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Triptolide alleviates isoprenaline-induced cardiac remodeling in rats via TGF-β1/Smad3 and p38 MAPK signaling pathway

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Triptolide (TPL) is a diterpene triepoxide with potent immunosuppressive and anti-inflammatory properties. It is the main effective component of the traditional Chinese herb Tripterygium wilfordii Hook F and has been used in China for centuries to treat immune-related disorders. The present study was conducted to investigate the effects of TPL on cardiac remodeling in rats. Age matched male Wistar rats were used in this study. Cardiac remodeling rat model was established by hypodermic injection of isoprenaline for ten days. The rats were treated with TPL (20 or 100 μg/kg/d) for six consecutive weeks. At the end of the study, the cardiac function, collagen volume fraction, perivascular collagen area and hydroxyproline concentration were studied. Echocardiography, Masson staining, immunohistochemistry, western blot and real-time polymerase chain reaction were performed. The protein and mRNA expression of transforming growth factor-β1 (TGF-β1), drosophila mothers against decapentaplegic protein 3 (Smad3) and p38 mitogen activated protein kinase (p38 MAPK) were analyzed. The results indicated that TPL treatment significantly reduced the collagen volume fraction, perivascular collagen area, ventricular weight/body weight ratio and hydroxyproline concentration in myocardial tissue compared with the model group. In addition, it also improved the cardiac function. TPL attenuated cardiac remodeling in rats by down-regulating the p38 MAPK and TGF-β1/Smad3 signaling pathways. TPL treatment significantly attenuated cardiac fibrosis and improved cardiac function through suppressing the p38 MAPK and TGF-β1/Smad3 signaling pathway in isoprenaline-induced cardiac remodeling rats. Our findings suggested that TPL might be a novel complementary medicine in the treatment of chronic heart failure.

1. Introduction

Heart failure is a complex clinical syndrome with an abnormality of cardiac structure or function and has become one of the most serious diseases threatening human health in the whole world (Yancy et al. 2013). Most cardiovascular diseases such as myocardial infarction, hypertension, myocarditis, dilated cardiomyopathy and congenital heart disease may lead to heart failure. The prevalence of heart failure in adult population is about 1-2% in developed countries. More than 10% of the people over 70 years old have developed heart failure (Mcmurray et al. 2012).

Previous studies have reported that immune-inflammatory activation may play an important role in the development and progression of chronic heart failure and cardiac remodeling (Nymo et al. 2014; Petersen and Felker 2006; Candia et al. 2007; Kaya et al. 2012). Based on this theory, anti-inflammatory and immunomodulation therapy is considered to be a promising strategy for the treatment of chronic heart failure. However, in the earlier studies, anti-inflammatory therapy targeting specific cytokines has failed to show clinical benefit (Ramasubbu et al. 2006). Zhang et al (2009) found that methotrexate and rapamycin can regulate inflammatory responses, alleviate cardiac fibrosis and improve cardiac function in rats. Moreover, in chronic heart failure patients, the addition of methotrexate to conventional therapy had significant anti-inflammatory effects and improved 6-minute walk test distance (Gong et al. 2006). As a result, broad-spectrum anti-inflammatory and immunomodulation strategy has become a hot research point in the study of chronic heart failure (Ramasubbu et al. 2006; Torre-Amione et al. 2007). Therefore, it's a central issue to find more potential broad-spectrum anti-inflammatory and immunoregulatory drugs for the treatment of chronic heart failure.

Triptolide (TPL), PG-490, is the main effective component of the traditional herb *Tripterygium wilfordii Hook F* which has been used in China to treat immune-related disorders for centuries (Zhang et al. 2013; Ma et al. 2013). TPL (molecular formula, $C_{20}H_{24}O_6$) is a diterpene triepoxide and has been proved to have significant immunosuppressive and anti-inflammatory properties (Zhang et al. 2009). TPL can reduce the inflammatory cytokines such as tumor necrosis factor α, interleukin-1β and interleukin-6, regulate matrix metalloproteinases and inhibit the

Table 1: Cardiac structure and function in different groups

Groups	Veh group (n = 8)	ISO group (n = 8)	LT group (n = 8)	HT group (n = 8)	Cap group (n = 8)
VW (mg)	1354.13 ± 292.10	2689.80 ± 884.23*	1821.15 ± 278.98#	1240.23 ± 221.84#	1385.65 ± 307.19#
BW (g)	388.25 ± 29.34	415.00 ± 88.20	382.00 ± 37.91	372.25 ± 23.80	385.00 ± 38.67
VW/BW (mg/g)	3.48 ± 0.62	6.39 ± 1.03*	4.79 ± 0.76*#	3.32 ± 0.38#	3.62 ± 0.83#
LVIDd (mm)	5.67 ± 0.93	8.40 ± 0.52*	6.88 ± 0.66*#	6.11 ± 0.58#	6.57 ± 1.11#
LVIDs (mm)	3.89 ± 0.32	6.44 ± 0.93*	4.95 ± 0.43*#	4.49 ± 0.38#	4.82 ± 0.28#
EF (%)	74.11 ± 4.67	46.44 ± 3.50*	53.22 ± 5.60*	60.89 ± 3.10*#	63.44 ± 1.64*#
FS (%)	38.00 ± 3.84	23.00 ± 2.08*	27.78 ± 5.19*	29.89 ± 1.68*#	32.00 ± 1.45*#

Veh = vehicle; ISO = isoprenaline; LT = low dose triptolide; HT = high dose triptolide; Cap = captopril; LVIDd = left ventricular internal diameter at end-diastole; LVIDs = left ventricular internal diameter at end-systole; EF = ejection fraction; FS = fractional shortening fraction; VW = ventricular weight; BW = body weight;
* $P < 0.05$ *vs*. Veh group; # $P < 0.05$ *vs*. ISO group.

expression of cycloxygenase-2 and prostaglandins (Li et al. 2014; Matta et al. 2009). TPL can also act as an effective immunosuppressor by regulating the activity of T cells and dendritic cells (Li et al. 2014; Xin et al. 2010). However, whether TPL has protective effects on cardiac remodeling *via* its immunosuppressive and anti-inflammatory properties is still unknown. We hypothesized that TPL alleviates myocardial fibrosis and improves cardiac function in isoprenaline-induced cardiac remodeling rats. Therefore, this study was conducted to examine this hypothesis.

