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Suppression of TGP on myocardial remodeling by regulating the NF-κB pathway

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ABSTRACT

Myocardial remodeling is one of the main mechanism which leads to chronic heart failure (CHF). Thus, the drugs which suppressed the process of myocardial remodeling showed better clinical outcomes to deal with CHF. Total glucosides of paeony (TGP) which is used in many traditional Chinese medicines (TCM) exhibited promising ethno-pharmacological effects such as immunosuppressant, anti-inflammatory, analgesia, anti-stress, liver disease, allergies, anticoagulant, and cardiovascular activities. This study aims to investigate the effects of TGP on myocardial remodeling by regulating the nuclear factor kappa B cells (NF-κB) pathway. SD rats were selected and divided into five groups (n = 8), control, sham-operated, Captopril, low dose TGP and high dose TGP respectively. The pressure-overload method was adopted by abdominal aorta ligation to induce the CHF. Furthermore, collagen fibers detected by picrosirius red staining and expression of NF-kB, TGF-β1 by immunohistochemistry and observed under a polarized microscope and assessed by image-pro plus 6.0. Matrix metalloproteinase's (MMP)-2, -9 mRNA levels by reverse transcription PCR (RT-PCR), the concentration of angiotensin II was determined by radioimmunoassay and ELISA was employed to determine the cytokine IL-1β. It was observed that TGP could relieve myocardial remodeling in rats induced by abdominal aorta ligation and decrease the level of angiotensin II and I/III collagen ratio, pathogenic cytokines and inhibit the expression and activities of MMPs. Consequently, the observations suggested that myocardial remodeling was mediated by the NF-κB pathway.

1. Introduction

Chronic/congested heart failure (CHF), is a kind of myocardial overload disease caused by multiple diseases with many manifestations and may cause high mortality and disability in clinics [1]. In developed countries, epidemiological research suggested that 1 out of 3 CHF patients died within a year and more than half of the patients had a survival rate of less than five years [2]. CHF has become a leading cause of disease in clinics with a high mortality rate among elderly patients. Recently, the incidence of CHF increased with aging, high blood pressure and coronary heart disease (CHD). Thus, CHF has become hygienically a great public problem. The nosogenesis of CHF could be called as myocardial remodeling [3,4] such as myocardial hypertrophy, myocyte apoptosis, collagen deposition of extracellular matrix (ECM), fibrosis, and change of metabolic as well as electrophysiological characters [5]. On a cellular level, myocardial remodeling could be a) Hypertrophy, dysfunction, and death. b). ECM sedimentary and composition changes, known as myocardial fibrosis [6]. Generally, ECM includes substances between and outside of myocytes [7]. Indeed, the collagen fibers perform a critical role in myocardial remodelings such

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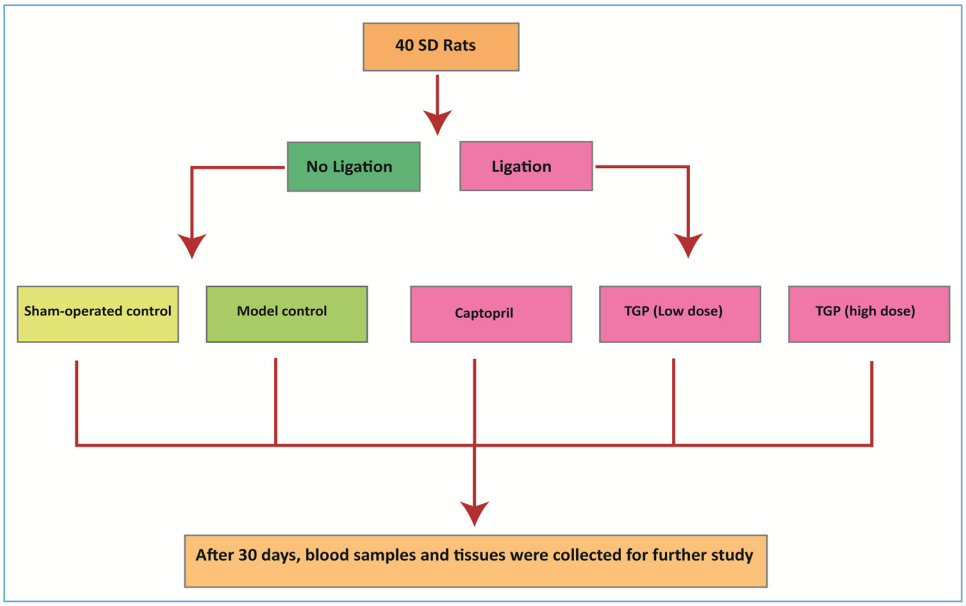

Fig. 1. Study design of experiments.

as providing structural support for morphology, arranging, and contraction of myocytes [8]. On the other hand, by pathological assessment, ECM remodeling was mainly presented by collagen deposition, phenotypic change and degree of cross-linking [9]. Collagen deposition is a kind of adaptive response of tissue injury to support and strengthen damaged myocytes, thus the increase of collagen level within a certain range is also beneficial. We can see widely collagen deposition in the process of myocardial fibrosis caused by pressure overload [10]. It was reported that, after Captopril, the angiotensin-converting enzyme inhibitors (ACEIs), succeeded in the market, emphasis on exploiting new CHF therapeutic drugs gradually focuses on these drugs which can delay and reverse myocardial remodeling. Current clinical and basic experiments have confirmed that ACEIs, angiotensin II receptor blockers (ARBs), β-blockers, aldosterone antagonists, matrix metalloproteinase inhibitors (MMPIs) perhaps inhibit myocardial remodeling to some extent [11,12]. From ancient times to nowadays, the medicinal herbal plants played an important role in various ancient remedies and modern medication therapies. These days, the extractions of active ingredients are economical, potent and have fewer side effects. Thus, reagents derived from medicinal herbs offered smart treatment options that could be beneficial to the world's population. Pharmacological investigations of the use of medicinal plants in anti-nephrolithic therapy revealed therapeutic potential in both *in-vitro* and *in-vivo* models [13]. Radix Paeoniae Alba (RPA), dried root of *Paeonia lactiflora* pall without bark, has been used as a medicinal herb in TCM for centuries based on its wide range of pharmacological activities and used in many herbal preparations [14,15]. RPA mainly contain phenolic acids, monoterpenoid tannins, triterpenes, glucosides and is used in reviving the blood circulation [16]. Meanwhile, various pharmacological studies suggested that the TGP extracted from RPA have fundamental pharmacological effects such as immunosuppressant, anti-inflammatory, analgesic, anti-depressant, rheumatoid arthritis, antimicrobial, antioxidant and protect hepatotoxicity [16–18]. The main components of TGP include paeoniflorin, hydroxy-paeoniflorin, paeonin, albiflorin, benzoylpaeoniflorin. Furthermore, the RPA was used to relieve pain, regulate menstrual functions, nourish blood, wind, and suppress sweating as well [19]. Although, it effects on myocardial remodeling that are being considered as one of the main mechanism leads to CHF. Thus, this study was designed to explore the neurohormonal effects of TGP on a myocardial remodeling in a rat model as well as to investigate its possible mechanism.

