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Original Article

Tanshinon II A attenuates diabetic cardiomyopathy by down-regulation of mTOR/P70S6K signaling pathway

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Abstract: Introduction: To verify whether tanshinone II A (Tan II A) could inhibit the development of diabetic cardiomyopathy (DCM) by blocking the mTOR/P70S6K signaling pathway. Material and Methods: Diabetes mellitus (DM) on Wistar Rats were induced by streptozocin (STZ). Sixty four DM rats were randomly divided into 4 groups: (1) Normal control (NC) group; (2) DM group; (3) Tan II A group; (4) MHY-1485 group. After 6 weeks of intervention, cardiac function was assessed by echocardiography. Serum levels of TNF-α, IL-6 and IL-1β were measured with Elisa method. Masson trichrome stain was used to evaluate cardiac fibrosis and electron microscope was used to test the cardiomyocytes ultrastructure. Western blotting was employed to investigate the expressions of MMP-2, MMP-9, TIMP-1, TIMP-2, p-mTOR and p-P70S6K. Gelatin zymography was used to detect the activity of MMP2 and MMP-9. Results: Compared with the DM group, Tan II A effectively improved cardiac function of contraction ($P<0.05$), decreased serum inflammatory factor levels ($P<0.05$), attenuated cardiac fibrosis, increased the expression and activity of MMP-2/9, and blocked mTOR/P70S6K signaling pathway ($P<0.05$). Meanwhile, MHY-1485 reversed the effect of Tan II A ($P<0.05$). Conclusion: Tan II A attenuated DCM by down-regulation of mTOR/P70S6K signaling pathway.

Keywords: Tanshinone II A, diabetic cardiomyopathy, mTOR/P70S6K, cardiac fibrosis, MMPs

Introduction

Diabetic cardiomyopathy (DCM), which was defined as left ventricular dysfunction independent of hypertension, atherosclerosis or coronary heart disease, is becoming the leading causes of mortality in the diabetic population [1, 2].

It has already been demonstrated by a lot of studies that cardiac fibrosis is the key pathological change which finally contributes to DCM [3-5]. Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) together keep the balance of ECM and changing in the composition of ECM result to cardiac fibrosis [6, 7]. Meanwhile, the increased serum inflammatory factors could also contribute to cardiac fibrosis [8]. The mTOR/P70S6K pathway had been reported to be involved in lots of pathological mechanisms of cardiovascular diseases. Previous studies found that suppression of mTOR/P70S6K pathway inhibited vascular smooth muscle dedifferentiation, induced autophagy in cardiomyocytes and inhibited cardiomyocytes apoptosis [9-11].

Tanshinone II A (Tan II A), a commonly used Chinese traditional drug, exhibits a variety of cardiovascular protective effect including attenuating and stabilizing atherosclerotic plaques, reducing blood press and improving the LVEF level of heart failure rats [12, 13]. Tan II A has been widely used in China for treatment of arrhythmia, angina pectoris and acute ischemic stroke. However, the effects of Tan II A on DCM is still unclear. Therefore, we aim to determine whether Tan II A could inhibit the progress of DCM in diabetic rat and further identify the role of mTOR/P70S6K pathway in the mechanism which is responsible for the effects of Tan II A.

Table 1. Baseline characteristics of Wistar Rats after 6 weeks intervention (x±s)

	Control (n=16)	DM (n=14)	Tan II A (n=16)	MHY-1485 (n=15)
Glucose (mmol/l)	4.6±1.4	22.5±4.6*	23.1±3.9	24.7±4.1
Weight (g)	389.3±37.5	318.5±35.6*	327.7±29.6	319.6±34.1
Heart/body (%)	1.95±0.17	2.14±0.19*	1.98±0.15	2.18±0.12
TNF-α (pg/ml)	2.01±0.12	6.76±1.09*	4.31±0.69**	6.89±1.27#
IL-6 (pg/ml)	3.44±0.55	6.14±1.19*	4.61±0.82**	5.99±0.97#
IL-1β (pg/ml)	0.31±0.04	0.94±0.12*	0.65±0.10**	0.99±0.18#
LVEDD (mm)	6.2±0.53	10.5±1.69*	8.1±1.03**	10.2±1.22#
LVEDS (mm)	2.5±0.29	7.6±0.81*	4.2±0.66**	6.9±0.93#
LVEF (%)	72.7±6.2	61.9±10.8*	67.4±10.6**	61.0±8.1#

DM: diabetes mellitus; Tan II A: tanshinone II A; LVEDD: left ventricular end-diastolic diameter; LVEDS: left ventricular end-systolic diameter; LVEF: left ventricular ejection fraction. *P<0.05 vs Control, **P<0.05vsDM, #P<0.05vs Tan II A.

