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Original Article / Özgün Makale

Osthole down-regulates miR-30a and promotes autophagy to protect rats against myocardial ischemia/reperfusion injury

Osthol sıçanları miyokardiyal iskemi/reperfüzyona karşı korumak için miR-30a'yı azaltarak düzenler ve otofajiyi destekler

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ABSTRACT

Background: This study aims to investigate the changes in miR-30a expression and myocardial autophagy following osthole treatment in a myocardial ischemia/reperfusion injury model.

Methods: Thirty male Wistar rats (weighing 170 to 220 g, aged 8 weeks) were randomly divided into three groups as control (sham) group, ischemia/reperfusion (model) group, and ischemia/reperfusion + osthole post-treatment (osthole) group. Masson's trichrome staining was used to detect myocardial collagen changes. Apoptotic cardiomyocytes in the ischemic area were labeled *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP (2'-deoxyuridine 5'-triphosphate) nick end labeling assay. Levels of autophagy markers light chain 3 beta (LC3β) and Beclin-1 in myocardial tissue were detected by western blotting. Expression of miR-30a was detected by quantitative reverse transcription-polymerase chain reaction.

Results: Compared with the sham group, ischemia/reperfusion significantly increased collagen contents. Osthole significantly inhibited the ischemia/reperfusion-increased collagen contents. Osthole inhibited the ischemia/reperfusion-increased myocardial fibrosis, myocardial swelling, necrosis, and myocardial atrophy. Osthole also significantly inhibited the ischemia/reperfusion-increased apoptosis of myocardial cells. Moreover, the conversion of LC3β-I to LC3β-II and the Beclin-1 expression were significantly inhibited by ischemia/reperfusion. Osthole treatment significantly increased the conversion of LC3β-I to LC3β-II and Beclin-1 expression in ischemia/reperfusion rats. Finally, the expression of miR-30a was significantly increased in ischemia/reperfusion rats, while Osthole suppressed the expression of miR-30a.

Conclusion: Osthole promoted autophagy, thereby alleviating myocardial ischemia/reperfusion injury. Osthole protects the myocardium during autophagy by down-regulating miR-30a expression.

Keywords: Autophagy, Beclin-1, miR-30a, myocardial ischemia/reperfusion injury, osthole.

ÖZ

Amaç: Bu çalışmada bir miyokardiyal iskemi/reperfüzyon hasarı modelinde osthol tedavisi sonrası miR-30a ekspresyonu ve miyokardiyal otofajideki değişiklikler araştırıldı.

Çalışma planı: Otuz erkek Wistar sıçanı (ağırlık, 170-220 g, yaş 8 hafta) randomize şekilde kontrol (taklit) grubu, iskemi/reperfüzyon (model) grubu ve iskemi/reperfüzyon + osthol tedavi sonrası (osthol) grubu olmak üzere üç gruba ayrıldı. Miyokardiyal kollajen değişiklilerini saptamak için Masson'un trikrom boyaması kullanıldı. İskemik alandaki apoptotik kardiyomiyositler terminal deoksinükleotidil transferaz aracılı dUTP (2'-deoxyuridine 5'-triphosphate) çentik-uç etiketleme testi ile *in situ* etiketlendi. Miyokardiyal dokuda LC3β (light chain 3 beta) ve Beclin-1 otofaji belirteçlerinin düzeyleri western blotlama ile saptandı. miR-30a ekspresyonu kantitatif ters transkripsiyon polimeraz zincir reaksiyonu ile saptandı.

Bulgular: İskemi/reperfüzyon, taklit grubuna kıyasla kollajen içeriklerini anlamlı şekilde artırdı. Osthol, iskemi/reperfüzyonun artırdığı kollajen içeriklerini anlamlı şekilde engelledi. Osthol, iskemi/reperfüzyonun artırdığı miyokardiyal fibrozu, miyokardiyal şişkinliği, nekrozu ve miyokardiyal atrofiyi engelledi. Osthol, miyokardiyal hücrelerde iskemi/reperfüzyonun artırdığı apoptozu da anlamlı şekilde engelledi. Ayrıca, LC3β-I'in LC3β-II'ye dönüşümü ve Beclin-1 ekspresyonu iskemi/reperfüzyon tarafından anlamlı şekilde engellendi. Osthol tedavisi iskemi/reperfüzyon sıçanlarında LC3β-I'in LC3β-II'ye dönüşümünü ve Beclin-1 ekspresyonunu anlamlı şekilde artırdı. Son olarak, miR-30a ekspresyonu iskemi/reperfüzyon sıçanlarında anlamlı şekilde artarken osthol, miR-30a ekspresyonunu baskıladı.