2. Investigations and results

2.1. Effects of TPL on cardiac structure and function in rats

Compared to the vehicle (Veh) group, rats in other groups became depressive, inactive and shaggy. The ISO group displayed significant cardiac dilatation, lower cardiac function and higher VW/BW ratio (3.48 ± 0.62 mg/g *vs*. 6.39 ± 1.03 mg/g, $P < 0.05$) than those of the Veh group. TPL treatment significantly alleviated cardiomegaly and improved cardiac function (Table 1).

2.2. Effects of TPL on cardiac fibrosis in rats

According to the HE and Masson staining (Fig. 1), collagen fibers were stained blue and myocardial tissue were stained red in Masson staining. The myocardial tissue of the Veh group were arranged in order (Fig. 1A) and stained well with few stained collagen fibers (Fig. 1F). In the ISO group, the myocardial tissue was arranged irregularly and infiltrated with inflammatory cells (Fig. 1B). The collagen fibers were significantly increased in the ISO group, especially in subendocardial myocardium and perivascular areas (Fig. 1G). In the immunohistochemistry of collagen I, the positive tissue was stained brown (Fig. 2). Collagen I was found thinly distributed in Veh group (Fig. 2A). However, the positive area was increased significantly in the ISO group (Fig. 2B). TPLtreatment can significantly reduce inflammatory cells (Fig. 1C∼D), collagen fibers (Fig. 1H∼I) and collagen I expression (Fig. 2C∼D) in myocardial tissues.

2.3. Effects of TPL on CVF and PVCA

As shown in Fig. 3, ISO significantly increased CVF (6.85 ± 1.48% *vs*. 30.97 ± 4.22%, $P < 0.05$) and PVCA (8.97 ± 3.16% *vs*. 28.31 ± 3.16%, $P < 0.05$) of myocardial tissues, compared with the Veh group. However, compared to the untreated group (ISO group), TPL treatment can significantly decrease CVF and PVCA ($P < 0.05$).

2.4. Effects of TPL on HYP concentration in myocardial tissue

The HYP concentrations in myocardial tissue were detected to reflect the degree of cardiac fibrosis. Compared to the Veh group, the HYP concentration was increased markedly in the ISO group (0.27 ± 0.10 μg/mg *vs*. 0.66 ± 0.10 μg/mg, $P < 0.05$). TPL treatment decreased the HYP concentration in myocardial tissue compared with ISO group (Fig. 4).

2.5. Safety of TPL treatment in rats

No rats died during the six weeks' treatment of TPL. To assess the safety of TPL treatment, the serum levels of ALT, BUN and Scr were detected. As shown in Table 2, the testing results indicated that no significant damage of kidney and liver function was observed during the administration of TPL in this study ($P > 0.05$).

2.6. Effects of TPL on protein expression of TGF-β1, Smad3 and p38 MAPK

According to the results of Western blot analysis (Fig. 5), the protein levels of TGF-β1 (0.09 ± 0.03 *vs*. 0.66 ± 0.07, $P < 0.05$), Smad3 (0.09 ± 0.05 *vs*. 0.40 ± 0.22, P = 0.008) and p38 MAPK (0.12 ± 0.13 *vs*. 0.81 ± 0.39, P = 0.002) were significantly increased in ISO group than in the Veh group. However, compared to the ISO group, TPL treatment markedly decreased the protein expressions of TGF-β1 (Fig. 5B), Smad3 (Fig. 5C) and p38 MAPK (Fig. 5D) in myocardial tissues in rats (n = 3).

2.7. Effects of TPL on mRNA expression of TGF-β1, Smad3 and p38 MAPK

Quantitative real-time PCR was performed to determine the mRNA expression in this study (n = 3). The results indicated that the expressions of TGF-β1, Smad3 and p38 MAPK mRNA were significantly higher in ISO group than in the Veh group ($P < 0.05$). On the other hand, the mRNA levels of TGF-β1 (Fig. 6A), Smad3 (Fig. 6B) and p38 MAPK (Fig. 6C) in TPL-treated group were markedly lower than those in the ISO group, which indicating that TPL suppressed the mRNA expression of TGF-β1, Smad3 and p38 MAPK in rats with cardiac remodeling (Fig. 6).

3. Discussion

In the present study, we investigated the effects of TPL on cardiac remodeling following 6 weeks of treatment in rats. The results indicated that TPL treatment significantly reduced CVF, PVCA, VW/BM ratio and HYP concentration in myocardial tis-