Materials and methods

Animal group and diet

Animal studies conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-27, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Zhejiang university. After one week of an adaptation, at age 7 weeks, sixty four wistar rats were randomly divided into 4 groups (n=16, each group): (1) Normal control (NC) group; (2) DM group; (3) Tan II A group; (4) MHY-1485 group. Diabetes was induced in 48 rats in DM, Tan II A and MHY-1485 gruops by injection of STZ (50 mg/kg) dissolved in 0.1 mol/l sodium citrate, pH 4.5 [8], and Vehicle-treated rats served as normal controls (n=16). Blood glucose >20 mmol/l was confirmed as DM rats. After two weeks STZ injection, the blood glucose in all rats raise beyond 20 mmol/l in the DM, Tan II A, and MHY-1485 groups. MHY-1485 (10 μg/kg) was injected via the tail vein 10 min before Tan II A injection in the MHY-1485 group. Then, Tan II A (5 mg/kg) was administered by i.p. injection for four weeks in the rats from Tan II A group and MHY-1485 group [14]. Meanwhile, rats in control group received the same volume of 0.9% saline by i.p. injection. After all, two rats in the DM group and one rats in the MHY-1485 group died during the intervention. The general health and activity of the mice were monitored closely. In the study, the mice were allowed free access to food and water. Liquid consumption, food intake, animal body weight, blood glucose were monitored weekly.

Determination of cardiac function

One day before sacrificed, left ventricular end-systolic diameter (LVEDS) and left ventricular end-diastolic diameter (LVEDD) of the rat heart were measured from M-mode images with an echocardiography system (Sequoia Acuson, Siemens), and subsequently, LVEF and fractional shortening (FS) were calculated based on LVEDS and LVEDD.

Serum analysis

After 6 weeks of intervention, the rats were deprived of food for 12 h and sacrificed after being anaesthetized by inhaling 1.5% isoflurane as previously described. Blood was drawn from the right ventricle into tubes and the serum was isolated by centrifugation at 2000 × g for 15 mins at 4°C. The isolated serum was stored at -80°C for analysis. The TNF-α, IL-6 and IL-1β was measured with Elisa method.

Electron microscopy

After blood was drawn from the rats, hearts were rapidly removed and washed with PBS solution. The weight of the heart was calculated by the total weight minus the weight of culture dish. The ratio of the heart weight to body weight was used as an indicator of cardiac hypertrophy. Then, ophthalmic scissors were used to cut the left ventricular myocardium into a 1 mm tissue mass at a low temperature. After fixation, soaking, stepwise alcohol dehydration, displacement, embedding, polymerization, sectioning, and staining, the images were taken and observed under an electron microscope (JEM-2000EX, Japan).

Masson stain

Along the long axis, the heart tissue was cut into three thick slices from the apex to base. Then, the tissue was embedded in paraffin and a 5-μm-thick section was obtained from each slice. After the tissue slice was stained with

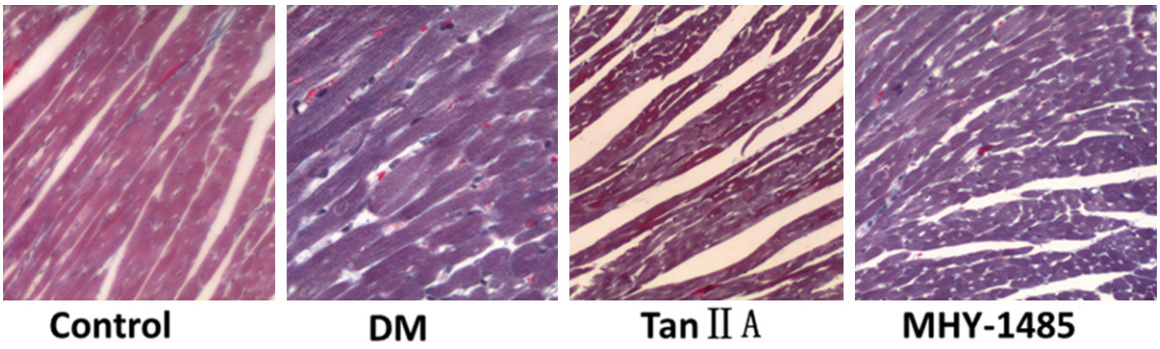

Figure 1. Effects of Tan II A on cardiac fibrosis. DM: diabetes mellitus; Tan II A: tanshinone II A.

