

## Original Article

# Protective role of Osthole on myocardial cell apoptosis induced by doxorubicin in rats

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**Abstract:** Objective: To explore the effect of Osthole on protecting myocardial cell apoptosis induced by doxorubicin during cardiac failure in rats. Methods: Myocardial cells isolated from the newborn SD rats were separated into three groups: cells treated with 1  $\mu$ mol doxorubicin, cells treated with Osthole at three concentrations of 10, 20, and 40  $\mu$ mol, cells treated neither with Osthole nor with doxorubicin were the control groups. Consequently, cell apoptosis of myocardial cells in each group was analyzed using TUNEL assay. Also, expressions of oxidase, NADPH, and ROS in myocardial cells were analyzed using different biological methods. Moreover, expressions of cell apoptosis associated proteins were analyzed using Western blotting. Results: Compared with the controls, the results showed that cells received Osthole and doxorubicin treatments performed high percentage of cell apoptosis, suggesting that Osthole could anesis myocardial cell apoptosis induced by doxorubicin ( $P < 0.05$ ). Osthole of 10  $\mu$ mol depressed the expressions of cell apoptosis associated proteins including Caspase-3 and CytC, and enhancing expression of Bcl-XL expression ( $P < 0.05$ ). Osthole of 20  $\mu$ mol significantly decreased the generation of intracellular superoxidase, NADPH, and NADPH activity in myocardial cells treated with doxorubicin ( $P < 0.05$ ). Moreover, Osthole of 20  $\mu$ mol could significantly increase phosphorylated eIF2 $\alpha$  level in cells. Conclusion: Our study suggested that Osthole may play a protective role in suppressing myocardial apoptosis induced by doxorubicin through inhibiting NADPH and superoxidase production and downstream phosphorylated eIF2 $\alpha$ .

**Keywords:** Myocardial cells, osthole, doxorubicin, cell apoptosis, cardioprotection

## Introduction

Anthracyclines are some kinds of pivotal drugs for acute lymphoblastic leukemia an cancer chemotherapy in clinical, and whose application is limited by their toxicity on hearts [1]. Doxorubicin is an alternative medicine of daunorubicin for cancer patients in clinical, which could brought toxicity including oxidative stress and cell damage to hearts during chemotherapy [2, 3]. Many papers have referred that toxicities such as myocardial cell apoptosis and myocardial cell damage induced by cancer therapy drugs could brought huge damage to patients life [4, 5]. Therefore, it will be of great significance to explore several effective protective medicines for heart in clinical.

Intracellular reactive oxygen species (ROS) generation could depress the liquidity of unsatu-

rated fatty acid and mitochondrial membrane, which results in mitochondria damage [6]. It has been reported that ROS was involved in myocardial cells induced by doxorubicin [7]. Recently, studies refer that myocardial cell apoptosis was coexisted with cardiac failure and may be a cause for cardiac failure development and progression [8]. Over the years, several pharmacological compounds have been reported to have cardioprotective effects in animal experiments, but only a few of these have been successfully translated to the clinical use. For instance, Li et al. refers paeoniflorin protected myocardial cells from doxorubicin-induced cell apoptosis through inhibition of NADPH oxidase [9], and Jyotirmoy et al. reported that arjunolic acid played protective roles in cardiac cell apoptosis induced by doxorubicin [10].

Osthole is a kind of coumarin that extracted from the traditional Chinese medicine of snake bed [11]. In vitro and in vivo experiments showed that Osthole performed strong cardiovascular pharmacological activities [12, 13], and previous papers have demonstrated that Osthole played crucial roles in arrhythmia, vessel blood, depressing blood pressure, and protecting cardiovascular [14]. Wand and his colleagues proved that Osthole reduces myocardial injury and improved functional recovery following myocardial I/R injury via significantly increased activities of SOD [15]. Shokoohinia et al. proved that Osthole attenuated doxorubicin-induced myocardial cell apoptosis in PC12 cells via inhibiting mitochondrial dysfunction and ROS production [16]. In spite of many studies have searched for useful drugs on protecting myocardial apoptosis induced by doxorubicin, the protective role and mechanism of Osthole on influencing doxorubicin-induced myocardial apoptosis still remain incomplete described.

