

## Original Article

# Effects of cryptotanshinone on the expression levels of inflammatory factors in myocardial cells caused by Ang II and its mechanism

Shu Tang<sup>1\*</sup>, Qiang Wen<sup>2\*</sup>, Peiqing Liu<sup>3</sup>, Zhenfeng Zhu<sup>1</sup>, Na Li<sup>1</sup>, Xiaojian Zhang<sup>1</sup>, Quancheng Kan<sup>1</sup>

<sup>1</sup>Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China; <sup>2</sup>Department of Clinical Pharmacology, Basic Medical College of Zhengzhou University, Zhengzhou 450002, Henan, China; <sup>3</sup>School of Pharmaceutical Science, Sun Yat-sen University, Guangzhou 510006, Guangdong, China. \*Equal contributors.

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**Abstract:** Objective: This study aims to explore the effects of the traditional Chinese medicine monomer cryptotanshinone (CTS) on the expression levels of inflammatory factors in myocardial cells caused by Ang II and its mechanism. Methods: The neonatal rat myocardial cells were cultured in vitro in this study. Their purities were identified by immunocytochemical method. The cellular viability in different groups was determined by MTT assay. The levels of TNF- $\alpha$  and IL-6 in the supernatant of cell culture were detected with ELISA method. The levels of intracellular reactive oxygen species (ROS) were detected by Dihydrogen ethidium (DHE) staining method. The location changes of NF- $\kappa$ B in cells were detected by immunofluorescence method. Results: The purity of primary cultured neonatal rat myocardial cells was over 95%, CTS had no obvious effect on the viability of cells while it inhibited the increased levels of TNF- $\alpha$ , IL-6 and ROS caused by Ang II with dose dependent. NF- $\kappa$ B mainly distributed in the cytoplasmic region in normal cells, it translocated to the nucleus after Ang II stimulation while CTS inhibited the translocation. Conclusions: CTS could inhibit the inflammatory factors such as TNF- $\alpha$  and IL-6 in myocardial cells induced by Ang II with dose dependent, its mechanism may be related with that CTS could decrease the levels of ROS in myocardial cells and inhibit NF- $\kappa$ B translocation into the nucleus.

**Keywords:** Cryptotanshinone (CTS), Ang II, TNF- $\alpha$ , IL-6, NF- $\kappa$ B

## Introduction

Inflammation plays an important role in the occurrence and development of cardiovascular disease (CVD). Inflammatory cytokines have dual effects, on the one hand they play an important protecting role in the immune regulation and anti infection, on the other hand, they may cause inflammatory reaction cascade which is harm to the cells if sustained release or produce too much. The sustained release of tumor necrosis factor-alpha (TNF- $\alpha$ ) could directly damage myocardial cells and increase the susceptibility of patients to myocarditis [1]. Kanda reported that interleukin-6 (IL-6) in the blood had close correlation with the left ventricular dysfunction [2]. Oxidative stress is a feature of inflammatory reaction [3-7] and reactive oxygen species (ROS) include the small

molecules closely related to oxidative stress. The nuclear transcription factors sensitive to activated oxygen in cells include nuclear factor kappa B (NF- $\kappa$ B) and activated protein-1 (AP-1) etc. Studies showed that NF- $\kappa$ B was strongly activated in the process of heart failure [8], myocardial ischemic reperfusion, unstable angina, atherosclerosis and others, it plays the role in CVD by regulating the expression of adhesion molecules and inflammatory molecules, etc [9].

Angiotensin II (Ang II) binding with its receptor can increase intracellular ROS [10] and activate multiple transcription factors, such as NF- $\kappa$ B, AP-1, signal transcription and activation factor (STAT) and cAMP reactive binding protein (CREB) [11, 12], which mediated the production of TNF- $\alpha$ , IL-6, IFN- $\gamma$  and other inflammatory

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mediators [13-15]. Study on the relationship between Ang II and ROS and NF- $\kappa$ B may provide a new theoretical basis for the pathogenesis and treatment of CVD.

