Early intervention with Di-Dang Decoction prevents macrovascular fibrosis in diabetic rats by regulating the TGF-β1/Smad signalling pathway

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[ABSTRACT] Macroangiopathy is a complication of Type II Diabetes Mellitus (T2DM), which is mainly caused by fibrosis of blood vessels. Using T2DM rat models, we investigated whether the traditional Chinese medicine, Di-Dang Decoction (DDD), exhibited anti-fibrotic actions on great vessels. T2DM rats were randomly divided into non-intervention group, early-, middle-, late-stage DDD intervention groups and control groups, including pioglitazone group and aminoguanidine group. After administration of DDD to T2DM rats at different times, we detected the amount of extracellular matrix (ECM) deposition in the thoracic aorta. The results showed that early-stage intervention with DDD could effectively protect great vessels from ECM deposition. Considering that TGF-β1 is the master regulator of fibrosis, we further validated at the molecular level that, compared to middle- and late-stage intervention with DDD, early-stage intervention with DDD could significantly decrease the expression levels of factors related to the activated TGF-β1/Smad signalling pathway, as well as the expression levels of downstream effectors including CTGF, MMP and TIMP family proteins, which were directly involved in ECM remodelling. Therefore, early-stage intervention with DDD can reduce macrovascular fibrosis and prevent diabetic macroangiopathy.

KEY WORDS Di-Dang Decoction; Macroangiopathy; Fibrosis; Diabetes; TGF-β1; CTGF; MMP; TIMP


Introduction

Diabetes is a metabolic disorder characterised by chronic hyperglycaemia. Diabetic macroangiopathy is a major complication of T2DM, which is prevalent in approximately 20%–30% of patients with T2DM [1]. Diabetic macroangiopathy mainly refers to atherosclerosis characterised by fibrosis of aortic intima and aortic tunica media [2]. Considering that fibrosis may result in an irreversible injury of great vessels, the effective prevention of macrovascular fibrosis is the most critical way to inhibit the occurrence of vascular lesions in diabetic patients. TGF-β1 is mainly involved in the regulation of fibrosis via activation of both Smad-dependent and Smad-independent signalling pathways. Overexpressed TGF-β1 could result in excessive production of ECM and inhibition of ECM degradation. Smad proteins exhibit competing pro-fibrotic and anti-fibrotic actions and are involved in the regulation of fibrosis [3]. Moreover, the downstream effectors of TGF/Smad signalling, such as MMPs, TIMPs and CTGF, are also associated with fibrosis. The TGF-β1/Smad signalling pathway has been widely reported in bioactivity study of traditional Chinese medicines. ZHAO et al. discovered that some natural products exhibit therapeutic effect against renal fibrosis through direct or indirect suppression of TGF-β1/Smad signalling pathway [4-9]. Hence, it is necessary to find an effective agent to prevent vascular fibrosis through regulation of the TGF-β1/Smad signalling pathway.

DDD is recorded in the “Treatise on Febrile Diseases”
and “Synopsis of Golden Chamber”, and is composed of leech, gadfly, peach seed and rhubarb \cite{10, 11}. DDD is a traditional Chinese medicine prescription for the elimination of blood stasis and the promotion of blood circulation. In previous studies, we found that early-stage intervention with DDD prevented aortic vessels from inflammatory injury and inhibited apoptosis of vascular endothelial cells, which could effectively maintain the integrity of great vessels \cite{10, 11}. Here, we compared the expression levels of vascular fibrosis-related factors at different stages of intervention with DDD, which could be used to evaluate the preventive effects of DDD on macrovascular fibrosis and reveal molecular mechanisms of DDD against fibrosis.

Materials and Methods

Drugs

Pioglitazone tablets were purchased from Takeda Pharmaceutical Co., Ltd. (Takeda, Japan). Aminoguanidine was purchased from Sigma (A8835, Sigma, USA). The ingredients of DDD were purchased from the pharmacy service in the first affiliated hospital of Tianjin University of traditional Chinese medicine (Tianjin, China). Raw DDD herbs include rhubarb (6 g), leech (10 g), peach seed (10 g) and gadfly (10 g). According to the traditional preparation process of the decoction, all the herbs were decocted for 1 h, three times in total. The filtrates were concentrated to 1 g mL$^{-1}$ of crude drug under reduced pressure and preserved at 4 °C.