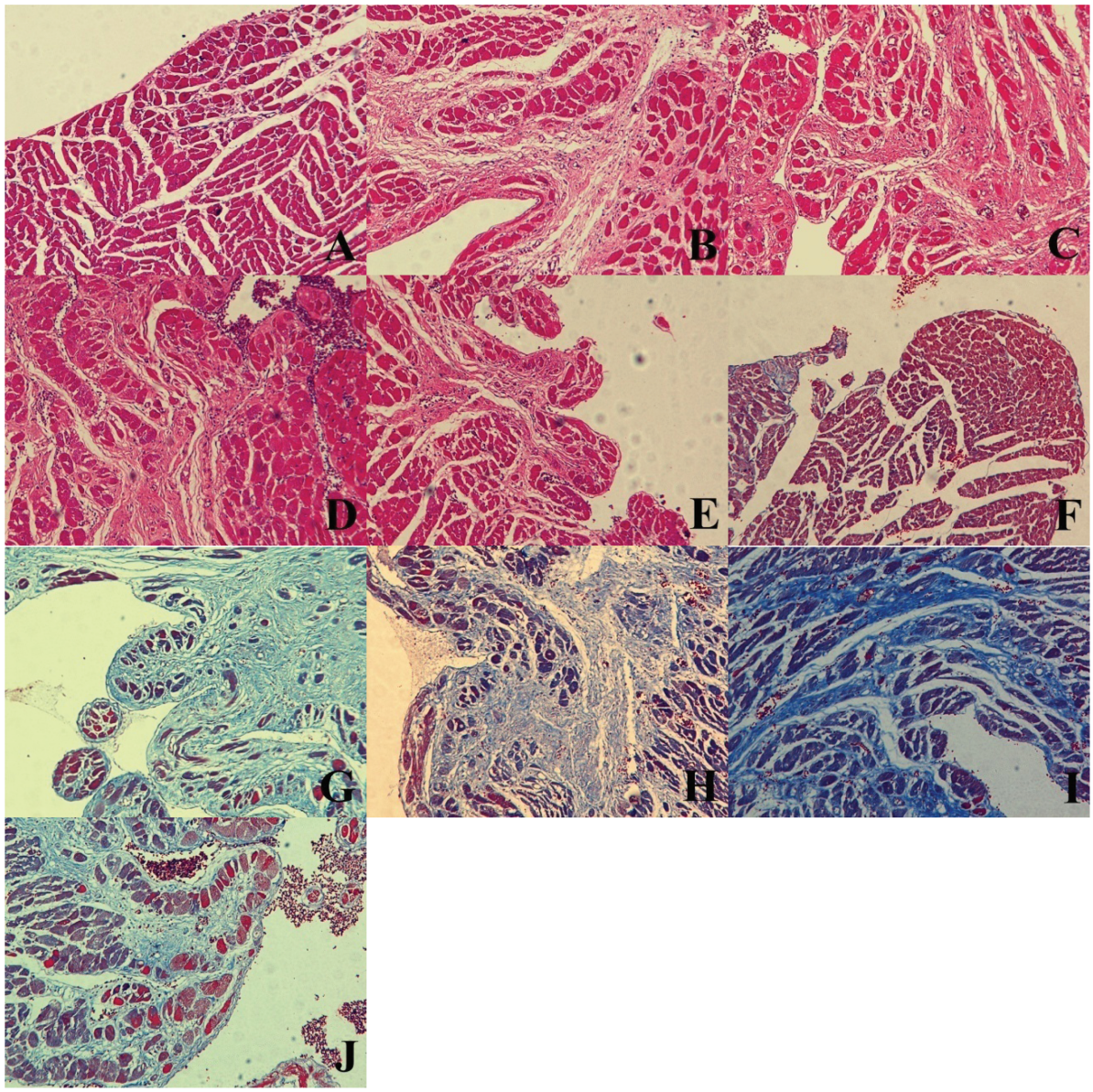

Fig. 1: Histopathology of myocardial tissues in rats. HE staining (100 ×): Veh group(A), ISO group (B), LT group (C), HT group (D) and Cap group (E); Masson staining (100 ×): Veh group (F), ISO group (G), LT group (H), HT group (I), Cap group (J).

Table 2: Safety of TPL treatment

Groups	Veh group (n = 8)	ISO group (n = 8)	LT group (n = 8)	HT group (n = 8)	Cap group (n = 8)
ALT(U/L)	79.67 ± 8.50	83.50 ± 35.71	117.75 ± 58.61	86.75 ± 32.82	87.00 ± 39.61
BUN (mmol/L)	6.46 ± 0.32	7.10 ± 0.94	6.74 ± 0.40	6.13 ± 0.74	6.41 ± 0.60
Scr (μmol/L)	25.00 ± 2.65	25.50 ± 2.65	23.75 ± 1.26	27.25 ± 11.44	23.25 ± 0.96

Veh = vehicle; ISO = isoprenaline; LT = low dose triptolide; HT = high dose triptolide; Cap = captopril; ALT = alanine aminotransferase; Scr = serum creatinine; BUN = blood urea nitrogen.

sue. In addition, it also effectively improved the cardiac function of isoprenaline-induced cardiac remodeling rats. No increase in Scr, ALT and BUN levels was observed during this study, which indicated that TPL treatment in cardiac remodeling rats may be safe, without additional damage of liver and renal fuction. Furthermore, TPL may alleviate cardiac fibrosis in rats by down-regulating the p38 MAPK and TGF-β1/ Smad3 signaling pathway.

In the management of chronic heart failure, the strategy to reverse cardiac remodeling is of great importance (Mcmurray et al. 2012). Previous studies have reported that immune-inflammatory activation is involved in the pathophysiological progression of chronic heart failure and cardiac remodeling (Candia et al. 2007; Kaya et al. 2012; Mao et al. 2014; Wen et al. 2013). Compared to normal controls, patients with chronic heart failure have increased levels of pro-inflammatory factors such as interleukin-1β, interleukin-6, tumor necrosis factor-α and C reactive protein (Candia et al. 2007). Accordingly, anti-inflammatory and immunosuppressive therapy is thought to be promising in the treatment of chronic heart failure (Ramasubbu et al. 2006; Torre-Amione et al. 2007). Therefore, it's of great necessity to look for safe and effective immunomodulators for