*Salvia miltiorrhiza* is a kind of traditional Chinese medicine and was widely used in the treatment of coronary heart disease and other cardiovascular and cerebrovascular diseases. Tanshinone is the effective active component of *salvia miltiorrhiza* including tanshinone I, tanshinone IIA, tanshinone IIB, cryptotanshinone (CTS) and other more than 10 monomer. Studies showed that CTS can reduce the elevated expression of MMP-9 induced by TNF- $\alpha$  and the increased activity of COX-2 induced by LPS [16, 17]. Our previous study found that CTS could inhibit the inflammatory response of macrophages [18]. There were no reports on the intervention effect of CTS on the inflammatory factors. Therefore, we studied the intervention effects of CTS on myocardial inflammatory reaction induced by Ang II and its possible mechanism in this study.

### Materials and methods

#### *Culture and identification of neonatal rat primary myocardial cells*

Heart was taken out from 2~3 days SD rat and put into dish with cooling PBS. The heart was cut into 1 mm<sup>3</sup> pieces after removing the connective tissue on the heart. The pieces were washed and put into a flask containing a rotor. They were digested with 10~15 ml 0.1% trypsin and 200  $\mu$ l DNA enzyme (0.05 mg/ml) on ice for 20 min. Then the flask was put on a magnetic stirrer at 37°C for 2 h. They were centrifuged at 1000 rpm for 5 min and washed with PBS. The isolated cells were cultured in DMEM medium containing 10% fetal bovine serum at 37°C with 5% CO<sub>2</sub>. The culture medium was collected after 1 hours and 0.1 mmol/l BrdU (10 mL/mL) was added to inhibit fibroblast proliferation. Then they were cultured in plates for 3-9 days. The morphology, adherent and pulse rhythm of myocardial cells were observed under inverted phase contrast microscope. The purity of cultured myocardial cells was identified using the anti- $\alpha$ -sarcomeric actin antibody. They were performed using immunohistochemistry staining kit according to the manual.

#### *Detection of the vitality of myocardial cells by MTT assay*

Myocardial cells ( $2 \times 10^5$ /ml) were inoculated in 96-well plate and cultured for 72 h. Then they were treated with 0.1% DMSO, 1  $\mu$ M Ang II and 10 mM CTS respectively and cultured for 24 h. 20  $\mu$ l MTT (5 mg/ml) was added into each well and cultured at 37°C for 4 h. The medium was discarded and 150  $\mu$ l DMSO was added into them. OD values at 570 nm were determined.

#### *Detection of TNF- $\alpha$ and IL-6 levels with enzyme linked immunosorbent assay (ELISA)*

The myocardial cells were treated with CTS (2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M) for 1 h respectively after culture for 72 h. Then they were treated with 100 nM Ang II and cultured for 24 h. The culture supernatant was collected and TNF- $\alpha$  and IL-6 levels were detected with ELISA kit according to the manual. OD values at 450 nm were determined by ELISA detector. Standard curve was drawn to calculate the concentration of TNF- $\alpha$  and IL-6 in the samples.