2. Materials and methods

TGP capsules (Lot No. 081202) were produced by Ningbolihua Pharmaceutical Co., Ltd., of Zhejiang Province China; it contains 40% of paeoniflorin, 10% of hydroxy-paeoniflorin and 50% other components which mainly include paeonin, albiflorin, and benzoylpaeoniflorin. The TGP (95% purity) was diluted into 10 g/L and 5 g/L suspension (1% CMC) and stored at 4 °C for further use. While, Captopril (95% purity) was purchased from Shanghai Pukang Pharmaceutical Co., Ltd., China and diluted into 2.25 g/L (Distilled water) and store at 4 °C for further use. Coomassie (Bradford) Protein Assay Kit and IL-1β ELISA kit purchased from Shanghai Bogoo Biotechnology Co., Ltd., China. SYBR Green real-time PCR Master MIX and NanoDrop purchased from Thermo Fisher Scientific, USA.

Male SD (Sprague-Dawley) rats (210–250 g) were purchased from B &K Universal Group Limited Shanghai, China. All animals were kept at 23 ± 2 °C, 12-h light and dark cycle. Furthermore, all the animals used in this study were received care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 86-23, revised 1996), and the protocol was approved by the China Pharmaceutical University Institutional Animal Care and Use Committee.

2.1. Animal model

A total of 40 SD rats were divided into 5 groups (n = 8) and named as, a model group, sham-operated group, Captopril group, lose dose TGP group and high doge TGP group, respectively as shown in Fig. 1. Usually, myocardial remodeling was induced by the pressure-overload method by occlusion of the abdominal aorta to induce CHF [20,21], a model for further study. Briefly, the rats were anesthetized with 10% chloral hydrate, 300 mg/kg, i/v. after a 12-hour fasting and free drinking. Rats were placed in a supine position. The skin was prepared aseptically and a midline laparotomy was performed. The abdominal aorta was found, and the tissue between a left renal artery and a right renal artery was bluntly separated, then, the abdominal aorta was partially occluded with a silver clip (0.6 mm), next the wound was sutured layer by layer. The animals in the model groups appeared centripetal hypertrophy showing thick ventricular wall and increased myocardial index [22]. On the other hand, the Sham-operated group underwent the same procedure except for ligation of the abdominal

Table 1
Primers used for Rt-PCR to detect the expression of MMP-9, MMP-2 gene expression.

Gene	Forward Primer	Reverse Primer	Product Size (bp)
MMP-9	CCACCGAGCTATCCACTCAT	GTCCGGTTTCAGCATGTTTT	159
MMP-2	CTGATAACCTGGATGCAGTCGT	CCAGCCAGTCCGATTTGA	135
GAPDH	AAGGTCATCCCAGAGCTGAA	ATGTAGGCCATGAGGTCCAC	326

The RT-PCR reaction conditions for each target gene were as follows: MMP-2: 94 °C 3 min; 94 °C 30 s; 54 °C 30 s; 72 °C 30 s; 45 cycles. MMP-9: 94 °C 5 min; 94 °C 30 s; 56 °C 45 s; 72 °C 45 s; 35 cycles. GAPDH: 94 °C 2 min; 94 °C 30 s; 54 °C 30 s; 72 °C 30 s; 25 cycles.

aorta. The surviving model rats have received i/m injection of 5000U penicillin G sodium respectively to prevent infection. One week after the ligation, drugs were administrated by intragastric (i.g.) once a day up to 30 days. Sham-operated group and model group were administered with an equal amount of (20 mL/kg/d) distilled water. After 30 days of blood samples and tissues were collected for further examination.

2.2. Determination of CVF and the collagen type I/III ratio

The collagenous fiber was detected by Picrosirius red staining solution. Briefly, the paraffin slices were dewaxed by dimethylbenzene and gradient ethanol, then the samples were washed three times (3 min every time) with normal saline, they were stained with Harris Hematoxylin for 5 min and were washed three times with NS (5 min every time), they were stained with Picrosirius red staining for 20 min. Finally, color segmentation and dehydration were done with absolute alcohol. Slices were cleared with xylene and fixed with neutral resins, then observed under 400× microscopes. ICVF (Interstitial collagen volume fraction = collagen area/total area ×100%) and PVCF (Perivascular collagen volume fraction = collagen in the perivascular space/Arterial lumen area) were observed under a polarized microscope and detected by Image Analysis Software (image pro plus 6.0).

Similarly, type I and III collagen was also observed under a polarization microscope. CVF (collagen volume fraction = collagen area/total area ×100%) and the collagen type I/III ratio was measured by Image Analysis Software (image pro plus 6.0).

2.3. Angiotensin II assays in myocardial tissue

To examine the Ang. II, tissues from the left ventricle (100 mg) were taken, chopped and quickly homogenized in 1 mL of NS on ice. All samples were centrifuged (4 °C, 3500 rpm, 10 min). The supernatant was diluted 10 times with normal saline (NS) to detect the concentration of protein by Coomassie (Bradford) Protein Assay Kit, and meanwhile, the supernatant was diluted 5 times with NS to detect the concentration of Ang. II by Radioimmunoassay (RIM) determination. The results were expressed as the concentration of Ang. II/ mg myocardial tissue.

