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Puerarin prevents isoprenaline-induced myocardial fibrosis in mice by reduction of myocardial TGF-β1 expression☆

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Abstract

It has been reported that soy isoflavones could significantly increase peroxisome proliferator-activated receptor α/γ gene expressions, while the activation of peroxisome proliferator-activated receptor α/γ may attenuate myocardial fibrosis. Puerarin is the main isoflavone isolated from the root of the wild leguminous creeper *Pueraria lobata* (Willd) Ohwi, so we thought that puerarin could inhibit myocardial fibrotic formation. A mouse myocardial fibrotic model was induced by hypodermic injection of isoprenaline when these mice were simultaneously treated with puerarin 600 and 1200 mg/kg by gavage for 40 days, respectively. The results showed that puerarin could significantly improve myocardial fibrosis and decrease the collagen accumulation, collagen volume fraction, hydroxyproline content in myocardial tissue and cardiac weight index. The results from reverse transcription polymerase chain reaction indicated that the messenger RNA (mRNA) expression of transforming growth factor-β1 in myocardial tissue was decreased, while the mRNA expressions of peroxisome proliferator-activated receptor α/γ were increased, in the puerarin groups as compared with the model group. Importantly, puerarin could significantly decrease the protein expressions of transforming growth factor-β1 and nuclear factor-κB in myocardial tissue. These results suggested that puerarin could prevent isoprenaline-induced myocardial fibrosis in mice, and its mechanisms might be related to reduction of transforming growth factor-β1 expression via activation of peroxisome proliferator-activated receptor α/γ and subsequent inhibition of nuclear factor-κB in myocardial tissue.

Keywords: Puerarin; Myocardial fibrosis; Peroxisome proliferator-activated receptor α/γ; Nuclear factor-κB; Transforming growth factor-β1

1. Introduction

Myocardial fibrosis occurs with hypertension, myocardial infarction and heart failure [1] and so on. It results from disruption of the equilibrium between synthesis and degradation of collagen, which leads to an excessive accumulation of collagen fibers within the myocardium. Because the myocardial fibrosis is an amazingly complicated process, the ideal therapeutic drugs have not been found as yet. Although angiotensin-converting enzyme inhibitors, angiotensin receptor 1 blockers, calcium channel blockers, β-adrenoceptor blockers and aldosterone antagonists have been proven to be effective in modulating the process of remodeling and in reducing the occurrence of adverse cardiovascular events [2–6], these drugs fail to completely stop the progression of myocardial fibrosis. So, it is very important to find new therapeutic drugs for myocardial fibrosis.

Prevention and cure of myocardial fibrosis with natural products are valuable and applicable research fields because plant sources are considered to be less toxic, with fewer side effects than synthetic medicines. Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β-D-gluco-pyranoside)] is the main isoflavone isolated from the root of the wild leguminous creeper *Pueraria lobata* (Willd) Ohwi. It has multiple pharmacological activities and has been used to treat many cardiovascular diseases such as hypertension [7], angina pectoris [8] and myocardial infarction [9]. It has been reported that soy isoflavones could significantly increase peroxisome proliferator-activated receptor (PPAR) α/γ gene expressions [10]. Peroxisome proliferator-activated receptors belonging to the nuclear receptors superfamily are ligand-activated transcription factors and have many biological effects on cardiovascular system [11], such as anti-inflammation and antiproliferation [12,13]. There are three subtypes, namely, PPARα, PPARβ/δ and PPARγ. Intriguingly, it has been reported that PPARα and PPARγ ligands might attenuate myocardial fibrosis in various models of myocardial hypertrophy and infarction [14–17]. Some literature data had shown that puerarin could reverse chemical-induced liver fibrosis and isoprenaline (ISO)-induced myocardial fibrosis in rats [18,19], but its possible mechanisms were not completely understood. In the present study, we firstly observed the effect of puerarin on myocardial fibrotic formation induced by ISO in mice and further investigated its possible mechanisms.

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2. Materials and methods

2.1. Materials

Puerarin was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (purity ≥99%). The assay kit for hydroxyproline was purchased from Nanjing Jiancheng Bioengineering Institute. Trizol was a product of Invitrogen. Taq DNA polymerase and reverse transcriptase were products of Sangon Gene Company and Fermentas, respectively. The primers used for amplification by reverse transcription polymerase chain reaction (RT-PCR) were synthesized by Sangon Gene Company. The antibodies to nuclear factor (NF)-κB subunit p65 and transforming growth factor (TGF)-β1 were purchased from CST Company and Abcam Company, respectively. The immunohistochemistry assay kits were purchased from GBI Company. All other reagents used in this study were of analytical grade.