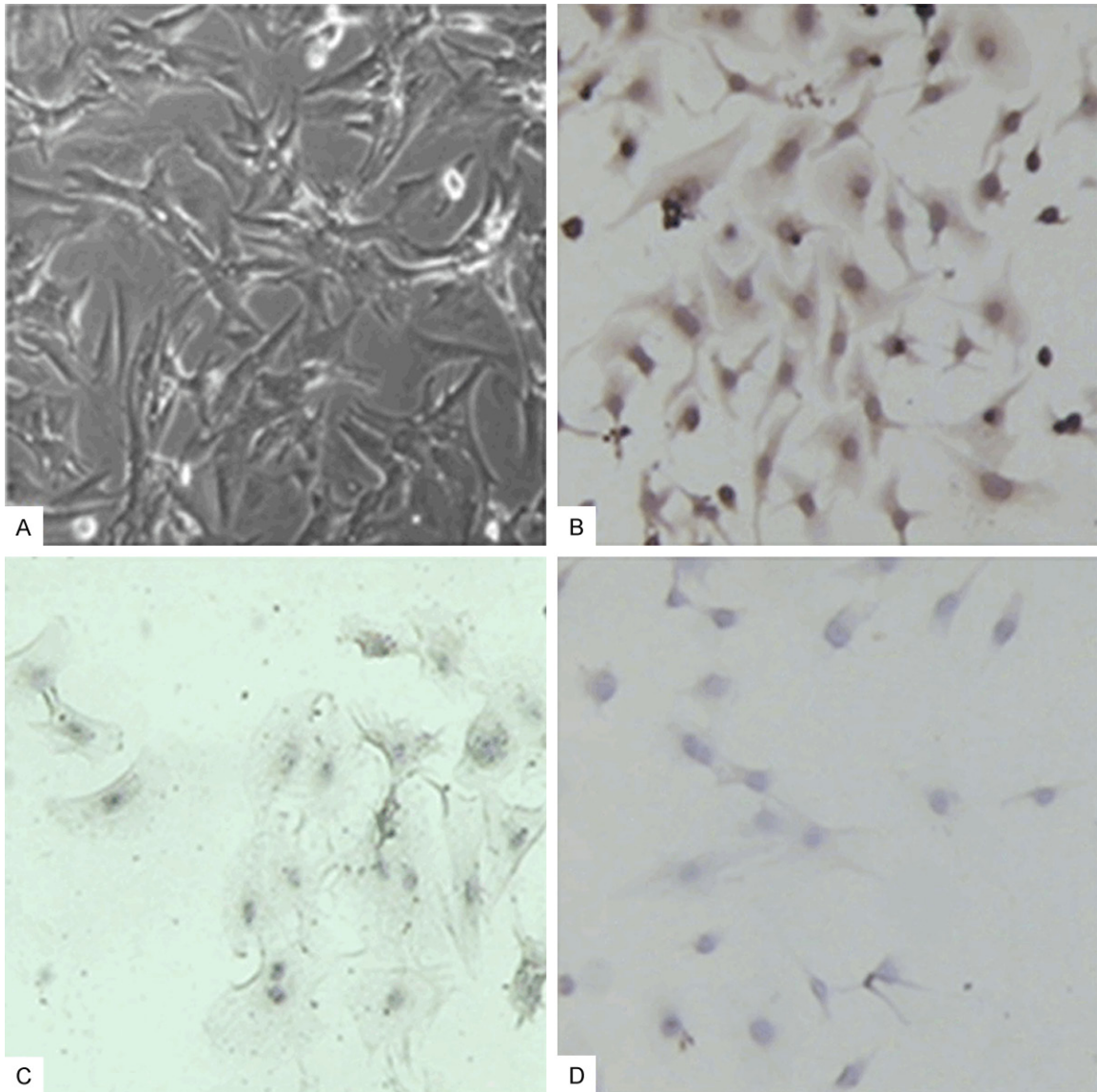
#### *Detection of intracellular ROS*

The myocardial cells were treated with CTS (2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M) for 1 h respectively after culture for 72 h. Then they were treated with 100 nM Ang II and cultured for 24 h. 10  $\mu$ mol/L dihydrogen ethidium (DHE) was added into them and incubated in dark at 37°C for 30 min. The cells were washed two times with PBS and ROS were observed under fluorescence microscope.

#### *Detection of NF- $\kappa$ B location with immunofluorescence method*

Myocardial cells ( $2 \times 10^5$ /ml) were inoculated in 6-well plate and cultured for 72 h. They were treated with CTS (2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M) for 1 h respectively, Ang II was added into them after that and incubated for 30 min. The medium was discarded and the cells were washed with PBS. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Discard the paraformaldehyde and air dry for 10 min. They were treated with PBS containing 0.5% Triton X-100 for 15 min and normal non-immune animal serum was added into them and incubated for 10 min at room temperature.

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**Figure 1.** The culture and identification of myocardial cells. A. Myocardial cells cultured for 72 h ( $\times 200$ ); B. The immunohistochemical staining of  $\alpha$ -sarcomeric actin in myocardial cells (72 h,  $\times 200$ ); C. The immunohistochemical staining of  $\alpha$ -sarcomeric actin in myocardial fibroblasts (72 h,  $\times 200$ ); D. The negative control (72 h,  $\times 200$ ).