2.4. Assays of interleukin 1 beta in serum

The serum was diluted at different concentrations with the following ratio: 1000, 500, 250, 125, 62.5, 31, 15.6 pg/mL, then 0.1 mL of serum was added to examine the concentration of IL-1β, respectively. The dilution liquid sample was used as a control. Wells were covered with preservative film and were incubated for 90 min at 37 °C. Water was thrown off and slapped with absorbent paper several times. 0.1 mL antibody (1:100) was added to each well at 37 °C for 60 min. Wells were washed with 0.01 M PBS 3 times. 0.1 mL (dilution:1:100) was added at 37 °C for 30 min and was washed with 0.01 M PBS for 5 times and was sink for 1 min each time. Then 90 μl TMB chromogenic solution which was balanced for 30 min at 37 °C was added to each well and was incubated at 37 °C for 20 min under dark condition. An OD value was measured at 450 nm with ELISA after stopping with 0.1 mL TMB stop buffer. Curve Expert version 1.3 was used to produce the standard curve and it achieves computational formula, and then the OD values of a sample were put into the formula to get its concentration.

2.5. Determination of MMP-2, -9 mRNA levels by reverse transcription PCR (Rt-PCR)

50-100 mg cardiac tissue was fully homogenized in a mortar and pestle with a small volume of liquid nitrogen. After fully homogenization RNA from single cell suspension was extracted by using RNA-iso plus according to the manufacturer instructions. The concentration of RNA was measured by using NanoDrop.

Reverse transcription of the total RNA into cDNA was done by using Master cycler Nexux gsx1 (Eppendorf, USA) according to the manufacturer instructions by using 1ug of extracted RNA. Denaturing and annealing reaction was done in the PCR at 35 °C for 5 min, 65 °C for 15 min and 94 °C for 5 min. The condition for reverse transcription was, 30 °C 10 min, 42 °C 15 min, 95 °C 5 min. The Rt-PCR was performed by using SYBR Green real-time PCR Master MIX and 50 ng cDNA (0.4 μL) and the total reaction system was 25 μL. The primer (0.4 μl) used for MMP-2, -9 and GAPDH in the reaction system is described in the table below, where GAPDH was used as housekeeping gene (Table 1).

After Rt-PCR the electrophoresis was performed by using 2% agarose gel. Next, the gel images were taken by GEL imaging system, and optical density of each band was detected by Gelpro Analyzer 4.0. The ratio of OD values of target genes to the internal reference gene (GAPDH) of every sample was used as a measurable indicator.

2.6. Expression levels of NF-kB and TGF-β1 in myocardial tissues

The expression of NF-κB; TGF-β_1 in myocardial tissues was determined by immunohistochemistry, generally, dewax 5um-paraffin sections and were washed three times by distilled water for five minutes, three times and inactivate endogenous enzymes by the treatment of 3% H_2O_2 for 10 min. They were washed with 0.01 M PBS three times for five minutes, and antigen retrieval was achieved by 0.1 M citrate buffer in 95℃ water bathing for 15 min. Dropped 5% BSA, incubated at 37℃ for 20 min and threw liquid off without washing. Dropped an appropriate concentration of the first antibody into a wet box for overnight at 4℃. Dilution concentration ratio of NF-κB was 1:50, meanwhile TGF-β_1 1:50 and washed by 0.01 M PBS three times for five minutes. Drop biotinylated IgG (goat anti-rabbit) and incubate at 37℃ for 15 min and wash by 0.01 M PBS for five minutes, three times. Drop SABC and incubate 37℃ for 15 min and washed by 0.01 M PBS four times for five minutes. DAB coloration (prepared when needed) for 5 min, then stopped reaction by sinking the sections into distilled water. Redyed with Haematoxylin for 1 min and differentiated with hydrochloric ethanol for 10–15 sec as well as flush with water for 30 min. Tissue blocks were dehydrated in ascending series of ethanol, cleared in xylene and embedded in neutral resins. Image Analysis was carried out by Image-Pro Plus 6.0. the reaction rate of the activated NF-κB positive cells was calculated by the previously reported method [23]. The reaction rate of activated NF-κB cells = Nuclear positive cell number/ total cell number. The content of TGF-β_1 was expressed by log (IOD). IOD = the logarithm of the integrated optical density value in TGF-β_1