Sonuç: Osthol otofajiyi destekleyerek miyokardiyal iskemi/reperfüzyon hasarını azalttı. Osthol miR-30a ekspresyonunu azaltarak düzenleyerek otofaji sırasında miyokardı korur.

Anahtar sözcükler: Otofaji, Beclin-1, miR-30a, miyokardiyal iskemi/reperfüzyon hasarı, osthol.

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Myocardial ischemia/reperfusion (I/R) injury is a major obstacle against the optimal therapeutic effect of reperfusion therapy. Preventing myocardial cell death after ischemia is the best treatment for controlling damage caused by ischemic myocardium. Cardiac myocytes are terminally differentiated cells that cannot be renewed. Therefore, it is particularly important to maintain the cell's survival and function by autophagy to remove overly-existent or excess proteins and aged organelles.[1,2]

Autophagy is regulated by multiple signaling pathways.[3] Mechanisms that promote autophagy during reperfusion include increased Beclin-1 expression, oxidative stress injury, mitochondrial permeability transition, and mitochondrial damage.[4,5] In addition, the down-regulation of B-cell leukemia/lymphoma 2 (Bcl-2) in the reperfusion phase can affect the activity of Beclin-1 and formation autophagy.[6,7] Beclin-1 overexpression can improve ischemia and reperfusion autophagic activity *in vitro*.[5] Conversely, the autophagic activity of the myocardium is significantly reduced during reperfusion following the interference of micro-ribonucleic acids (RNAs) or in Beclin-1 knockout mice. The miR-30a binding sequence in the 3'UTR of Beclin-1 contributes to the regulation of Beclin-1 expression by miR-30a. miR-30a might be involved in the regulation of myocardial I/R injury.[8]

The natural coumarin derivative 7-methoxy-8-isopentenoxycou marin, also known as osthole, was isolated from Cnidium monnieri (L.) Cusson.[9] Studies show that osthole can improve cerebral ischemic stroke and intestinal I/R injury.[10,11] Osthole suppressed the formation of lipid peroxidation products, enhanced the capacities of antioxidant enzymes, and inhibited the expression of inflammatory cytokines following myocardial I/R injury, thus providing the protective effects in rats.[12] Moreover, osthole attenuated myocardial I/R injury in rats by inhibiting apoptosis and inflammation.[13] Therefore, in this study, we aimed to investigate the changes in miR-30a expression and myocardial autophagy following osthole treatment in a myocardial I/R injury model.

MATERIALS AND METHODS

Thirty male Wistar rats (weighing 170 to 220 g, aged 8 weeks), provided by the Experimental Animal Center of Sichuan Academy of Medical Sciences, were randomly divided into three groups as control (sham) group, I/R (model) group, and I/R + osthole post-treatment (osthole) group. Rats in each group were anesthetized with 25% urethane (40 mL/kg) intraperitoneally and fixed in a supine position. The skin was cut on the left side of the sternum, three-five ribs were cut off, the heart was exposed, and the proximal end of the left anterior descending coronary artery was ligated with a 6-0 silk thread. After myocardial ischemia for 30 minutes, ligature was released to restore blood supply, and perfused for 120 minutes. Electrocardiograms were recorded one time before ligation and after perfusion. ST-segment elevation or T-wave height after ligation and myocardial darkening were the criteria for successful myocardial ischemia. In osthole group, immediately after ischemia, rats were intravenously injected with osthole (25 mg/kg) into the internal jugular vein. The study protocol was approved by the Ethics Committees of the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, China.

