institue of Biological Engineering (Nanjing, China). All antibodies were acquired from cell signaling technology (Boston, USA).

Serum preparation

Male Wistar rats weighing 200-250 g were used for this study. They were housed in clean polypropylene cages with an air-conditioned room at 24 ± 1°C and were fed with standard pellet diet and water *ad libitum*. All the studies were approved by Inner Mongolia University for Nationalities Animal Ethical Committee, Tongliao, China.

Rats were grouped to blank serum group and SD serum group, referring to previous studies with slight changes.[20] The rats of blank serum group were treated with 1 mL/100 g saline orally twice each day for 5 days in succession each rat. SD serum group was treated with 1 g/ kg SD orally twice each day for 5 days in succession each rat. After 2 h of treatment with SD or saline for 5 days, rats were anaesthetized under light ether anesthesia. The blood was obtained germ freely form main ventral artery. The serum was acquired by centrifugation and filtrated with 0.22 μm cellulose acetate membrane. The serum samples were left standing on ice and stored-70°C until required estimation.

Cell culture and treatment

h9c2 cardiomyocytes were cultured in Dulbecco's modified Eagle's medium with 10 % (v/v) fetal bovine serum and 100 U/ml penicilin-streptomycin. Cells were cultured in a humidified incubator at 37 °C in a 5% CO_2 atmosphere. H9c2 cardiomyocytes were pretreated with various doses (25, 50, and 100 μ mol/L) of serum of SD and then stimulated with ISO for 24 h.

Analysis of cell viability

MTT assay was used to measure cell viability, Sigma. First of all, H9c2 cardiomyocytes were seeded at 1 × 105 cells/well in 96-well plates. Then, the cells were pretreated with different doses serum of SD for different times and incubated with different doses ISO for an additional 24 h. Finally, 1 mg/mL MTT solution was added to 96-well plates and incubated for 4 h at 37 °C. After removing cell culture medium, DMSO was added to dissolve the insoluble MTT-formazan salt. The results were detected at 570 nm on a microplate reader (Thermo, USA).

Detection of myocardial enzymatic activity

The levels of GSH-Px, SOD, and MDA in the H9c2 cardiomyocytes were measured according to the manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China).

TUNEL assay

DNA fragmentation of apoptotic cells were detected by TUNEL staining following the manufacturer's instructions. First of all, cells were cultured on cover slips for 24 h. After drug treatment, the cardiomyocytes were fixed by 4% paraformaldehyde solution for 30 min at room temperature. Then, the cells were incubated with 0.3% H_2O_2 for 30 min at room temperature in order to block endogenous peroxidase activity. After incubated in the TUNEL reaction mixture for 60 min, the cells were visualized by microscopy (Nikon, Japan).

Flow cytometric detection of apoptosis

The annexin V-FITC/PI apoptosis kit was used for detected the percentage of early apoptosis and necrosis. After treating with drugs, H9c2 cardiomyocytes were collected and washed by cold phosphate-buffered saline. Then, cells were incubated with annexin V FITC and PI in the dark for 15 min. Finally, the samples were measured by flow cytometry analysis with a FACS Calibur Flow Cytometer (Becton Dickinson, USA). A total of 10,000 events were counted. The annexin V-/PI- were considered as viable cells, the annexin V+/PI- were considered as early stage apoptotic cells and annexin V+/PI+ were considered as late apoptotic or necrotic cells.

Western blot assay

Western blot was performed as described previously.[21] Atibodies for anti-p53 (1: 1000), anti-Bax (1 : 1000), anti-Bcl-2 (1: 1000), anti-caspase-9 (1: 1000), anti-caspase-3 (1: 1000), anti-cleaved PARP (1: 1000), anti-ERK (1: 1000), anti-p-ERK (1: 1000) , and anti-P38 (1: 1000), anti-p-P38 (1: 1000) were purchased from cell signaling technology. Protein was extracted and mixed in loading buffer, and then equal amounts were fractionated on gel and transferred onto Hybond-C extra nitrocellulose membrane using a semidry transfer apparatus. Last, the protein was blocked with nonfat dry milk, added antibodies, and detected with supersignal west pico chemiluminescent substrate (Invitrogen, USA). The blots were identified for band densities using Image J 1.45s software (National Institute of Health, USA).