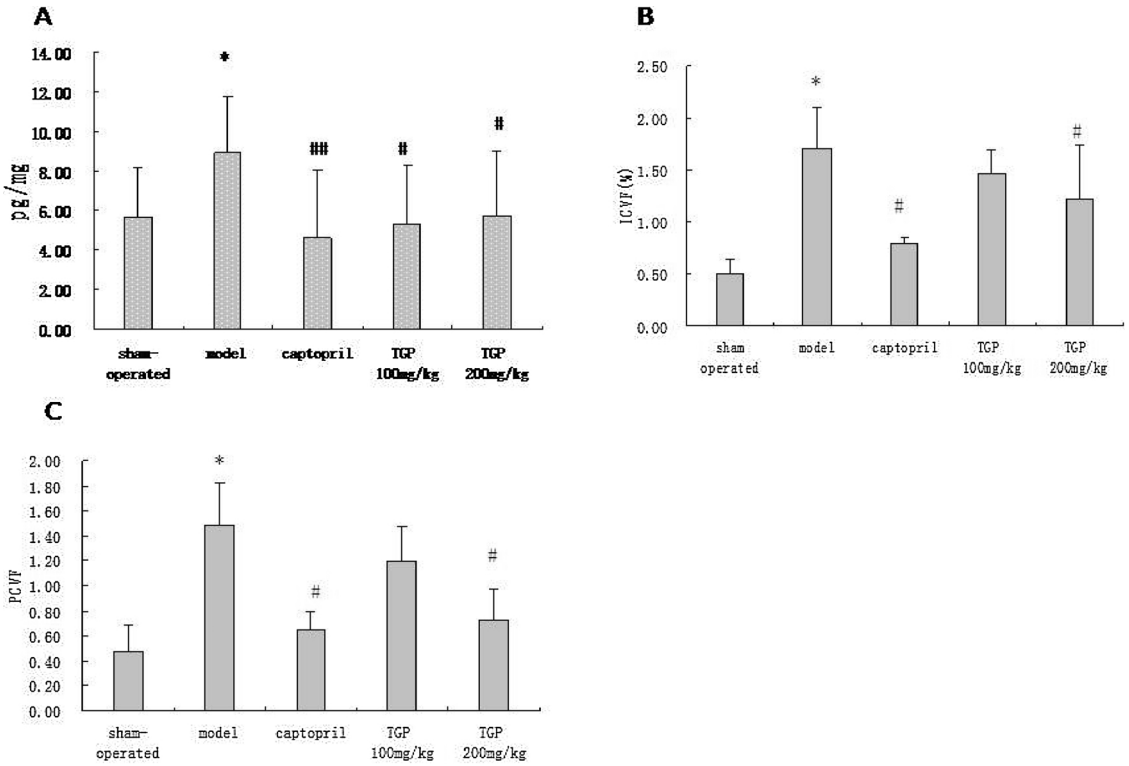

Fig. 2. A, Effects of TGP on Ang-II concentration of ventricular tissue in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$; compared with model: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **B,** Effects of TGP on ICVF in rats with myocardial remodeling by abdominal aorta ligation (n = 5) $^{*}P < 0.05$ (compared with sham-operated); $^{\#}P < 0.05$ (compared with model). **C**, Effects of TGP on PCVF in rats with myocardial remodeling by abdominal aorta ligation (n = 3) $^{*}P < 0.05$ (compared with sham-operated); $^{\#}P < 0.05$ (compared with model).

positive expression area.

2.7. Statistical analysis

All the data were calculated as a mean ± standard error. The differences among multiple groups were assessed by One-way ANOVA using SPSS (version 15.0). The differences between two groups were assessed by the LSD test for homogeneity of variances, meanwhile the Dunnett's T_3 test for non-homogeneity of variances. Enumeration data were analyzed by Mann-Whitney U test. Where, $P < 0.05$ was considered statistically significant.

3. Results and discussions

3.1. Effects of TGP on angiotensin II concentration

The level of Ang. II in ventricular tissues of a model group was much higher than the sham-operated and control groups. Ang. II level in low dose Captopril group and high dose TGP group was significantly low ($P < 0.01$) as compared to control and sham-operated group (Fig. 2A).

The neurohormonal factors in CHF include sympathetic nervous system (SNS) renin-angiotensin-aldosterone system (RAAS) and cell factor system, which could make up the decrease of cardiac function to maintain the homeostasis of cardiovascular function in the early disease stage [24]. However, the long-term effects of these compensatory changes could become vicious circles, worsen the structure and function of the heart to a decompensatory stage and finally induce CHF [25]. Generally, the drugs used for HF can affect the above three systems to some extent. Thus, the activation of RAAS can be observed by pressure overload and volume overload animal model [3], senile disease and myocardiopathy. *in vivo*, *in vitro* and clinical studies indicated that Angiotensin II, as a main effective peptide in RAAS, played an important role in the myocardial remodeling [26]. It could induce hypertrophy and necrosis of cardiac myocytes, formation of collagen and fibrosis degeneration of myocardial matrix [12,27,28]. Ang. II could induce expression of immediate early genes through mitogen-activated protein kinase (MAPK) signaling pathway to regulate cardiomyocyte hypertrophy, promote nerve endings in the heart to secrete NA and induce endothelial cells to secrete endothelin. Ang. II had multiple effects on myocardial fibroblasts such as promoting proliferation of myocardial fibroblasts, synthesis, and secretion of adhesion molecules, ECM related protein and expression of integrin adhesion receptors [29].

In this context, the results depicted that TGP can reduce Ang. II level in myocardial tissue and restrains the activity of RAAS to some extent.

3.2. Effects of TGP on interstitial and perivascular CVF

It was found that the collagen sections were red under the Light microscope after picrosirius red staining. There is little collagen in the interstitial space and the perivascular space of the Sham-operated and control groups. Meanwhile, collagen in the interstitial space and coronary surrounding was observed hyperplastic, it confirmed that the Captopril (45 mg/kg/d) and TGP (200 mg/kg/d) could produce change (Fig. 2B-C). Half quantitative analysis of images showed that ICVF and PCVF values in the model group were significantly upgraded whereas, Captopril and TGP groups significantly decreased the ICVF and PCVF values, as shown in Fig. 3A & B.

3.3. Effects of TGP on CVF I, III, and I/III collagen ratio

Most collagen molecules could orderly parallel alignment, so collagen had birefringent character [30]. It could have strong binding to acidic dye such as Picrosirius-red and obviously strengthened the birefringent character of collagen. Under the Polarized light microscopy, it was observed that, type I collagen fiber was red, tightly arranged and

