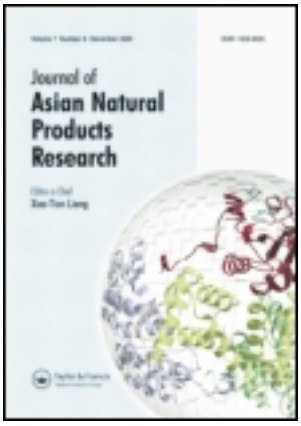


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# Protective effect of oxymatrine on myocardial fibrosis induced by acute myocardial infarction in rats involved in TGF-$\beta_1$-Smads signal pathway

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Oxymatrine (**1**), a component extracted from a traditional Chinese herb *Sophora japonica* (*Sophora flavescens* Ait.), has been demonstrated to have a variety of pharmacological actions. Abundant experimental evidence indicates that **1** may exert a protective effect on the cardiovascular system. This study was designed to explore the possible role of **1** against myocardial fibrosis induced by acute myocardial infarction (AMI) and its modulation on transforming growth factor beta 1 (TGF-$\beta_1$)-Smads signaling pathways. Rats with AMI induced by ligation of left anterior descending branch were randomly assigned to receive **1** 50 and 25 mg/kg intragastrically, and model group which were further compared with sham-operated group, and positive group treated with captopril. The effects of 4-week therapy with **1** starting 24 h after infarction had been investigated based on (1) hemodynamics, (2) tissue weights, (3) biochemical indicator (hydroxyproline contents in left ventricle), and (4) TGF-$\beta_1$, TGF-$\beta_1$ receptor (T$\beta$R$_1$), *Smad3, Smad4, Smad7, Col1, and Col3* expression by semi-quantitative reverse transcription PCR. Treatment with **1** significantly ameliorated hemodynamics, inhibited the expression of T$\beta$R$_1$ mRNA and Smad3 mRNA, and reduced the left ventricle weight/body weight. The results of this research indicated that **1** might protect against myocardial fibrosis and the mechanism may be involved in modulating TGF-$\beta_1$-Smads signal pathway.

**Keywords:** oxymatrine; acute myocardial infarction; cardiac fibrosis; TGF-$\beta_1$-Smads signal

## 1. Introduction

Cardiovascular disease accounts for nearly 40% of all deaths annually in developed countries. Acute myocardial infarction (AMI) is the leading cause of congestive heart failure (CHF) and death in the industrialized world [1]. Myocardial infarction (MI) often leads to adverse ventricular remodeling resulting in changes involving the size, shape, and function of heart, and the subsequent development of heart failure. Therefore, investigation of novel treatments to improve the prognosis of CHF is an area of intense activity. In the long pathological period of hepatic fibrosis to heart failure, transforming growth factor beta 1 (TGF-$\beta_1$) is one of the strongest pro-fibrotic cytokines [2,3] responsible for inducing the proliferation of cardiac fibroblasts, their phenotypic transformation to myofibroblasts, and the deposition of extracellular matrix (ECM) [4], particularly collagen types I and III [5], and its sustained production underlies the development of tissue fibrosis particularly

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after MI. Recently, TGF-$\beta_1$-Smads signaling is the cardinal signal transduction pathway, which has been verified by several related studies [6].

The Smad proteins consist of a large family of transcription factors, which are also found in vertebrates, insects, and nematodes. To date, Smads are the only TGF-β receptor substrates with a demonstrated ability to propagate signals. TGF-β receptor types I and II, two different transmembrane protein serine/threonine kinases, are brought together by the ligand that acts as a receptor assembly factor [7]. During the TGF-β signal transduction, receptor II is activated first, then the receptor I. The type I receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads), which are Smad2 and Smad3 [8]. Then R-Smads are activated and form a complex consisting of R-Smads and Smad4, which belongs to Co-Smad. The Smads complex then accumulates in the nucleus. After transferring into the nucleus, the transcriptional complex binds to the certain domain of the target gene and causes the gene expression like collagen production. The excess collagen production would lead to collagen deposition in heart tissue and myocardial fibrosis or heart failure at last. There are also two inhibitant Smads (I-Smads), named as Smad6 and Smad7, which could combine to the Smads complex in cytoplasm. Smad6 and Smad7 could prevent the Smads complex to transfer into the nucleus; thus prevent the stimulating signal being transferred from outside into the cell nucleus [9,10]. This signal pathway has been paid great attention by several researchers, and we also make an effort to explore the effective drugs to inhibit this adverse pro-fibrotic signaling way.

