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Sevoflurane ameliorates adriamycin-induced myocardial injury in rats through the PI3K/Akt/GSK-3β pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of sevoflurane (SEV) pretreatment on Adriamycin (ADR)-induced myocardial injury through the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/glycogen synthase kinase-3β (GSK-3β) pathway.

MATERIALS AND METHODS: A total of 24 rats weighing 200-250 g were divided into four groups, including: control group (C group), ADR injection group (ADR group), SEV pretreatment group (ADR + SEV group) and inhibitor group (ADR + SEV + LY group). H9c2 cells were cultured in vitro and were divided into control group (C group), ADR treatment group (ADR group), and SEV pretreatment group (ADR + SEV group) and inhibitor group (ADR + SEV + LY group) as well. Next, the content of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) in the serum was detected via Enzyme-Linked Immunosorbent Assay (ELISA). Hematoxylin-eosin (HE) staining assay was performed to determine the severity of myocardial injury. Meanwhile, the apoptosis rate of cells was detected through terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay. Additionally, Western blotting (WB) was employed to measure the protein expression levels of phosphorylated (p)-GSK-3β, p-PI3K, Akt and p-Akt.

RESULTS: Compared with C group, ADR significantly increased the content of AST, LDH and CK in the serum (p<0.01), reduced protein expression levels of p-GSK-3β, p-PI3K and p-Akt (p<0.01), increased apoptosis rate (p<0.01), and induced myocardial injury. SEV pretreatment significantly alleviated the effect of ADR, manifested as significantly lowered content of AST, LDH and CK in the serum (p<0.01), distinctly elevated protein expression levels of p-GSK-3β, p-PI3K and p-Akt (p<0.01), notably reduced apoptosis rate (p<0.01), and relieved myocardial injury. LY294002 remarkably inhibited the protective effect of SEV against myocardial injury (p<0.01)

CONCLUSIONS: SEV is able to prominently ameliorate ADR-induced myocardial injury by regulating the phosphorylation level of the PI3K/Akt/GSK-3β signaling pathway.

Key Words:
Sevoflurane, Adriamycin, PI3K/Akt/GSK-3β pathway, Myocardial injury, Cell apoptosis.

Introduction

Adriamycin (ADR) is one of the most commonly used anti-anticancer drugs. Currently, it has been widely applied in the treatment of different types of malignant tumors, including ovarian cancer, thyroid cancer, breast cancer, and leukemia, making satisfactory clinical effect[1-3]. However, the usage of ADR is limited because it is prone to cause side effects of acute cardiotoxicity in patients during or after treatment. This can eventually lead to fatal congestive heart failure (CHF), abnormal electrophysiology and arrhythmia[4,5]. Since the strong cardiotoxicity of ADR was first discovered, many studies have focused on determining its underlying mechanism. However, the etiology of myocardial injury induced by ADR has not been fully elucidated.

Sevoflurane (SEV) has been clinically used for inhalation anesthesia for more than 20 years. Meanwhile, SEV has also been tested in many studies in around 900 million patients, whose safety and efficacy are generally recognized[6]. Inhalational SEV can improve early brain injury

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caused by subarachnoid hemorrhage (SAH)[7]. It can also be used as a sedative for acute respiratory distress syndrome to improve gas exchange and reduce edema and inflammation in pulmonary alveoli[8]. In addition, SEV protects rats from myocardial injury caused by ischemia and reperfusion, and may ameliorate ADR-induced myocardial injury by affecting the phosphorylation status of proteins in the phosphatidylinositol 3-hydroxy kinase (P13K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway[9,10]. In this study, the effects of SEV on ADR-induced myocardial injury were investigated by using rat models of ADR cardiotoxicity. Our findings might help to better elucidate the molecular mechanism of SEV in relieving myocardial injury induced by ADR.

Materials and Methods

Main Materials

Rats weighing 200-250 g were selected as research subjects in this study. H9c2 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). SEV was bought from Beijing MREDA Technology Co., Ltd. (Beijing, China). LY294002 was purchased from Selleck (Houston, TX, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin + 0.02% EDTA (ethylenediaminetetraacetic acid) and phosphate buffered solution (PBS) were provided by Gibco (Rockville, MD, USA). A one step terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay kit (green fluorescence) was bought from Beyotime (Shanghai, China). Phosphorylated glycogen synthase kinase-3β (p-GSK-3β) antibody was obtained from CST (Danvers, MA, USA). P-PI3K, Akt, p-Akt and β-Actin antibodies were purchased from Abcam (Cambridge, MA, USA).