2.2. Animals and treatments

Kunming mice (male, 25±2 g) were obtained from the Animal Breeding Center of Soochow University (Suzhou, China), housed in regular cages in a room with controlled temperature and humidity, and allowed free access to food and water. The animals were allowed to acclimatize to the laboratory environment for 3 days prior to the study. The animal study was approved by the University Ethic Committee and conducted according to the regulations for the use and care of experimental animals at Soochow University.

The mice were randomly divided into four groups with 10 mice in each group, namely, the control group, the myocardial fibrotic model group and the puerarin 600- and 1200-mg/kg groups. The doses of puerarin were designed according to the literature [18]. The medicine-treated mice were orally given puerarin 0.1 ml/10 g (body weight per day) by gavage based on different dose in the morning for 40 days, whereas control and model mice were treated with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution. The mice in the medicine-treated groups and the model group were given ISO 5 mg/kg by hypodermic injection on day 4 after administration in the afternoon. From the following day, the dose of ISO was reduced to 2.5 mg/kg, which lasted for 30 days [20]. Finally, all of the mice were sacrificed on day 41 by stunning and cervical dislocation, and the hearts were taken and weighed. Partial ventricles were homogenized (10%, wt/vol) in cold normal saline for measurement of hydroxyproline, fixed in 10% formaldehyde solution for 24 h and then embedded in paraffin for hematoxylin and eosin (H&E) staining, Masson's trichrome staining and immunohistochemistry assay, respectively. The rest of the ventricles were quickly frozen in liquid nitrogen and stored at −80°C for measurement of RT-PCR.

2.3. Calculation of cardiac weight index (CWI)

Hearts were rapidly excised and rinsed in cold normal saline. Heart was then weighed, and the CWI was calculated according to heart weight (mg) divided by body weight (g).

2.4. Determination of hydroxyproline level in myocardial tissue

In order to estimate the collagen content in heart, the hydroxyproline level in myocardial tissue was determined by a colorimetric method according to the manufacturer's kit instruction.

2.5. Histopathological observation

The ventricle samples were fixed in 10% formaldehyde solution and embedded in paraffin. Sections were stained with H&E for histopathological examination under a light microscope. The extent of myocardial fibrosis was graded according to the Fishbein et al. method [21] and expressed as 0, +, ++ and +++. On the other hand, Masson's trichrome stain was also used for determination of collagen fibers. The collagen volume fraction (CVF) was determined by quantitative morphometry with an automated image analysis system (Visilog 4.1.5, Noesis).

2.6. RT-PCR

Reverse transcription PCR was used to measure the messenger RNA (mRNA) expressions of PPARα/γ, TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in myocardial tissue. The ventricle samples were immediately placed into Trizol reagent, and total RNA was extracted according to the manufacturer's instruction. The final RNA pellet was resolved by 0.1% diethyl pyrocarbonate-treated water. The concentration and purity of the RNA were determined spectrophotometrically by the absorbance ratio 260:280 nm. Total RNA (2 μg) was used as the RT reaction following the manufacturer's introduction. After RT, 22 μl of a PCR master mix, including all PCR components and the primers (Table 1), was added to tubes containing 3 μl of complementary DNA. These tubes were then placed in the DNA thermal cycler. The PCR conditions were as follows: 33 cycles of denaturation at 94°C for 30 s, annealing (the temperature is seen in Table 1) for 45 s and extension at 72°C for 45 s after an initial step of 94°C for 5 min. A final extension was 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide (0.5 g/L) and quantitated by densitometry using the Image Master VDS system and associated software (Pharmacia, USA). Data were expressed as a ratio of the signals of interest band to that of GAPDH band; the latter acted as the internal control in the experiment.