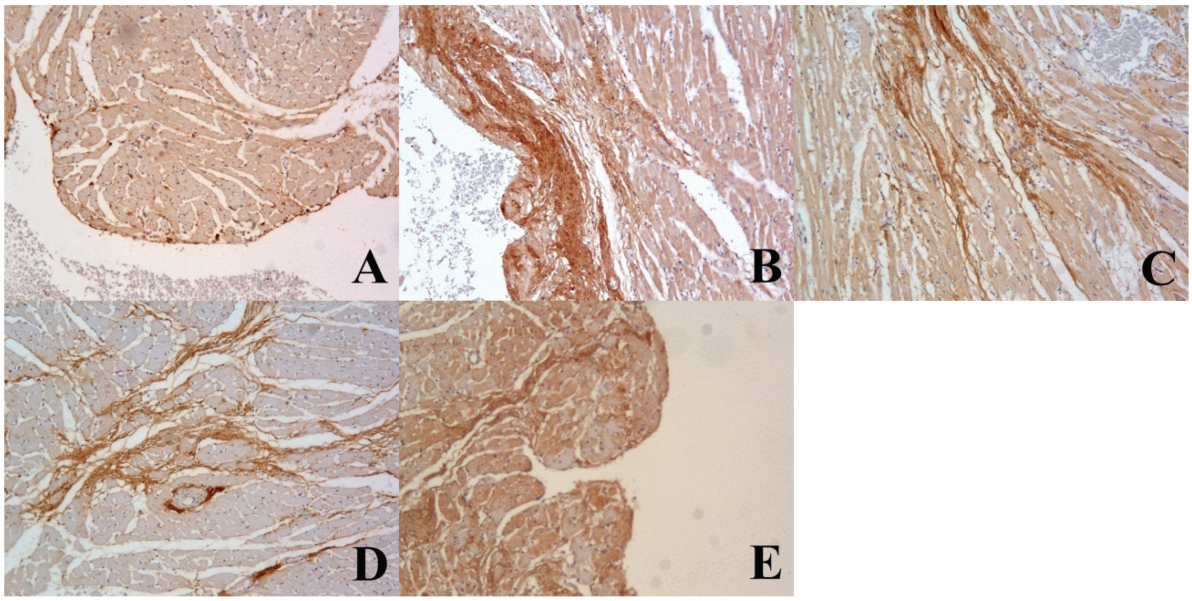

Fig. 2: Immunohistochemistry of collagen I (100 ×). Veh group(A), ISO group (B), LT group (C), HT group (D) and Cap group (E).

the treatment of chronic heart failure and cardiac remodeling. TPL is a monomeric compound extracted from the traditional Chinese herb *Tripterygium wilfordii Hook F* with immunosuppressive and anti-inflammatory properties. In recent years, TPL has been proved to be effective to alleviate renal interstitial fibrosis, collagen-induced arthritis and asthmatic airway remodeling in rats (Zhang et al. 2013; Mo et al. 2007; Yuan et al. 2011). However, the effect of TPL on chronic heart failure and cardiac remodeling remains unknown.

In 2013, Zhang et al. firstly reported that TPL may inhibit myocardial pro-fibrogenic factor production and inflammatory activation in the pressure overloaded rat heart (Zhang et al. 2013). However, the study mainly focused on a few pro-inflammatory cytokines (interleukin-1β, interleukin-6) in hypertensive rats. Fibrosis related molecular mechanisms were not discussed. Compared to the previous research, some improvements were made in our study. Firstly, the rat model induced by ISO selected in this study is more suitable for the study of myocardial fibrosis in chronic heart failure. As we know, myocyte loss (necrosis or apoptosis) is an important etiologic factor of wall thinning and chamber dilation in progression of chronic heart failure (Anversa et al. 1996). In the pressure overloaded rat model, the main pathological change in the early stage is myocardial hypertrophy, which does not follow the course of myocyte loss and appears to be a response to altered cardiac loading. However, myocardial fibrosis induced by ISO administration appears to be more closely related to myocyte necrosis with respect to collagen accumulation in the same areas of the heart (Benjamin et al. 1989). Besides, kidney and liver functions were detected to assess the safety of TPL treatment in our study. High dose TPL administration may have potential nephrotoxicity and hepatotoxicity (Li et al. 2014). The drug concentrations used in this study were relatively low and no significant damage of kidney and liver function were observed. Moreover, TPL is insoluble in water and not suitable to be used as an injection in clinical practice. Consequently, the ideal administration method of TPL should be intragastric administration, rather than intravenous injection or peritoneal injection.

Furthermore, the underlying mechanisms by which TPL attenuates myocardial fibrosis in rats were also explored in this study. As we know, the p38 MAPK signaling pathway plays an important role in inflammation (Schieven 2009). Both clinical and animal studies have found that sustained p38 MAPK activation is associated with chronic heart and cardiac remodeling (See et al. 2004). On the other hand, lots of studies have suggested that the canonical TGF-β1/Smad3 pathway is critically involved in the pathogenesis of fibrosis in many tissues (Biernacka et al.

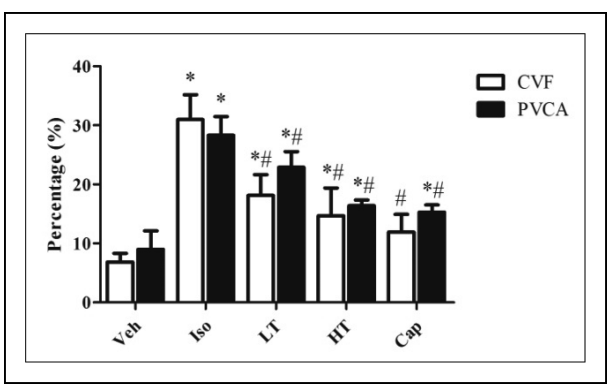

Fig. 3: Effects of TPL on CVF and PVCA. * $P < 0.05$ *vs.* Veh group; # $P < 0.05$ *vs.* ISO group.

