

Original Article

Osthole attenuated myocardial ischemia/reperfusion injury via a mitochondrial apoptosis

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Abstract: The mitochondrion plays an important role in myocardial ischemia/reperfusion (MI/R) injury. Osthole, a bioactive coumarin derivative extracted from medicinal plants, has been shown to have protective properties in ischemic disease. The aims of the present study were to explore whether Osthole could attenuate myocardial I/R injury and to investigate the potential mechanisms involved. The I/R model was established in vivo in SD rats. To evaluate the protective effects of Osthole on I/R injury, we measured the hemodynamics, myocardial pathological change and the expression of CK, LDH, BCL-2, Bax, BCL-XL, Caspase3, Cleaved-Caspase3, Caspase9 and Cleaved-Caspase9. We also explored the potential mechanisms by investigating mitochondrial function as demonstrated by the ROS, ATP content, translocation of cytochrome c and AIF. Pretreatment with Osthole can significantly decrease the serum CK and LDH level, the pathological and hemodynamic changes, the ROS level, the expression level of cleaved-Caspase3, cleaved-Caspase9, Bax, cytoplasmic cytochrome C and AIF in MI/R injury with the up-regulation of Bcl-2, BCL-XL, Caspase3 and Caspase9. Our results implied that Osthole may protect rats against MI/R injury by preventing ROS-induced mitochondrial apoptosis.

Keywords: Osthole, mitochondrion, apoptosis, myocardial ischemia reperfusion injury

Introduction

Myocardial ischemia reperfusion (MI/R) injury is a common and serious complication in clinical practice, which seriously impairs myocardial function and significantly increases morbidity and mortality [1, 2]. The exact molecular mechanisms of MI/R injury is still not completely understood, but there are substantial convincing evidence that apoptosis plays a vital role in the MI/R injury [3, 4]. Therefore, It may be an effective strategy to alleviate myocardial ischemia reperfusion injury by anti-apoptosis.

The mitochondrion plays an important role in energy metabolism which provide ATP for various cellular activities by oxidative phosphorylation [5]. But in the hypoxic and ischemic tissues, the oxidative phosphorylation activity of mitochondrion is blocked. Which will lead to ROS generation, calcium overload, the change of mitochondrial structure and function in proper sequence, ultimately triggering mitochondrial apoptotic pathway [5-7].

Osthole, a bioactive coumarin derivative extracted from traditional Chinese medicine has been showed to have multiple of pharmacological activities, such as anti-inflammatory, anti-apoptotic, anti-tumor, as well as anti-hepatic and anti-allergic effect [8, 9]. In addition, previous study have demonstrated that Osthole can reduce renal ischemic reperfusion injury [10]. Therefore, the aim of the present study was to explore the effect of Osthole in an animal model of MI/R injury and its underlying mechanism.

Materials and methods

Drug and animals

Osthole (>98% purity) was purchased from the Yi Yue Biotechnology (Xi An, China) and dissolved in dimethyl sulfoxide (DMSO, Sigma) (<0.1%, which have no toxicity).

50 Male SD rats (8 wk-old, 220-250 g) were purchased from Shandong University Experimental Animal Center, Shang dong, China. The rats

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were placed in a specific pathogen-free (SPF) facility and fed with laboratory chow and water. After 7 days of acclimation, the rats were randomly equally divided into three groups with each containing 10 rats: (1) MI/R-saline group (MI/R), in which the rats were pretreated with saline (50 mg/kg, intraperitoneally) at 0.5 h prior to MI/R induction; (2) MI/R-Osthole groups (Osthole), in which the rats were pretreated with Osthole (12.5, 25, 50 mg/kg, intraperitoneally) at 0.5 h prior to MI/R induction; (3) Sham-operated group (Sham), in which the rats were pretreated with saline (50 mg/kg, intraperitoneally) prior to Sham operation. The dose of Osthole was according to previous study [11]. All studies were approved by the Institutional Animal Care and Use Committee at Sichuan Provincial People's Hospital, Chengdu, China.