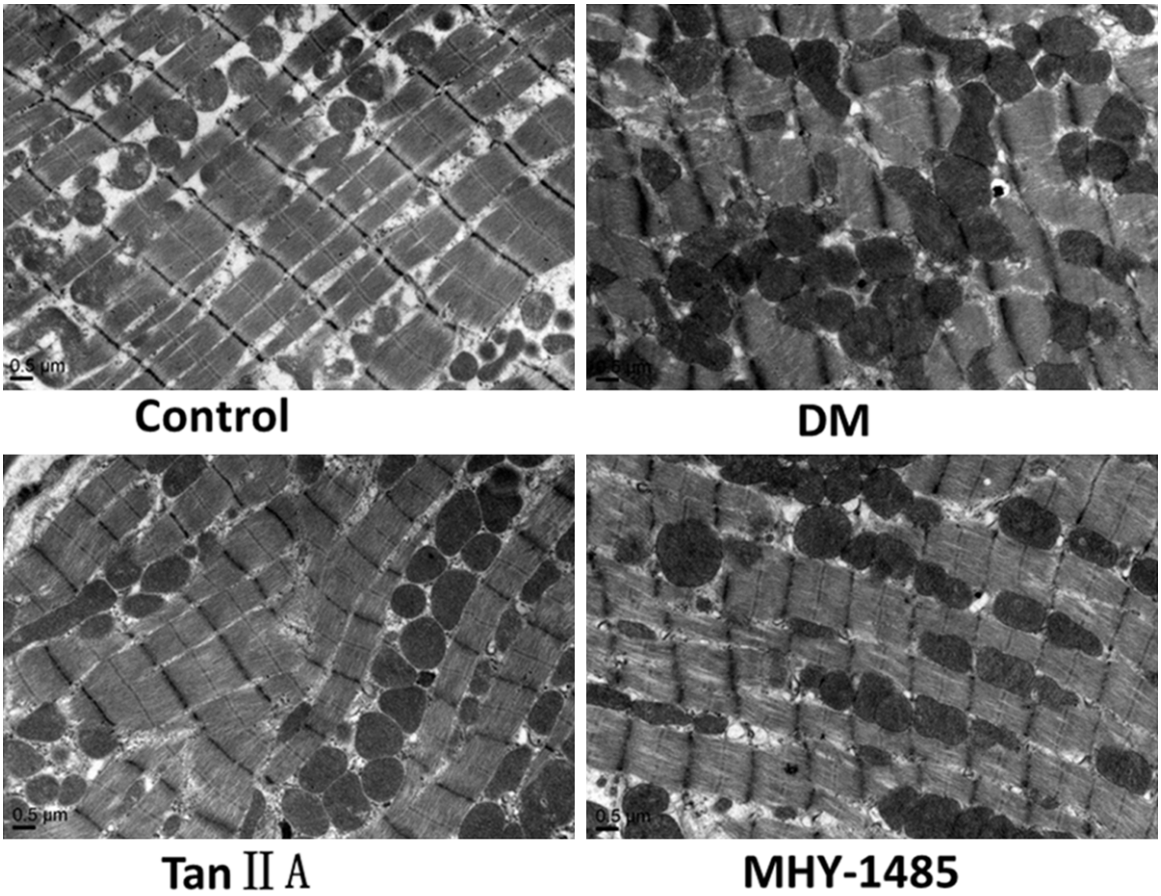

Figure 2. Effects of Tan II A on cardiomyocytes ultrastructure. DM: diabetes mellitus; Tan II A: tanshinone II A.

Masson trichrome, images were taken by light microscope and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) [15].