The serum was discarded and washed with PBS, 200  $\mu$ L (1:100 dilution) mouse anti NF- $\kappa$ B (p65) was added into them and incubated at 4°C overnight. They were washed with PBS and fluorescein labeled second antibody (100  $\mu$ L, 1:200) was added into them and incubated at 37°C for 30 min. The nucleus was stained with Hoechst 33342 (1:500). NF- $\kappa$ B was stained green fluorescence and nucleus was blue.

### Statistical analysis

All statistical analyses were performed with SPSS 13.0 statistical software. Data were exp-

ressed as means  $\pm$  standard deviations (SD). Differences among groups were analyzed using t test and one-way ANOVA.  $P < 0.05$  were considered statistically significant.

### Results

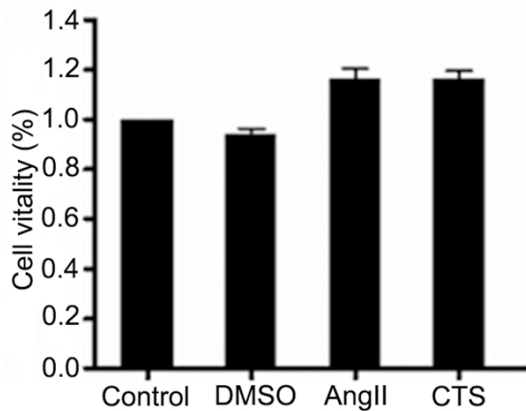
#### *The identification of myocardial cells and determination of vitality*

The cultured adherent myocardial cells were spindle, triangular or irregular polygon, the nucleus was elliptic, the pseudopodia among cells contact with each other and interwoven

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**Table 1.** Vitality test of cultured Myocardial cells ( $\bar{x} \pm s$ , n = 20)

Time (day)	Pulse (times/min)
3	85.20 $\pm$ 2.27
5	85.30 $\pm$ 2.61
7	84.20 $\pm$ 2.41
9	84.30 $\pm$ 1.75



**Figure 2.** The effects of drugs on the activity of myocardial cells ( $\bar{x} \pm s$ , n = 6).

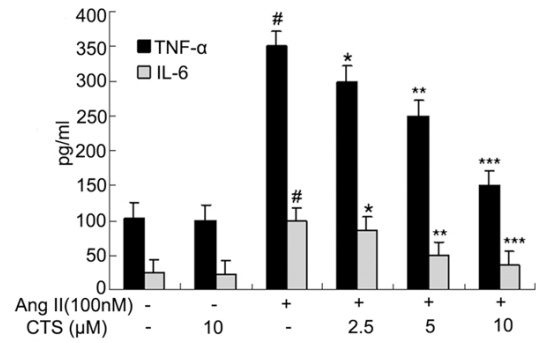
**Table 2.** The effects on activity of myocardial cells ( $\bar{x} \pm s$ , n = 6)

Group	OD values
Control	0.715 $\pm$ 0.065
0.1% DMSO	0.679 $\pm$ 0.047
1 $\mu$ M Ang II	0.822 $\pm$ 0.051
10 $\mu$ M CTS	0.815 $\pm$ 0.067

into a network and gradually formed a cell cluster or cell monolayer (**Figure 1A**). The primary myocardial cells began to beat rhythmically after culture for three days, there was no difference in the beat frequency between the third day and the ninth day (**Table 1**,  $P > 0.05$ ). The immunohistochemical staining of  $\alpha$ -sarcomeric actin showed that it was positive (**Figure 1B**). There was no brown positive cell in control group (**Figure 1C, 1D**).