In this present study, we detected the effect of Osthole on protecting myocardial cells apoptosis induced by doxorubicin from newborn Sprague-Dawley (SD) rats. Comprehensive experimental methods were used to analyze the oxidase level, NADPH, and ROS level and their mechanism in myocardial cells that treated with Osthole and doxorubicin. This study aimed to investigate the effect of Osthole on protecting doxorubicin-induced myocardial apoptosis and its mechanism. Our study may provide theoretical basis on illustrating the biological role of Osthole on myocardial cell apoptosis induced by doxorubicin in clinical.

### Materials and methods

#### *Cell culture and cell treatment*

All the experimental procedures were approved by the local animal ethics committee.

The newborn SD (Sprague-Dawley) rats ageing at 1-2 days (Purchased from Animal center, Zhengzhou University) were sacrificed to collect the heart tissues with approximately 1-3 mm pieces. Fresh heart tissues were digested with 0.25% trypsin (Sigma, USA) collagenase for 1 h, and then were cultured in Dullbecco's modified eagle (DMEM, Invitrogen, USA) medium supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in an atmosphere of 5% CO<sub>2</sub> at 37°C. After being cultured for 2-4 days, cells

which were anchorage dependent growth were trypsinized and subcultured. Consequently, myocardial cells cultured in DMEM medium with cell density of  $1 \times 10^5$  cells were separated into three groups, which were as follows: myocardial cells treated with Osthole with three concentration of 10, 20, and 40  $\mu\text{mol}$  for 4 h; myocardial cells mixed with 1  $\mu\text{mol}$  doxorubicin for 24 h; after being treated with Osthole of three concentration for 4 h, myocardial cells were then treated with 1  $\mu\text{mol}$  doxorubicin for 24 h; myocardial cells treated neither with doxorubicin nor with Osthole were considered as the control group.