Masson's trichrome staining was used to observe collagen deposition in myocardium, according to the manufacturer's instructions (Masson's Trichrome Stain Kit, Solarbio, Beijing, China). Myocardial collagen volume fraction was the ratio of the collagen area to the total area of the recording area. The collagen area did not include the collagen area around the blood vessels. Each specimen took an average of five areas, and data were normalized to sham group.

Apoptosis of myocardial cells in each group was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling following the kit instructions (Roche, Basel, Switzerland). The data were normalized to sham group.

Proteins were extracted by the total protein extraction kit instructions (Thermo Fisher, Massachusetts, United States) and protein concentration was measured with bicinchoninic acid method. Proteins were separated by 8% to 15% polyacrylamide gel electrophoresis with loading of 30 to 40 mg protein and then transferred to polyvinylidene difluoride membrane. The blots were incubated with 5% bovine serum albumin for one hour at room temperature and incubated with primary antibodies Beclin-1, light chain 3 beta (LC3β) overnight at 4°C. The internal reference was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). After incubated with the secondary antibody at room temperature for one hour, the blots were imaged with enahnced chemiluminescence reagent and analyzed by ImageJ software (Wayne Rasband, National Institutes of Health, USA). Beclin-1, LC3β, and GAPDH antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Conventional Trizol extraction of total RNA from myocardium was performed. Reverse transcription (RT) was performed by DBI Bestar® quantitative polymerase chain reaction (qPCR) real-time kit (DBI Bioscience, Ludwigshafen, Germany). Quantitative RT-PCR was performed using DBI Bestar® SybrGreen qPCR Mastermix. Primers were DBI Bestar® SybrGreen qPCR Mastermix. Rno-miR-30a quantitative detection primers and U6 small nuclear RNA were used as internal references. Reaction parameters were: Predegeneration 95°C 20 sec, 95°C 10 sec, 60°C 20 sec, 70°C 10 sec, 40 cycles. The data of miR-30a and U6 were calculated as 2-ΔΔCt. The primers used in the study were listed as U6 forward: CTCGCTTCGGCAGCACA; U6 reverse: AACGCTTCACGAATTTGCGT; all R, CTCAACTGGTGTCGTGGA; rno-miR-30a TGTAAACATCCTCGACTGGAAG; rno-miR-30a RT CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT TGAGCTTCCAGT; rno-miR-30a forward ACACTCC AGCTGGGTGTAAACATCCTCGACTG.

Statistical analysis

Statistical analysis was performed using the GraphPad 6.0 software (GraphPad Software, San Diego, CA, USA) Each group of data were analyzed by one-way analysis of variance. Data were expressed as mean ± standard deviation. The significance level α was set at 0.05.

RESULTS

After Masson's trichrome staining of rat myocardial tissue, compared with the sham group, the total collagen content of model group increased significantly (normalized area 12.6±2.4 in model group vs. 0.8±0.3 in sham group; $p<0.01$). Compared with the model group, the total collagen content of the myocardial tissue in the osthole group was decreased (normalized area 4.1±2.1 in osthole group vs. 12.6±2.4 in model group; $p<0.01$). Compared with the sham group, myocardial swelling, necrosis, inflammatory cell infiltration, and myocardial atrophy were significantly increased in the model group, while muscle swelling, necrosis, inflammatory cell infiltration, and myocardial atrophy were significantly reduced in the osthole group compared with the model group (Figure 1).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay showed normal nuclei