Grouping and Modeling

This study was approved by the Animal Ethics Committee of Xi'an Jiaotong University Animal Center. A total of 24 healthy rats weighing 200-250 g purchased from the Laboratory Animal Center were adaptively fed in the laboratory for 7 days. All rats were divided into 4 groups, including: control group (C group), ADR injection group (ADR group), SEV pretreatment group (ADR + SEV group), and inhibitor group (ADR + SEV + LY group). The rats in ADR group were intraperitoneally injected with ADR at a dose of 3 mg/kg/d every three days for 8 times, with a cumulative dose of 24 mg/kg. In ADR + SEV group, 1.5 MAC SEV treatment was adopted at 2 h before ADR injection. Rats in ADR + SEV + LY group were subjected to 1.5 MAC SEV treatment and injected with 0.5 mg/kg LY294002 *via* the tail vein at 2 h before ADR injection. In C group, the same volume of normal saline was injected. After the experiment, serum samples and heart tissues were timely collected from rats for later use.

Culture of H9c2 Cells

H9c2 cells were cultured in DMEM containing 10% FBS in an incubator containing 5% CO_2 at 37°C. All these cells were divided into control group (C group), ADR injection group (ADR group), SEV pretreatment group (ADR + SEV group), and inhibitor group (ADR + SEV + LY group). Cells in ADR group were cultured with 2 μM ADR medium for 2 h. Meanwhile, the cells in ADR + SEV group and ADR + SEV + LY group were cultured with 2 mM SEV medium and 2 mM SEV medium + 20 μM LY294002 for 2 h before ADR treatment, respectively. In C group, the cells were cultured with DMEM + 10% FBS.

Detection of Serum Via Enzyme-Linked Immunosorbent Assay (ELISA)

After separation of rat serum, ELISA was conducted as per the instructions of the kit (R&D Systems, Minneapolis, MN, USA) to examine aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK). Myocardial injury was finally evaluated.

Hematoxylin-Eosin (HE) Staining Assay

Heart tissues were prepared into paraffin sections. Then, the sections were sequentially placed in xylene I and xylene II for 10 min each, and anhydrous ethanol I, anhydrous ethanol II, 95% alcohol, 90% alcohol, 80% alcohol and 70% alcohol for 5 min each for deparaffinization. After washing with distilled water, the sections were stained with hematoxylin (Harris) for 5 min. Next, the sections were washed with running water, and differentiated with 1% hydrochloric acid alcohol for a few seconds. After washing with running water, the sections were blued with 0.6% ammonia water, followed by washing again with running water. Thereafter,

the sections were stained in eosin staining solution for 1-3 min, dehydrated and mounted. Finally, the sections were observed and photographed under a microscope.

Detection of Apoptosis Through One Step TUNEL Assay

Cell apoptosis was determined in accordance with the one step TUNEL assay kit (green fluorescence). Specifically, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 30 min. After washing once with PBS, the cells were incubated with PBS containing 0.1% Triton X-100 on ice for 2 h. Next, the cells were washed with PBS twice and incubated with 50 μL of TUNEL detection solution in the dark at 37°C for 60 min, followed by washing with PBS for 3 times. Afterwards, the cells were mounted with an anti-fluorescence quenching mounting solution, observed and photographed under a fluorescence microscope.

Western Blotting (WB)

Myocardial tissues or cells were lysed with cell lysis buffer at 4°C overnight, followed by centrifugation at 13,000 rpm to extract total proteins. The concentration of extracted protein was determined by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Next, the protein samples were separated by 8% sodium lauryl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk powder and 0.1% Tween-20 in Tris-Buffered Saline, the membranes were incubated with p-GSK-3β, p-PI3K, Akt, p-Akt and β-Actin primary antibodies *via* gentle shake at 4°C overnight. On the next day, the membranes were incubated with horse radish peroxidase horse radish peroxidase (HRP)-labeled secondary antibody. Immuno-reactive bands were finally exposed by the enhanced chemiluminescence (ECL) reagent.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was employed for statistical analysis. Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Independent sample *t*-test was adopted for comparison between groups. $p<0.05$ was considered statistically significant.