*Sophora flavescens* Ait., commonly known as Kushen in Chinese medicine, grows widely in Northeastern and Northern China. Radix Sophorae Flavescentis (Kushen) [11], a popular Chinese herb, has been widely and successfully used for treating angina pectoris, MI, and stroke. Oxymatrine (OMT) (**1**, Figure 1) is a constituent extracted from Kushen and was assigned as the marker species for Kushen in the 2005 edition of Chinese Pharmacopoeia. The bioactivities of **1**, such as anti-inflammatory, antitumor, immunomodulatory, and antiproliferative activities, have been reported. There are many reports about the prophylactic effect of **1** on liver fibrosis and its function to suppress activation of hepatic stellate cells [12–14]. In this study, a rat cardiac fibrosis model was produced by AMI induced by coronary artery ligation, and then the anti-fibrotic effects and probable mechanism of **1** on cardiac fibrosis were investigated.

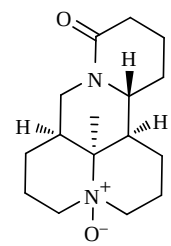


Figure 1. Chemical structure of OMT (**1**).

## 2. Results

### *2.1 1 Ameliorated left ventricular (LV) hemodynamics of cardiac fibrosis*

We evaluated LV hemodynamics using a Millar pressure catheter before sacrificing the rat. As illustrated in Table 1, after ligation of left anterior coronary for 4 weeks, the LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), maximal rate of LV pressure ($+ dp/dt_{max}$), and minimal rate of LV pressure ($- dp/dt_{min}$) were remarkably decreased compared with sham group ($p < 0.01$). After successive administration of **1**, especially the 50 mg/kg dose, within 4 weeks, these indicators had significantly ameliorated ($p < 0.05$–$0.01$ vs. model group). Captopril (Cap) showed some better effects in

Table 1. Effect of **1** on left ventricle function in rats with myocardial fibrosis after AMI induced by coronary artery ligation after 4 weeks.

| Group | Dose (mg/kg) | LVSP (mmHg) | LVEDP (mmHg) | $+dp/dt_{max}$ (mmHg/s) | $-dp/dt_{min}$ (mmHg/s) |
|---|---|---|---|---|---|
| Sham | – | 100.60 ± 11.91 | 1.82 ± 4.56 | 3410.62 ± 900.15 | − 3466.82 ± 313.54 |
| Model | – | 59.64 ± 12.50$^{\#\#}$ | 7.25 ± 7.47 $^{\#}$ | 1613.47 ± 152.08$^{\#}$ | − 1154.79 ± 167.08$^{\#\#}$ |
| Cap | 50 | 79.60 ± 12.18* | 2.09 ± 2.27* | 2646.85 ± 285.28* | − 3392.31 ± 602.24* |
| OMT | 50 | 84.95 ± 24.33* | 5.75 ± 1.64 | 2819.20 ± 257.75* | − 2236.35 ± 600.08* |
| | 25 | 77.53 ± 10.41* | 6.32 ± 7.01 | 2346.46 ± 890.68 | − 1948.41 ± 860.82 |

Notes: LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; $+dp/dt_{max}$, maximal rate of LVSP; $-dp/dt_{min}$, minimal rate of LV systolic pressure. Data are expressed as means ± SD ($n = 8$).
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. model.

some cases. These findings strongly suggested that **1** could ameliorate the heart function.

### 2.2 Change of hydroxyproline content in the LV

Hydroxyproline is a character amino acid of collagen protein, and to detect the content of hydroxyproline means the level of total collagen in myocardial tissue, which can be responsed the fibrosis degree of left ventricle. The sham group has low proportions of hydroxyproline. In the model group, the content of hydroxyproline was significantly increased ($p < 0.01$ vs. sham group), as shown in Figure 2. With the administration of **1**, the content of hydroxyproline was decreased remarkably in both groups ($p < 0.01$ vs. model group), which means **1** can significantly ameliorate myocardial fibrosis induced by ligated left anterior coronary. At this point, Cap also exerted therapeutic effects ($p < 0.01$ vs. model group).

### 2.3 Observation of anatomy and histopathologic examinations

Typical morphological photographs of hearts in the 4th week and infarct size as a proportion of LV size are shown in Figure 3(A) and (B); the infarct size was significantly reduced in the hearts treated with **1** and Cap.