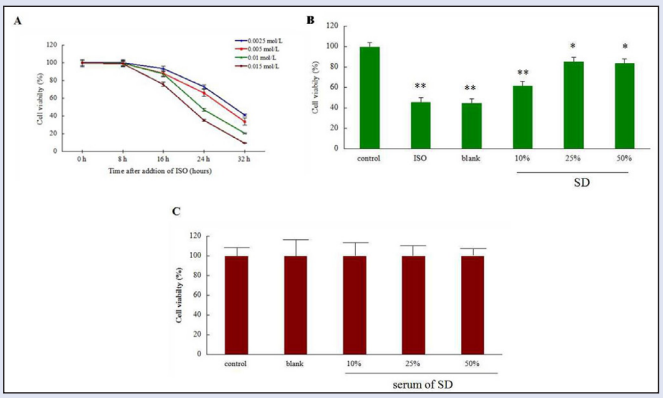

Figure 1: Effects of ISO and serum of SD on cell viability. (A): Cells were treated with different doses of ISO for different times; (B/C): Cells were treated with different doses of serum of SD or blank rats' serum. All data are shown as mean ± SE (n = 3 per group). **P<0.01 *vs* control; *P<0.05 *vs* control.

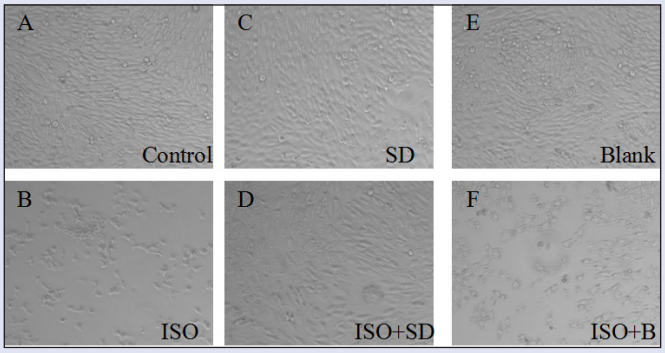

Figure 2: The effective ingredients of serum of SD protects H9c2 cardiomyocytes.

Statistical analysis

All values were expressed as mean ± standard error (SE). Multigroups comparisons of the means were carried out by matched t-test (Microsoft Excel®) using Statistical Package for the Social Sciences 11.5.

RESULTS

Serum of SD protective effect ISO-induced myocardial injury in H9c2 cardiomyocytes

To evaluate the protective effect of serum of SD on ISO-induced myocardial injury in H9c2 cardiomyocytes, the effect of ISO was detected using MTT assay. Cells were treated with different doses (0.015, 0.01, 0.005, and 0.0025 mol/L) ISO for 8, 16, 24, and 32 h. ISO-induced a dose- and time- dependent toxicity in H9c2 cardiomyocytes by the decreased cell viability Figure 1(A). Cells were treated with 0.01 mol/L of ISO for 24 h for 47.1 ± 1.2 % of cell death. Therefore, 0.01 mol/L of ISO for 24 h was used in subsequent experiments.

The protective effect of serum of SD on H9c2 cardiomyocytes was assessed. H9c2 cardiomyocytes pretreated with different doses serum of SD (10%, 25%, and 50%). From the Figure 1(B), the cell viability increased with serum of SD compared with exposure to on 0.01 mol/L ISO for 24 h, and 25 % serum of SD is the optimum dose. Then the cytotoxic effect of serum of SD was measured. After treatment with different doses serum of SD (10%, 25%, and 50%), no change in H9c2 cardiomyocytes was measured using MTT assay Figure 1(C). From these