Table 1
The primers used for the RT-PCR amplification

Gene	Sequence	Length (bp)	Annealing (°C)
PPARα	5′-CCTGGAAAGTCCCTTATCT-3′(sense) 5′-GCCCTTACAGCCTTCACAT-3′(anti-sense)	319	56
PPARγ	5′-CTCACAATGCCATCAGGTTT-3′(sense) 5′-CTCTTGCACGGCTTTCTACGG-3′ (anti-sense)	359	51
TGF-β1	5′-CGGAAGCGCATCGAAGCCATCC-3′ (sense) 5′-GCAAGCGCAGCTCTGCACGG-3′ (anti-sense)	350	60
GAPDH	5′-GTATGACGTGGAGTCTACTG-3′ (sense) 5′-TACTCCTTGGAGGCCATGTA-3′ (anti-sense)	728	56

2.7. Immunohistochemical studies

The paraffin-embedded tissue sections (5 μm) were deparaffinized with xylene, rehydrated through a graded series of ethanol to phosphate-buffered saline (PBS) and then incubated in blocking solution (3% H_2O_2) at room temperature for 10 min. After three washes in PBS (containing 0.1% Tween 20), the sections were incubated overnight at 4°C with the primary antibodies of rabbit anti-mouse NF-κB-p65 (1:100 dilution) and mouse anti-mouse TGF-β1 (1:75 dilution), respectively. The sections were then washed in PBS and incubated with corresponding reagents in the immunohistochemistry assay kits at room temperature for 30 min. All sections were then stained with diaminobenzidine reagent and hematoxylin, dehydrated, mounted and viewed under a light microscope. The area ratios of positive expressions of NF-κB-p65 and TGF-β1 were assessed using the image analysis software Sigma Scan Pro 5.0 (SPSS Inc., Chicago, IL, USA).

2.8. Statistical analysis

Data are expressed as mean±S.D. Statistical analysis was performed by one-way analysis of variance for comparisons between groups. χ^2 test was used for histopathological evaluation. Statistical differences were considered at a value of $P<.05$.

3. Results

3.1. Reduction of CWI and myocardial hydroxyproline level by puerarin

In the present experiment, the results showed that CWI and hydroxyproline levels in myocardial tissue were significantly higher in the model group than in the control group ($P<.01$). In the puerarin 600- and 1200-mg/kg groups, CWI and myocardial hydroxyproline levels were obviously lowered ($P<.01$) and decreased by 7.6%–9.9% (Fig. 1) and 18.3%–19.5% (Fig. 2), respectively.

3.2. Histopathological observation of mouse myocardial fibrosis with H&E and Masson's trichrome stains

The mouse myocardium in the model group showed myocyte hypertrophy and excessive collagen accumulation (Figs. 3b and 4b), and the CVF was significantly increased (Fig. 4B, $P<.01$). In the control

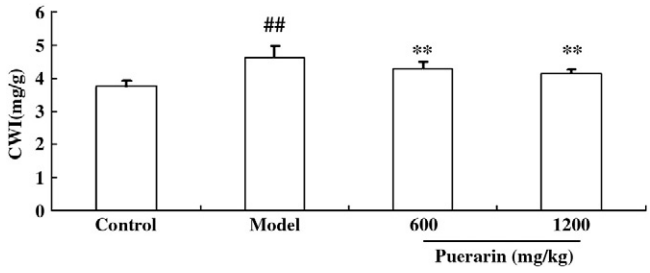

Fig. 1. Cardiac weight index after administration of puerarin for 40 days in ISO-induced myocardial fibrotic mice. Data were presented as mean±S.D., $n=10$. $^{\#\#}P<.01$ versus control; $^{**}P<.01$ versus model.

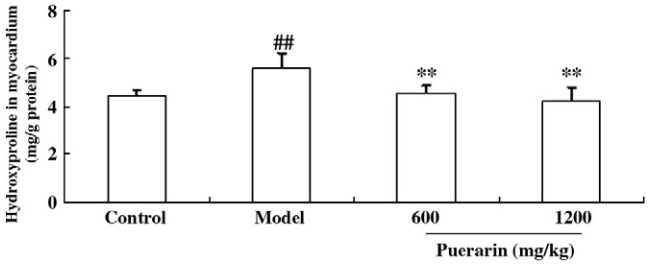

Fig. 2. Hydroxyproline level in myocardial tissue after administration of puerarin for 40 days in ISO-induced myocardial fibrotic mice. Data were presented as mean±S.D., $n=10$. $^{\#\#}P<.01$ versus control; $^{**}P<.01$ versus model.

group, the structure of myocardium was normal (Figs. 3a and 4a). After administration of puerarin 600 and 1200 mg/kg for 40 days, the degree of myocardial fibrosis was significantly improved (Figs. 3c–d and 4c–d, Table 2, $P<.01$), and the CVF was decreased by 77.9% and 90.3%, respectively (Fig. 4B). These results demonstrated that puerarin could dramatically decrease the collagen accumulation induced by ISO.