IR induction in the heart

The rats were anaesthetized by intraperitoneal injection of 1% sodium pentobarbital solution at the concentration of 65 mg/kg at first. Myocardial I/R injury was induced as follows: The rats were fixed in the supine position and secured in the dissection tray. Myocardial ischemia was induced by exteriorizing the heart with a left thoracic incision followed by making a slipknot (4-0 silk) around left anterior descending coronary artery. After 0.5 hour of ischemia, the slipknot was relaxed and the myocardium was reperfused for 4 h. The rats in the Sham group underwent the same surgical procedures without the exception of left anterior descending coronary artery occlusion.

Hemodynamic assessment

The right common carotid artery was exposed and cannulated with a 2 F Millar Catheter into the left ventricle through the ascending aorta to monitor heart function, including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), heart rate (HR), mean arterial pressure (MAP) and first derivative ($\pm dp/dt_{max}$) of left ventricular pressure in each group. To eliminate the confounding factor in which the loading conditions of the heart may influence cardiovascular parameters, additional rats were used to examine whether Osthole on its own has an effect on LVSP, LVEDP, HR and $\pm dp/dt_{max}$ in normal hearts under sham-operated conditions.

Assessment of myocardial injury

Blood samples were obtained from the inferior vena cava at 4 h after reperfusion. Serum creatine kinase (CK) and serum Lactate dehydrogenase (LDH) levels were assayed in the core laboratory of the Sichuan Provincial People's Hospital, Chengdu, China for assessment of myocardial injury.

Histological analysis

At 4 h reperfusion, the excised myocardial were fixed with 10% formalin, paraffin-embedded, and stained with hematoxylin and eosin (HE). MI/R injury was scored by morphologic criteria [12]: 0, no damage; 1 (mild), interstitial edema and localized necrosis; 2 (moderate), widespread myocardial cell swelling and necrosis; 3 (severe), necrosis with contraction bands and compressed capillaries, or 4 (highly severe), diffuse necrosis with contraction bands, compressed capillaries, and hemorrhage.

Measurement of ROS

ROS Kits were used to measure ROS level of myocardial according to the manufacturer's instructions. Techniques to measure ROS were performed as previously described [13]. Briefly, Protein extracts from myocardial were incubated with the 1:500 ROS-sensitive dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) dilution, and then incubated for 20 min at 37°C.

Measurement of ATP content

ATP concentration was examined by using an ATP determination kit (Beyotime, Nanjing, China). Myocardial tissues were lysed and centrifuged at 10000 g for 15 min.

10 mL supernatants (10 IL) were mixed with a reaction buffer (100 mL) containing 0.5 mM luciferin, 1 mM dithiothreitol, and 12.5 mg/mL luciferase. Luminance (RLU) of the mixtures was examined by using a Varioskan Flash microplate reader. An ATP standard curve was established for the calculating the ATP content.

Western blot analysis

Myocardial tissue were lysed with RIPA buffer (Beyotime, Nanjing, China) in the presence of a cocktail of protease inhibitors Roche, Indian-

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Table 1. The effect of Osthole on the CK and LDH in the MI/R injury

Group	Number	CK (mU/mL)	LDH (mU/mL)
Sham	10	67.7 ±9.1	356.7±52.7
MI/R	10	213.5±15.3*	1980.5±165.3***
Osthole (12.5 mg/kg)	10	162.5±13.2#	1285.42.5±132.2#
Osthole (25 mg/kg)	10	110.4±10.7##	1075.4±114.4##
Osthole (50 mg/kg)	10	82.6±9.9##	732.6±99.5##

Note: *Compared MI/R group with Sham group (* $P<0.05$, *** $P<0.001$); #Compared MI/R group with Osthole group (# $P<0.05$, ## $P<0.01$). CK, Serum creatine kinase; LDH, serum Lactate dehydrogenase.