#### *Cell apoptosis assay*

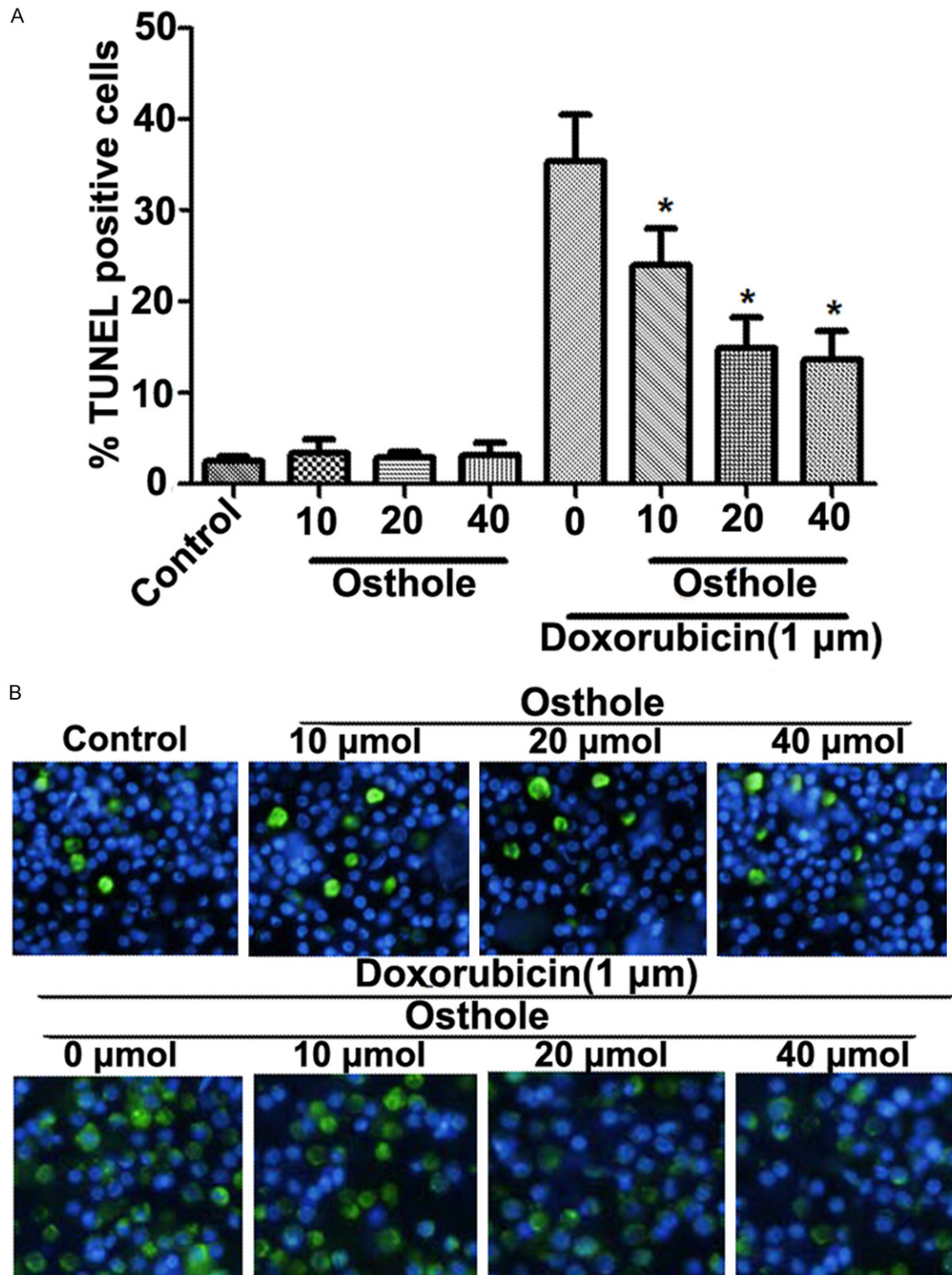
Effect of Osthole with different concentration on myocardial cell apoptosis was quantified using terminal deoxynucleotidyl transferase mediated dUTP nick (TUNEL) assay with TUNEL cell apoptosis kit (Roche, USA) according to the manufacturer's instructions [17]. Myocardial cells were plated on 40 mm round glass cover slips in 60 mm culture dishes. TUNEL was conducted after cells were fixed with 4% paraformaldehyde in PBS buffer (PH 7.4). Nuclear staining was determined via green unclear fluorescence and was observed through laser scanning confocal microscopy in more than 300 cells. Cells were observed in five fields, and cells with brown color were considered as positive cells while others were negative cells. Percentage of myocardial cell apoptosis was calculated as the following formula:  $100\% \times \text{total positive cells in 5 fields} / \text{total cells in 5 fields}$ .

#### *Oxidase assay*

Oxidase production in myocardial cells were measured as previously described [18]. Briefly, myocardial cells in each group with cell density of  $1 \times 10^5$  cells were mixed with the addition of 30  $\mu\text{mol}$  2',7'-dichloro fluorescein diacetate salt (DCFDA) (Invitrogen, USA). After being cultured for 1 h, cells were washed with PBS buffer for 3 times, followed by detecting the fluorescence intensity using a fluorescence microscope (Olympus, Japan).

#### *Detection of superoxide production*

Superoxide production in myocardial cells treated with Osthole and doxorubicin was detected using lucigenin-amplified chemiluminescence as