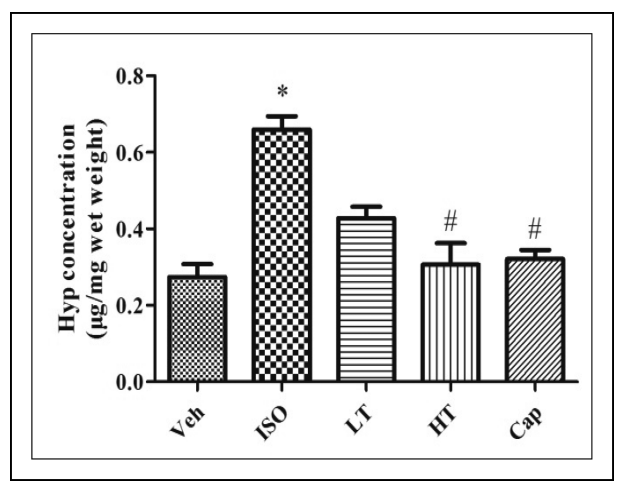

Fig. 4: Effects of TPL on HYP concentration in myocardial tissue. * $P < 0.05$ *vs.* Veh group; # $P < 0.05$ *vs.* ISO group.

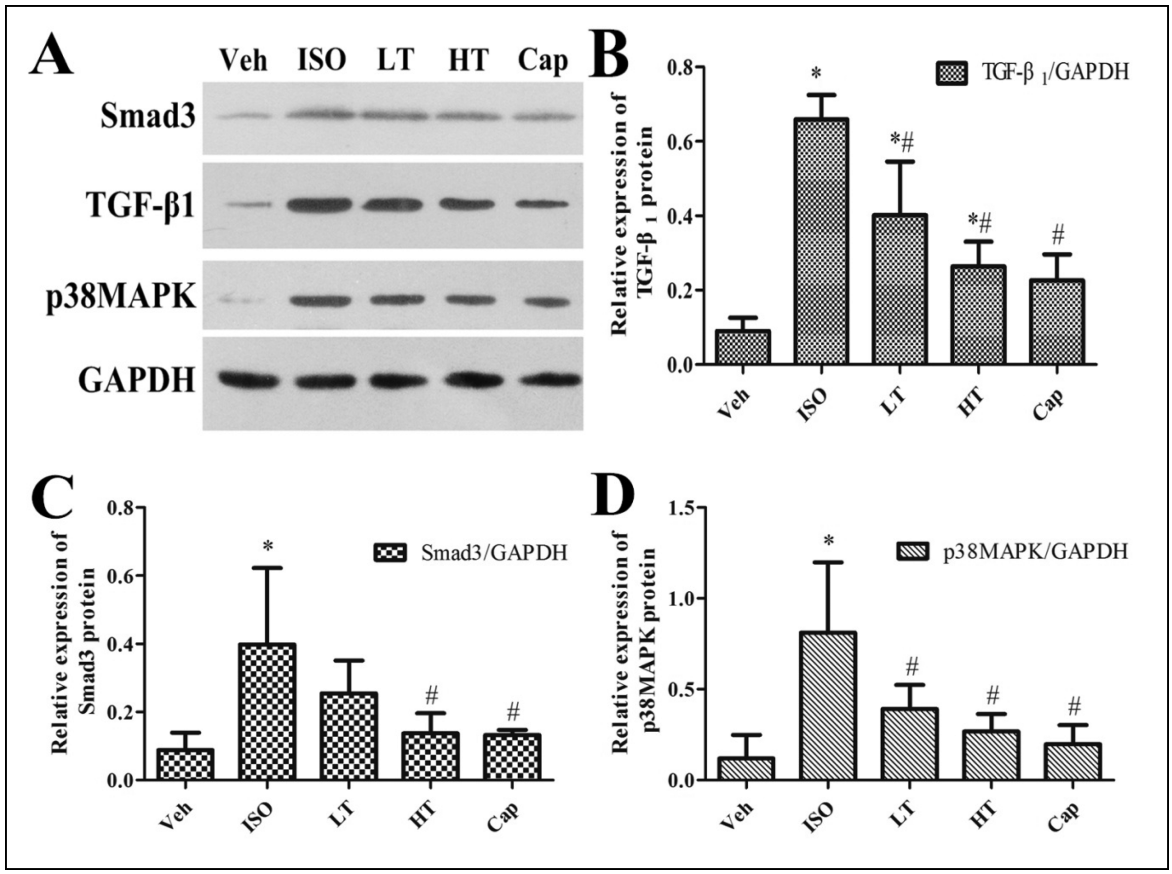

Fig. 5: Effects of TPL on protein expression of TGF-β1, Smad3 and p38 MAPK. Fig. 5A is the electrophoresis pattern; Fig. 5B, 5C and 5D is the relative expression of TGF-β1, Smad3 and p38 MAPK protein, respectively. * $P < 0.05$ *vs*. Veh group; # $P < 0.05$ *vs*. ISO group.

2011; Zhang et al. 2014; Zhu et al. 2013). TGF-β1 and Smad3 are up-regulated in fibrotic diseases and modulates fibroblast phenotype and function (Zhang et al. 2013; Biernacka et al. 2011). The activation of TGF-β1/Smad3 pathway may promote myofibroblast differentiation, increase the extracellular matrix protein deposition and connective tissue growth factor production. Therefore, TGF-β1 has been considered as an attractive therapeutic target in the treatment of fibrotic diseases (Biernacka et al. 2011). In the present study, the protein and mRNA expressions of p38 MAPK, TGF-β1 and Smad3 were significantly lower in the TPL treated rats compared with ISO group, indicating that the down-regulation of p38 MAPK signaling and TGF-β1/Smad3 pathway may be an important mechanism for the protective effects of TPL in cardiac remodeling.