Figure 3(C) and (D) showed the hematoxylin–eosin (HE) and Massion micrographs. In HE staining, the model group indicated augmentation and loose arrangement of the myocardial fibers, staining asymmetry, monstrosity of partly nucleolus, and marked edema and infiltration. The continuous administration of **1** and Cap markedly improved the pathological changes compared with model group. Collagen, the main contents of ECM, was stained blue in Massion staining, which indicated the fibrosis degree of myocardium. The model group presented

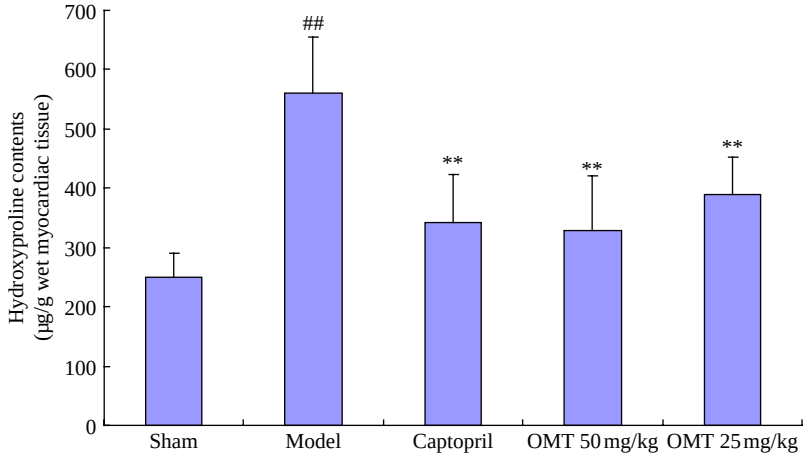


Figure 2. Effects of **1** on hydroxyproline in myocardial fibrosis induced by ligated left anterior coronary. The myocardial fibrosis was reproduced in rats by ligation of the left anterior descending coronary artery for 4 weeks. Sham-operated rats underwent the identical surgical procedure as described except that the suture was not tightened around the coronary artery. The AMI surviving rats were divided randomly into four groups: model group; OMT 50 mg/kg, OMT 25 mg/kg, and Cap 50 mg/kg. Model and sham operation groups were treated with vehicle. All animals were treated 24 h after operation. Model, ligated left anterior coronary for 4 weeks; OMT, oxymatrine. $*p < 0.05$ and $**p < 0.01$ compared with model group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with sham group. Data are expressed as mean ± SEM, $n = 8$.

blue staining that meant the cardiac tissue was extensive fibrosis, and **1** and Cap could ameliorate the fibrosis degree.

### *2.4 The expression of related mRNA in cardiac fibrosis via reverse transcription PCR (RT-PCR) assay*

Four weeks after AMI, as shown in the Table 2 and Figure 4, the expression of TGF-$\beta_1$ receptor (T$\beta$R$_1$) mRNA significantly increased ($p < 0.01$ vs. sham group), and the expression of TGF-$\beta_1$, Smad3, Smad4, Col1, and Col3 mRNA also increased in model group ($p < 0.05$ vs. sham group). However, the expression of Smad7 mRNA had no significant change. After 4 weeks of treatment with successive **1** 25 and 50 mg/kg, compared with model group, the expression of TGF-$\beta_1$, T$\beta$R$_1$, Smad3, Smad4, Col1, and Col3 mRNA decreased, while the expression of Smad7 mRNA increased. Especially, the expression of T$\beta$R$_1$ mRNA had remarkably changed in all doses of **1**-treated groups ($p < 0.01$ vs. model group), as well as in TGF-$\beta_1$ and Smad3 mRNA in **1** 50 mg/kg group ($p < 0.05$ vs. model group). The expression of T$\beta$R$_1$ mRNA decreased significantly ($p < 0.01$ vs. model group), and the expression Col1 mRNA decreased remarkably as well in Cap group ($p < 0.01$ vs. model group).

## 3. Discussion

In this study, we demonstrated that **1** elicited cardioprotective effects in the improvement of heart function, the reduction of infarct size, and the amelioration of ventricular remodeling. These findings supported the concept that **1** could be an effective preventive and therapeutic candidate against cardiac hypertrophy and heart failure.