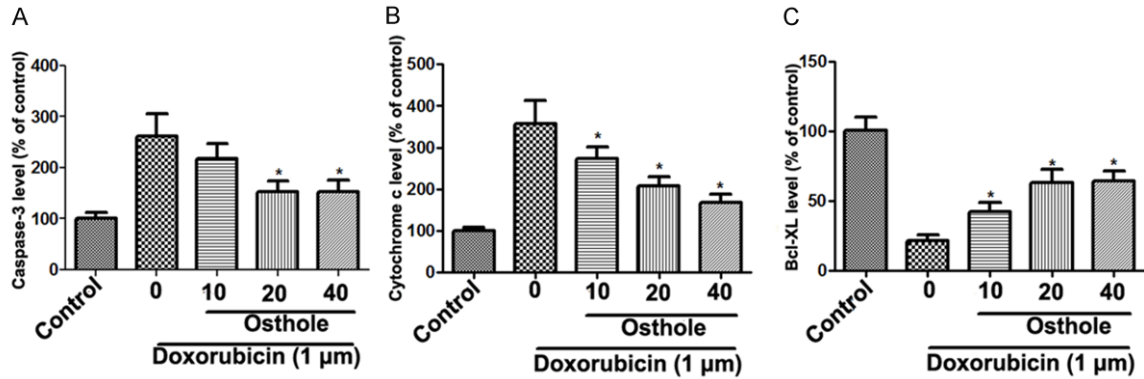


**Figure 1.** Myocardial cell apoptosis treated with doxorubicin and Osthole of different concentration (10, 20, and 40 μmol). \*P<0.05, compared with cells treated with 0 μmol doxorubicin.

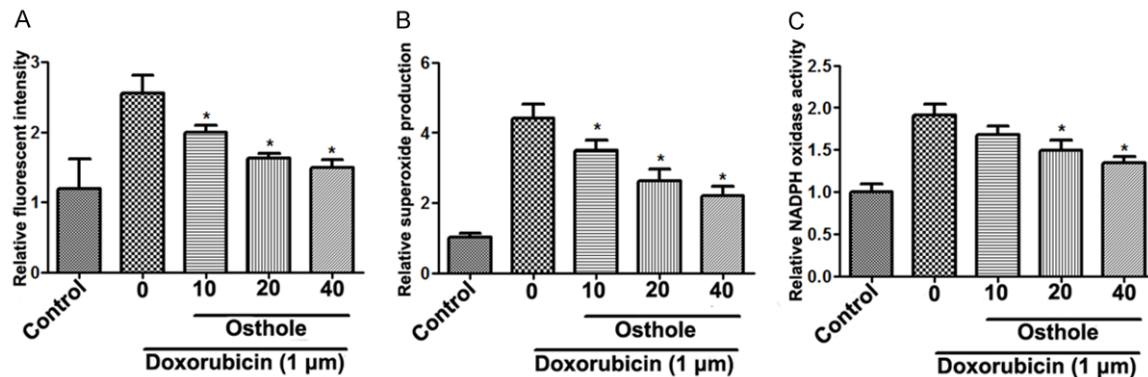
previously described [19]. In a word, myocardial cells that treated with Osthole and doxorubicin

were lysed with lysis buffer supplemented with 200 μmol lucigenin. After that, absorbance of

## Role of Osthole in myocardial cell apoptosis



**Figure 2.** Expressions of myocardial cell apoptosis associated proteins. A. Expression of Caspase-3 in different groups; B. Expression of Cytc in different groups; C. Expression of Bcl-XL in different groups. \*P<0.05, compared with myocardial cells treated with 0 μmol doxorubicin.



**Figure 3.** ROS and NADPH oxidase assay in myocardial cells treated with Osthole and doxorubicin. A. ROS production in myocardial cells; B. NADPH oxidase production in myocardial cells; C. NADPH oxidase activity assay in myocardial cells. \*P<0.05, compared with myocardial cells treated with 0 μmol doxorubicin.

cells was read using a microplate scintillation counter (Topcount, Packard). In addition, cells mixed with SOD were the blank controls.