Although the results of this study indicated that TPL may alleviate myocardial fibrosis and improve cardiac function in ISO-induced cardiac remodeling rats, some limitations should be taken into consideration. Firstly, only two different drug concentrations of TPL were studied. As a result, we cannot determine the optimal therapeutic dosage of TPL in the treatment of cardiac remodeling. Besides, we failed to study the optimal period of TPL treatment because no variant-time (6, 8, 12 weeks or more) subgroups were set. Moreover, inflammatory cytokines and parameters of immune function such as lymphocyte counts and interleukin-6 levels are not detected. More detailed experimental studies will be performed in our subsequent research.

In conclusion, TPL treatment significantly alleviated cardiac fibrosis and improved cardiac function through suppressing the p38 MAPK and TGF-β1/Smad3 signaling pathway in ISO-induced cardiac remodeling rats. Our findings suggested that TPL may be a novel complementary treatment option in chronic heart failure.

4. Experimental

4.1. Animals and reagents

Forty 8-week-old male Wistar rats (weight 200∼230 g) were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). The animal license number was SCXK (Yue) 2011-0029. The rats were allowed to access to water and food in a 12-hour light/dark cycled room. Isoprenaline (ISO) and captopril ($C_9H_{15}NO_3S$, purity > 98% by HPLC) were purchased from Meilun Biotech Co., Ltd. (Dalian, China). TPL ($C_{20}H_{24}O_6$, purity > 98% by HPLC) was obtained from Gold Wheat Biotech Co., Ltd. (Shanghai, China). TPL was dissolved in dimethyl sulfoxide (DMSO). Other reagents such as Masson staining kit (PanEra, Guangzhou, China), TRIzol (Invitrogen, CA, USA), All-in-One ™ first strand cDNA synthesis kit and All-in-One TM quantitative real-time polymerase chain reaction (PCR) mix (SYBR Green Method, GeneCopoeia, MD, USA) were also used. The protocol of this study was approved by the Animal Research Committee of Sun Yat-sen University. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications, No. 85-23, revised 1985).

4.2. Animal model and TPL treatment

According to previous studies (Garjani et al. 2011), rats were hypodermically injected with isoprenaline (ISO) 5 mg/kg/d (dissolved in normal saline) from the first day to tenth day at an interval of 24 h to establish the cardiac remodeling model. The controls received normal saline only. Rats were randomly divided into five groups by computer: vehicle group (Veh group), model group (ISO group), low dose TPL group (LT group),

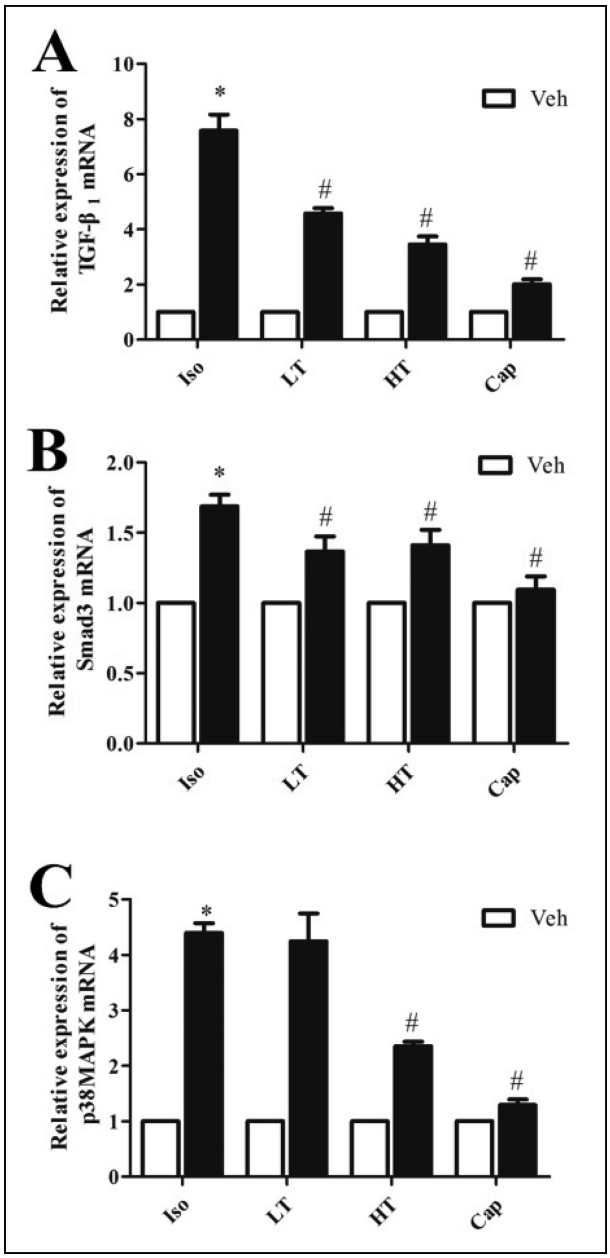

Fig. 6: Effects of TPL on mRNA expression of TGF-β1, Smad3 and p38 MAPK. Fig. 6A, 6B and 6C is the relative expression of TGF-β1, Smad3 and p38 MAPK mRNA, respectively. * $P < 0.05$ *vs*. Veh group; # $P < 0.05$ *vs*. ISO group.

high dose TPL group (HT group) and captopril group (Cap group). After establishment of cardiac remodeling model, rats in ISO group, LT group, HT group and Cap group were given DMSO 2 ml/d, TPL 20 μg/kg/d, TPL 100 μg/kg/d and captopril 15 mg/kg/d by intragastric administration for six weeks, respectively. All of them were sacrificed on the 53th day.

4.3. Ultrasonic cardiogram (UCG)

Rats were anaesthetized with 2% pentobarbital sodium (50 mg/kg). Then, the chest area was shaved for UCG study. The left ventricular internal diameter at end-systole (LVIDs), left ventricular internal diameter at end-diastole (LVIDd), left ventricular fractional shortening fraction (FS) and left ventricular ejection fraction (EF) were measured with a GE Ultrasound System (Vivid 7, NY, USA). Three consecutive cardiac cycles were saved for each measurement and the average of them was taken for final analysis.