3.3. Increase of PPAR α/γ mRNA expressions and reduction of TGF-β1 mRNA expression by puerarin

The mRNA expressions of PPARα/γ in myocardial tissue in the model group were significantly decreased than those in the control group ($P<.01$), whereas the TGF-β1 mRNA expression was increased markedly ($P<.01$). In the puerarin 600- and 1200-mg/kg groups, the mRNA expressions of PPARα/γ were significantly increased ($P<.01$), and the TGF-β1 mRNA expression was significantly reduced (Fig. 5, $P<.01$).

3.4. Reduction of NF-κB subunit p65 and TGF-β1 protein expressions by puerarin

Compared with the control group, the protein expressions of NF-κB subunit p65 and TGF-β1 in myocardial tissue in the model

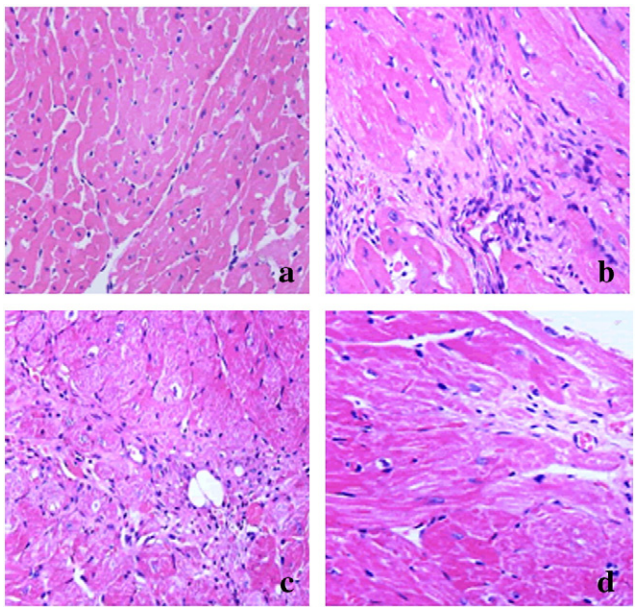

Fig. 3. Histopathological changes in mouse myocardium (H&E staining, 40×). No myocardial fibrosis was seen in the control group (a) and more than one half of myocardial tissues filled with fibers was seen in the model group (b). The degree of myocardial fibrosis was significantly alleviated after administration of puerarin 600 mg/kg (c) and 1200 mg/kg (d) for 40 days.