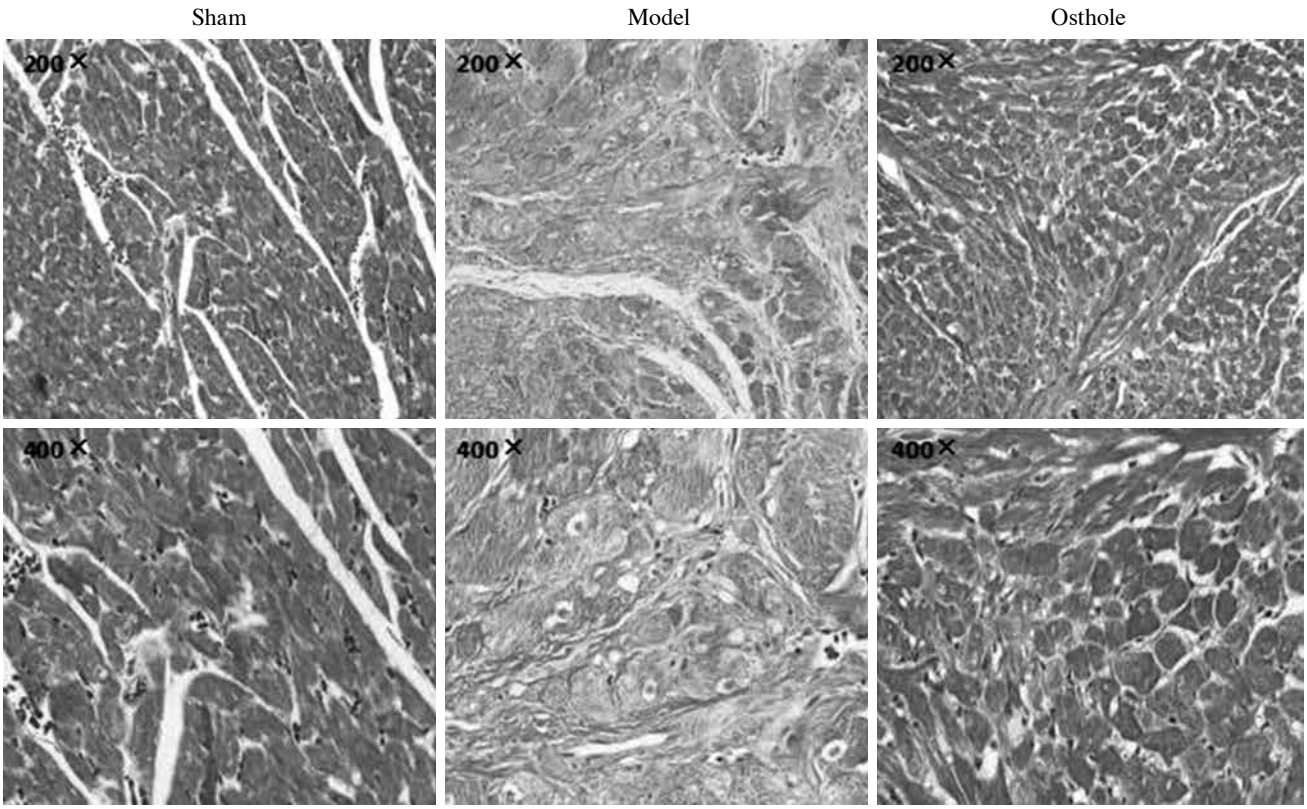

Figure 1. Masson's trichrome staining images of heart tissue samples in rat. Rats were treated with dimethyl sulfoxide solution (vehicle) or osthole at 25 mg/kg concentrations upon initiation of myocardial ischemia/reperfusion. First line of figure shows a 200× picture, while second line is 400×. Second line is a magnified view of first line typical areas; blue for collagen and red for muscle fibers.

