
Effects of two myricetin on renal fibrosis in diabetic nephropathy rats

Abstract: Objective: To investigate the protective effect of two - myricetin (DMY) on diabetic nephropathy (DN) and its effect on renal fibrosis. **Methods:** the DN model was established by intraperitoneal injection of high-dose ($55 \text{ mg} \cdot \text{kg}^{-1}$) streptozotocin (STZ). The model was successfully established with blood glucose $> 16.7 \text{ mmol} \cdot \text{L}^{-1}$. The rats were randomly divided into normal group, model group and low, medium and high dose DMY group ($125, 250$ and $500 \text{ mg} \cdot \text{kg}^{-1}$). DMY group was administered by gavage, and the model group was administered by normal saline for 12 weeks. The contents of 24h urinary protein (24h-Pro), blood urea nitrogen (BUN) and blood creatinine (SCR) were detected by biochemistry. He staining was used to observe the morphological changes of kidney in each group, PAS staining was used to observe the changes of basement membrane thickness of renal tissue in each group, and Masson staining was used to observe the degree of glomerulosclerosis and renal interstitial fibrosis in each group. TGF in renal tissue of rats in each group was detected by Western blot- $\beta 1$. Expression level of Smad2 and Smad7. **Results:** compared with the normal group, the contents of 24h pro, bun and SCR in DN model group were significantly increased ($P < 0.01$). Compared with the model group, the contents of 24h pro, bun and SCR in each dose of DMY group decreased ($P < 0.01$). The pathological results showed that the renal tissue of the model group showed increased glomerular volume and glue more

Key words: Diabetic nephropathy; Dihydromyricetin; Renal fibrosis; TGF- $\beta 1$ /Smads;

Diabetic nephropathy (DN) is a common microvascular complication of diabetes, and it is the main cause of end-stage renal disease. With the increase of incidence rate, DN is the main cause of chronic renal failure and death in [1]. Renal fibrosis is one of the most important features of diabetic nephropathy. Many factors are involved in the process of diabetic renal fibrosis, such as transforming growth factor- β 1 (Transforming Growth Factor- β 1, TGF- β 1), cytokines, chemokines, growth factors, etc., including TGF- β 1 is the most important cytokine to promote renal fibrosis [2]. Studies have shown that TGF- β 1. It can promote the transformation from renal epithelium to stroma by activating Smads signal transduction, resulting in renal fibrosis and eventually progression to renal insufficiency [3]. So far, in addition to controlling blood sugar and blood pressure to slow down the progress of DN, there are no better measures to prevent and treat diabetic nephropathy. Therefore, it is of great practical significance to find new methods for effective prevention and treatment of DN.

Dihydromyricetin (DMY) is one of the main active components in rattan tea. Previous studies have shown that DMY has various biological activities such as anti-inflammatory, antioxidant, anti-fibrosis, and anti-tumor [4]. In addition, different diabetic animal models have confirmed that DMY has a hypoglycemic effect and can effectively improve the state of impaired glucose tolerance [5]. However, the mechanism of action of DMY on high glucose-induced renal fibrosis remains unclear [6]. In this paper, a DN rat model was established to explore the effect of DMY on high

glucose-induced rat renal fibrosis, and to elucidate the molecular mechanism of DMY in the treatment of DN from the TGF- β 1/Smads signaling pathway.

1 Materials and methods

1.1 Animals and reagents 40 healthy male SD rats (200 ± 20) g were selected and provided by the animal center of Gannan Medical College. The animals were raised at 25 °C with normal ventilation, free drinking water, conventional feed and adaptive feeding for 1 week. Streptozocin (STZ), the staining kit, Masson staining kit and PAS staining kit were purchased from Beijing solebao company, TGF- β 1, Smad2 and Smad7 antibodies were purchased from Wuhan bode company, secondary antibody was purchased from Fuzhou Maixin company, and dihydromyricetin was purchased from Chengdu Dexter company. 1.2 preparation and experimental grouping of DN model after fasting for 12 hours, all rats dissolved streptozotocin (STZ) in 0.1 M sodium citrate buffer (pH=4.5) to prepare high-dose STZ. Except for the normal control group, 55 mg · kg⁻¹ STZ was injected intraperitoneally and fed with common feed to establish DN model. 72 hours after modeling, the blood of rat tail vein was taken to measure fasting blood glucose. The modeling was regarded as successful when the blood glucose value was > 16.7 mmol · L⁻¹. The rats were randomly divided into model group and low, medium and high dose DMY group (125, 250 and 500 mg · kg⁻¹), and the normal group was set up. DMY group was administered by gavage, and the model group was administered by gavage with equal volume of normal saline once a day for 12 weeks.