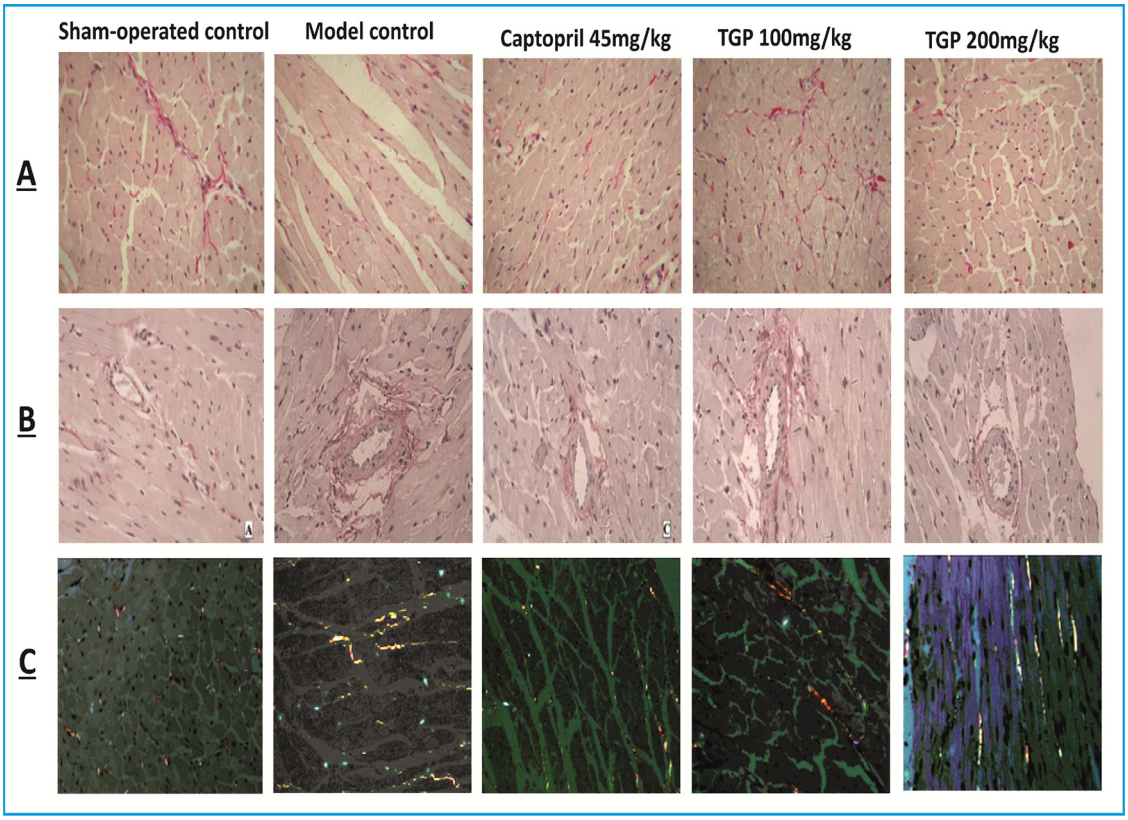

Fig. 3. A, Effects of TGP on cardiac collagen in the interstitial space of the left ventricle in rats with myocardial remodeling by abdominal aorta ligation (Sirius red stain and Light microscope. × 400). **B**, Effect of TGP on cardiac collagen in the perivascular space of the left ventricle in rats with myocardial remodeling by abdominal aorta ligation (Sirius red stain and Light microscope. × 400). **C,** Effect of TGP on cardiac collagen in the interstitial space of the left ventricle in rats with myocardial remodeling by abdominal aorta ligation (Sirius red stain & polarized light, ×400).

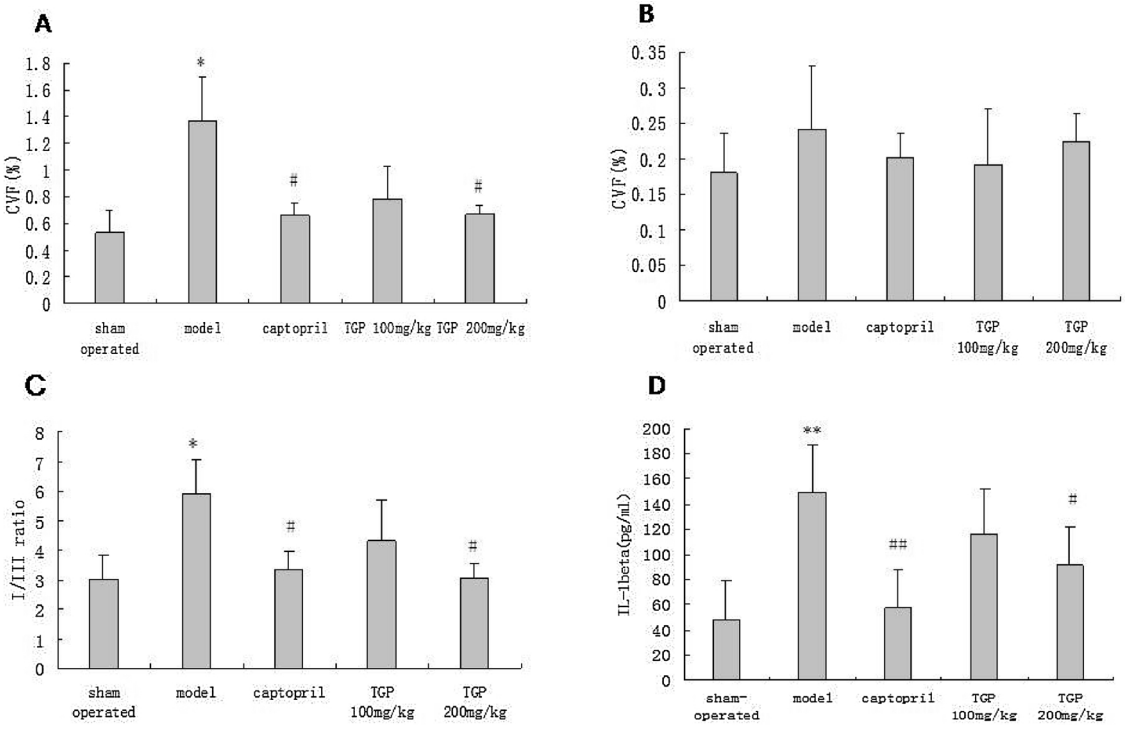

Fig. 4. A, Effect of TGP on type I collagen CVF, **B,** Effect of TGP on type III collagen CVF. **C,** Effect of TGP on type I/III collagen ratio of the left ventricle in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$; compared with model: $^{\#}P < 0.05$. **D**, Effects of TGP on an IL-1β concentration of serum in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$, $^{**}P < 0.01$; compared with model: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