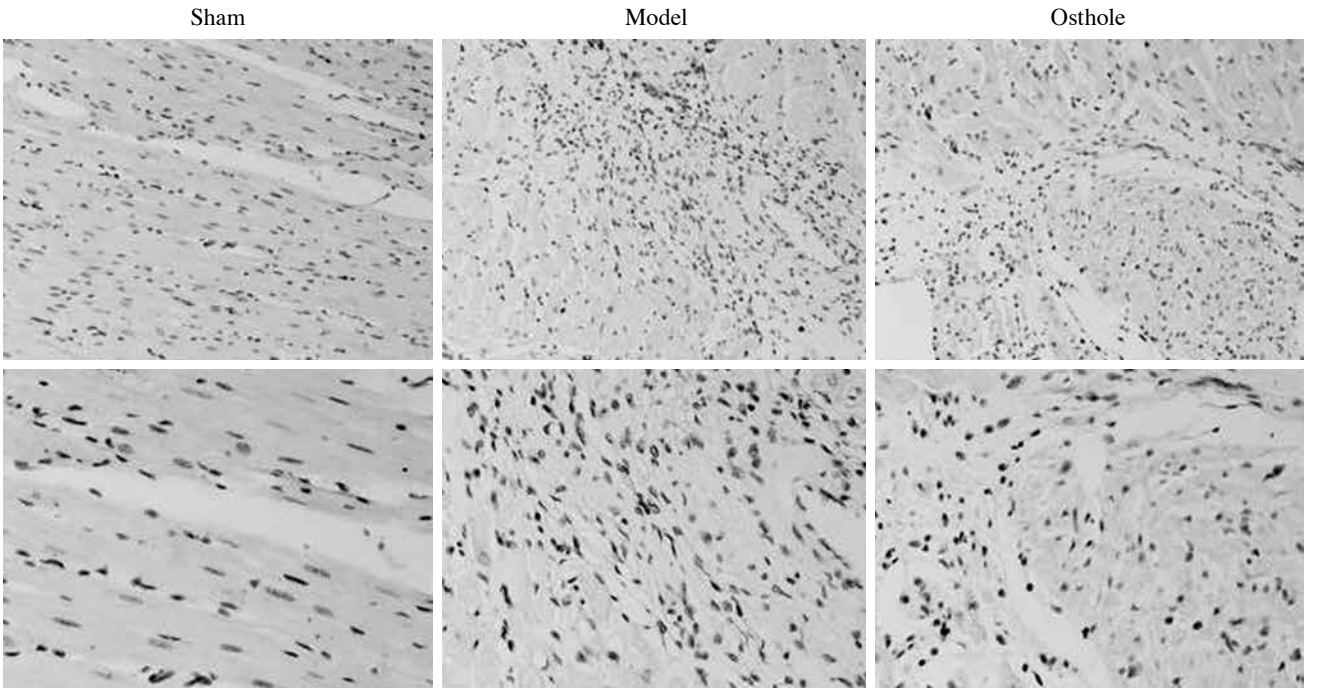

Figure 2. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay detects myocardial cell apoptosis. Rats were treated with dimethyl sulfoxide solution (vehicle) or osthole at 25 mg/kg concentrations upon initiation of myocardial ischemia/reperfusion. First line of figure shows a 200× picture, while second line is 400×. Second line is a magnified view of first line typical areas; blue for normal cells and brown for apoptotic cells.

blue and apoptotic nuclei brown. Scattered apoptotic cells were observed in the sham group. Apoptotic cells increased in the model group, significantly higher than the sham group (normalized apoptotic cell 6.1±1.2 in model group vs. 0.9±0.2 in sham group; $p<0.001$). It indicated that after myocardial I/R, myocardial cells were damaged and their apoptosis increased. After osthole treatment, apoptosis in the osthole group was less than that in the model group (normalized apoptotic cell 1.6±0.5 in osthole group vs. 6.1±1.2 in model group; $p<0.01$) (Figure 2).

Beclin-1 protein expression in the myocardial tissue of the model group was decreased compared with the sham group (458.1±18.8 in model group vs. 696.6±31.4 in sham group; $p<0.05$). Compared with the model group, Beclin-1 protein expression was significantly increased in the osthole group (1,123.4±156.9 in osthole group vs. 458.1±18.8 in model group; $p<0.01$). Comparison of the proportions of LC3β-II/LC3LC3β-I in each group revealed that the proportion of myocardial tissue in the model group was decreased compared to the sham group (1.9±0.2 in model group vs. 3.6±0.5 in sham group; $p<0.001$), the proportion of LC3β-II/LC3β-I in the osthole group was increased (3.8±0.5 in osthole group vs. 1.9±0.2 in model group; $p<0.001$) (Figure 3).

To explore the mechanism by which osthole induces autophagy, we attempted to detect the expression of miR-30a using real-time PCR. The expression of miR-30a in the model group was significantly up-regulated compared with the sham group (4.0±0.2 in model group vs. 1.0±0.1 in sham group; $p<0.001$). The expression of miR-30a in the osthole group was inhibited compared with the model group (2.0±0.1 in osthole group vs. 4.0±0.2 in model group; $p<0.001$), but the expression was increased compared with the sham group (2.0±0.1 in osthole group vs. 1.0±0.1 in sham group; $p<0.01$) (Figure 4).