Type 2 diabetes mellitus rat model

Four-week-old male Sprague-Dawley (SD) rats (180–210 g) were purchased from the Experimental Animal Center (Tianjin, China). The rats were housed under a 12 : 12 h light/dark cycle at an ambient temperature of 20–28 °C for one week. Fourteen rats were randomly assigned to the normal group fed regular chow diet, whereas the remaining 72 rats were fed 36.2% high fat diet (HFD) for 8 weeks. After being fasted for 12 h with free access to water, the fasting blood glucose (FBG) and serum insulin (FINS) of the rats were detected to calculate the insulin resistance index (IRI) \[ IRI = (FBG \times FINS)/22.5 \]. Then, HFD fed rats were injected with streptozotocin (STZ, S0130, Sigma, USA) via the tail vein to induce T2DM on the 14th week. The rats in the normal group and the non-intervention group \((n = 8)\) and non-intervention \((n = 14)\), late-stage DDD intervention \((n = 14)\), middle-stage DDD intervention \((n = 14)\), and early-stage DDD intervention \((n = 8)\) and non-intervention \((n = 8)\). The rats in the normal group and the non-intervention group received equal volumes of sterile drinking water daily. The rats in the early-stage DDD intervention group were administered DDD five weeks before T2DM was induced. The rats in the middle-stage DDD intervention group were administered DDD right after T2DM was induced. The rats in the late-stage DDD intervention group were administered DDD four weeks after T2DM was induced. The rats in the intermediate group were administered aminoguanidine right after T2DM was induced. The animals were sacrificed at the 24th week. Blood was collected from femoral arteries and thoracic aorta tissues were cryopreserved in liquid nitrogen for later use.

RT-qPCR

Real-time quantitative PCR analysis was employed to determine mRNA levels in thoracic aorta tissues. In brief, total RNA from aorta tissues was extracted using RNAiso Plus (D9108A, TaKaRa, Japan). One microgram of RNA was reverse transcribed with the Reverse Transcription Kit (DRR047A, TaKaRa, Japan). Using real-time quantitative PCR kit (DRR420A, TaKaRa, Japan) and ABI7500 Detection System (Thermo, USA), we obtained triplicate \( C_{\Delta \Delta \text{t}} \) values for each sample and normalised these values to \( \beta\)-actin expression level. Expression levels were calculated using the 2$^{-\Delta\Delta C_{\text{t}}}$ method. Primers were designed as follows: TGF-$\beta1$ (forward) 5’-GCTAACTTGAGGACACCG-3’, (reverse) 5’-CTCCCTCTCTTGTGTACGC-3’; Smad2 (forward) 5’-AGGAAGAAAGTGTGTG-3’, (reverse) 5’-AGTCTGGTGTAGTGACAC-3’; Smad3 (forward) 5’-CAACCTTCTCCCGGAAT-3’, (reverse) 5’-CGTGGATGCTCTCCCAA-3’; Smad7 (forward) 5’-GAATGGATGGTCGTGG-3’, (reverse) 5’-TGAACCTCGTGTCATTGGG-3’; CTGF (forward) 5’-CCCCGCAACCCGCAAG-3’, (reverse) 5’-ACACAGCCCACGGCCCAT-3’; \( \beta\)-Actin (forward) 5’-GCAAATTCCATGGCACCAGTC-3’, (reverse) 5’-AGCATAAGGCCCCACT-TGAATTT-3’.