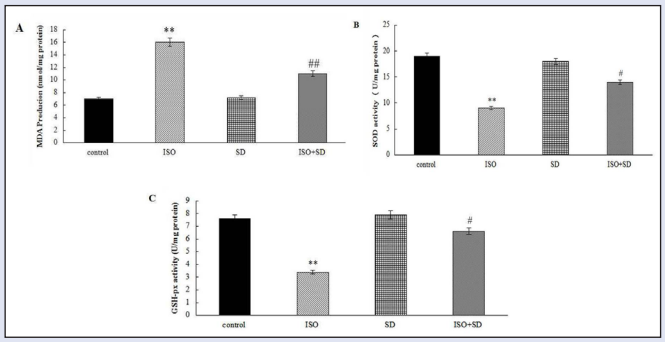

Figure 3: Serum of SD prevents ISO-induced oxidative stress. (A/B/C): ELISA examines the level of MDA, SOD, and GSH-px. All data are shown as mean ± SE (n = 3 per group). **P<0.01 *vs* control; #P<0.05 *vs* ISO-induced group, ##P<0.01 *vs* ISO-induced group.

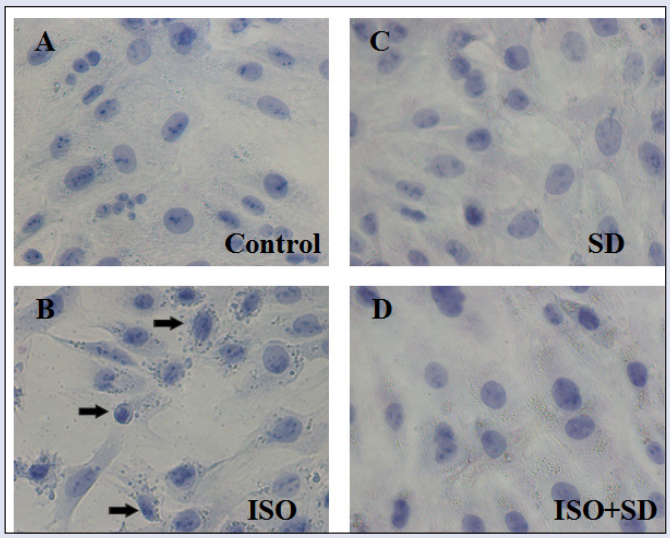

Figure 4: Apoptosis of cells were detected by TUNEL

results, we propose serum of SD could protect ISO-induced myocardial injury in H9c2 cardiomyocytes.

The effective ingredients of serum of SD protect ISO-induced myocardial injury in H9c2 cardiomyocytes to analyze whether the effective ingredients of serum of SD play a crucial role, we measured the statement of H9c2 cardiomyocytes which pretreated with serum of SD or blank serum of rats and ISO- inducted. As shown in [Figure 2], the statement of H9c2 cardiomyocytes treated with serum of SD and ISO is better than ISO or serum of blank and ISO. This result indicated the effective ingredients of serum of SD play a vital role, not serum of rats.

Serum of SD prevent oxidative stress

The effect of serum of SD on the activation of SOD, GSH-px, and production of MDA in H9c2 cardiomyocytes were analyzed by ELISA. As an indicator of lipid peroxidation, the levels of MDA/SOD were analyzed Figure 3(A) and Figure 3(B). The levels of MDA were found to be higher in H9c2 cardiomyocytes after treating with ISO, whereas these changes were effectively improved by serum of SD. The levels of GSH-px or SOD showed a significant reduction in H9c2 cardiomyocytes after treating with ISO compared with control group, while serum of SD increased the levels of GSH-px or SOD [Figure 3(C)].

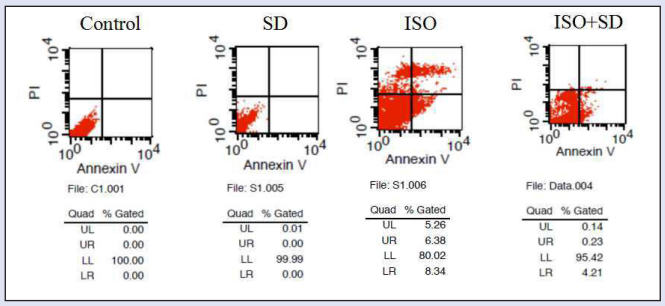

Figure 5: Effects of serum of SD were measured with annexin V-FITC/PI by flow cytometry.