Results

SEV Relieved ADR-Induced Myocardial Injury

The expression levels of AST, LDH and CK in the serum are important biochemical indicators of myocardial injury. Compared with those in C group, the expression levels of AST, LDH and CK in the serum increased significantly after ADR treatment ($p<0.01$). However, they were remarkably reduced after SEV pretreatment ($p<0.01$). Meanwhile, LY294002 treatment significantly inhibited the effect of SEV ($p<0.01$) (Figure 1).

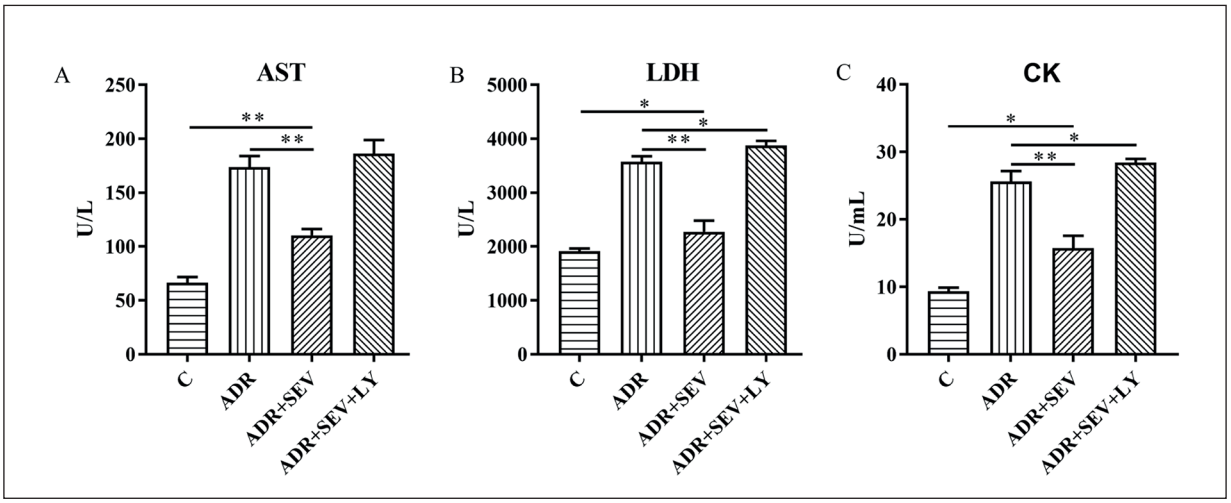

Figure 1. Expression levels of AST, LDH and CK in rat serum detected *via* ELISA. **A,** AST expression level. **B,** LDH expression level, and (**C**) CK expression level. C: control group, ADR: ADR treatment, SEV: SEV pretreatment, and LY: LY294002 (PI3K inhibitor) injection. *$p<0.05$ and **$p<0.01$: significant differences compared with other groups.

Results of HE Staining Assay on Myocardial Tissues

According to the results of HE staining assay, there were neatly and densely arranged myocardial fibers in C group. ADR treatment led to myocardial fiber fracture, loosely and irregularly arranged myocardial fibers, and pyknotic cell nuclei. SEV pretreatment alleviated ADR-induced myocardial injury. However, myocardial injury in ADR + SEV + LY group was severer than that in ADR group (Figure 2).

Effect of SEV on Expression of PI3K/Akt/GSK-3β Pathway in Rat Myocardial Tissues

WB results demonstrated that ADR led to remarkably reduced protein expressions of p-PI3K, p-Akt and p-GSK-3β in rat myocardial tissues compared with C group (p<0.01). However, SEV pretreatment markedly upregulated the protein expressions of p-PI3K, p-Akt and p-GSK-3β (p<0.01). Meanwhile, LY294002 could reverse the effect of SEV (p<0.01) (Figure 3).

Effect of SEV on Apoptosis of H9c2 Cells Cultured In Vitro

The results of TUNEL assay uncovered that compared with C group, the apoptosis rate rose remarkably after treatment with ADR (p<0.01). However, such an increase was significantly reduced by SEV (p<0.01). Meanwhile, the effect of SEV was evidently reversed by LY294002 (p<0.01) (Figure 4).