1.3 Renal function test before the rats were killed in the 12th week, 24 h urine was collected and 24 h urinary protein (24 h-pro) was measured. The blood was taken from abdominal aorta. After the serum was naturally precipitated, $3000 \times g$. After centrifugation at $4^\circ C$ for 10 min, the insoluble matter was discarded, the serum was collected, and the contents of blood urea nitrogen (BUN) and serum creatinine (SCR) were measured in strict accordance with the experimental instructions.

1.4 HE staining of renal embedded tissue $4 \mu M$ -thick slice, dewaxing the slice to water, dyeing with hematoxylin for 5 minutes, and rinsing with running water; Differentiation with hydrochloric acid and alcohol for 3 s and rinsing with running water for 1 min; Return to blue with ammonia and rinse with running water for 1 min; Eosin staining for 1 min and rinsing with running water for 1 min; Gradient alcohol dehydration, xylene transparent, neutral gum seal.

1.5 PAS stained sections are routinely dewaxed to water, oxidized with periodic acid solution for 10 min, and rinsed with distilled water for 1 min; Stain with Schiff reagent for 20 min and rinse with running water for 1 min; Hematoxylin staining for 5 min, rinsing with running water for 1 min, conventional dehydration, transparent, neutral gum seal.

1.6 Masson stained sections are routinely dewaxed to water, stained with fuchsin for 3 minutes and washed with weak acid for 1 minute; Phosphomolybdic acid differentiation for 1 min, weak acid washing for 1 min; Aniline blue staining for 2 min, weak acid washing for 1 min; Conventional dehydrated transparent, neutral gum seal.

1.7 The level of protein detected by Western blot was taken from the homogenate of the kidney tissue and lysed for 1 h, and the protein was quantified by BCA. The protein was added to the sample buffer to boil and denaturate. After SDS-PAGE gel electrophoresis, the protein was transferred by PVDF membrane. After 5% skim milk was closed, TGF- $\beta 1$, Smad2 and Smad7 primary antibodies were incubated at $4^\circ C$ overnight, and the secondary antibodies were incubated at room temperature for 1 h. Finally, the protein bands were scanned by bio rad software and the gray value was measured, and the results were compared with those of the control group.

1.8 Statistical analysis was performed by SPSS 16.0 software. All data were expressed by $X \pm s$. The comparison of measured indexes between multiple groups was analyzed by one-way ANOVA, and the comparison between two groups was performed by LSD test. $P < 0.05$ was statistically significant.

2 Results

2.1 Effect of DMY on renal function of DN rats biochemical test results showed that the contents of 24 h pro, bun and SCR in the model group were significantly higher than those in the normal group ($P < 0.01$); After 12 weeks of DMY administration, the levels of 24h pro, bun and SCR in each dose of DMY group were significantly lower than those in the model group ($P < 0.01$). See Table 1.

2.2 Effect of DMY on renal histopathology of DN rats HE staining showed that the renal tissue of model group showed increased glomerular volume, vasodilation and congestion,