CHF resulting from cardiac fibrosis is a complex clinical syndrome with poor prognosis, and its development is a long-term and complex process involving many factors such as the sympathetic nervous system, rennin–angiotensin system,

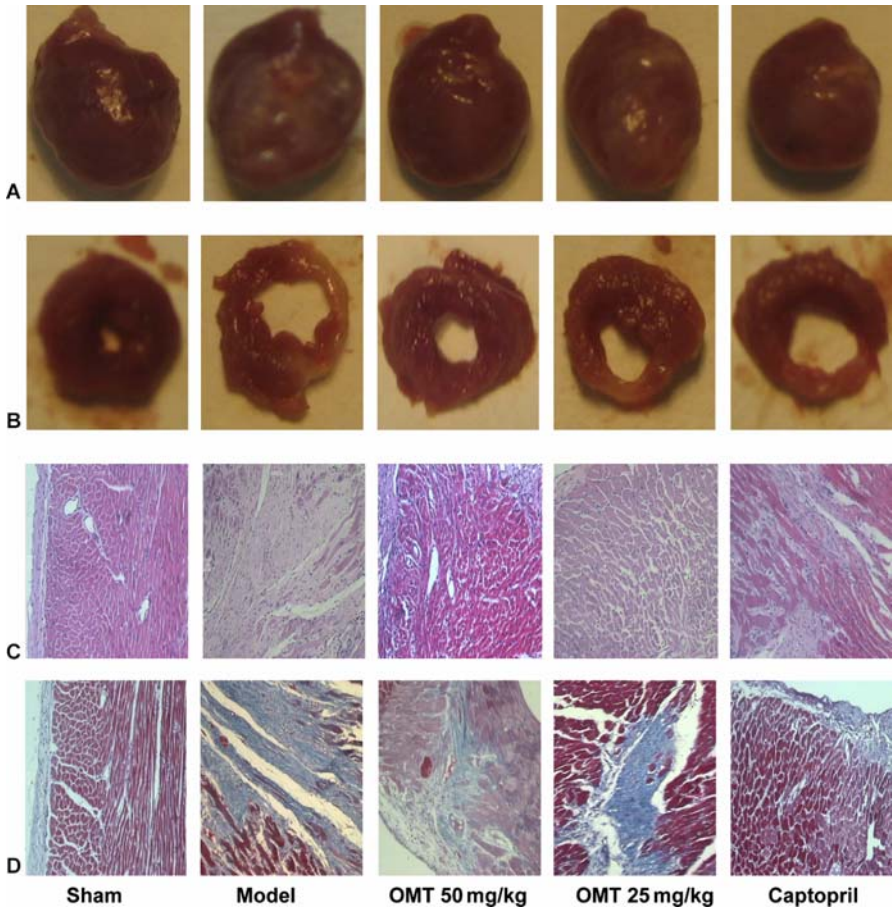


Figure 3. Effect of **1** on myocardial fibrosis assayed by anatomy and histopathologic examination. The animals were treated as per the procedure described in Figure 2. (A) representative pictures of the whole heart; (B) representative pictures of transverse section of heart; (C) the cardiac histopathology assayed by hematoxylin and eosin staining; and (D) the cardiac histopathology assayed by Massion staining.



reactive oxygen species, and apoptosis [15]. Despite considerable scientific data on the biochemical and molecular characteristics of CHF, the precise molecular mechanisms responsible for CHF still remain disputed. At this stage, diminution of infarct size, improvement of heart function, and prevention of ventricular remodeling are important end-points in the treatment of cardiovascular disorders induced by infarction.

Many research data find that **1** has been shown to possess many of the actions of the Kushen herb (such as antioxidant, hepatoprotective and so forth) [16,17]. Many previous studies primarily concentrated on hepatic fibrosis, anti-inflammatory process, etc. In this study, the results indicated that the chronic treatment with **1** could ameliorate cardiac function, reverse remodeling, and attenuate cardiac fibrosis in rats with AMI, and also showed that **1** caused a significant prevention of cardiac fibrosis as evidenced by the reduction of collagen deposition. In this research, **1** did not affect blood pressure (data are not shown at present), and this indicated that the primary target of **1** action was cardiac

Table 2. Effect of **1** on cardiac fibrosis-related gene mRNA expression of myocardial remodeling rats after AMI induced by coronary artery ligation after 4 weeks.