4.4. Kidney and liver function

After UCG measurement, blood samples were collected from the eye orbit. To assess the safety of TPL, serum alanine aminotransferase (ALT), creatinine (Scr) and blood urea nitrogen (BUN) were measured by a HITACHI automatic biochemistry analyzer (7170S, Tokyo, Japan).

4.5. Ventricular weight/body weight (VW/BW) ratio

Rats were sacrificed after blood collection. Their hearts were immediately cut out and washed with cold normal saline to remove the blood. Cardiac atrium, vessels and adipose tissue were removed and only ventricular tissues were retained. The samples were dried with filter papers and weighed with electronic balance. VW/BM ratio = ventricular weight (mg)/body weight (g).

4.6. Histopathology and immunohistochemistry

The ventricular samples were fixed with 10% formaldehyde solution and embedded with paraffin. The paraffin embedded tissue sections were used for hematoxylin-eosin (HE) staining, Masson staining and immunohistochemistry of collagen type I. Masson staining sections were also used to calculate collagen volume fraction (CVF) and perivascular collagen area (PVCA). Based on the positive stained area, which scattered between the surviving myocytes and around the blood vessels, collagen volume fraction (CVF) and perivascular collagen area (PVCA) were measured (Zhang et al. 2013). CVF = collagen area/total area. PVCA = collagen area around the arterial lumen/arterial lumen area. Six visions under the microscope of each sample were randomly chosen and the average of them was taken for analysis. The histological specimens were observed under microscope (ZEISS Axio Scope A1, Oberkochen, Germany). Image Pro Plus 6.0 software (Media Cybernetics, MD, USA) was used for image analysis.

4.7. Hydroxyproline (HYP) concentration

Put 30∼100 mg (wet weight) ventricular tissue into 1 ml hydrolytic agent, 95 °C constant temperature bath for 20 min, adjust the PH to 6.0∼6.8. According to the instruction of HYP testing kit (alkaline method) provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China), optical density (OD) of each sample was measured. HYP concentration in ventricular tissue (μg/mg) = (sample tube OD-blank tube OD) × standard sample concentration (5 μg/ml) × total volume (10 ml)/ [(standard tube OD- blank tube OD) × tissue wet weight (mg)].

4.8. Western blot

Western blots were used to quantify the protein levels of transforming growth factor-β1 (TGF-β1), drosophila mothers against decapentaplegic protein 3 (Smad3) and p38 mitogen activated protein kinase (p38 MAPK). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control for western blots in this study. Protein concentration was measured with a microplate reader (Thermo Fisher, MA, USA). Protein samples (from ventricular tissue) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electropheresis and transferred to polyvinylidene difluoride membranes (MILLIPORE). Blots were probed with anti-TGF-β1 (Santa Cruz Biotechnology), anti-Smad3 (Cell Signaling Technology) and anti- p38 MAPK (Abcam), respectively. Horseradish peroxidase-conjugated anti-immunoglobulin G

(Southern Biotechnology) was used as a secondary antibody. The bands were detected by use of Gel Image System (Tanon, Shanghai, China).

4.9. Quantitative real-time PCR

Total RNA was prepared from ventricular tissue by using TRIzol reagent and reverse transcribed to cDNA by using the All-in-One TM first strand cDNA synthesis kit. Two microliter cDNA was amplified by quantitative real-time PCR using the All-in-One TM quantitative real-time PCR mix. The primers used in this study were provided by GENEWIZ Co., Ltd. (Suzhou, China). The sequences were as follows: TGF-β1(F) 5'-TGCTTCAGC TCCACAGAGAA-3', TGF-β1(R) 5'-TGGTTGTAGAGGGCAAGGAC-3', Smad3(F) 5'-G GCAGGATGTTTCCAGCTA-3',Smad3(R) 5'-GCAGTCCACAGACCATGTCA-3', p38 MAPK (F) 5'-GGGACCTCCTTATAGACGAA-3', p38 MAPK (R) 5'-GGCACTTGAATG GTATTTGG-3', β-actin(F) 5'-AGGGAAATCGTGCGT GACAT-3', β-actin(R) 5'-GAACC GCTCATTGCCGATAG-3'. Each experiment was performed at least three times. In this study, β-actin was used as an internal control gene to normalize the mRNA levels of TGF-β1, Smad3 and p38 MAPK in ventricular tissues.

4.10. Statistical analysis

The data were presented as mean ± standard deviation. Normality tests and homogeneity of variance tests were performed. One way ANOVA followed by LSD tests was used in the multiple comparisons of quantitative data. While Kruskal-Wallis test was performed if nonnormal distribution or heterogeneity of variance was found. A two-tale $P < 0.05$ was considered to indicate a significant difference. All data were analyzed with IBM SPSS software Version 20.0 for Windows (SPSS Inc., Chicago, USA).

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References

Anversa P, Kajstura J, Olivetti G (1996) Myocyte death in heart failure. Curr Opin Cardiol 11:245–251.

Benjamin IJ, Jalil JE, Tan LB, Cho K, Weber KT, Clark WA (1989) Isoproterenol-induced myocardial fibrosis in relation to myocyte necrosis. Circul Res 65:657–670.

Biernacka A, Dobaczewski M, Frangogiannis NG (2011) TGF-beta signaling in fibrosis. Growth Factors 29:196–202.

Candia AM, Villacorta HJ, Mesquita ET (2007) Immune-inflammatory activation in heart failure. Arquiv Brasil Cardiol 89:183–190, 201–208.

Garjani A, Andalib S, Biabani S, Soraya H, Doustar Y, Garjani A, Maleki-Dizaji N (2011) Combined atorvastatin and coenzyme Q10 improve the left ventricular function in isoproterenol-induced heart failure in rat. Eur J Pharmacol 666:135–141.