Effect of SEV on Expression of PI3K/Akt/GSK-3β Pathway in H9c2 Cells In Vitro

Based on WB, ADR prominently decreased the protein expressions of p-PI3K, p-Akt and p-GSK-3β in H9c2 cells compared with C group (p<0.01). However, SEV pretreatment dramatically elevated the protein expressions of p-PI3K, p-Akt and p-GSK-3β in cells (p<0.01). In addition, LY294002 distinctly suppressed the effect of SEV (p<0.01) (Figure 5).

Discussion

Cardiotoxicity of ADR is a major factor that limits its wide therapeutic application in oncology. It is caused by a variety of mechanisms, such as interference with DNA repair, the induction of DNA damage, the generation of free radicals, lipid peroxidation, and mitochondrial damage, etc.[11-13]. This may eventually reduce the activity of Na^+ K^+ ATPase[14]. Previous studies[15,16] have shown that ADR at 15-25 mg/kg can trigger cardiotoxicity. ADR at 24 mg/kg and higher cumulative doses can cause severe heart disease in mice[17]. Insufficient supply of oxygen or glucose may destruct cardiac sarcolemma, thereby resulting in the leakage of AST, LDH, and CK from cardiac mitochondria[18]. Therefore, these serum enzymes are regarded as the best biomarkers of myocardial injury. Meanwhile, their increases denote that cardiac damage is occurring. The expression levels of AST, LDH and CK in the serum of rats were analyzed in this study. The results showed that the expression levels increased significantly due to ADR treatment. Combined with the outcomes of hematoxylin-eosin (HE) staining of myocardium, it was indicated that ADR could cause myocardial injury in rats. Volatile anesthetics affect the cardiovascular system by acting on cardiac muscles or reducing systemic vascular resistance[19]. SEV can weak coronary vessel dilatation, thereby

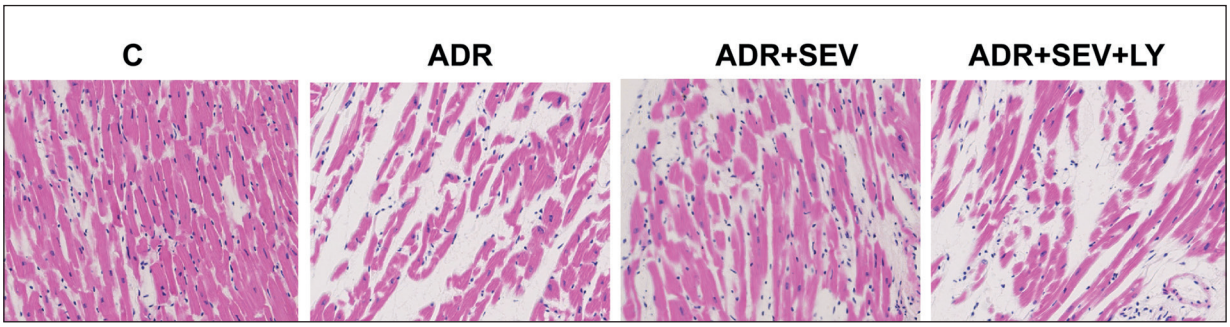

Figure 2. Severity of myocardial injury in rats determined through HE staining assay. (magnification: 200×) C: control group, ADR: ADR treatment, SEV: SEV pretreatment, and LY: LY294002 (PI3K inhibitor) injection.