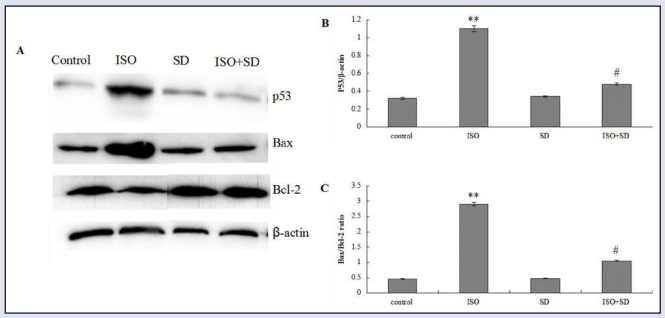

Figure 6: Effects of ISO and serum of SD on expression of Bcl-2 family proteins *in vitro*. Figure 6 (A, C) The expression levels of Bcl-2, Bax, and p53 were detected by western blot (A) and expression as the fold changes over the control (B and C). All data are shown as mean ±SE (n = 3 per group). **P<0.01 *vs* control; #P<0.05 *vs* ISO-induced group.

Serum of SD mitigate ISO-induced apoptotic damage *in vitro*

To exam whether the protective effect of serum of SD related with apoptosis, TUNEL staining was used in H9c2 cardiomyocytes. ISO significantly increase apoptotic rate compared with control group. While the number of TUNEL-positive cells decreased after dealing with serum of SD, at the same time, the serum of SD- induced apoptosis was detected [Figure 4].

We next assessed whether ISO-induced apoptosis, but not by serum of SD. In the stage of apoptosis, phosphatidylserine was translocated from the inside of the cell membrane to the outside. As the calcium-dependent phospholipid-binding protein associated with a high affinity for phosphatidylserine, annexin V-FITC was used to detect early apoptotic cells. And PI was used to detect the cells which have lost membrane integrity. H9c2 cardiomyocytes were stained with annexin V-FITC/PI. As shown in [Figure 5], ISO caused apoptosis in H9c2 cardiomyocytes (14.72% of apoptotic cells), whereas pretreatment with serum of SD reduced the population of apoptosis cells. Serum of SD alone showed no obvious effects on these processes.

Antiapoptotic effects of serum of SD against ISO were related with mitochondria-dependent apoptotic pathway

To measure the antiapoptotic effects of serum of SD, the expression levels of mitochondria-related proteins (Bax, Bcl-2, p53) in H9c2 cardiomyocytes were evaluated by western blot. In our study, the expression level of proapoptotic protein Bax and p53 [Figure 6 (A,B,C)] in H9c2 cardiomyocytes treated with ISO were significantly higher

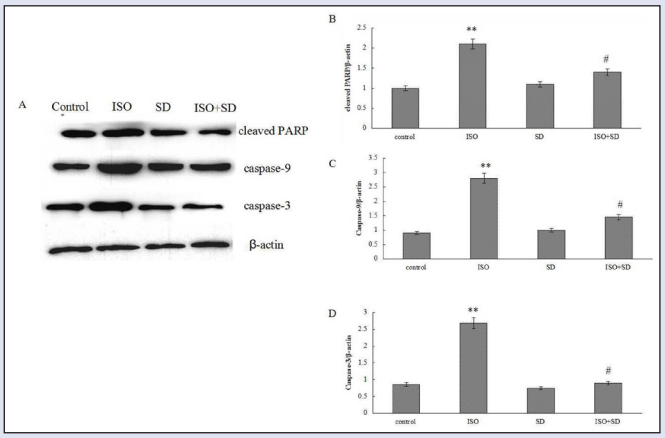

Figure 7: Effects of ISO and serum of SD on expression of apoptotic related with proteins *in vitro*. The expression levels of Caspase-3, Caspase-9, and cleaved PARP were detected by western blot (A) and expression as the fold changes over the control (B, C, and D). All data are shown as mean ±SE (n = 3, per group) **P<0.01 *vs* control; #P<0.05 *vs* ISO-induced group.