apolis, IN, USA) and centrifuged at 12,000 g and 4°C for 30 min. Cytosolic fractions were prepared using a commercially available cytosol/mitochondria fractionation kit, following the manufacturer's recommendations (Beyotime, Nanjing, China). After protein concentration determination using a BCA kit (Beyotime, Nanjing, China), the proteins extracted from myocardial tissue were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following protein transfer, nitrocellulose membranes were blocked, followed by detection with different primary antibodies Caspase3 (1:1000), cleaved-Caspase3 (1:500), Caspase9 (1:1000), cleaved-Caspase9 (1:500), Bax (1:500), BCL-2 (1:500), Bcl-XL (1:500), cytochrome c (1:500) and AIF (1:1000) at 4°C overnight. As described previously [13], followed by incubation with an HRP-conjugated secondary antibody (Jackson ImmunoResearch Lab-oratories, West Grove, PA). β -actin (Abmart, China, 1:300) was used for normalization. The reactive bands were visualized using the ECL-Plus reagent (Amersham, Piscataway, NJ) as instructed. The density of each reactive band was quantified using the Labworks image acquisition platform and its related analytic software (UVP, USA).

Statistical analysis

Experiments were repeated three times in biological replicates to obtain mean values. Data are presented as the mean \pm standard deviation. Differences among groups were assessed using a one-way analysis of variance. Least significant difference t-tests were used when a single control group was compared with all other groups. Statistical significance ($P<0.05$) as compared to control group is denoted with an asterisk (*). All statistical analyses were

conducted using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Osthole pretreatment decreased IR-induced myocardial damage

We first sought to establish the impact of on myocardial function following MI/R insult. For this purpose, we examined the expression levels of CK and LDH in the serum.

As compared with the Sham group, the MI/R group exhibited a 3-fold higher levels of CK and a 6-fold higher levels of LDH ($P<0.05$), implying that MI/R insult induced serious myocardial injury. Notably, rats pretreated with Osthole can significantly reduce the myocardial injury in a dose-manner as manifested lower level of CK and LDH in Osthole group than MI/R group ($P<0.05$) as shown in **Table 1**. Altogether, our data support that pretreatment with Osthole can provide protection for rats from MI/R injury in a dose manner and the best concentration of Osthole for protection is 50 mg/kg.

Osthole pretreatment attenuated MI/R-induced the hemodynamic changes

In order to further ascertain the protective effect of Osthole on MI/R injury, we evaluated the effect of Osthole on hemodynamic changes at the best concentration of Osthole. As shown in **Table 2**, compared with the Sham group, the MI/R group displayed higher level of LVEDP and HR with the lower LVSP, MAP and $\pm dp/dt_{max}$. However, Compared with the MI/R group, the Osthole groups showed lower levels of LVEDP and HR with the higher level of LVSP, MAP and $\pm dp/dt_{max}$. This result demonstrated that pretreatment with Osthole can attenuate MI/R-induced the hemodynamic changes.

Osthole pretreatment reduced MI/R-induced myocardial pathological changes As shown in **Figure 1**, compared with the Sham Group, the MI/R group have more significant pathological changes as manifested more atrophy of myocardial fibers, coagulation necrosis, colliquative necrosis and inflammatory cell infiltration ($P<0.05$) However, compared with the MI/R Group, the Osthole group have less pathological changes as manifested less atrophy of myo-

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Table 2. The effect of Osthole on hemodynamic changes in MI/R injury

Group	N (n)	LVSP (mmHg)	LVEDP (mmHg)	+dp/dt _{max}	-dp/dt _{max}	HR (bpm)	MAP (mmHg)
Sham	10	112±9	8.6±1.5	9142±756	8372±719	312±19	86±7
MI/R	10	55±6*	13.4±2.2*	5537±536*	5015±598*	395±26*	65±6*
Osthole	10	88±7#	9.4±1.2#	8284±567#	7458±637#	325±21#	78±6#

Note: *Compared MI/R group with Sham group (* $P<0.05$); #Compared MI/R group with Osthole group (* $P<0.05$). LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dp/dt_{max}, indice of left ventricular contraction; -dp/dt_{max}, indice of left ventricular relaxation; HR, heart rate; MAP, mean arterial pressure.