Western blot analysis

Frozen heart were homogenized and lysed by radio immunoprecipitation assay buffer (RIPA Lysis Buffer, Beyotime company, China) which contained protease and phosphatase. The protein was subjected to SDS-PAGE for electrophoresis and then transferred to the PVDF blotting membranes. The membranes were blocked with blocking buffer for 30 mins at room temperature and then incubated with the primary antibody overnight at 4°C. After the mem-

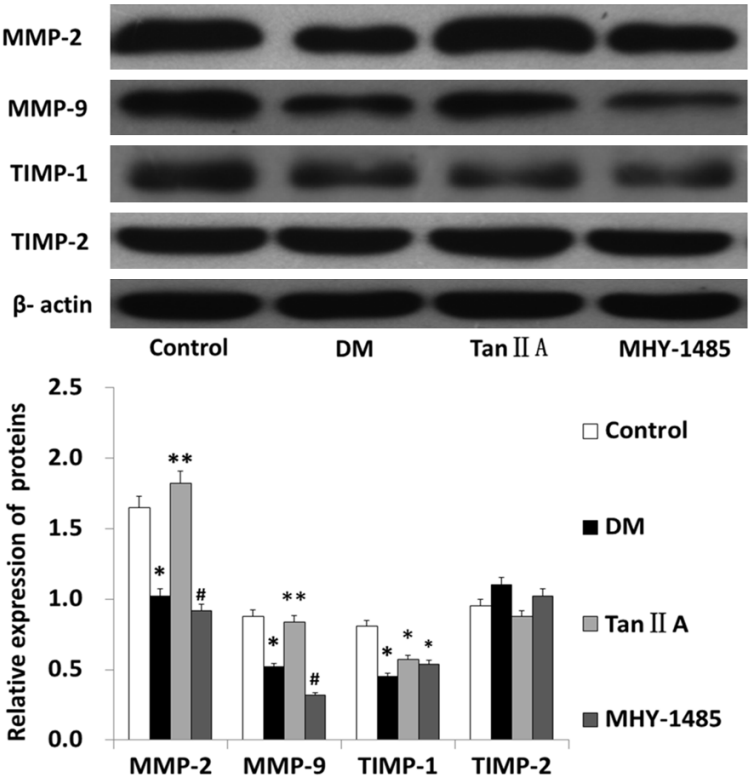

Figure 3. Effects of Tan II A on the expression of MMP-2/9 and TIMP-1/2. DM: diabetes mellitus; Tan II A: tanshinone II A. *P<0.05 vs Control, **P<0.05 vs DM, #P<0.05 vs Tan II A.

branes were incubated in horseradish peroxidase-linked secondary antibody, the standard chemical luminescence method was used to detect the antigen by exposing the membranes to Kodak X-Omat AR film. At last, the films were scanned on a gel imaging and analysis system and analyzed by Quantity One 4.4 (Bio-Rad, Hercules, CA, USA).

Gelatin zymography analysis

Zymography was used to measure the activity of MMP-2 and MMP-9 in the supernatants. The supernatant was collected and mixed with 5 × SDS sample buffer without a reducing agent as described in Western blot. Then, equal amounts (30 mg) of the sample were loaded onto the SDS-PAGE gel for electrophoresis. After that the gels were washed and incubated in the renaturation buffer, 0.1% Coomassie blue R-250 in 10% glacial acetic acid/45% methanol were used to stain the gels. Gels were destained (50% methanol, 10% acetic acid, and 40% water solution) until clear bands of gelatinolysis appeared on a dark background, then transferred to water for rehydration before acquiring images. At last, the images of the bands were analyzed by Quantity One 4.4 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data were analyzed by SPSS 20.0. All values were expressed as mean ± S.D. Differences among all data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tukey's Studentized Range (HSD) post-hoc test for multiple comparisons. Statistical probability of P less than 0.05 was considered to be significant.

Results

Baseline characteristics of the rats

As show in **Table 1**, after 6 weeks intervention, body weight decreased in the STZ injected rats. Meanwhile, heact/body mass ratio increased in the STZ injected rats (**Table 1**).

Effects of Tan II A on cardiac function

LVEDD, LVEDS were increased and LVEF was decreased in the DM group compared with the control group (P<0.05). Tan II A decreased the LVEDD, LVEDS and increased the LVEF of the DM rats. Pre-treatment with MHY-1485 partly abolish the effect of Tan II A (**Table 1**).