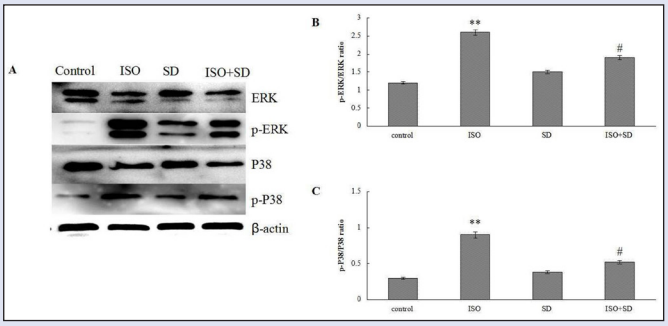

Figure 8: Effects of ISO and serum of SD on expression of MAPK *in vitro*. The expression levels of ERK, JNK, P38, p-ERK, p-JNK, p-P38 were detected by western blot (A) and expression as the fold changes over the control (B, C, and D). All data are shown as mean ± SE (n = 3, per group) **P<0.01 *vs* control; #P<0.05 *vs* ISO-induced group.

than the control group, while the antiapoptotic protein Bcl-2 [Figure 6 (A)] in H9c2 cardiomyocytes treated with ISO lower than the control group; however, pretreatment with serum of SD blocked these effects. To further, activation of Caspase-3/9 and cleavage of PARP were analyzed by western blot [Figure 7 (A,B,C,D)]. From the results indicated, serum of SD prevented ISO-induced mitochondria- dependent apoptotic pathways.

MAPK signaling pathway was related with anti-apoptotic effects of serum of SD

MAPK signaling pathway related with apoptosis has been reported. In general, the pathways (JNK, ERK, P38) were involved with MAPK. In our study, we detected total and phosphorylated (active form) JNK, ERK, P38 MAPK by western blot. As shown in Figure 8 (A, B, C) the expression levels of p-ERK and p-P38 were markedly increased with treatment with ISO. However, this effect was inhibited in H9c2 cardiomyocytes before pretreated with serum of SD. No changes were observed in total protein levels of p-ERK and p-P38 were detected. However, this phenomenon was not detected in expression of p-JNK.

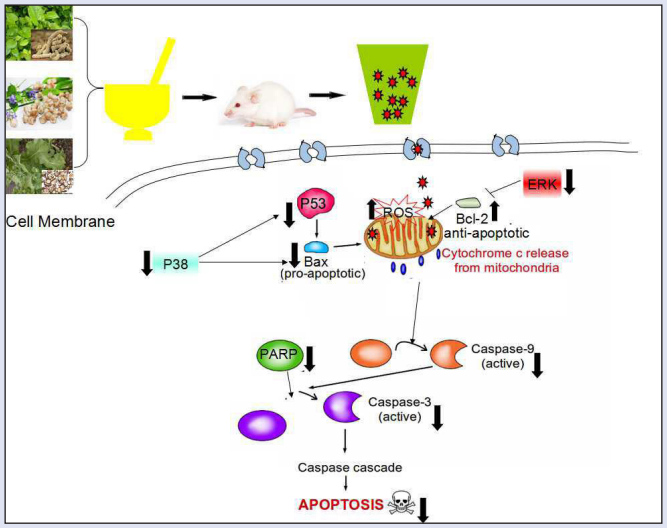

Figure 9: Schematic of serum of SD preventing ISO-induced apoptosis.

DISCUSSION

HF is one of the leading pathological causes of mortality worldwide.[22] Apoptosis involved with HF has recently attracted increased attention; however, no effective treatment has been developed.

Traditional Chinese medicine (TCM) is a healthcare-focused medical system with its rich experience over 3000 years.[23] As the TCM, SD is frequently used in the prevention and treatment of cardiovascular diseases. In our research, we evaluated the protective mechanism of SD on ISO-induced H9c2 cardiomyocytes. This is the first report that SD, as a potential candidate, prevented and treated HF.