DISCUSSION

Cardiomyocytes are highly differentiated terminal cells that are difficult to replenish after injury. Therefore, it is important to prevent myocardial cell damage. Autophagy is a metabolic mechanism of cells and is thought to be closely related to diseases such as neural degeneration, tumorigenesis, aging, and diabetes. Insufficient or excessive autophagy can cause myocardial cell damage, excessive autophagy during I/R, and myocardial cell damage.[14] Osthole provided the protective effects and attenuated myocardial I/R

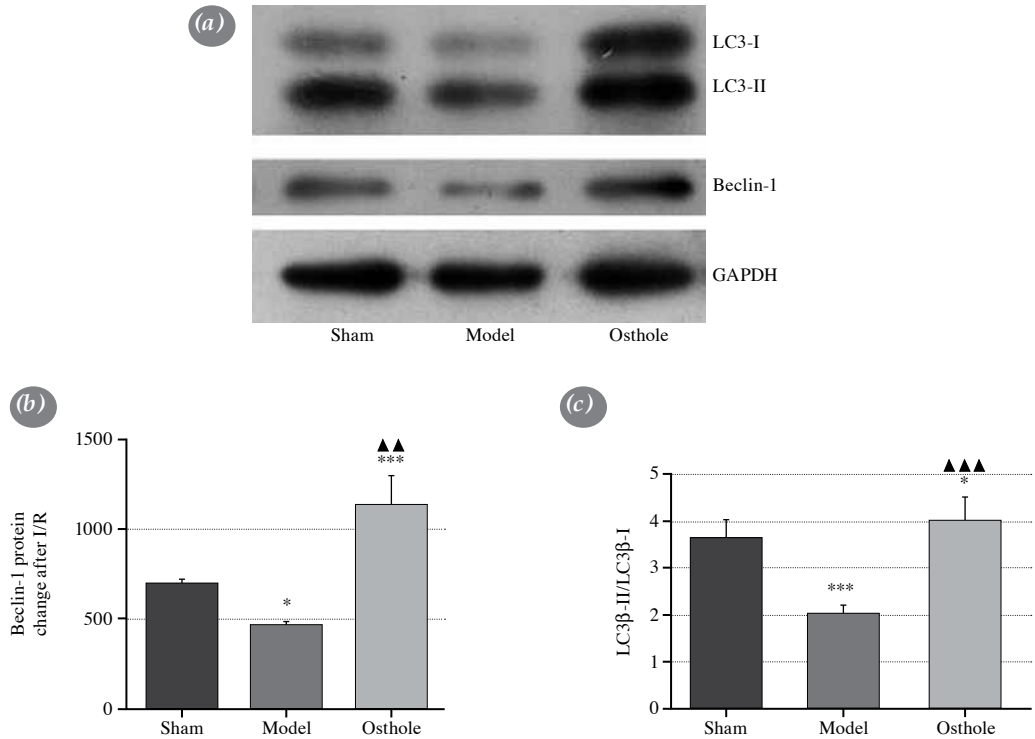

Figure 3. Western blotting detection of Beclin-1 and LC3β protein expression. Rats were treated with dimethyl sulfoxide solution (vehicle) or osthole at 25 mg/kg concentrations upon initiation of myocardial ischemia/reperfusion. **(a)** Expression of Beclin-1 and LC3β (LC3β-I and LC3β-II) was detected by western blotting analysis. **(b)** ImageJ software was then used to quantify optical density of Beclin-1 band. **(c)** ImageJ software was then used to quantify ratio of LC3-II/LC3-I. Data were presented as mean ± standard deviation.

* p<0.05; *** p<0.001 vs. sham; ▲▲ p<0.01; ▲▲▲ p<0.001 vs. model; LC3: Light chain 3; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; I/R: Ischemia/reperfusion; LC3β: Light chain 3 beta.