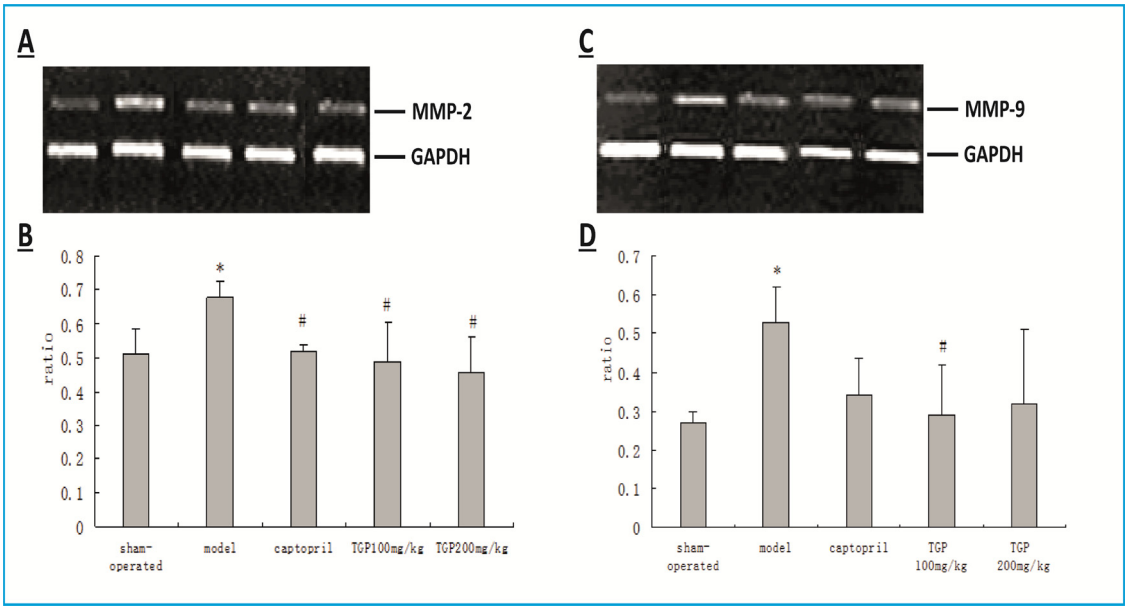

Fig. 5. A, Effects of TGP on MMP-2 mRNA expression in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$; compared with model: $^{\#}P < 0.05$. **B,** Effects of TGP on MMP-9 mRNA expression in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$; compared with model: $^{\#}P < 0.05$.

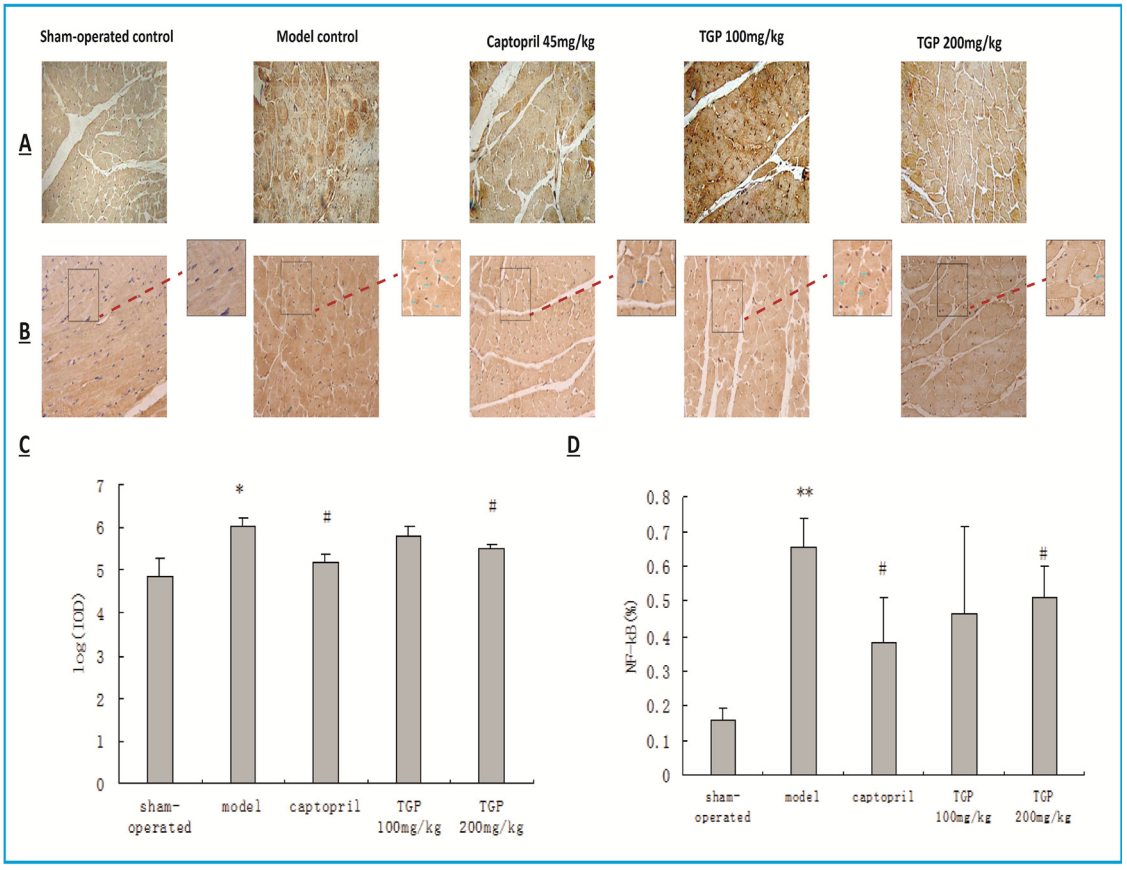

Fig. 6. A, Effects of TGP on TGF-β1 expression in rats with myocardial remodeling by abdominal aorta ligation (400×). **B,** Effects of TGP on rats of positive labeling for NF-κB in the nuclei of myocardial cells in rats with myocardial remodeling by abdominal aorta ligation (400×). **C,** Effects of TGP on TGF-β1 expression in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$; compared with model: $^{\#}P < 0.05$. **D,** Effects of TGP on rates of positive labeling for NF-κB in the nuclei of myocardial cells in rats with myocardial remodeling by abdominal aorta ligation, n = 3. **Note:** compared with sham-operated: $^{*}P < 0.05$; compared with model: $^{\#}P < 0.05$.