| Relation mRNA | Sham | Model | Cap 50 (mg/kg) | OMT 50 (mg/kg) | OMT 25 (mg/kg) |
|---|---|---|---|---|---|
| TGF-$\beta_1$ | 0.020 ± 0.001 | 0.047 ± 0.015 | 0.031 ± 0.016 | 0.034 ± 0.007 | 0.029 ± 0.015 |
| T$\beta$R$_1$ | 0.011 ± 0.005 | 0.152 ± 0.021$^{\#\#}$ | 0.030 ± 0.001** | 0.053 ± 0.014** | 0.065 ± 0.012** |
| Smad2 | 0.143 ± 0.005 | 0.373 ± 0.243 | 0.117 ± 0.045 | 0.304 ± 0.102 | 0.315 ± 0.165 |
| Smad3 | 0.032 ± 0.024 | 0.115 ± 0.036$^{\#}$ | 0.051 ± 0.014 | 0.034 ± 0.013* | 0.062 ± 0.028 |
| Smad4 | 0.382 ± 0.052 | 1.586 ± 0.673$^{\#}$ | 0.593 ± 0.048 | 0.575 ± 0.045 | 0.741 ± 0.201 |
| Smad7 | 0.218 ± 0.051 | 0.083 ± 0.032 | 0.160 ± 0.042 | 0.449 ± 0.072 | 0.344 ± 0.039 |
| Col1 | 0.095 ± 0.031 | 1.065 ± 0.328$^{\#}$ | 0.130 ± 0.032* | 0.425 ± 0.210 | 0.471 ± 0.159 |
| Col3 | 0.143 ± 0.073 | 1.262 ± 0.607$^{\#}$ | 0.310 ± 0.175 | 0.548 ± 0.392 | 0.503 ± 0.168 |

Note: Data are expressed as means ± SD ($n = 8$).
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. sham; $^{*}p < 0.05$, $^{**}p < 0.01$ other group vs. model.

protection, rather than lowering blood pressure.

Previous studies have indicated a significant relationship between hemodynamics and the severity of coronary heart disease. The deteriorated hemodynamics in rats with large AMI would diminish the coronary flow, exacerbate the microcirculation disorder of the ischemic myocardium, and enlarge the ischemic and hypoxic area. In this study, a significant improvement of hemodynamics was found in 50 mg/kg **1**-treated rats ($p < 0.05$ and 0.01).

TGF-$\beta_1$ plays an indispensable role in the long progress of myocardial fibrosis and serves as a major pro-fibrogenesis factor. In addition, TGF$\beta$1-Smad signaling pathway is the main pathway of TGF-$\beta_1$ [18–20]. Since the TGF-$\beta_1$-Smad signaling pathway is very important in the formation of myocardial fibrosis, inhibiting its transduction may prevent cardiac fibrosis. As shown in some researches, inhibiting the TGF-$\beta_1$-Smad signaling pathway or modulating the gene expression of certain Smads could effectively interfere with myocardial fibrosis [21]. In this study, treatment with **1** leads to the inhibition of TGF-$\beta_1$-Smads signaling, and thus significantly reduced ventricular collagen deposition and improved hemodynamics.

In conclusion, this work demonstrated that **1** exerted beneficial cardioprotective effects by impacting related gene expression in the TGF-$\beta_1$-Smads signaling pathway so as to improve significantly post-MI cardiac function and reduce hemodynamics, infarct size, cardiac collagen volume, and hypertrophy. The data confirmed that cascade of TGF-$\beta_1$-Smads signaling pathway may be the target of **1**'s inhibitory actions. This study is relevant to the inhibitory effect of **1** on cardiac hypertrophy and related molecular mechanisms. Future studies should examine the hypothesis that **1** may be a safe and effective approach to prevent and treat