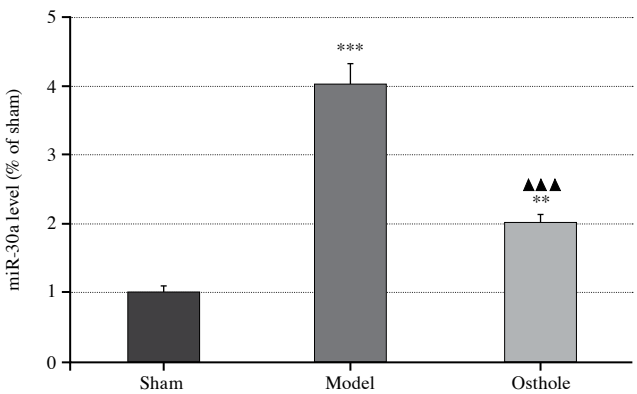

Figure 4. Expression of miR-30a in cardiomyocytes after myocardial ischemia/reperfusion. Rats were treated with dimethyl sulfoxide solution (vehicle) or osthole at 25 mg/kg concentrations upon initiation of myocardial ischemia/reperfusion. Expression of miR-30a was detected by quantitative-polymerase chain reaction. Relative expression level between treatments was then calculated using following equation: relative gene expression = $2^{-(\blacktriangle Ct\ sample\ -\ \blacktriangle Ct\ control)}$.

Data were presented as mean ± standard deviation; ** p<0.01; *** p<0.001 vs. sham; ▲▲▲ p<0.001 vs. model.

injury in rats by inhibiting apoptosis.[12,13] Our results showed that autophagy plays an important role in the protective effect of osthole in rats. Regulating autophagy may be one of the potential targets for the prevention and treatment of cardiovascular diseases.[15]

The autophagosome formation is mainly regulated by two ubiquitin-like binding systems: the LC3β and Atg12-Atg5 systems.[16] LC3β (microtubule-associated protein 1 light chain 3) is the first mammalian protein associated with autophagosome formation and can be divided into cytoplasmic LC3β-I and membrane LC3β-II. The amount and ratio of the two proteins can indicate the number of autophagosomes.[17] During reperfusion, Beclin-1-induced autophagy increases leading to autophagic cardiomyocyte death, increased myocardial damage, and reduced cardiac function.[18] Beclin-1 is a key gene that regulates the autophagy process. The Beclin-1 mediated autophagy/apoptotic feedback signaling pathway is one of the classical autophagy signaling pathways,[19] indicating that

osthole protects I/R injury by promoting autophagy in cardiomyocytes. Osthole increased the ratio of LC3β-II/LC3β-I and induced the expression of Beclin-1, suggesting that osthole induced autophagy.

In the absence of nutrients, autophagy can promote cell survival by providing metabolically-maintaining substances and removing toxic or damaged proteins and organelles.[20] In the hypoxia-reoxygenated cardiomyocyte model and the I/R heart model, autophagic activity has been shown to increase cell survival.[21] In this study, apoptotic cells were increased in the model group, but were decreased by osthole, indicating that osthole may promote autophagy to stimulate cell survival. This is consistent with the literature. Osthole inhibits apoptosis by upregulating Bcl-2 protein expression.[22] Similarly, Wang et al.[12] found that osthole can effectively reduce the apoptotic degree and myocardial I/R injury in rats.

The literature indicates that miR-30a regulates the Beclin-1 gene in many human tumor cells, down-regulates the classical autophagy process, and participates in the regulation of metabolic processes in tumor cells.[22] We found that during myocardial I/R, the expression of miR-30a was increased, while LC3β-I conversion to LC3β-II was decreased, and Beclin-1 expression was decreased, indicating that osthole inhibited the autophagy. In the treatment of osthole, the expression of miR-30a was inhibited, while the expression of Beclin-1 was increased, and LC3β-I was converted to LC3β-II, suggesting that osthole can increase autophagy of myocardial cells during myocardial I/R injury to protect the myocardium.

This study has some limitations. The roles of autophagy and miR-30a in the protection of osthole in myocardial I/R injury were not investigated. The target gene of miR-30a was also not investigated, requiring future studies.

In conclusion, we demonstrated that osthole may enhance autophagy in myocardial ischemia and reperfusion by down-regulating miR-30a expression, reducing myocardial cell apoptosis, and protecting the myocardium from damage. Targeting on autophagy and miR-30a might be helpful to protect the myocardium against ischemia/reperfusion injury.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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