### NADPH assay

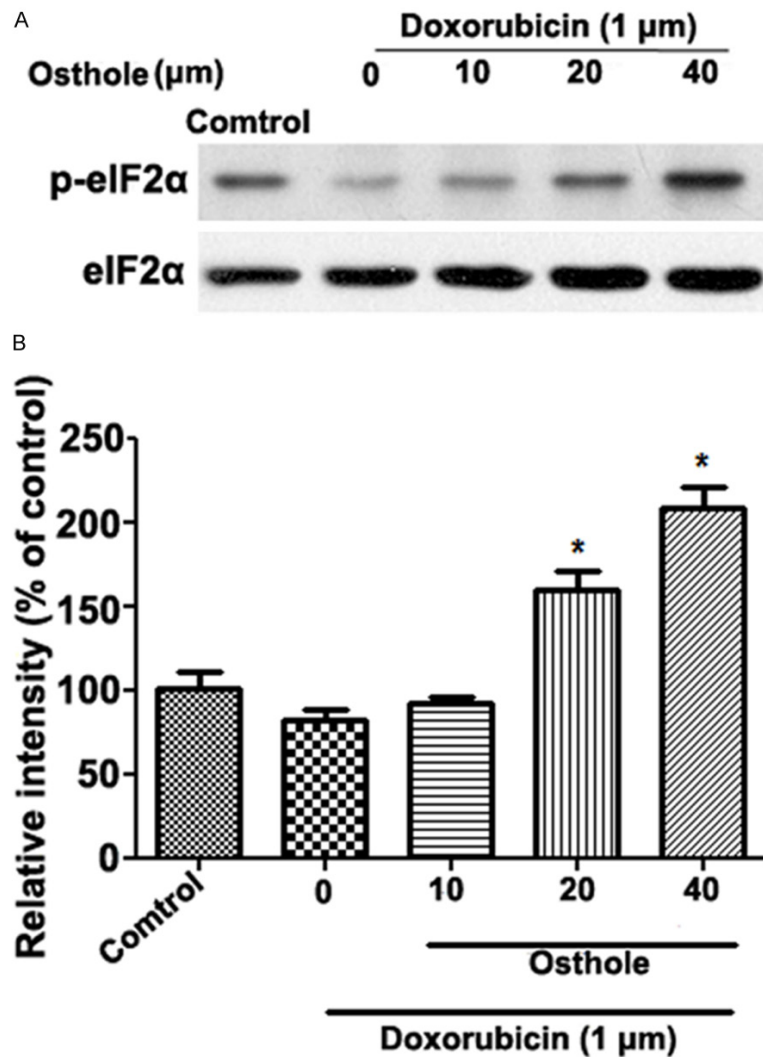
Myocardial cells that treated with Osthole and doxorubicin were centrifuged at 12,000 rpm at 4°C for 5 min. Harvested cells were lysed in 150 μL PBS buffer (50 mM K<sub>3</sub>PO<sub>4</sub> PH 7.0, 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 mM NADPH). Fluorescence intensity of cells was read using the Orion MPP fluorescent reading meter per 60 s. Then concentration of proteins was measured using the BCA method, and NADPH oxidase activity was considered as the ratio of optical unit and protein concentration.

### Western blotting analysis

Myocardial cells treated with 20 and 40 μmol Osthole and 1 μmol doxorubicin at 24 h were

lapped in RIPA (radioimmunoprecipitation assay, Sangon Biotech) lysate containing phenylmethanesulfonyl fluoride (PMSF) and then were centrifuged at 12,000 rpm at 4°C for 5 min [9]. The supernatant was collected to determine the concentration of lysed proteins using bicinchoninic acid (BCA) protein assay kit (Pierce, Rochford, IL) [20]. After that, a total of 20 μg protein per cell lysate was subjected into a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a Polyvinylidene difluoride (PVDF) membrane (Mipore). Membrane was blocked in Tris Buffered Saline Tween (TBST) supplemented with 5% non-fat milk for 1 h, and subsequently incubated with monoclonal antibodies (1:100 dilution, cleaved Caspase-3, phosphorylated eIF2α, eIF2α, Cytc, and Bcl-XL) overnight at 4°C, followed by incubation with horseradish peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Then PVDFs were washed with 1 × TBST





**Figure 4.** Western blotting analyzes of myocardial protection associated proteins. A. Expression of eIF2 $\alpha$ , B. Relative expression of eIF2 $\alpha$  in different groups. \*P<0.05, compared with myocardial cells treated with 0  $\mu\text{mol}$  doxorubicin.

buffer for 10 min for 3 times. Finally, detection of PVDFs was performed using the development of X-ray after chromogenic substrate with an enhanced CEL (chemiluminescence) method. Phosphoglyceraldehyde dehydrogenase (GAPDH, Sigma, USA) was considered as the internal control.