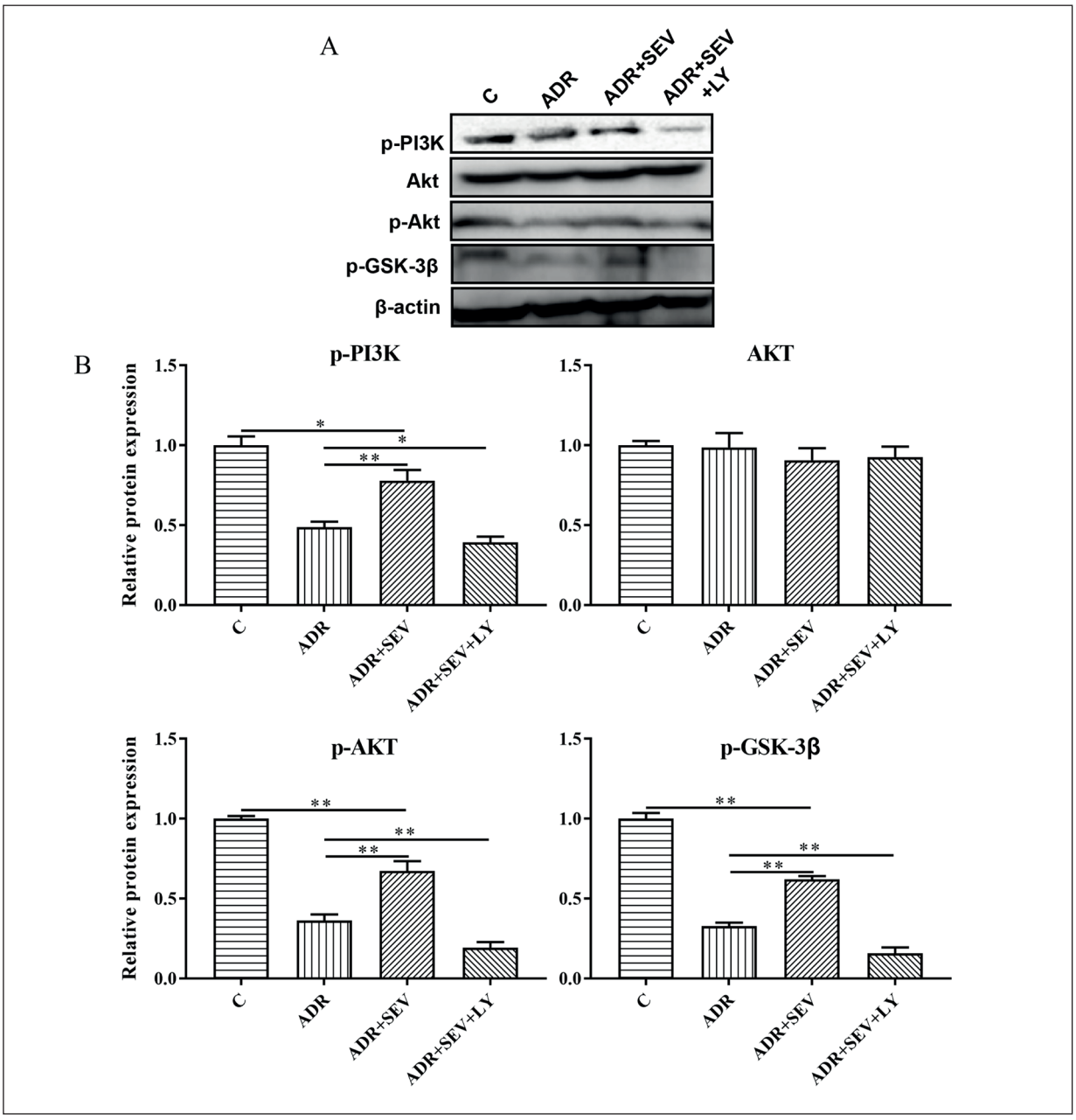

Figure 3. Expression of PI3K/Akt/GSK-3β pathway in rat myocardial tissues measured *via* WB. (**A**) Expressions of the PI3K/Akt/GSK-3β pathway-related proteins p-PI3K, p-Akt, p-GSK-3β and Akt, and (**B**) Relative quantitative analysis of the expression levels of p-PI3K, p-Akt, p-GSK-3β and Akt. C: control group, ADR: ADR treatment, SEV: SEV pretreatment, and LY: LY294002 (PI3K inhibitor) injection. *p<0.05 and **p<0.01: significant differences compared with other groups.

maintaining blood flow in the coronary artery[20] Belhomme et al[21] have indicated the beneficial effects of SEV on the reperfusion injury of myocardial ischemia of rabbits. Meanwhile, its protective effect on endothelial cells has also been confirmed in the human body, which may generate additional beneficial effects[22]. SEV administration can decrease inflammatory markers, and its pretreatment significantly lowers the concentration of interleukin-6 (IL-6) and IL-8 of proinflammatory cytokines[23]. In the present study, we found that pretreating ADR rats with SEV significantly lowered the expression levels of AST, LDH and CK and reduced the severity of myocardial injury in rats, confirming the protective effect of SEV on myocardium.