Western blot

Thoracic aorta tissues were lysed in the lysis buffer [sodium dodecyl sulphate (0.1%), W/V], 150 mmol L$^{-1}$ NaCl, 50 mmol L$^{-1}$ Tris-Cl (pH 8.0)]. Aliquots of equal levels of proteins from the lysates were separated using SDS-polyacrylamide gel electrophoresis and transferred to a 0.45 µm PVDF membrane (Millipore, USA). The primary antibodies were diluted in 5% W/V non-fat dry milk in Tris-buffered saline Tween-20. The membranes were incubated with primary antibodies: TGF-$\beta1$ (1 : 300, bs-20411R, BIOSS, China), Smad2 (1 : 200, bs-0718R, BIOSS, China), Smad3 (1 : 200, bs-3484R, BIOSS, China), Smad7 (1 : 200, bs-0566R, BIOSS, China), and \( \beta\)-actin (1 : 1000, bs-0061R, BIOSS, China) overnight at 4 °C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 3000) (bs-0295G-HRP, BIOSS, China). After exposure, the final bands were quantified with Image J 1.52v software.

Enzyme-linked immunosorbent assays

Serum levels of MMP2, MMP3, MMP8, MMP9, TIMP1 and TIMP2 were quantified using separate ELISA kits (Fusheng, Shanghai, China), according to the manufacturer’s instructions. In brief, 10 µL of serum was added to the pre-coated micro-well in 1 : 5 dilution in dilution buffer. The well was incubated at 37 °C for 1 h followed by incubation
with 100 μL of secondary antibody conjugated to peroxidase at 37 °C for an hour. Next, washing buffer was added. The well was incubated for 1 min and washed another four times. Then, 100 μL of TMB (3, 3’, 5, 5’-tetra methyl benzidine) substrate was added and the well was incubated in the dark at 37 °C for 15 min. Finally, 50 μL of stop solution was added to the well and optical density (OD) was measured using a microtiter plate reader (Molecular Devices, USA) at 450 nm.

**Histological analysis**

Rat aortic tissues were fixed in 4% PFA and embedded in paraffin. Tissue sections (5-μm thick) were prepared for haematoxylin and eosin staining using a standard protocol.

**Statistical analysis**

All statistical analyses were carried out using the GraphPad Prism 4.0 software (GraphPad, USA). Difference between groups was assessed using the Student’s t-test or one-way ANOVA followed by Tukey’s test; the P value was adjusted by family-wise error correction, and considered significant when *P < 0.05, **P < 0.01 or ***P < 0.001. Data were shown as mean ± SEM.

**Results**

**HFD/STZ induces T2DM rat models**

The overall schematic workflow of the study was shown in Fig. 1A. To evaluate the interventional effects of DDD on diabetic macroangiopathy at different times, we established rat models of T2DM using HFD and STZ. At the 9th week, we randomly selected six HFD rats to detect the IRI in order to determine the time point of administration of rats in the early-stage DDD intervention group. The IRIs of HFD rats

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**Fig. 1** Establishment of the T2DM rat model. A. The schematic workflow; B. Determination of the insulin resistance. Data are expressed as mean ± SEM (n = 6), *P < 0.05 vs Normal; C. The successfully established T2DM rat model. Data are expressed as mean ± SEM (n = 14 in normal, DDD-early, DDD-middle, Aminoguanidine and Pioglitazone groups; n = 8 in T2DM and DDD-late groups)
were significantly higher than those of rats in the normal group, which indicated that the incidence risk of diabetes in HFD rats was higher than that of rats in normal group (Fig. 1B). One week after STZ injection, T2DM rats showed random blood glucose levels higher than 16.7 mmol·L$^{-1}$, which indicated that the T2DM rat models were successfully established, whereas random blood glucose levels of the rats in normal group were lower than 16.7 mmol·L$^{-1}$ in the fasting or fed state (Fig. 1C).

**Early-stage intervention with DDD inhibits the deposition of ECM in thoracic aorta**

To observe the effect of intervention with DDD on diabetic macroangiopathy at different times, haematoxylin and eosin (HE) staining was performed on thoracic aortic vessels of each group, followed by light microscopy analyses. In the normal group, the arterial intima was relatively intact and the arterial smooth muscle cells (SMCs) were arranged neatly (Fig. 2A). In non-intervention group, the arterial intima was not intact, most of the endothelial cells were absent and the arterial SMCs exhibited a disordered arrangement (Fig. 2B). Compared with middle- and late-stage intervention with DDD, early-stage intervention with DDD showed the relatively intact intima of the great vascular artery and the arterial SMCs were arranged neatly (Figs. 2C–2E).