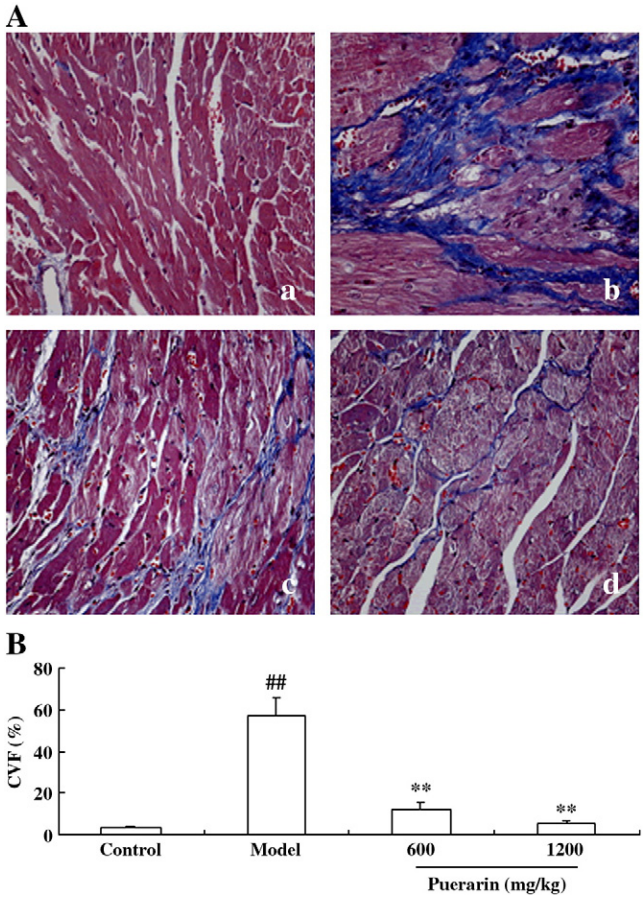

Fig. 4. Histopathological changes in mouse myocardium. (A) is Masson staining (40×). No collagen accumulation was seen in the control group (a), and numerous collagen fibers were seen in the model group (b). The degree of collagen accumulation was significantly alleviated after administration of puerarin 600 mg/kg (c) and 1200 mg/kg (d) for 40 days. (B) is the CVF, which was determined and calculated by quantitative morphometry with an automated image analysis system. Data were presented as mean±S.D., $n=10$. $^{\#\#}P<.01$ versus control; $^{**}P<.01$ versus model.

group were higher ($P<.01$). In the puerarin 600- and 1200-mg/kg groups, the protein expressions of NF-κB subunit p65 and TGF-β1 were decreased by 55.2%–80.8% (Fig. 6) and 49.7%–78.2% (Fig. 7), respectively ($P<.01$).

4. Discussion

Our present experimental results showed that excessive collagen accumulation in myocardium was seen in the model group; the CVF, CWI and hydroxyproline level in myocardial tissue in the model group were significantly higher as well. These results suggested that the model of ISO-induced myocardial fibrosis was developed, and

Table 2
Histopathological changes of myocardial fibrosis after administration of puerarin for 40 days in ISO-induced myocardial fibrotic mice

Degree of myocardial fibrosis	Control	Model	Puerarin	
			600 mg/kg	1200 mg/kg
0	10	0	0	6
+	0	1	7	3
++	0	2	3	1
+++	0	7	0	0
P		<.01	<.01	<.01

$n=10$. *P* value for puerarin-treated or control groups versus model group by χ^2 test.

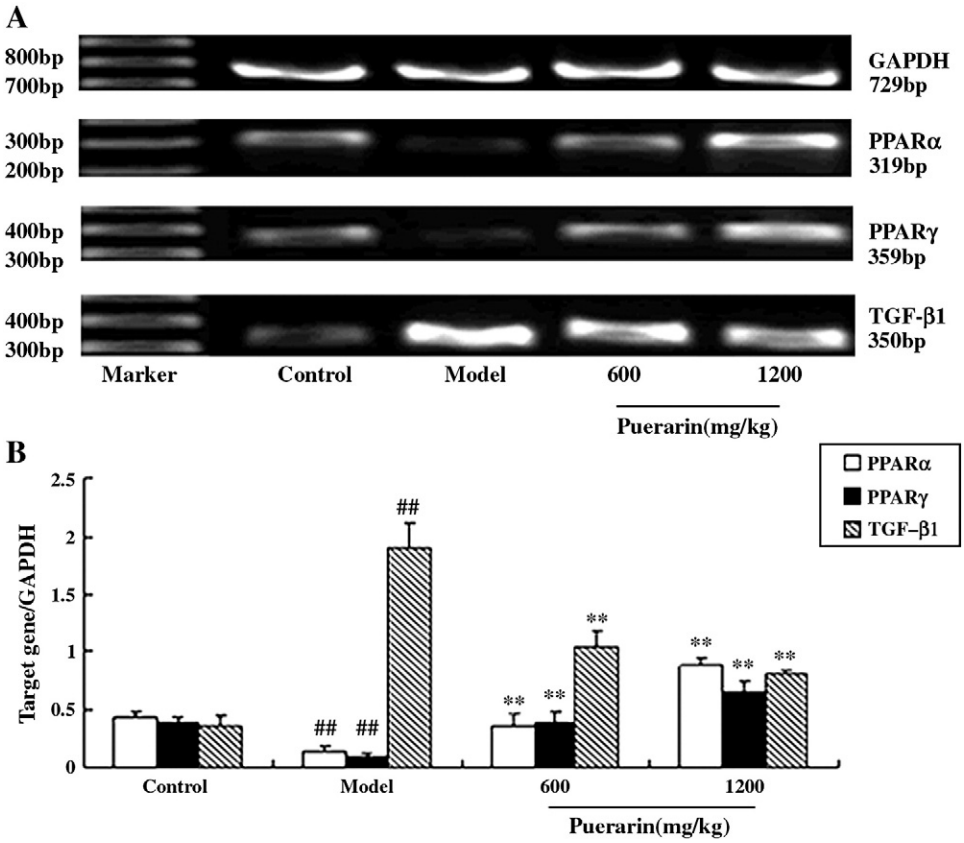

Fig. 5. Effects of puerarin on mRNA expressions of PPARα/γ and TGF-β1 in myocardial tissue were determined by RT-PCR after administration for 40 days in ISO-induced myocardial fibrotic mice. Data are expressed as mean±S.D., n=6. (A) Electrophoresis gel photo of PCR products. (B) Ratio of PPARα/γ or TGF-β1/GAPDH. $^{\#\#}P<.01$ versus respective control; $^{**}P<.01$ versus respective model.