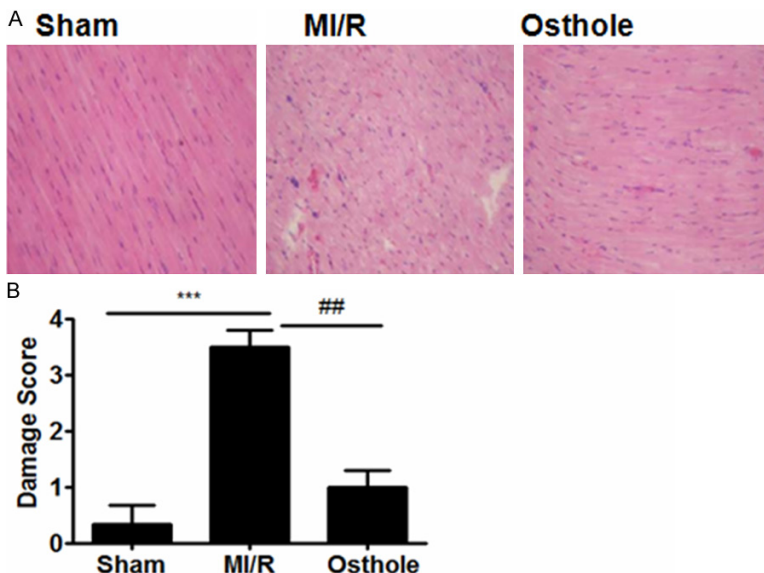


Figure 1. Histological analysis of myocardial tissues. A: Representative HE staining results for myocardial sections originated from Sham group, MI/R group and Osthole group; B: Semi-quantitative analysis of HE staining for all rats included. Ten rats were analyzed in each group, and the images are presented at 200× magnification. *** $P<0.001$ (MI/R vs. Sham); ## $P<0.001$ (MI/R vs. Osthole).

cardial fibers, coagulation necrosis, colliquative necrosis and inflammatory cell infiltration ($P<0.05$). This indicated that Osthole pretreatment can significantly attenuate the MI/R-induced myocardial pathological changes.

Osthole pretreatment decreased ROS in the MI/R injury

Compared with the Sham Group, the MI/R group have higher level of ROS ($P<0.05$). However, compared with the MI/R Group, the Osthole group have less level of ROS ($P<0.05$), as shown in **Figure 2**. This indicated that Osthole pretreatment can significantly reduce ROS by promoting oxidative phosphorylation activity of mitochondrion in the MI/R injury.

Osthole pretreatment suppressed mitochondrial dysfunction induced by MI/R injury

Mitochondrial dysfunction plays an important role in MI/R injury [5-7]. To explore the effects of Osthole on mitochondrial function, ATP content is an indication

of mitochondrial activity in the rats heart. As shown in **Figure 3**, compared with the Sham Group, the ATP content were significantly reduced in the MI/R group. In contrast, pretreatment with Osthole can increase the ATP content as manifested higher level of ATP concentration in Osthole group than MI/R group ($P<0.05$). This indicated that Osthole pretreatment can significantly suppress mitochondrial dysfunction in the MI/R injury.

Osthole pretreatment attenuated the translocation of cytochrome c and AIF induced by MI/R injury

Compared with the Sham Group, the MI/R group have higher level of cytochrome c and AIF in the cytosolic ($P<$

0.05). Notably, pretreatment with Osthole can lead to a decrease in the cytosolic cytochrome c and AIF as manifested lower level of cytosolic cytochrome c and AIF in Osthole group than MI/R group ($P<0.05$), as shown in **Figure 4**. This indicated that Osthole pretreatment can significantly attenuate the translocation of cytochrome c and AIF in the MI/R injury.

Osthole pretreatment suppressed the activation of apoptotic pathway in the MI/R injury

In order to ascertain the effect of Osthole on apoptotic pathway in the MI/R injury, we evaluated the effect of Osthole on the expression of apoptosis related proteins by western blotting examining. As shown in **Figure 5**, compared

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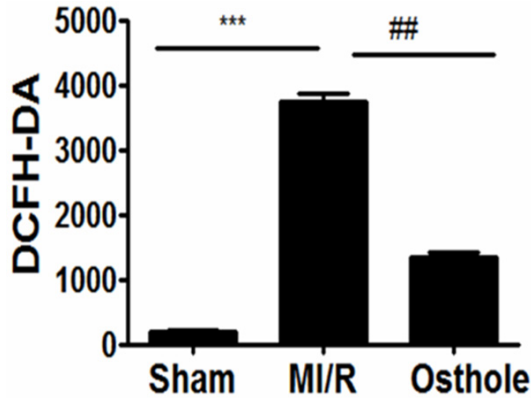


Figure 2. Effect of Osthole pretreatment on the expression level of ROS after MI/R injury. Myocardial tissue homogenates of all the groups were respectively incubated with DCFH-DA at 37°C for 20 minutes. The fluorescence in each group was assessed by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 535 nm. Data were represented as mean ± SEM (n = 10). ****P* < 0.01 (MI/R vs. Sham); #*P* < 0.05 (MI/R vs. Osthole).