### The effects on the vitality of myocardial cells

The effects on the activity of myocardial cells were detected by MTT assay. We found that 0.1% DMSO had no obvious effect on the activity of myocardial cells, 1  $\mu$ M Ang II and 10  $\mu$ M CTS also had no obvious effect on the activity



**Figure 3.** The effects on the TNF- $\alpha$  and IL-6 levels in the cell culture supernatant. Compared with control group, # $P < 0.05$ ; Compared with Ang II group, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Table 3.** The levels of TNF- $\alpha$  and IL-6 in the cell culture supernatant ( $\bar{x} \pm s$ , n = 6, pg/mL)

Group	TNF- $\alpha$	IL-6
Control	100 $\pm$ 3.72	30 $\pm$ 1.52
10 $\mu$ M CTS	99 $\pm$ 3.49	29 $\pm$ 1.43
Ang II	345 $\pm$ 10.82	100 $\pm$ 4.96
Ang II + 2.5 $\mu$ M CTS	305 $\pm$ 8.74	88 $\pm$ 3.49
Ang II + 5 $\mu$ M CTS	251 $\pm$ 7.84	50 $\pm$ 2.47
Ang II + 10 $\mu$ M CTS	156 $\pm$ 4.67	39 $\pm$ 2.38

of myocardial cells (**Figure 2; Table 2**,  $P > 0.05$ ). So the drug concentration in this experiment was safe without cytotoxicity.

### Detection of the TNF- $\alpha$ and IL-6 levels in the cell culture supernatant

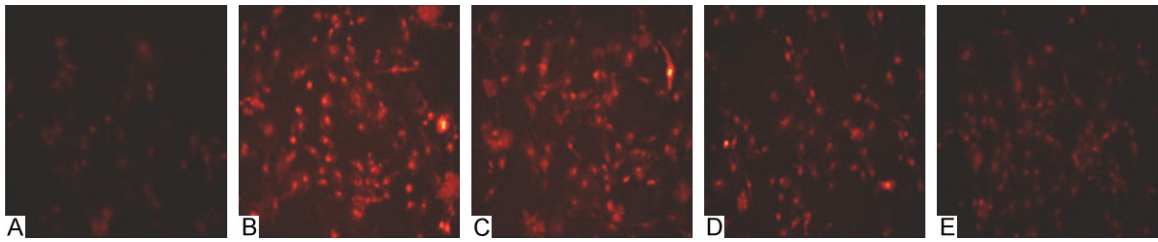
The results of ELISA showed that the TNF- $\alpha$  and IL-6 levels in control group was low while they increased significantly in Ang II group ( $P < 0.05$ ), the TNF- $\alpha$  and IL-6 levels in 2.5  $\mu$ M, 5  $\mu$ M and 10  $\mu$ M CTS group were significantly lower than that of Ang II group ( $P < 0.05$ ), it suggested that CTS could inhibit the increased TNF- $\alpha$  and IL-6 levels induced by Ang II with dose dependent (**Figure 3; Table 3**).