were in line with previous reports [20]. Compared with the rat myocardial fibrosis induced by ISO, the mouse model might be a more practical model because it is technically simple and acceptable.

After administration of puerarin 600 and 1200 mg/kg for 40 days, the degree of myocardial fibrosis was significantly improved, and partial myocardial tissues did not find fibrosis in the puerarin 1200-mg/kg group. The CWI, CVF and hydroxyproline content in myocardial tissue were significantly lowered as compared with the model group. These results confirmed that puerarin was effective in inhibiting myocardial fibrotic formation induced by ISO in mice.

TGF-β is an important fibrogenic cytokine in the fibrotic response of heart [22] and includes three subtypes (TGF-β1, TGF-β2 and TGF-β3) in mammalian cells. In the heart, TGF-β1 may induce the proliferation of cardiac fibroblasts and increase the phenotypic conversion of cardiac fibroblasts to myofibroblasts and the extracellular matrix generation. TGF-β1 may simultaneously block matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors. In the present experimental study, the TGF-β1 mRNA and protein expressions in the model group were obviously increased. The results might be from angiotensin II-mediated mechanism [23] due to its increased secretion by ISO stimulation [24]. Importantly, we found that puerarin could reduce the mRNA and protein expressions of TGF-β1 in myocardial tissue in ISO-induced myocardial fibrotic mice. Therefore, we thought that the reduction of myocardial fibrosis by puerarin might be from its inhibitory effect on TGF-β1 expression in myocardial tissue.

It is known that the activation of PPARα/γ may inhibit fibrotic gene expressions through interference with NF-κB [15]. NF-κB is a nuclear transcription factor that mediates the inflammatory response. Under normal conditions, NF-κB, together with its inhibitory proteins-IκB family, is retained in the cytosol. In response to an inflammatory insult, IκB proteins are degraded, and the free NF-κB translocates to the nucleus where it initiates the gene transcription of proinflammatory and profibrogenic mediators [25], such as TGF-β1. It has been reported that soy isoflavones could increase PPAR α/γ gene expressions [10]. Puerarin, as one of the main isoflavones, might also affect multiple pathophysiological processes by activation of PPARγ signal pathway [26,27]. In the present study, we examined the effects of puerarin on PPARα/γ gene expressions and NF-κB protein expression. The results indicated that, in the puerarin-treated groups, the mRNA expressions of PPARα/γ were significantly increased, while the protein expression of NF-κB subunit p65 was decreased. From these literature data and our present results, we assumed that the effect of puerarin on reduction of TGF-β1 expression was related to its inhibition of NF-κB via the activation of PPARα/γ in myocardial tissue, which might be one of its mechanisms. But further research is needed to clarify the exact molecular signal pathways.

In conclusion, our experimental results demonstrated that puerarin might decrease the expression of TGF-β1 through activation of PPARα/γ and subsequent inhibition of NF-κB in myocardial tissue. These effects of puerarin might be beneficial for the prevention of myocardial fibrosis.