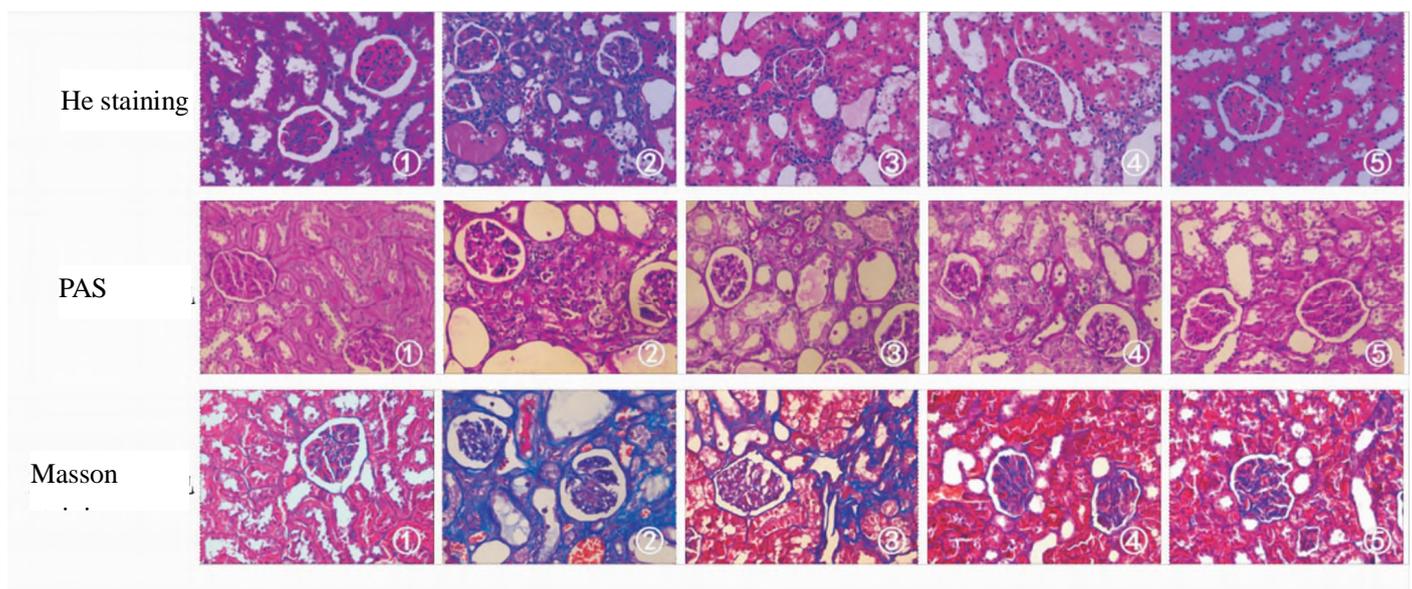
mild atrophy or lumen expansion of some renal tubules, swelling of epithelial cells and abscission of some epithelial cells. The renal tissue lesions of rats in each dose of DMY group were less than those in the model group, especially in the medium and high dose groups. PAS staining showed that

DMY could reduce glomerular basement membrane thickening after intervention. Masson staining showed the proliferation of glomerular collagen fibers in the model group, and the collagen fibers decreased in different degrees in each dose of DMY group after treatment. See Figure 1.

Table 1 Effects of DMY on renal function of rats in each group/ $\bar{x} \pm s$

group	n	24 h-Pro / mg · 24 h ⁻¹	BUN / mmol · L ⁻¹	Scr / μ mol · L ⁻¹
Normal group	5	7.70±1.24	4.58±0.64	35.24±6.51
Model group	4	84.17±14.78**	17.90±1.18**	127.57±15.74**
Low dose DMY group	5	62.48±12.75 ^{##}	10.14±0.83 ^{##}	98.93±11.58 ^{##}
Medium dose DMY group	4	59.70±8.98 ^{##}	8.56±0.76 ^{##}	86.75±9.49 ^{##}
High dose DMY group	5	36.38±8.62 ^{##}	7.97±0.73 ^{##}	88.27±10.72 ^{##}

Note: ** P < 0.01, compared with the normal group^{##} P < 0.01, compared with the model group.



① Normal group; ② Model group; ③ Low dose DMY group; ④ Medium dose DMY group; ⑤ High dose DMY group.