Arterial SMCs secrete elastic fibres and collagen fibres to the extracellular environment and provide nutrition support for vascular endometrial cells, which maintain normal vascular structure [12]. Under normal conditions, SMCs have aligned arrangement with continuously distributed elastic fibres and collagen fibres. During diabetic macroangiopathy, SMCs are transformed to phenotypes that tend to secrete more ECM. The reduction of elastic fibres and the compensatory disordered hyperplasia of collagen fibres result in the breakdown of the regular arrangement of SMCs and disordered alignment of the tunica media [12–14]. Therefore, we concluded that early intervention with DDD could effectively inhibit the changes in ECM and maintain the original structure of blood vessels by regulating the related signalling pathway.

**Early-stage intervention with DDD prevents over-activation of the TGF-β1/Smad signalling pathway**

Next, we investigated the effect of intervention with DDD at different times on the TGF-β1/Smad signalling pathway associated with fibrosis by comparing the expression levels of the molecules in this pathway. Compared to middle- and late-stage intervention with DDD, early-stage intervention with DDD could effectively inhibit the overexpression of TGF-β1 and downstream Smad2, Smad3 and Smad7 (Figs. 3A–3F). Notably, Smad2 and Smad3 are positive feedback factors of the TGF-β1/Smad canonical signalling pathway, while Smad7 is a negative feedback factor. Smad2/3 and Smad7 together regulate the TGF-β1/Smad canonical signalling pathway [3]. The non-significant difference in expression levels of Smad2/3 between the early-stage DDD intervention group and the normal group indicated that early-stage intervention with DDD could effectively maintain the balance between the levels of Smad2/3 and Smad7 in diabetic macroangiopathy, and therefore, prevent the excessive activation of the TGF-β1/Smad signalling pathway (Figs. 3B–3F).

Because extracellular TGF-β1 binds to membrane surface receptors to activate downstream signalling pathways, we quantified the levels of extracellular TGF-β1. The results showed that the level of extracellular TGF-β1 in early-stage DDD intervention group is significantly lower than that in the middle- and late-stage DDD intervention groups, which also indicated that early-stage intervention with DDD could inhibit the TGF-β1/Smad signalling pathway from being excessively activated (Fig. 3G).

**Early-stage intervention with DDD inhibits the increase in the extracellular levels of ECM regulators mediated by TGF-β1**

Further, we explored the effect of the intervention with...
DDD on levels of ECM regulators including CTGF, TIMPs and MMPs, which are regulated by TGF-β1. CTGF promotes the secretion of ECM. Compared to middle- and late-stage intervention with DDD, early-stage intervention with DDD could effectively decrease the extracellular levels of CTGF (Fig. 4A).
Fig. 4  Expression levels of factors related to ECM regulation. The levels of seven factors, including CTGF (A), MMP1 (B), MMP2 (C), MMP8 (D), MMP9 (E), TIMP1 (F) and TIMP2 (G), were measured in the serum by ELISA. Data are expressed as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs Normal; *P < 0.05, **P < 0.01, ***P < 0.001 vs T2DM; *P < 0.05, **P < 0.01, ***P < 0.001 vs DDD early-stage. The seven groups are normal group (Normal) (n = 14), non-intervention group (T2DM) (n = 8), early-stage DDD intervention group (DDD-early) (n = 14), middle-stage DDD intervention group (DDD-middle) (n = 14), late-stage DDD intervention group (DDD-late) (n = 8), aminoguanidine group (Aminoguanidine, DDD positive control) (n = 14) and pioglitazone group (Pioglitazone, DDD negative control) (n = 14)
MMPs and TIMPs are two important factors involved in the regulation of ECM synthesis and degradation. ECM remodelling and fibrosis are mainly caused by the imbalance between the levels of extracellular MMPs and TIMPs in diabetic macroangiopathy. Compared to middle- and late-stage intervention with DDD, early-stage intervention with DDD could effectively decrease extracellular levels of MMP1/2/8/9 and TIMP1/2, and thus, maintain the balance between the levels of extracellular MMPs and TIMPs, and prevent ECM from being drastically changed (Figs. 4B–4G).