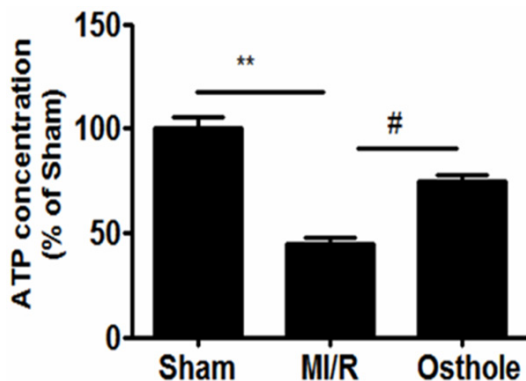


Figure 3. Effect of Osthole pretreatment on the ATP concentration after MI/R injury. Myocardial tissue homogenates of all the groups were respectively incubated with ATP determination kit. The fluorescence in each group was assessed by flow cytometry with an excitation wavelength of 488 nm and an emission wave length of 535 nm. Data were represented as mean ± SEM (n = 10). ****P* < 0.01 (MI/R vs. Sham); #*P* < 0.05 (MI/R vs. Osthole).

with the Sham Group, the MI/R group have higher level of Cleaved Caspase-3, Cleaved caspase-9 and Bax expression with lower level of Caspase-3, Caspase-9, Bcl-2 and Bcl-xL expression (*P* < 0.05). However, Compared with the MI/R Group, the Osthole group have less level of Cleaved Caspase-3, Cleaved Caspase-9 and Bax expression with higher level of Caspase-3, Caspase-9, Bcl-2 and Bcl-xL expression (*P* < 0.05). This indicated that Osthole pre-

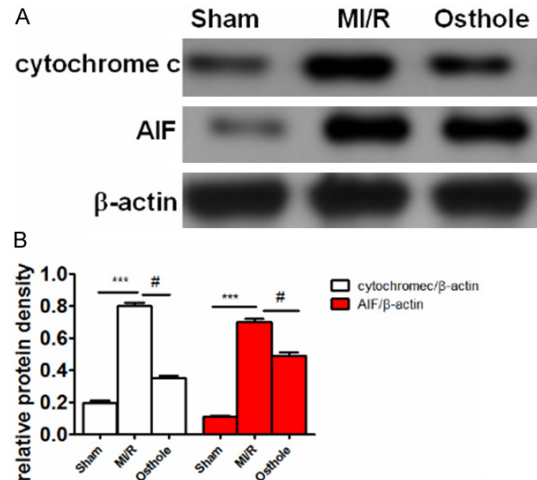


Figure 4. Effect of Osthole treatment on the release of cytochrome C and AIF proteins. A: Representative Western blots for cytoplasmic cytochrome C and AIF from each group. B: Semi-quantitative analysis of 10 animals studied in each group. The relative amount of cytoplasmic cytochrome C and AIF were normalized by β-actin and presented as a ratio between cytochrome C/β-actin and AIF/β-actin. ****P* < 0.001 (MI/R vs. Sham); #*P* < 0.05 (MI/R vs. Osthole).

treatment can significantly suppress the activation of apoptotic pathway in the MI/R injury and the structure of Osthole is as shown in **Figure 6**.