Acknowledgments

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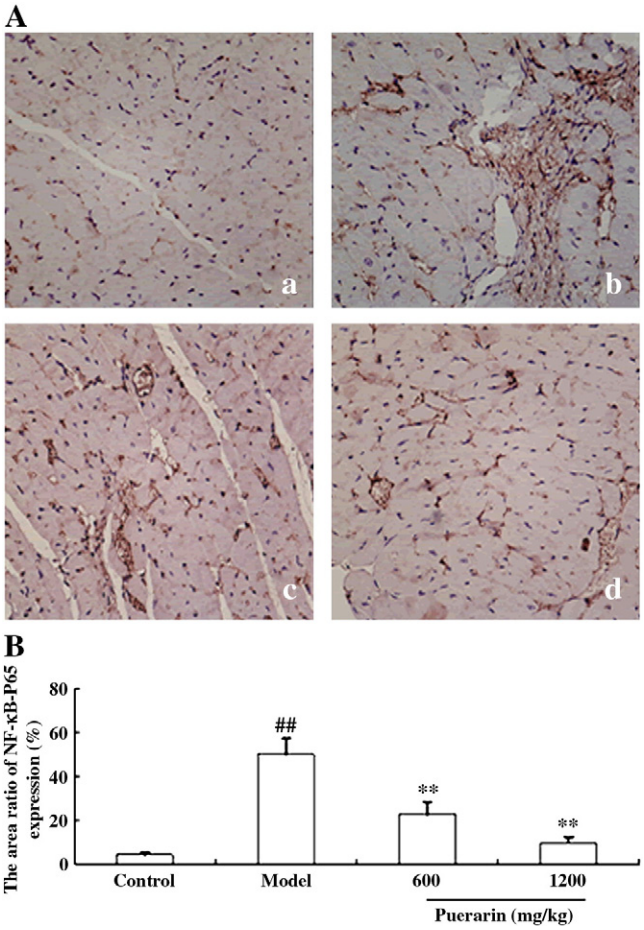

Fig. 6. The protein expression of NF-κB-p65 in myocardial tissue was determined by immunohistochemistry method after administration of puerarin for 40 days in ISO-induced myocardial fibrotic mice. Each section was examined under a light microscope, and the results were regarded as the mean of five different fields on each section. Data are expressed as mean±S.D., n=6. (A) Immunohistochemical photo of NF-κB-p65. (a) Control; (b) model; (c) puerarin 600 mg/kg; (d) puerarin 1200 mg/kg. (B) The area ratio of NF-κB-p65. $^{\#\#}P$<.01 versus control; $^{**}P$<.01 versus model.

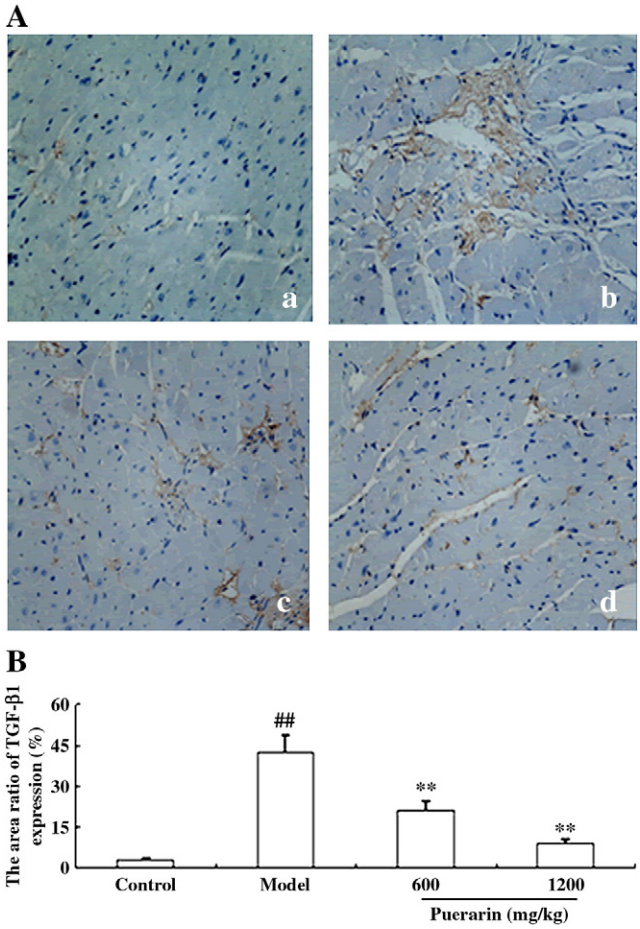

Fig. 7. The protein expression of TGF-β1 in myocardial tissue was determined by immunohistochemistry method after administration of puerarin for 40 days in ISO-induced myocardial fibrotic mice. Each section was examined under a light microscope, and the results were regarded as the mean of five different fields on each section. Data are expressed as mean±S.D., n=6. (A) Immunohistochemical photo of TGF-β1. (a) Control; (b) model; (c) puerarin 600 mg/kg; (d) puerarin 1200 mg/kg. B. The area ratio of TGF-β1. $^{\#\#}P$<.01 versus control; $^{**}P$<.01 versus model.

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