Discussion

Diabetic macroangiopathy is a common and serious complication of diabetes, which is mainly caused by the deposition of ECM and the subsequent development of vascular fibrosis [1, 2]. Therefore, we speculated that targeting the factors related to ECM remodelling and vascular fibrosis could effectively prevent the occurrence of diabetic macroangiopathy.

Based on current knowledge that activation of the TGF-β1/Smad signalling pathway results in the excessive production of ECM, followed by the promotion of fibrosis, we mainly focused on the effect of intervention with DDD at different times on the TGF-β1/Smad signalling pathway [3]. The regulatory mechanism of TGF-β1 for vascular fibrosis, which is intervened by DDD, was depicted in Fig. 5. TGF-β1 binds to specific membrane receptors of the target cells to activate Smad proteins. The activated Smad proteins, as transcription factors, are transferred into the nucleus to promote the expression of effectors associated with ECM remodelling and vascular fibrosis [3].

CTGF is a downstream factor of the TGF-β1/Smad signalling pathway and is directly regulated by TGF-β1 [9]. Over-expressed CTGF is secreted by vascular SMCs and is involved in the pro-fibrotic action of TGF-β1, including the proliferation of fibroblasts and the deposition of ECM [15, 16].

MMPs and TIMPs are other two categories of downstream factors of the TGF-β1/Smad signalling pathway and are directly involved in the regulation of ECM [17-19]. The MMP family primarily comprises proteases involved in the degradation of ECM. MMP1 and MMP2 are mainly responsible for the degradation of collagen I, collagen II and collagen III, which constitute the main components of the ECM [20]. MMP8 and MMP9 are mainly responsible for the degradation of collagen IV, which constitutes the main component of the basement membrane [20, 21]. The TIMP family constitutes a group of natural inhibitors of MMPs [20, 21]. Therefore, the balance between the expression levels of MMPs and TIMPs has a critical influence on the synthesis and degradation of ECM. Previous studies have showed that over-activated TGF-β1 induces the imbalance between the expression levels of MMPs and TIMPs in a variety of pathologies [17-19]. Increased activity of TGF-β1 can result in ECM remodelling and fibrosis through the dysregulation of balance between MMPs and TIMPs [1, 18].

The four components of DDD play important roles in the prevention of macroangiopathy. Recent pharmacological researches have proven that rhubarb is involved in the TGF-β1/Smad signalling pathway and resists cell fibrosis. The main active components of rhubarb include rhein, emodin, catechin and epicatechin. These active components can regulate abnormal accumulation of ECM synergistically [22, 23]. The extract of leech contains hirudin, which can inhibit platelet formation, prevent the formation of blood clots and alleviate atherosclerosis. In addition, hirudin can also reduce the proliferation rate of fibroblasts [24, 25]. The extract of gasfly contains polysaccharides, which can reduce the activity of blood coagulation factors, increase the activity of fibrinolytic system, and prevent the formation and development of thrombus [26]. Peach seed mainly contains amygdalin, which can increase the activity of collagenase, promote the decomposition of collagen, and suppress fibrosis development via downregulation of the expression of α-SMA and fibronectin [27-28].

In the present study, we have demonstrated that early-stage intervention with DDD can decrease the activity of TGF-β1/Smad singling pathway, and thus, inhibit the increase in the extracellular level of CTGF and maintain balance between MMPs and TIMPs. Therefore, early-stage intervention with DDD can prevent ECM remodelling and fibrosis in great vessels. Our previous studies have showed that early-stage intervention with DDD can maintain the integrity of vascular endothelial cells and protect great vessels from inflammatory injury [16, 11]. In conclusion, early-stage intervention with DDD can strengthen the defence function of great vessels and prevent the development of macroangiopathy in T2DM.
References