Effect of Tan II A on plasma inflammatory factors

Elisa analysis results showed that TNF-α, IL-6 and IL-1β were increased in the serum of DM mice (P<0.05), and decreased in the mice treated with Tan II (P<0.05). Meanwhile, the levels of TNF-α, IL-6 and IL-1β were increased in the rats of MHY-1485 group compared with Tan II A group (P<0.05). These results indicated that high blood glucose induced inflammatory response in the body and Tan II A inhibited

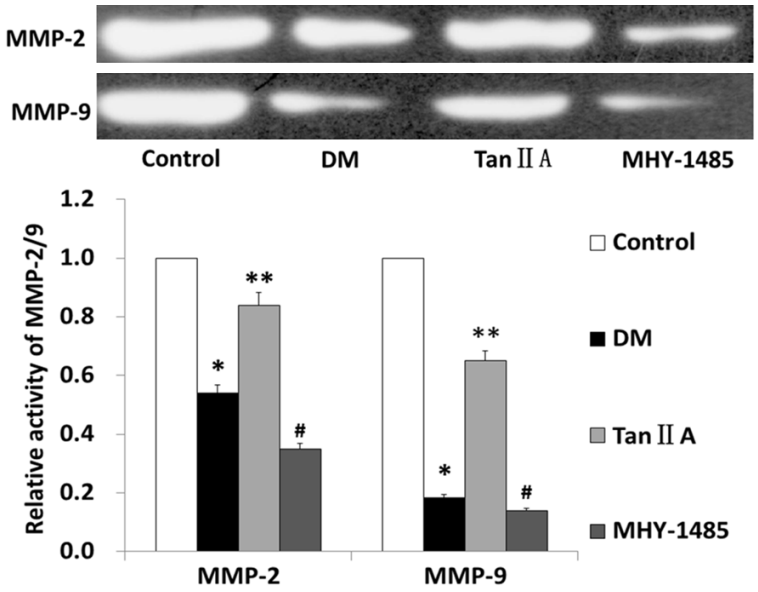

Figure 4. Effects of Tan II A on the activation of MMP-2/9. DM: diabetes mellitus; Tan II A: tanshinone II A. *P<0.05 vs Control, **P<0.05 vs DM, #P<0.05 vs Tan II A.

inflammatory response, pre-treatment with MHY-1485 partly abolish the effect of Tan II A (**Table 1**).

Effects of Tan II A on cardiac fibrosis

The results of masson stain showed that the myocardial fibrosis was increased in the DM group compared with control group (P<0.05). Tan II A decreased the myocardial fibrosis induced by DM (P<0.05) and pre-treatment with MHY-1485 partly abolished the inhibitory effect of Tan II A on myocardial fibrosis (P<0.05) (**Figure 1**).

Effects of Tan II A on cardiomyocytes ultrastructure

Electron microscope demonstrated that the number of cardiomyocyte mitochondria was increased in the DM group. The mitochondria in DM were found to be much bigger and with the destruction of cristae compared with the control group. Meanwhile, as shown in **Figure 2**, Tan II A alleviated mitochondria ultrastructure changes with smaller size and intact membrane and cristae.

Effects of Tan II A on MMP-2/9 and TIMP-1/2

After 6-weeks treatment, both the expression and activity of MMP-2/9 in the heart were increased in the DM group (P<0.05) compared with control group. Tan II A increased the expression and activity of MMP-2/9 which could be reversed by MHY-1485 (**Figures 3**, **4**). The expression of TIMP-1 was decreased in the DM group (P<0.05) and both Tan II A and MHY-1485 had no effect on it. There was no statistical difference between each group on the expression of TIMP-2 (**Figure 3**).

Effects of Tan II A on mTOR pathway

Compared with control group, the expression of p-mTOR and p-P70S6K were increased in the DM group (P<0.05). Compared with DM group, the expression of p-mTOR and p-P70S6K decreased in the Tan II A group (P< 0.05). There was no statistical difference between each group on the expression of mTOR and P70S6K (**Figure 5**). These data indicated that DM promoted the phosphorylation of mTOR and P70S6K, induced activation of mTOR pathway, while Tan II A could partly inhibit the activation of mTOR pathway.