Gong K, Zhang Z, Sun X, Zhang X, Li A, Yan J, Luo Q, Gao Y, Feng Y (2006) The nonspecific anti-inflammatory therapy with methotrexate for patients with chronic heart failure. Am Heart J 151:62–68.

Kaya Z, Leib C, Katus HA (2012) Autoantibodies in heart failure and cardiac dysfunction. Circul Res 110:145–158.

Li XJ, Jiang ZZ, Zhang LY (2014) Triptolide: Progress on research in pharmacodynamics and toxicology. J Ethnopharmacol 155:67–79.

Ma R, Liu L, Liu X, Wang Y, Jiang W, Xu L (2013) Triptolide markedly attenuates albuminuria and podocyte injury in an animal model of diabetic nephropathy. Experim Therap Med 6:649–656.

Mao L, Jian C, Dan H, Jianting H, Wei W (2014) A meta-analysis of proinflammatory cytokines in chronic heart failure. Heart Asia 6:130–136.

Matta R, Wang X, Ge H, Ray W, Nelin LD, Liu Y (2009) Triptolide induces anti-inflammatory cellular responses. Am J Translat Res 1:267–282.

McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA, Jaarsma T, Kober L, Lip GY, Maggioni AP, Parkhomenko A, Pieske BM, Popescu BA, Ronnevik PK, Rutten FH, Schwitter J, Seferovic P, Stepinska J, Trindade PT, Voors AA, Zannad F, Zeiher A (2012) ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in Collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 33:1787–1847.

Mo BW, Wang CM, Zhang ZX, Xu YJ, Xiong WN, Liu XS, Fang CS (2007) Effect of triptolide on airway remodeling and the expression of phosphoinositide 3-kinases in asthmatic rats. Zhongguo Ying Yong Sheng Li Xue Za Zhi 23:359–364.

Nymo SH, Hulthe J, Ueland T, McMurray J, Wikstrand J, Askevold ET, Yndestad A, Gullestad L, Aukrust P (2014) Inflammatory cytokines in chronic heart failure: interleukin-8 is associated with adverse outcome. Results from CORONA. Eur Heart J 16:68–75.

Petersen JW, Felker GM (2006) Inflammatory biomarkers in heart failure. Congest Heart Failure 12:324–328.

Ramasubbu K, Oliveira G, Torre-Amione G (2006) Novel therapies for heart failure: focus on anti-inflammatory strategies. Congest Heart Failure 12:153–159, 160–161.

Schieven GL (2009) The p38alpha kinase plays a central role in inflammation. Curr Top Medicin Chem 9:1038–1048.

See,F, Thomas W, Way K, Tzanidis A, Kompa A, Lewis D, Itescu S, Krum J (2004) p38 mitogen-activated protein kinase inhibition improves cardiac function and attenuates left ventricular remodeling following myocardial infarction in the rat. J Am Coll Cardiol 44:1679–1689.

Torre-Amione G, Bourge RC, Colucci WS, Greenberg B, Pratt C, Rouleau JL, Sestier F, Moye LA, Geddes JA, Nemet AT, Young JB (2007) A study to assess the effects of a broad-spectrum immune modulatory therapy on mortality and morbidity in patients with chronic heart failure: the ACCLAIM trial rationale and design. Canad J Cardiol 23:369–376.

Wen HL, Liang ZS, Zhang R, Yang K (2013) Anti-inflammatory effects of triptolide improve left ventricular function in a rat model of diabetic cardiomyopathy. Cardiovasc Diabetol 12:50.

Xin MJ, Cui SH, Liu S, Sun HC, Li F, Sun JB, Luo B (2010) Triptolide prolonged allogeneic islet graft survival in chemically induced and spontaneously diabetic mice without impairment of islet function. Hepatobil Pancreat Dis Int 9:312–318.

Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DJ, Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJ, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WH, Tsai EJ, Wilkoff BL (2013) 2013 ACCF/AHA Guideline for the Management of Heart Failure: Executive Summary: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol 62:1495–1539.

Yuan XP, He XS, Wang CX, Liu LS, Fu Q (2011) Triptolide attenuates renal interstitial fibrosis in rats with unilateral ureteral obstruction. Nephrology 16:200–210.

Zhang G, Liu Y, Guo H, Sun Z, Zhou YH (2009) Triptolide promotes generation of FoxP3 + T regulatory cells in rats. J Ethnopharmacol 125:41–46.

Zhang Y, Huang XR, Wei LH, Chung AC, Yu CM, Lan HY (2014) miR-29b as a therapeutic agent for angiotensin II-induced cardiac fibrosis by targeting TGF-beta/Smad3 signaling. Molecul Ther 22:974–985.

Zhang Y, Xu W, Li H, Zhang X, Xia Y, Chu K, Chen L (2013) Therapeutic effects of total alkaloids of Tripterygium wilfordii Hook f. on collagen-induced arthritis in rats. J Ethnopharmacol 145:699–705.

Zhang Z, Qu X, Ni Y, Zhang K, Dong Z, Yan X, Qin J, Sun H, Ding Y, Zhao P, Gong K (2013) Triptolide protects rat heart against pressure overload-induced cardiac fibrosis. Int J Cardiol 168:2498–2505.

Zhang Z, Zhao P, Li A, Lv X, Gao Y, Sun H, Ding Y, Liu J (2009) Effects of methotrexate on plasma cytokines and cardiac remodeling and function in postmyocarditis rats. Mediat Inflamm 2009:389720.

Zhu JN, Chen R, Fu YH, Lin QX, Huang S, Guo LL, Zhang MZ, Deng CY, Zou X, Zhong SL, Yang M, Zhuang J, Yu XY, Shan ZX (2013) Smad3 inactivation and MiR-29b upregulation mediate the effect of carvedilol on attenuating the acute myocardium infarction-induced myocardial fibrosis in rat. PLoS One 8:e75557.