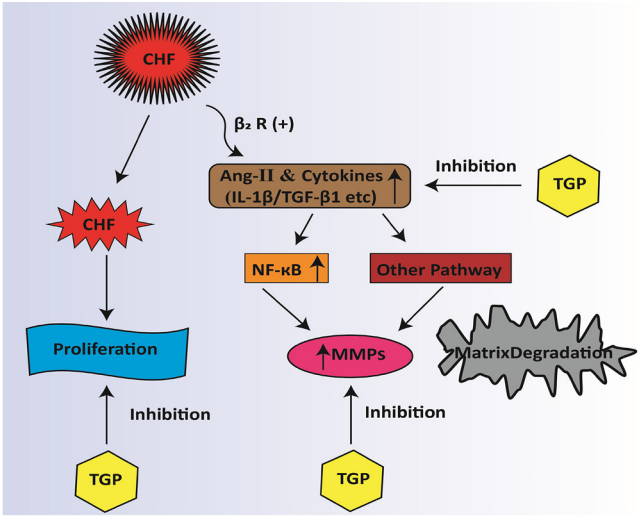

Fig. 7. Role of TGP in myocardial remodeling by regulating the NF-kB pathway.

showed strong birefringent, while, type III collagen fiber in model group was green fine fiber and had weak birefringent character (Fig. 3C). Comparing with the sham-operated and control group, CVF type I, III and ratio of I/III collagens in the control group increased significantly, meanwhile, low dose Captopril group and high dose TGP group significantly decreased (Fig. 4A-C).

Collagen phenotype can be divided into I, II, III, and V and the majority is I and III [31]. Previously, 90% of the ECM is type I and type III collagen to ensure structural integrity among myocytes and co-ordinate myocardial cell synchronous contraction to influence heart pumping function. Type I collagen has a strong anti-tensile strength and small extension resilience. Myocardial stiffness increases when the content of type I increase. Whereas, type III Collagen belongs to embryo-type collagen and has largely extended resilience [32,33].

The ratio of type I and type III changed in the process of myocardial remodeling, thus it influenced cardiac diastolic or contraction. In cardiac compensation period, the ratio of type I and type III rises while, in cardiac decompensation period, the ratio decreases. The time duration of our research was one month. The increased ratio of type I and type III showed that when the heart was in cardiac compensation period and the results of the blood flow dynamic test showed myocardial contractility of the model was a compensable enhancement. As the ratio of type I and type III collagen increased, myocardial stiffness increased, and then ventricular compliance was decreased manifesting diastolic dysfunction, systolic dysfunction and eventually led to the failure of pumping function [34].

The results of this study showed that TGP had the effect on inhibiting the increased ratio of type I and type III in the animals of abdominal aorta ligation group which can reduce cardiac muscle stiffness, improve compliance and delay the process of heart failure.

3.4. Effects of TGP on interleukin 1 beta concentration of serum

The IL-1β concentration in the serum in model groups was much higher than the sham-operated and control groups. Low dose Captopril group and high dose TGP group can significantly decrease the level of IL-1β ($P < 0.01$ and $P < 0.05$) respectively (Fig. 4D).

Cytokines possess pleiotropic functions and mediate systemic and local biological actions, which relate to cell growth and differentiation, immune function and inflammation [35]. Many clinical and laboratory research results showed that the local inflammatory reaction happened with CHF [36,37]. Invasive monocyte and local stress myocardial cells secrete large amounts of cytokines, such as TGF-β1, IL-1β, TNF-a, and so on. Cytokines triggered and involved in the infarction area scarring, cardiomyocytes hypertrophy, ECM structural changes and other related path physiological processes [38]. Interleukin-1 (IL-1) is a protein of the interleukin-1 family [35]. Previously, *in-vivo* experiments showed that IL-1β obviously increased in the myocardial tissue of pressure-overloaded animals [39]. Whereas, *in-vitro* experiments explored that IL-1β could promote myocardial cells growth [40], inhibit fibroblasts, collagen synthesis and promote collagen to degrade by enhancing the activity of MMP-2, -9 [41].

Recent research has demonstrated the recombinant human IL-1 receptor antagonist (IL-1RA) Anakinra can inhibit apoptosis in the myocardial infarction animal model [42], improve left ventricular remodeling [43]. Clinical trials demonstrate that Anakinra can improve the myocardial abnormity in patients with rheumatoid arthritis, perhaps, due to its protective effect on myocardium cell injury induced by oxidative stress and endothelial dysfunction [44]. These findings suggest that left ventricular remodeling can be attenuated by interfering with the specific inflammatory reaction pathways [45].

The results of our current study demonstrated that TGP had effects on the inhibition of serum IL-1β level caused by the abdominal aorta ligation. It was concluded that inhibiting the increase of inflammatory cytokine levels could be one of the mechanisms against myocardial remodeling associated with TGP.

3.5. Effects of TGP on MMP-2, -9 mRNA expressions

The Rt-PCR result showed that MMP-2, -9 mRNA expressions in the cardiac tissue in model groups were higher than the sham-operated and control group. The intragastric administration of TGP possibly lowered MMP-2, -9 mRNA expressions (Fig. 5A & B). Whereas, low dose Captopril and high dose TGP noticeably lowered the MMP-2, -9 mRNA expression ($P < 0.05$).