### Detection of the ROS levels

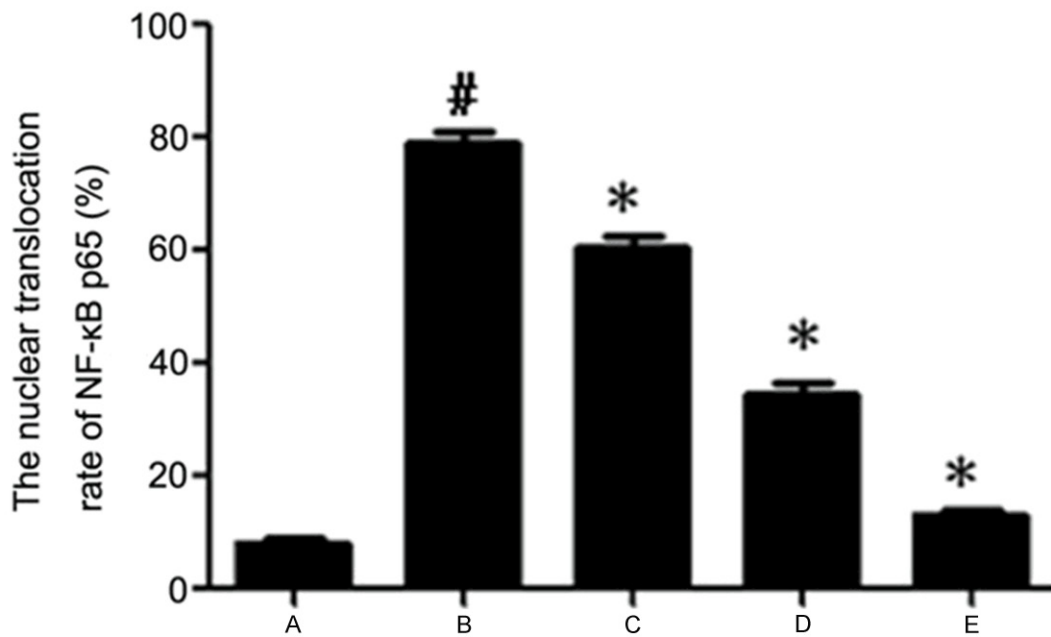
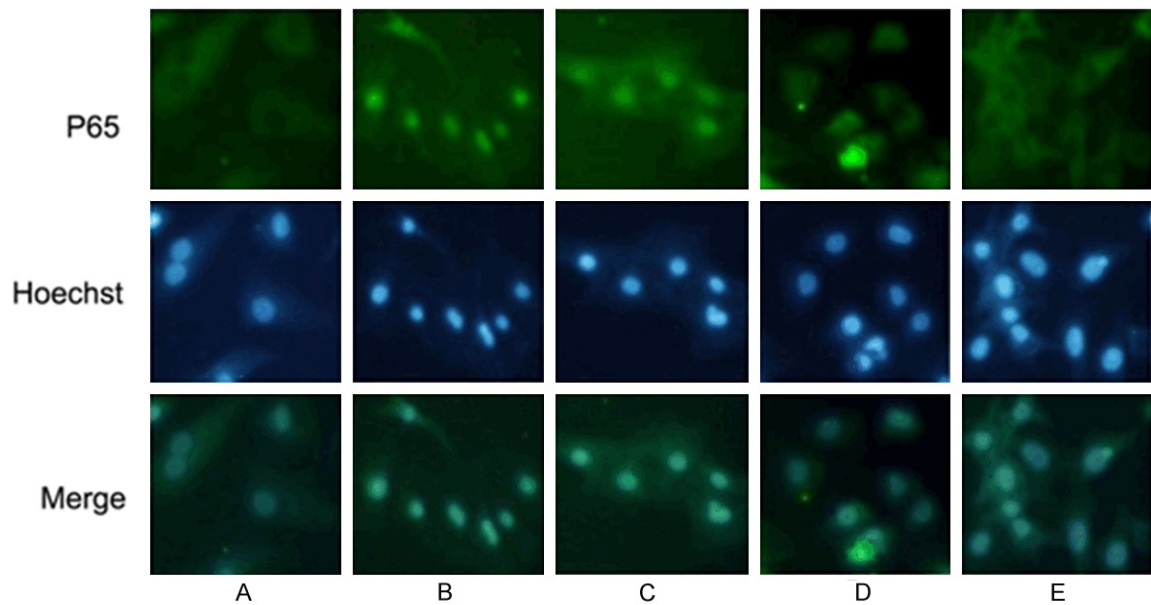
A small amount of red fluorescence can be detected in normal cell, it suggested that the intracellular ROS level is relatively low. The fluorescence in the cell increased significantly after the cell was treated with 100 nM Ang II for 24 h, which suggested that Ang II could increase the intracellular ROS level. The fluorescence in the



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**Figure 4.** The effects on the intracellular ROS levels. A. Control group ( $\times 200$ ); B. 100 nM Ang II group ( $\times 200$ ); C. 2.5  $\mu\text{M}$  CTS + 100 nM Ang II group ( $\times 200$ ); D. 5  $\mu\text{M}$  CTS + 100 nM Ang II group ( $\times 200$ ); E. 10  $\mu\text{M}$  CTS + 100 nM Ang II group ( $\times 200$ ).