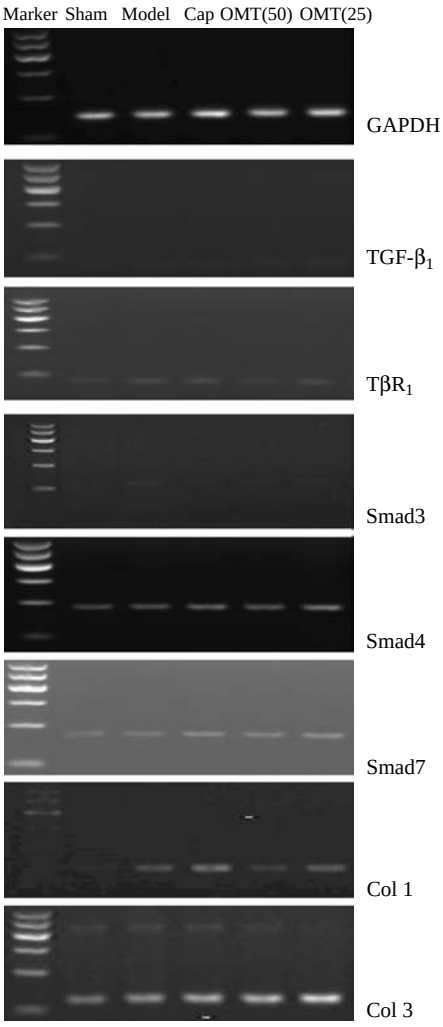


Figure 4. Effects of **1** on related gene expression in TGF-$\beta_1$-Smads signaling pathway. The animals were treated as per the procedure in Figure 2. Data are expressed as mean ± SEM, $n = 3$. Note: Makers from up to down represent 600, 500, 400, 300, 200, and 100 bp, respectively.

cardiac hypertrophy and the transition to failure.

## 4. Materials and methods

### *4.1 Materials*

#### *4.1.1 Drugs and reagents*

OMT (**1**, $C_{15}H_{24}N_2O_2$, purity >95%, determined by HPLC) was purchased from Green Valley Pharmaceutical Co., Ltd (Xi'an, China) and was purified in our lab; Cap was purchased from Shandong Huaxing Pharmaceutical Co., Ltd (Heze, China); hydroxyproline assay kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China); TaKaRa RT-PCR kit was purchased from TaKaRa Biotechnology (Dalian, China); trizol reagent was purchased from Invitrogen (Carlbad, CA, USA); chloral hydrate was purchased from Tianjin Taihe Pharmaceutical (Tianjin, China).

#### *4.1.2 Animals*

Sprague–Dawley (SD) rats (300–350 g) were provided by the experimental animal center of Guiyang Medical College and acclimated for at least 7 days. All animals were kept in separated cages with laboratory chow and tap water ad libitum. The protocol of animal experiments was approved by the University Laboratory Animal Research Committee.

### *4.2 Methods*

#### *4.2.1 Animal model reproduction*

Myocardial fibrosis was reproduced in rats by ligation of the left anterior descending coronary artery for 4 weeks. The surgical procedure was performed according to a previous study with minor modifications [22]. Briefly, SD rats of 300–350 g body weight were anesthetized with chloral hydrate (3%, 10 ml/kg, i.p., Tianjin Taihe Pharmaceutical, Tianjin, China) and then a left thoracotomy was performed. The incised area was extended using forceps and the pericardium was opened. After tracheal intubation, the rats were ventilated by a respirator (ALC-V8, Shanghai, China) with room air in a tidal volume of 25 ml/min and a respiratory rate of 70 times/min. The heart was exteriorized and ligated at the proximal left anterior descending coronary artery 2–3 mm from its origin between the pulmonary artery

Table 3. Primer sequences and annealing temperatures for RT-PCR assays.

| mRNA | Primers | Products of amplification (base pair) | Annealing temperature |
|---|---|---|---|
| Gapdh | *f*, GGCACAGTCAAGGCTGAGAATG | 143 | 63.6 |
| | *r*, ATGGTGGTGAAGACGCCAGTA | | |
| Tgfb | *f*, TGCGCCTGCAGAGATTCAAG | 82 | 65 |
| | *r*, AGGTAACGCCAGGAATTGTTGCTA | | |
| Tgfbr1 | *f*, GCTGACATCTATGCAATGGGCTTA | 87 | 64.5 |
| | *r*, AGGCAACTGGTAGTCTTCGTGGA | | |
| Smad2 | *f*, AGTGTTTGCCGAGTGCCTAAGTG | 146 | 64.8 |
| | *r*, GAGCAGCAAATTCTTGGTTGTTGA | | |
| Smad3 | *f*, GATGCTTCGTGACATTGGAACCTA | 145 | 64.4 |
| | *r*, TTCCCACGTTTAAATGCTGCTG | | |
| Smad4 | *f*, TGACGCCTGTCTGAGCATTGTA | 190 | 63.9 |
| | *r*, TCTCTGTATGGTGACACACTTGCTG | | |
| Smad7 | *f*, TGCTGTGCAAAGTGTTCAGGTG | 177 | 64 |
| | *r*, CCATCGGGTATCTGGAGTAAGGA | | |
| Col1a1 | *f*, CATCTCCATGGCCTCTGCAA | 137 | 64.6 |
| | *r*, CACATGTGTGGCCGATGTTTC | | |
| Col3a1 | *f*, TGGACAGATGCTGGTGCTGAG | 123 | 64.9 |
| | *r*, GAAGGCCAGCTGTACATCAAGGA | | |