Discussion

Nowadays, the pharmacological properties of Tan II A have attracted great interests. Variety of clinical trials and experimental studies have demonstrated the protective effect of Tan II A on hyperlipidemia, hypertension stroke, coronary artery disease, and other cardiovascular diseases [12, 16-18]. Xu et al. found that Tan II A attenuated and stabilized high cholesterol diet induced atherosclerotic plaques in Apolipoprotein-E knockout mice [12]. Besides, Liu et al. demonstrated that through inhibition of the extracellular signal-regulated kinase signaling pathway, Tan II A could attenuate IL-17A induced vascular smooth muscle cell activation [19]. What's more, Zheng et al. revealed that KV currents could be modulated by Tan II A which finally resulted in the reduction of hypoxic pulmonary hypertension. In spite of the widely use of Tan II A in the clinical treatment of cardiovascular diseases, its effect on DCM has not been

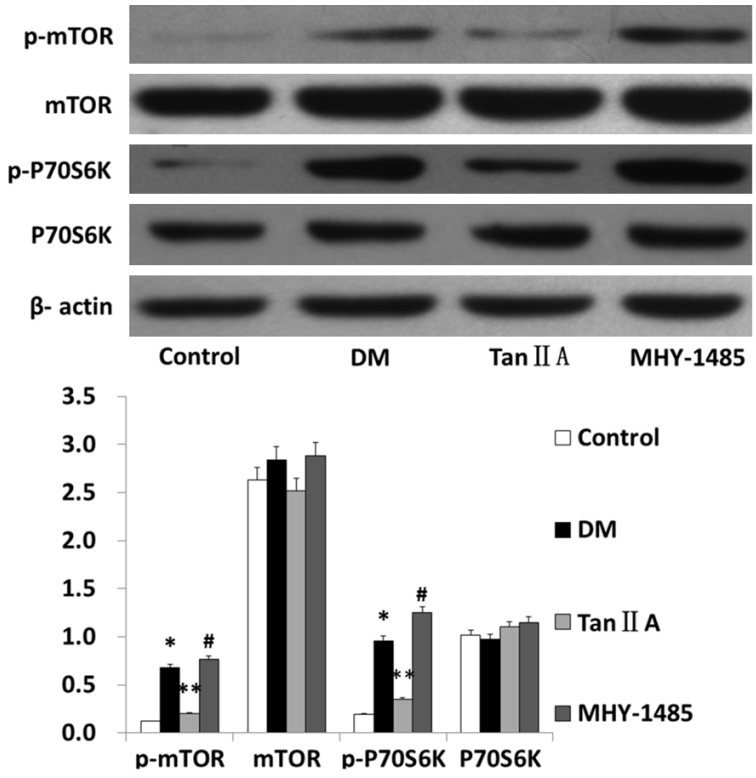

Figure 5. Effects of Tan II A on mTOR/P70S6Ksignaling pathway. DM: diabetes mellitus; Tan II A: tanshinone II A. *P<0.05 vs Control, **P<0.05 vs DM, #P<0.05 vs Tan II A.

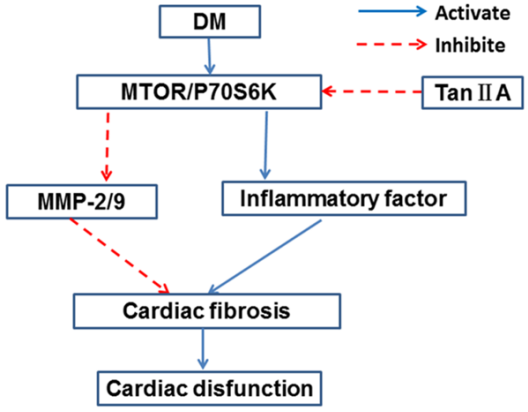

Figure 6. Tan II A induced anti-DCM effects through mTOR/P70S6K signaling pathway.

fully understood. In our study, we found that Tan II A could increase LVEF, decrease the serum inflammatory factor levels, alleviate mitochondria ultrastructure changes, inhibit the cardiac fibrosis and up-regulate the expression of MMP-2/9 in diabetic rat. What's more, our results further indicated that mTOR/P70S6K pathway played a key role in the mechanism responsible for the effects of Tan II A.