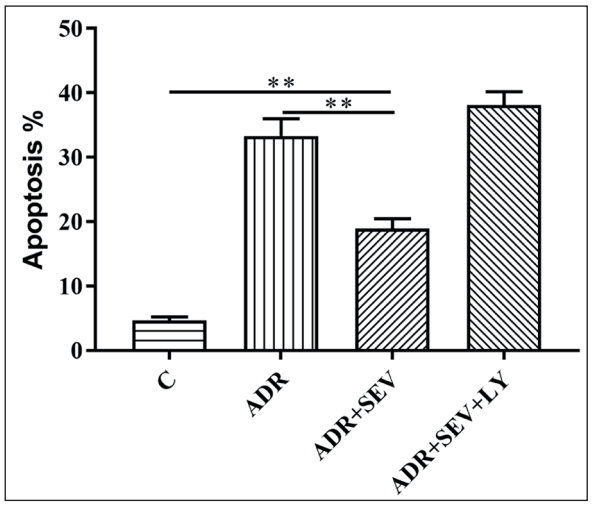

Figure 4. Apoptosis level of H9c2 cells cultured *in vitro* determined through TUNEL assay. Apoptosis rate of relatively quantified cells. C: H9c2 cells normally cultured, ADR: H9c2 cells cultured in medium containing ADR, SEV: H9c2 cells cultured after SEV pretreatment, and LY: H9c2 cells cultured in medium containing LY294002 (PI3K inhibitor). $^{**}p<0.01$: significant differences between groups.

There is some controversy on the relationship between ADR dose and apoptosis. ADR-induced cardiotoxicity can be ascribed to multiple mechanisms, the most extensively accepted one of which is increased myocardial apoptosis[24]. Hong et al[18] have found that there are increases in myocardial apoptosis, marked growing in apoptosis proteins (e.g., Bax and caspase-3), and decreases in anti-apoptotic protein Bcl-2 in rats treated by ADR. Arola et al[25] have proven that even a single peritoneal injection of ADR at 2.5 or 5 mg/kg can also rise cardiomyocyte apoptosis. Zhang et al[26] have found that there is apoptosis of endothelial cells in the absence of CHF clinical symptom within 12 weeks after intravenously injecting ADR with the highest dose of 12 mg/kg. In this study, cardiomyocytes were cultured *in vitro*. Subsequent experiments demonstrated that the apoptosis rate of cells increased strikingly after ADR treatment. However, SEV pretreatment remarkably reduced the increased apoptosis rate of cardiomyocytes caused by ADR.

In the current studies of myocardial injury model, the PI3K/Akt pathway plays an important role in alleviating myocardial injury. PI3K/Akt pathway is of vital importance in oxidative stress and cardiomyocyte apoptosis. Meanwhile, it can be activated to inhibit the occurrence of myocardial injury[27-29]. It has been found that urolithin-A can protect the heart by activating the PI3K/Akt signaling pathway[30]. Soy isoflavone exerts a protective effect against reperfusion injury in ovariectomized rats by activating estrogen receptor in the PI3K/Akt/eNOS signaling pathway[31]. Sufentanil processing can realize myocardial protection by activating the PI3K/Akt/GSK-3β pathway and regulating the expressions of Bax and Bcl-2[32]. SEV alleviates ADR-induced myocardial injury by affecting the phosphorylation of proteins in the PI3K/Akt/mTOR signaling pathway and reducing the damage to biomarkers[10]. In this study, ADR rats were pretreated with SEV. It was found that ADR could reduce the expressions of myocardial injury markers, generate less cardiomyocyte apoptosis, and upregulate the phosphorylation level of the PI3K/Akt/GSK-3β signaling pathway, Finally, this might weaken the myocardial injury caused by ADR in rats. However, treatment with LY294002, a P13K inhibitor, suppressed the protective effect of SEV on myocardium.