Discussion

Myocardial ischemia-reperfusion injury is a complicated pathophysiological process [14]. Previous research have displayed that the MI/R injury was associated with cascade reactions that is ischemic heart tissues leading to insufficiency of oxygen and ATP, in turn repressing oxidative phosphorylation activity of mitochondrion, producing oxygen radicals, accumulation of H⁺, calcium overload, then altering the mitochondrial permeability, impairing the functional of mitochondria, containing dissipation of the ΔΨ_m, release of cytochrome C and AIF into the cytoplasm, then activating Caspases, resulting in apoptosis, ensuing restoring the blood flow aggravating the above reaction, eventually inducing cardiac structural changes and loss of function [15, 16]. Therefore, it is a strategy to attenuating MI/R injury by promoting oxidative phosphorylation activity of mitochondrion in ischemia phase.

Over the past few years, a number of pharmacological compounds have been displayed to possess excellent protective effects on heart in some animal experiments; unfortunately, only a

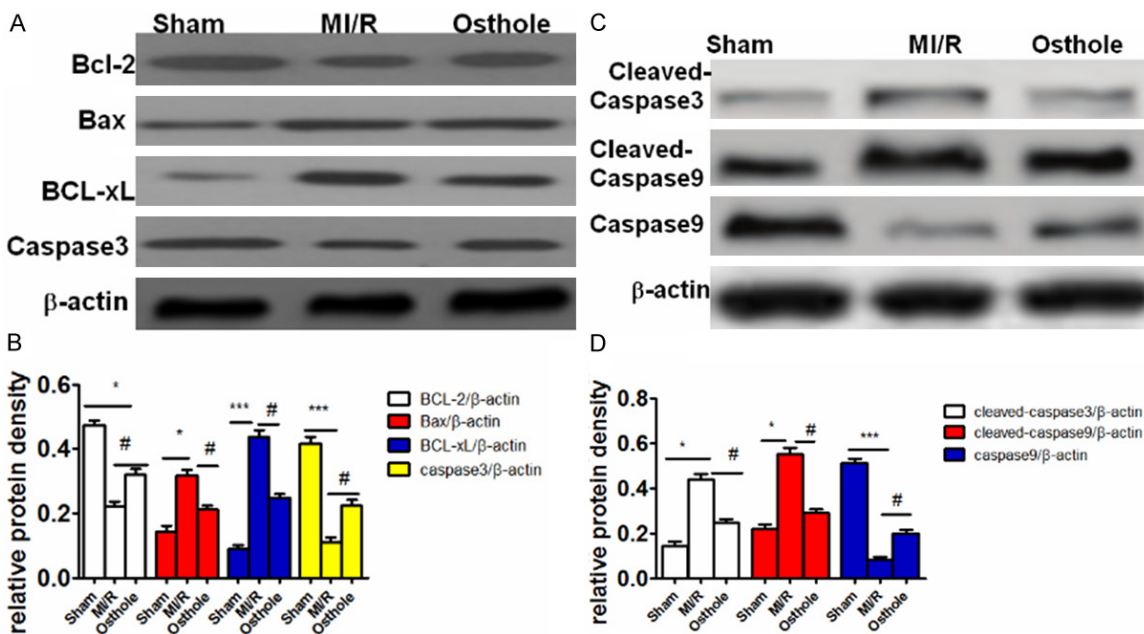


Figure 5. Effect of Osthole treatment on the expression of proteins regulating mitochondria-mediated apoptosis. A: Representative Western blots for Bcl-2, Bax, BCL-xL and caspase3 from each group. B: Semi-quantitative analysis of 10 animals studied in each group. The relative amount of Bcl-2, Bax, BCL-xL and caspase3 was normalized by β -actin and presented as a ratio between Bcl-2/ β -actin, Bax/ β -actin, BCL-xL/ β -actin and caspase3/ β -actin. * P <0.05, *** P <0.001 (MI/R vs. Sham); # P <0.05 (MI/R vs. Osthole). C: Representative Western blots for cleaved-Caspase3, cleaved-Caspase9 and caspase9 from each group. D: Semi-quantitative analysis of 10 animals studied in each group. The relative amount of cleaved-Caspase3, cleaved-Caspase9 and caspase9 was normalized by β -actin and presented as a ratio between cleaved-Caspase3/ β -actin, cleaved-Caspase9/ β -actin, and caspase9/ β -actin. * P <0.05, *** P <0.001 (MI/R vs. Sham); # P <0.05 (MI/R vs. Osthole).