**Figure 5.** The nuclear translocation rate of NF- $\kappa$ B (p65) in myocardial cells. A. Control group; B. 100 nM Ang II group; C. 2.5  $\mu\text{M}$  CTS + 100 nM Ang II group; D. 5  $\mu\text{M}$  CTS + 100 nM Ang II group; E. 10  $\mu\text{M}$  CTS + 100 nM Ang II group. Compared with control group,  $^{\#}P < 0.05$ ; Compared with Ang II group,  $^*P < 0.05$ .

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**Table 4.** The nuclear translocation rate of NF- $\kappa$ B (p65) in myocardial cells

Group	Nuclear translocation rate (%)
Control	8 $\pm$ 1.53
Ang II	80 $\pm$ 3.93
Ang II + 2.5 $\mu$ M CTS	64 $\pm$ 2.15
Ang II + 5 $\mu$ M CTS	38 $\pm$ 3.51
Ang II + 10 $\mu$ M CTS	15 $\pm$ 1.15

cell in 10  $\mu$ M CTS group was similar with that of control group, CTS could inhibit the increased ROS level induced by Ang II with dose dependent (**Figure 4**).

### *The translocation of NF- $\kappa$ B*

Immunofluorescence showed that NF- $\kappa$ B mainly existed in the cytoplasm in normal myocardial cells with little in nuclear area, which suggested that NF- $\kappa$ B (p65) was mainly in a static state. NF- $\kappa$ B obviously aggregated in the nucleus and near the nuclear membrane area after the cells were treated by Ang II. There was NF- $\kappa$ B both in cytoplasmic and nuclear area in 2.5  $\mu$ M CTS group, while in 5  $\mu$ M and 10  $\mu$ M CTS group, NF- $\kappa$ B mainly existed in cytoplasm. So CTS inhibited the translocation of NF- $\kappa$ B (**Figure 5**; **Table 4**).

### **Discussion**

The myocardial cells cultured in vitro can maintain certain characteristics of the structure and function of in vivo myocardial. They had the advantages of simple, quantitative, reproducible and not affected by neurohumoral factors. In this study, we used the improved myocardial cell culture method and cells had high yield and good active state. The use of in vitro cell model can confirm the direct effect of Ang II on myocardial cells.

Ang II as a factor with the roles of vascular contraction and promoting proliferation can increase blood pressure and activate mononuclear phagocyte system to release a variety of inflammatory factor, which could cause leukocyte adhesion, induce the apoptosis of endothelial cell and atherosclerosis and promote myocardial remodeling. In this study, we found that Ang II could increase the levels of TNF- $\alpha$  and IL-6 in the myocardial cell culture supernatant while CTS could inhibit this effect of Ang II

with dose dependent. Ang II could increase the levels of ROS in cells. Our previous study showed that CTS had obvious protective effects on glutamate toxicity of cultured nerve cells, its mechanism was related with CTS against the role of oxidative stress induced by glutamate [18]. In this study, we observed that CTS could reduce the increased ROS levels induced by Ang II with dose dependent. NF- $\kappa$ B binding with inhibition protein monomer I $\kappa$ B at resting state existed in cytoplasm as inactive forms. The protein kinase in cytosol can be activated by intracellular ROS and caused the dissociation of NF- $\kappa$ B and I $\kappa$ B, NF- $\kappa$ B transferred into the nucleus and combined specifically with the  $\kappa$ B sequence in immunoglobulin  $\kappa$  light chain, increased the expression of inflammatory factors such as TNF- $\alpha$  and IL-6 gene [13-15]. In this study, it is obvious of NF- $\kappa$ B into the nucleus after the myocardial cells were treated by Ang II for 30 min, while CTS could inhibit this effect of Ang II with dose dependent.

In a word, we concluded that CTS could reduce the TNF- $\alpha$  and IL-6 levels in the cell culture supernatant, this may be related with that CTS could decrease the levels of ROS in myocardial cells and inhibit NF- $\kappa$ B translocation into the nucleus.

### **Acknowledgements**

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### **Disclosure of conflict of interest**

None.

**Address correspondence to:** Shu Tang, Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China. Tel: 86-371-66913344; E-mail: shutangst@126.com

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