#### Statistical analysis

All data were expressed as mean  $\pm$  standard error of mean (SEM) in this study. Independent sample t-test was used to calculate the difference between two groups using the graph prism 5.0 software (GraphPad Prism, San Diego, CA). ANOVA (analysis of variance) was

used to calculate the difference for more than 3 groups. The P<0.05 was defined as statistically significant.

#### Results

##### *Protective effect of Osthole on myocardial cells*

The results showed that there were few apoptotic myocardial cells which were treated with Osthole lonely, indicating that Osthole is nontoxicity to myocardial cells. Otherwise, cells received Osthole and doxorubicin treatments performed high percentage of cell apoptosis, suggesting that Osthole could anesis myocardial cell apoptosis induced by doxorubicin (**Figure 1**).

##### *Effect of Osthole on myocardial cell apoptosis associated proteins*

The results showed that cell apoptosis associated protein of Caspase-3 and Cytc expressions were increased when treated with 1  $\mu\text{mol}$  doxorubicin but with 0  $\mu\text{mol}$  Osthole compared with the control group, while Bcl-XL expression was declined among the several groups. However, among the experi-

mental myocardial cells treated with 1  $\mu\text{mol}$  doxorubicin, Caspase-3 and Cytc expressions in groups that cells were treated Osthole were both gradually declined with Osthole concentration increasing from 10  $\mu\text{mol}$  to 40  $\mu\text{mol}$  compared with the 0  $\mu\text{mol}$  group (**Figure 2A** and **2B**). Otherwise, expression of Bcl-XL in experimental groups was gradually increased compared with the cells in 0  $\mu\text{mol}$  Osthole (**Figure 2C**).

##### *ROS and NADPH level assay*

The results showed that ROS and NADPH levels in myocardial cells treated with 1  $\mu\text{mol}$  doxorubicin were both significantly increased com-

pared with the control group. Besides, after being treated with Osthole, ROS and NADPH levels were significantly declined with Osthole concentration increasing from 10  $\mu\text{mol}$  to 40  $\mu\text{mol}$  ( $P < 0.05$ ) (**Figure 3A** and **3B**). In addition, after being centrifuged, NADPH were collected, the results showed that relative NADPH oxidase activity was significantly decreased when myocardial cells were treated with 20  $\mu\text{mol}$  or 40  $\mu\text{mol}$  Osthole ( $P < 0.05$ ) (**Figure 3C**).

### *Western blotting analyze*

In order to analyze the potential mechanism of Osthole on myocardial toxicity induced by doxorubicin, western blotting analysis was used to detect the proteins expressions in myocardial cells (**Figure 4**). After being treated with 20 and 40  $\mu\text{mol}$  Osthole, the phosphorylated eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) were significantly increased compared with the cells in 0 or 10  $\mu\text{mol}$  Osthole group ( $P < 0.05$ , **Figure 4A** and **4B**).

### **Discussion**

Myocardial cell toxicities such as myocardial cell apoptosis and myocardial cell damage induced by cancer therapy drugs including doxorubicin could brought about huge damage to patients life [4, 5]. Osthole, a kind of coumarin that extracted from the traditional Chinese medicine of snake bed, has been proved to present strong cardiovascular pharmacological activities [12, 13]. In this study, we analyzed the potential protective role of Osthole on myocardial cell apoptosis induced by doxorubicin in rats. Our data presented that doxorubicin significantly increased the expressions of Caspase-3, Cytc, NADPH activity, ROS level, and phosphorylated eIF2 $\alpha$ , and significantly decreased Bcl-XL expression compared with the controls ( $P < 0.05$ ). However, the effects of doxorubicin on myocardial cell apoptosis associated proteins expressions could be excellent inhibited by addition of Osthole with different concentration ( $P < 0.05$ ).