MMPs enzyme can degrade heart matrix components thus a powerful actuating factor in the process of heart matrix remodeling [46]. There is enough evidence which manifests that alterations in MMPs and its tissue inhibitors of metalloproteinase's (TIMPs) can make the process of ECM generation and degradation out of balance and it is one of the main causes of myocardial dysfunction. Collagen protein can also be degraded by MMPs [47]. At present, more than 10 kinds of MMPs are divided into collagen enzyme (MMP-1, -8, -13) such as, gelatinase (MMP-2, -9), Matrix-degrading enzymes (MMP-3, -10, -11), MT-MMP (MMP-14), others (MMP-7,-12) and so on [48]. The main function of gelatinases is degrading the denatured collagen fiber and elastin [49]. The expression and activity of MMP-2 and -9 mRNA were increased obviously in the myocardial remodeling caused by pressure overload aorta occlusion [50]. The higher expression represents more deposition of myocardial collagen and severe damage to myocardial diastole [51]. The expression of MMPs was controlled by various factors in the process of myocardial remodeling. Ang-II acted on an AT-1 receptor, activated JAK-STAT pathway and then activated transfer factors AP-1, STATs, NF-κB, etc. [52,53]. Because various MMPs have the binding sites of transfer factors, Ang. II can control the activity of MMPs [49], e.g. increasing the transcription of MMP-9 by promoting the nuclear shift of NF-κB [54].

In addition, cytokines have certain regulatory functions in controlling the expression levels of MMPs. The research showed that IL-1β can noticeably increase the activity of myocardial fibroblasts MMP-2, -9 cultivated *in vitro*. Although TNF-α had less effect on the activity of MMP compared with IL-1β, it had a synergistic function with IL-1β and increased selectively the activity of MMPs [55–58].

The results demonstrated that the expression of MMP-2 and MMP-9 mRNA were increased in our abdominal aorta ligation animal model and it is agreed with literature. The basic foundation of TGP is to inhibit the gene expression of MMP-2, -9 may be that it can inhibit collagen deposition and ECM remodeling. Furthermore, the effect of TGP on the expression of MMP-2, -9 may be in relation to reducing the Ang. II level of myocardial tissue and low IL-1 β level in serum.

3.6. Effects of TGP on TGF-β1 in the nuclei of myocardial cells

TGF-β1 was mainly expressed in the cytoplasm and highly articulated as brown granules. Expression of TGF-β1 in the sham-operated and control group is very low with weak intracellular dyeing. TGF-β1 was over-expressed in the cytoplasm of myocardial cells of model animals groups with a large number of brown granules. Whereas, low dose Captopril and high dose TGP decreased the expression of TGF-β1 respectively ($P < 0.05$), (Fig. 6A & C).

TGF-β1 is a dimer peptide with a molecular mass of 25 kD secreted by myocytes and fibroblasts. Large amounts of TGF-β1 were secreted in the process of stability myocardial hypertrophy to CHF [59,60]. TGF-β1 expression was noticeably increased in the pressure-overloaded animal model [61]. Previously, the experimental results *in-vitro* and *in-vivo* and indicated that Ang II and TGF-β1 did not exist independently but as a part of a signal transduction network to promote the myocardial remodeling together [62]. Ang. II promoted myocardial cells and fibroblasts to express TGF-β1 [63,64], up-regulate I, III collagen type gene expression [62,65], improving collagen synthesis [66,67] by activating AT1 receptors [63,64] to cause myocardial cell hypertrophy, promote ECM to degrade [62] and eventually lead to myocardial remodeling.

Interestingly, our results showed that TGP to some extent inhibits the raising expression of TGF-β1. The mechanism was partially for reducing the Ang. II level and then inhibiting the raising expression of TGF-β1.

3.7. Effects of TGP on NF-κB in the nuclei of myocardial tissue

NF-κB was mainly expressed in the cytoplasm and manifested as brown granules in normal cells. After cellular stimulation, NF-κB would shift into the cellular nucleus and make brown granules and can be seen in the nucleus. NF-κB expression in the sham-operated control group is very lower with little positive particles in the cytoplasm. The model groups showed the nuclear shifting of NF-κB, low dose of Captopril and high dose TGP showed a significantly low level of NF-κB, respectively (Fig. 6B & D).

NF-κB is a kind of ubiquitous transcription factor which does vital functions such as, in immune response, inflammation, regulation of cell growth, apoptosis, and embryonic development and so on. NF-κB is a common pathway of myocardial remodeling induced by RAAS, inflammatory cytokines (e.g. IL-1β) and oxidative stress reaction. Meanwhile, NF-κB activation is the most necessary link in myocardial remodeling which was induced by Ang. II and ISO [58].

NF-κB is a dimmer of p50 and p65 and mainly exists in the cytoplasm of quiescent cells. It can lose regulation activity when combining with IκBs to formed trimmers p50-p65-IκB [58]. When stimulated by factors such as cell factors TNF-α, IL-1β, the trimmer disintegrated into a dimmer and shifted into the nucleus to affect the expression of the specific gene such as MMPs [57]. For example, IL-1β can enhance the expression of proMMP-2 and proMMP-3 mRNA suggesting that it can increase the activity of MMP in relation to promoting transcription. The previous research showed that MMP-2, -9 could be down-regulated to inhibit ECM remodeling by enhancing the expression of IκBs to inhibit the activity of NF-κB [68,69]. Therefore, inhibiting the expression of NF-κB could be a more direct way to inhibit myocardial remodeling and improve heart function [70].

Specific anti-NF-κB p65 protein antibody was used for immunehistochemical identification to reflect the expression and the nuclear shift of NF-κB. The results showed that the nuclear shift of p65 was in the sham-operated model group. Thus, we concluded that NF-κB could participate in the gene regulation of myocardial remodeling, by modulating the expression of MMP-2, -9 mRNA. Furthermore, TGP can noticeably inhibit the nuclear shift of NF-κB. This may be the foundation of inhibiting expression of MMP-2, -9 mRNA and may also be one of the main mechanisms of inhibiting myocardium remodeling; the relationship between IL-1β, NF-κB, and MMPs can be seen in Fig. 7.

4. Conclusions

In concluding remarks, TGP improves the morphological and physiological changes of the heart and attenuate the myocardial remodeling by restraining rennin-angiotensin-aldosterone system (Ang. II) and decreasing the level of inflammatory cytokines (TGF-β1, IL-1β) and inhibiting the expression of MMP-2, -9 mRNA and it has been suggested that all these effects may have associated with NF-κB pathway. Meanwhile, the finding also revealed a new insight to understand the mechanism behind myocardial remodeling.

Conflict of interest

The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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