Fig. 1 pathological staining of kidney tissue of rats in each group (× 400)

2.3 effect of DMY on TGF in renal tissue of DN rats- β1. The expression of Smad2 and Smad7. The results of Western blot showed that compared with the normal control group, the renal tissue TGF in the model group- β1. The expression level of Smad2 protein increased and Smad7 protein

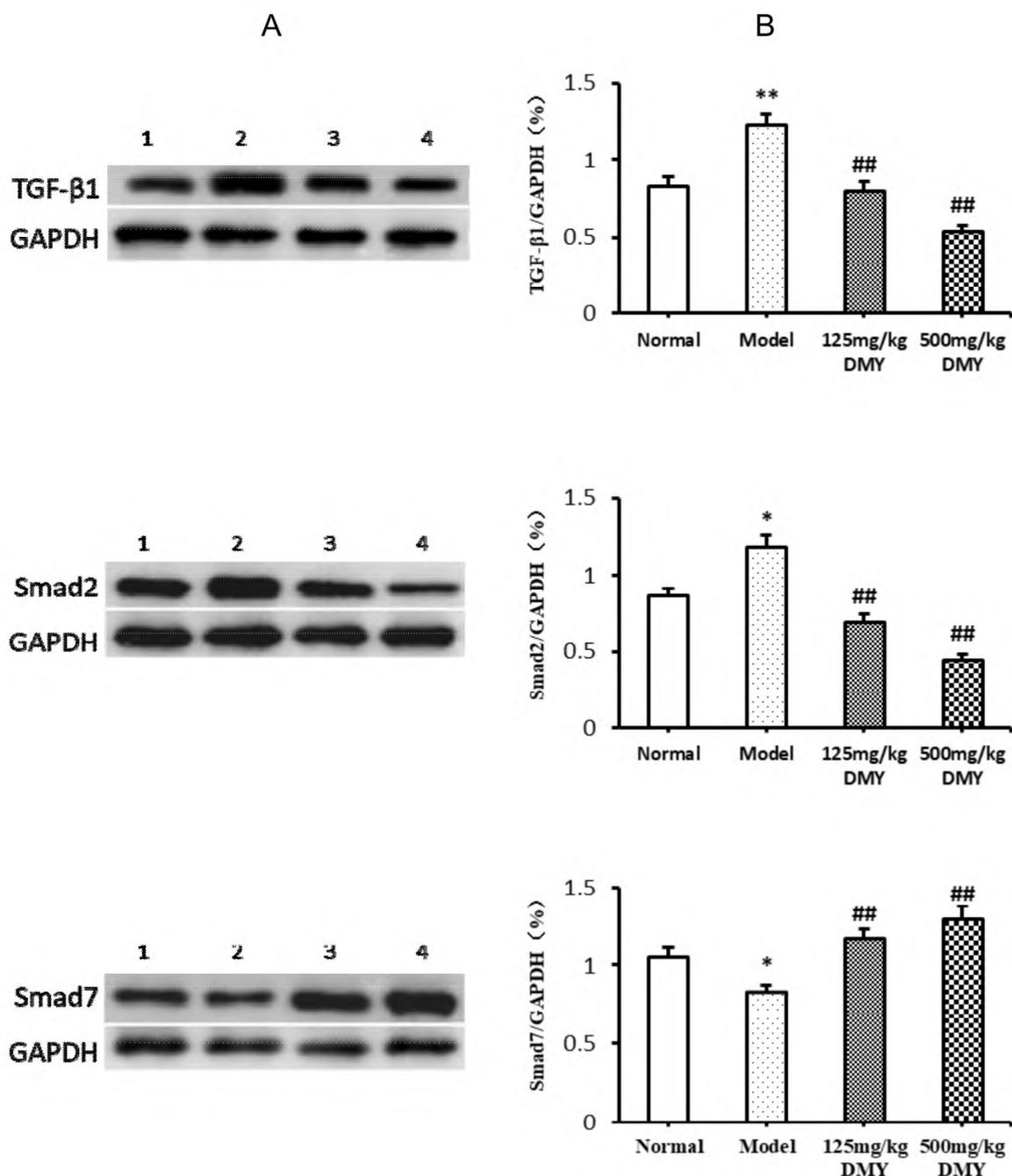
decreased (P < 0.05). Compared with the model group, TGF in low and high dose DMY group- β1. Smad2 protein decreased in varying degrees, while Smad7 protein expression increased (P < 0.05). See Figure 2.