ISO-induced myocardial hypertrophy in rats involves many similarities with human HF.[24] In the present study, we have observed a significant decrease in cell activation in ISO-induced H9c2 cardiomyocytes. While H9c2 cardiomyocytes pretreated with serum of SD can effectively increase the cell viability; however, serum of blank does not protect ISO-induced myocardial injury in H9c2 cardiomyocytes.

As indispensable role in cardiac energy metabolism, oxygen plays a crucial role in some biological processes that can be determinants of cardiac function,[25] and oxidative stress have been implicated in most processes thought to have a significant effect on cardiac function.[26] The activation of SOD and GSH-px reflects the cellular capability of scavenging/quenching free radicals.[27] The levels of MDA are used as an indicator of lipid peroxidation.[28] In our study, we primarily want to detect the effect of serum of SD on ISO-induced oxidative stress. From our study, the levels of SOD and GSH-px were downregulated as well as the levels of MDA was upregulated in ISO-induced H9c2 cardiomyocytes. However, pretreatment with serum of SD reduced this phenomenon.

Apoptosis is very rare in normal myocardium with a reported rate of 0.001%–0.002%; however, it's increase in both acute and chronic heart pathologies seems to play an important role.[29] In present study, serum of SD pretreatment to H9c2 cardiomyocytes followed by ISO significantly lowered ratio of apoptosis to ISO-induced cells. The present study suggests that serum of SD can inhibit ISO-induced H9c2 cardiomyocytes injury.

Two main molecular pathways (extrinsic and intrinsic pathways) relate with apoptosis.[30] Extrinsic apoptosis indicates a form of death induced by extracellular signals, while intrinsic pathway is activated in response to a number of stressing condition such as oxidative, DNA damage, and so on.[31] In intrinsic pathway, Bcl-2 family proteins are essential regulations. This family can be classified in antiapoptotic members (Bcl-2 and so on) and proapoptotic members (Bax).[32] As a transcription factor, p53 plays an important role in the cellular response to DNA damage and regulates Bcl-2 family. Intrinsic pathway involved in HF, to confirm whether the antiapoptotic effect of serum of SD was related to the mitochondrial-dependent intrinsic pathway, we detected the expression of Bax, Bcl-2, and p53. The ratio of Bax/Bcl-2 and p53 was downregulated in pretreatment of serum of SD cells following ISO. Our study showed that serum of SD significantly suppressed ISO-induced intrinsic apoptosis pathway. In addition, the expression of Caspase-3/-9 and cleavage PARP were detected. Our results demonstrated that serum of SD markedly prevented the activation of Caspase-3/-9 and PARP cleavage induced by ISO in H9c2 cardiomyocytes.

The MAPK signal pathway (ERK, JNK, P38) has been regarded as a central mechanism related with cardiac hypertrophy and failure.[33] In the present study, an increase of p-ERK, p-P38 in H9c2 cardiomyocytes after ISO was observed, which was significantly reduced by the pretreatment with serum of SD. These results suggest that ERK and p-38 signaling pathway is essential for ISO-inducted H92 cardiomyocytes injuries, serum of SD could alter p-ERK, p-P38 expression.

The present study provides experimental evidence [Figure 9] that serum of SD pretreatment significantly ameliorated ISO-induced H9c2 cardiomyocytes injuries, reduced apoptosis (mitochondrial-dependent intrinsic pathway). Inhibition of MAPK pathway was involved in serum of SD-induced cardioprotection. These findings might be helpful to understand the beneficial effects of serum of SD against myocardial injury.

Acknowledgement

This work was supported by Natural Science Foundation of Inner Mongolia (No. 2016BS0806), higher scientific research project of Inner Mongolia (No. NJZY16186), and the Mongolian medicine systems biology science and technology innovation team plan of Inner Mongolia.

Financial support and sponsorship

Nil

Conflicts of interest

There are no conflicts of interest

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