Cytc, exists between the inside and outside mitochondrial membrane, is the first proved cell apoptosis-induced factor in mitochondria [21], while Caspase-3 is a frequently activated protease, catalyzing specific cleavage of many key cellular proteins through activating several downstream signals [22]. However, Bcl-XL is the anti-cell apoptosis factor in Bcl-2 family

proteins that correlated to cell apoptosis induced by pathogen factors and others [23]. In Chandran's paper, doxorubicin inactivated Cytc oxidase in rats myocardial cells [24], and Wang and his partners demonstrated that doxorubicin increased Cytc release in cardiomyocytes [25]. Our data showed that doxorubicin significantly increased Caspase-3 and Cytc activation compared with the controls, which is in line with former evidences. On the other hand, Osthole protected myocardial ischemia/reperfusion in rats via inhibiting overexpression of Cytc and Caspase-3 [15]. High Bcl-XL level leads to a decreased cell apoptosis in mitochondria. Shokooahinia said that Osthole protected doxorubicin-induced PC12 cell apoptosis through suppressing Cytc and Caspase-3 release but increasing Bcl-XL level [16]. Our results performed that after being treated with Osthole of different concentration, expressions of Cytc and Caspase-3 in myocardial cells were both significantly declined while Bcl-XL was significantly increased compared with the controls, therefore, we speculated that Osthole may protect myocardial cells from apoptosis induced by doxorubicin through affecting the cell apoptosis proteins expression.

Meanwhile, ROS have been implicated in pathogenesis of cardiac disease including myocardial apoptosis and myocardial remodeling [26, 27], while NADPH oxidases are multi-subunit enzyme complexes that transfer electrons across biological membranes and catalyze the production of ROS [28]. Kotamraju and his colleagues proved that doxorubicin induced ROS release and nitrogen species in endothelial cells and cardiomyocytes [29]. Also, NADPH oxidase activity was increased in human heart failure [30]. Our study presented that doxorubicin significantly increased ROS level and NADPH oxidase activity compared with the controls, indicating that addition of doxorubicin lead to cell mitochondrial damage and resulted in ROS release and NADPH activity increasing. After being added with Osthole, the ROS level and NADPH activity in myocardial cells from each group significantly declined compared with the controls. Based on these results, we speculated that Osthole may play certain protect role on myocardial cell apoptosis via affecting the ROS release and NADPH activity.

Additionally, eIF2 $\alpha$  is an important regulator during protein translation in many biological

processes [31]. Phosphorylated eIF2 $\alpha$  reduced translation of total proteins and then resulted in pressure reducing of cells in stress state [32]. Application of doxorubicin resulted in mitochondrial DNA damage of myocardial cells [33]. Paper referred that DNA damage may induced cell response and then activated the downstream signals including eIF2 $\alpha$  to activate protection or reparation [34]. Our data performed that doxorubicin significantly increased phosphorylated eIF2 $\alpha$ , suggesting that mitochondrial DNA damage induced by doxorubicin resulted in activation of phosphorylated eIF2 $\alpha$ . However, this effect was inhibited by addition with Osthole of 20 and 40  $\mu$ mol, indicating that Osthole may function as a protector in myocardial cell damage through decreasing phosphorylated eIF2 $\alpha$  level in myocardial cells.

In conclusion, the data presented in this study suggests that Osthole may protect myocardial cells from damaging induced by doxorubicin. Osthole may protect myocardial cells from apoptosis through decreasing the expressions of Caspase-3, CytC, ROS release, and NADPH oxidase activity, and increasing Bcl-XL and phosphorylated eIF2 $\alpha$ . Our study may provide basis for the potential application of Osthole in myocardial protection in clinical. However, further experimental studies are still needed to investigate the mechanism of Osthole downstream signaling pathway in protecting myocardial apoptosis.

## Disclosure of conflict of interest

None.

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