Conclusions

The novelty of this study was that SEV can alleviate the myocardial injury caused by ADR by regulating the phosphorylation level of PI3K/Akt/GSK-3β signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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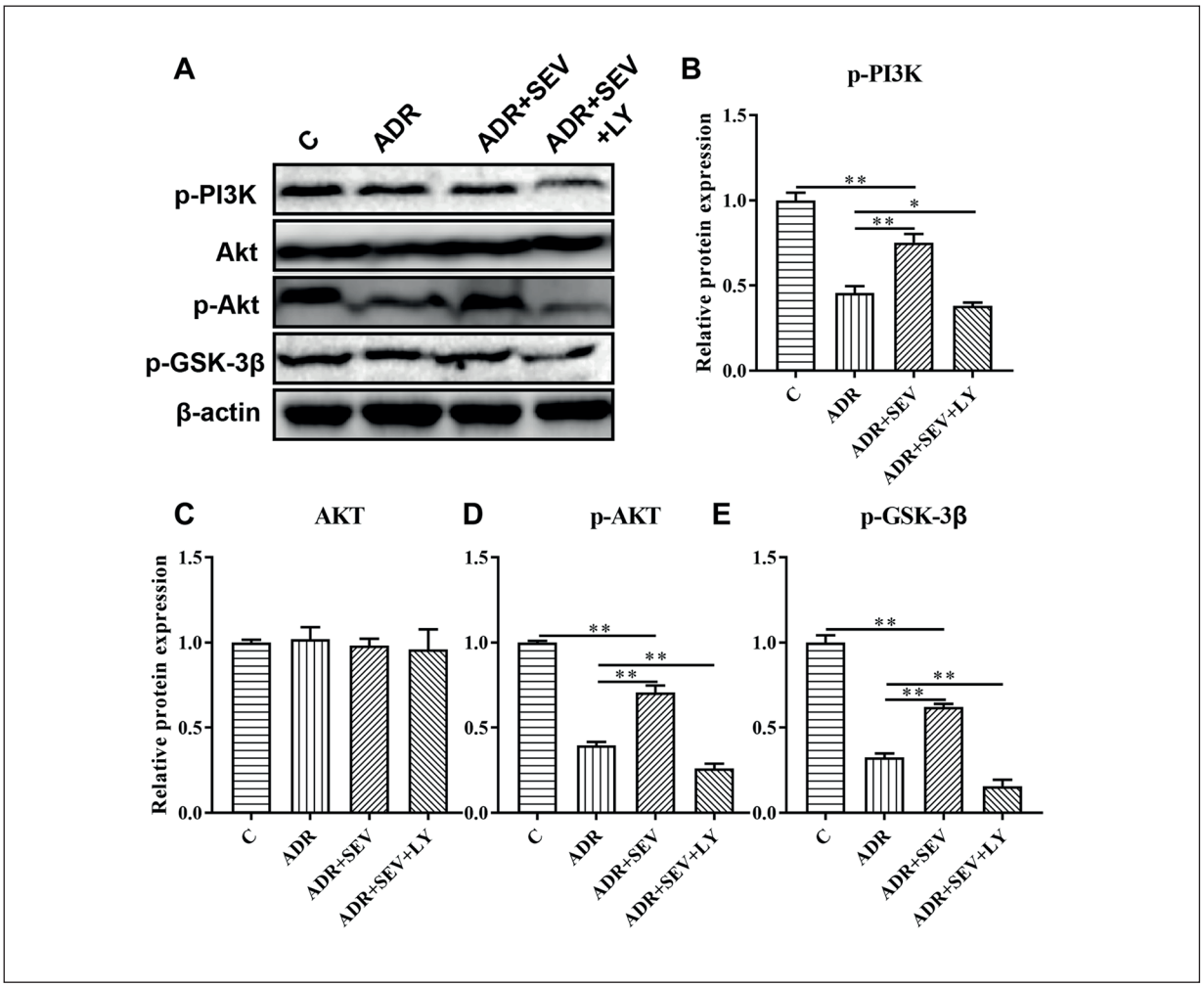

Figure 5. Expression of PI3K/Akt/GSK-3β pathway in H9c2 cells cultured *in vitro*. (**A**) Expressions of the PI3K/Akt/GSK-3β pathway-related proteins p-PI3K, p-Akt, p-GSK-3β and Akt detected *via* WB, and (**B-E**) Relative quantitative analysis of the expression levels of p-PI3K, p-Akt, p-GSK-3β and Akt. C: H9c2 cells normally cultured, ADR: H9c2 cells cultured in medium containing ADR, SEV: H9c2 cells cultured after SEV pretreatment, and LY: H9c2 cells cultured in medium containing LY294002 (PI3K inhibitor). **p*<0.05 and ***p*<0.01: significant differences compared with other groups.

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