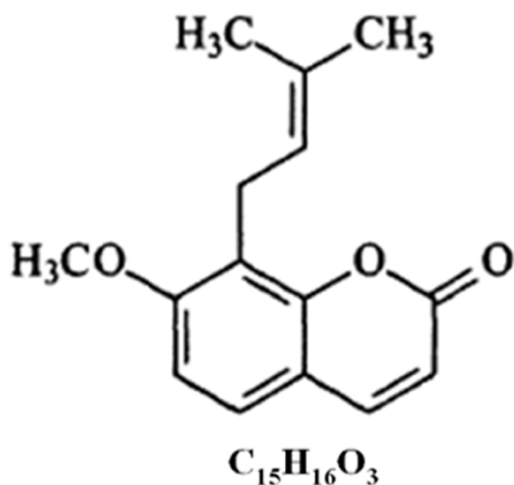


Figure 6. The structure of Osthole.

few have been used for clinic successfully [8]. The reason is that most of the compounds are either side-effects or wireless, as well as the limited window treatment. Last research displayed it may be an effective way to looking for safe and effective medicine for treating isch-

emic heart disease by study of natural drugs, especially Chinese herbal medicine [8, 9]. Osthole is an active ingredient extracted from nature plants, such as *Angelica pubescent* and *Cnidium monnieri* [8]. Because of its excellent tonic and aphrodisiac effect, the Osthole have been widely used in clinic in Chinese medicine for several centuries [8, 9]. Recently, the study pharmacological research have demonstrated that Osthole have a effect of suppressing inflammation, apoptosis and tumor, as well as promoting oxidative phosphorylation activity of mitochondrion [8, 9]. This implied that Osthole have underlying therapeutic applications in MI/R injury.

In this study, we found that Osthole significantly reduced the expression of serum LDH enzymes and CK isoenzyme in a dose-manner induced by myocardial I/R injury, and also attenuated the pathological changes in the MI/R injury. In addition, pretreatment with Osthole can significantly protect left ventricular function, as manifested lower level of LVEDP and higher level of LVSP and $\pm dp/dt_{max}$ in the Osthole group than

MI/R group. All above these document evidently confirmed that Osthole can protect rats against MI/R injury. we further explore the potential mechanism for the observed Osthole protective effect on MI/R injury.

It is well known that oxidative phosphorylation of mitochondrion plays an important role in the MI/R injury. If the activation of oxidative phosphorylation is damaged, it will increase ROS level, then altering the mitochondrial structure and permeability, impairing the function of mitochondria, such as releasing cytochrome C and AIF into the cytoplasm [15, 16]. In addition, the release of cytochrome C and AIF can induce apoptosis. In our study we found that MI/R injury can increase the ROS level and the expression of cytoplasmic cytochrome C and AIF and their downstream cleaved caspases with down-regulating ATP concentration which are in line with previous study. Pretreatment with Osthole can decrease the ROS level, the cytoplasmic cytochrome C and AIF and their downstream cleaved caspases with up-regulating the ATP concentration. These results demonstrated that Osthole provide cardioprotection partly by inhibiting ROS mediating mitochondrial apoptosis.

Notably, Osthole reduced apoptosis by another possible mechanism regulated by Bcl-2 family of proteins. Bcl-2 and Bcl-XL (anti-apoptotic proteins) can maintain the integrity of external mitochondrial membrane, impeding the release of AIF and cytochrome C from the mitochondria. Conversely, pro-apoptotic protein Bax mitochondrial induce cell death [19, 20]. In the study, we demonstrated that MI/R can decrease the expression of Bcl-2 and Bcl-XL (anti-apoptotic proteins) and increased significantly the expression of Bax. Osthole pretreatment can significantly enhance the expression of Bcl-2 and Bcl-XL and decrease the expression of Bax in the MI/R injury. These results further demonstrated that Osthole pretreatment can attenuate MI/R-induced apoptosis by a mitochondrial-dependent pathway.

Conclusion

In conclusion, our studies confirmed that Osthole can protect rats against MI/R injury by decreasing ROS-induced mitochondria apoptosis. Therefore, Osthole may be an useful agent for myocardial injury a clinical.

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Disclosure of conflict of interest

None.

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