Note: Sequences are listed $5'-3'$; forward primers are designated as *f* and reverse primers as *r*.



conus and the left atrium with 4-0 prolene suture. The heart was returned to its normal position and the thorax was closed. Sham-operated rats underwent the identical surgical procedure as described above except that the suture was not tightened around the coronary artery. In this study,the operation-related mortality was approximately 20% for 24 h after operation.

#### 4.2.2 *Experimental protocol*

The rats that survived after operation were divided randomly into four groups: model group; **1** (50 mg/kg intragastrically); **1** (25 mg/kg intragastrically); and Cap (50 mg/kg intragastrically). Beginning on the following day of surgery, the vehicle was given to sham and model groups once a day for 4 weeks.

#### 4.2.3 *Hemodynamic changes*

After 4 weeks, the rats were subjected to surgical procedures to assay hemodynamic parameters. Hemodynamic parameters (LVSP, LVEDP, $+ \mathrm{d}p/\mathrm{d}t_{max}$, and $- \mathrm{d}p/\mathrm{d}t_{min}$) were recorded as described previously [23]. The study was performed in a blinded manner. After measuring hemodynamic parameters, the hearts of the sacrificed rats were removed, washed with physiological saline, and then photographed. The LVs parts were freezed by liquid nitrogen and then stored at $-70°C$.

#### 4.2.4 *Pathological and histological changes*

LV tissues were arrested in diastole with phosphate buffered saline/20 mmol/L KCl solution and fixed with 4% paraformaldehyde or 10% neutral-buffered formalin. Paraffin-embedded tissues were sectioned (4 μm thick) and stained with hematoxylin and eosin staining to assay the infarct size and the morphology of myocardial cell or Massion staining to detect the degree of cardiac fibrosis. The sections were quantified morphometrically with an optical microscope.

#### 4.2.5 *Hydroxyproline contents*

Hydroxyproline was measured using the assayed kit according to the manufacturer's

instructions. Briefly, partly LV tissues were sliced and thoroughly washed with potassium phosphate buffer (50 mmol/L) to deprive of blood. Homogenization of the LV tissue was performed following the method described by Lee *et al.* [24]. The homogenates were used to determine hydroxyproline contents.

#### 4.2.6 RT-PCR analysis

RT-PCR analysis was performed on selected genes (*Gapdh, TGF-β, TGF-βR1, Smad2, Smad3, Smad4, Smad7, Col1a1, Col3a1*). Oligonucleotide primer sequences and PCR annealing temperatures for each gene studied are illustrated in Table 3. Total RNA was extracted using standard trizol RNA isolation method. Reverse transcription of 5 μg RNA was carried out according to the instruction of TaKaRa RT-PCR kit (TaKaRa Biotechnology, Otsu, Japan). The PCR amplification was conducted by a Biometra T-gradient thermal cycler (Biometra, Tampa, FL, USA). According to the protocol, solutions' predenaturation at 94°C for 5 min was performed before the following PCR cycling parameters: denaturation at 94°C for 30 s, annealing for 30 s at the gene-specific annealing temperature (Table 1), and extension at 72°C for 1 min. Each sample underwent 30 cycles and a final extension phase at 72°C for 8 min. PCR products were run on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. The density of bands was analyzed using a Bio-Rad GS-700 densitometer (Foster City, CA, USA). The expression of indicated genes was normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

#### 4.2.7 Statistical analysis

The values are expressed as mean ± SE. The basic comparative statistics were analyzed using a one-way ANOVA test or paired *t*-test. All statistical analyses were performed using SPSS version 11 (SPSS Inc., Chicago, IL, USA). *p*-Value of $<0.05$ and $<0.01$ was taken to indicate statistical significance, remarkable statistical significance, respectively.


## Acknowledgements

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