Westermann et al. first found the increased inflammatory cytokine levels was accompanied with diabetic cardiomyopathy in rat [8]. Besides, Nian's research demonstrated that inflammatory cytokines can decrease cardiomyocytes contractility by down-regulating the sarcoplasmic calcium ATPase expression [20]. What's more, Li et al. revealed that TGF-β acted as a profibrotic growth factor (PGF) by up-regulating the connective tissue growth factor (CTGF) through gene transcription which contributed to the increased collagen deposition and finally leaded to cardiac fibrosis [21]. Our previous research also found that IL-1β could stimulated over-expression of MMP-2 in human cardiac fibroblasts [22]. Consistently, the present study found that TNF-α, IL-6 and IL-1β were increased in the serum of DM mice, and Tan II A could decrease the TNF-α, IL-6 and IL-1β level. These results indicated that down-regulation of serum inflammatory cytokine levels maybe one of the mechanisms by which Tan II A played its anti-DCM role.

Evidence from human and animal models of DCM indicated that cardiac fibrosis was the key pathological substrate of diabetic cardiomyopathy. Cardiac fibrosis leaded to increased stiffness and decreased compliance of the ventricular wall, and resulted in systolic and diastolic dysfunction of the heart. In accordance with the previous researches, our results showed that accompanied with increased cardiac fibrosis, the LVEDD, LVEDS were increased and LVEF was decreased in the DM rats. Meanwhile, Tan II A could not only inhibit the cardiac fibrosis, but also decrease the LVEDD, LVEDS and increase the LVEF of the DM rats.

Our previous researches had already proved the important role of MMP-2 in the atheroscle-

rosis and cardiac fibrosis [23-25]. The decrease of MMP-2/9 expression and activity in the diabetic heart would contribute to the increased accumulation of collagen by digesting type I, II, and III collagen [26]. Besides, MMP-2 was proved to be a direct mediator of ventricular remodeling and systolic dysfunction by activation of Pi3k/akt or JNK/p38 signaling pathway [27, 28]. In the present study, we found that Tan II A reversed DM induced down-regulation of MMP-2/9 in the heart tissue of rats. This maybe another mechanism by which Tan II A play the anti-DCM role, besides down-regulation of serum inflammatory cytokine levels.

MTOR is a protein kinase which is ubiquitously expressed in the cells. Through the effectors P70S6K1 and 4E-BP1/eIF4E, mTOR regulates translation initiation of specific growth-related mRNA subsets. The mTOR signaling pathway regulates protein synthesis in response to amino acids, and its effectors integrate signals from mTOR with growth factor signals via PI3K to coordinately regulate cell cycle progression, protein synthesis, cell proliferation and migration [10]. In recent years, the effect of mTOR/P70S6K signaling pathway in cardiac fibrosis has been demonstrated by some studies [29-34]. Martinez et al. revealed that by activation of mTOR signaling pathway, leptin induced cardiac fibrosis [30]. Meanwhile, Ai et al. found that berberine could inhibit collagen synthesis of cardiac fibroblasts by down-regulation of mTOR/P70S6K signaling pathway [29]. Later, Zhao et al. demonstrated that NADPH oxidase 4 could induce cardiac fibrosis and hypertrophy mainly through activating NFkappaB and Akt/mTOR signaling pathways [33]. Besides, Chen et al. showed that by blocking the activation of mTOR/ERK signaling, angiotensin-converting enzyme (ACE) 2 could decrease cardiac fibrosis in apolipoprotein E-deficient mice [32]. In the present study, we found that Tan II A blocked the activation of mTOR/P70S6K signaling pathway. What's more, our results showed that MHY-1485 [14, 35], an special stimulator of mTOR/P70S6K signaling pathway, reversed the effect of Tan II A on serum inflammatory factor levels, expression of MMP-2/9 and cardiac fibrosis. These data strongly suggested that Tan II A attenuated DCM by inhibiting mTOR/P70S6K signaling pathway.

Our findings underscore the protective effects of Tan II A. Tan II A inhibits DCM by increasing LVEF, decreasing the serum inflammatory factor levels, alleviating mitochondria ultrastructure changes, inhibiting the cardiac fibrosis and up-regulating the expression of MMP-2/9 in experimental diabetic cardiomyopathy. Tan II A induced anti-DCM effects are mainly mediated through the mTOR/P70S6K signaling pathway (**Figure 6**).

Acknowledgements

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Disclosure of conflict of interest

None.

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