3 Discussion

Diabetic nephropathy is one of the most serious complications of diabetes. In the course of its development, glomerular hypertrophy, basement membrane thickening, extracellular matrix accumulation and podocyte loss are the main pathological features, leading to renal interstitial fibrosis and glomerulosclerosis. TGF- β 1 and its regulatory factors play a key role in the process of renal interstitial fibrosis in DN, but the methods that can effectively reduce renal fibrosis during DN need to be further studied.

DMY is a dihydroflavonol flavonoid compound, which has the effects of anti-tumor, antioxidant stress, anti-aging of skin and so on. In recent years, it has been found that DMY has various pharmacodynamic effects and has important medicinal value in reducing blood glucose, inhibiting fibrosis and protecting neurological, cardiovascular and cerebrovascular diseases [7-10]. Xu Yanyan [11] found that DMY has a significant protective effect on rats with liver fibrosis induced by carbon tetrachloride, which may be related to the inhibition of extracellular matrix formation. Li Jialin et al. [12] found that

DMY can inhibit the proliferation of glomerular mesangial cells induced by high glucose and reduce the expression of extracellular matrix proteins. Chen Hong and other [13] used 60 mg kg⁻¹ STZ intraperitoneal injection and normal feeding for 8 weeks to establish DN model. DMY was found to alleviate renal fibrosis in diabetic rats. However, the mechanism of DMY on DN has not been fully clarified. In this study, 55 mg · kg⁻¹ STZ was injected intraperitoneally and fed with ordinary diet for 12 weeks to establish DN model. He and PAS staining showed glomerular hypertrophy, atrophy of some renal tubules and thickening of basement membrane in the model group; Masson staining showed extensive deposition of collagen in kidney tissue of model group, which showed that DN rat model was successfully established in this paper. DMY intervention can significantly reduce the above pathological changes. In addition, this study found that DMY can effectively reduce the levels of 24 h-pro, bun and SCR. These results indicate that DMY has a significant protective effect on diabetic kidney injury.



A: TGF in each group- β1. Smad2 and Smad7 protein levels; B: TGF in each group- β1. Protein gray density value of Smad2 and Smad7, n=4.

1 normal group; 2 model group; 3 low dose group (125 mg · kg⁻¹); 4 high dose group (500 mg · kg⁻¹).

*P < 0.05, ** P < 0.01, compared with the normal group# P < 0.05, ##p < 0.01, compared with the model group.

Fig. 2 Effect of DMY on TGF in renal tissue of DN rats- β1. Effect of Smad2 and Smad7 expression

Renal fibrosis is one of the main pathological features of DN. Its pathological process is characterized by

glomerular mesangial expansion and extensive deposition of extracellular matrix^[14]. Many cytokines are involved in the

pathological process of renal fibrosis, TGF- β 1 is the most important cytokine to promote renal fibrosis [15]. TGF- β 1 participate in a variety of biological processes, including cell proliferation, differentiation, apoptosis, autophagy and the production of extracellular matrix [16]. The study found that TGF- β 1 can not only promote the synthesis of extracellular matrix, but also activate Smad2 protein to induce the transformation of renal epithelium to stroma, resulting in renal interstitial fibrosis [17-19]. In addition, TGF- β 1 neutralizing antibody can reduce the renal interstitial fibrosis and reduce the expression of extracellular matrix [20] in diabetic rats. These studies suggest that TGF- β 1 pathway plays an important role in the process of diabetic renal fibrosis. This study found that DMY could reduce the renal tissue TGF- β 1. Smad2 protein level and increase the expression level of Smad7 protein, suggesting that DMY can pass through TGF- β 1 / Smads pathway inhibits the expression of extracellular matrix and reduces renal fibrosis in rats.

In conclusion, DMY has an obvious protective effect on the renal injury of DN rats induced by STZ, and its mechanism may be related to the inhibition of TGF- β 1 / the over activation of Smads pathway can reduce the deposition of collagen in renal tissue and reduce the fibrosis of rat renal tissue. This study provides a new idea for the prevention and treatment of renal fibrosis in rats with DMY.

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