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Nephroprotective Effects and Its Mechanism of an Herbal Prescription on Diabetic Nephropathy Rats

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The work aims at investigating protective effects and mechanism of an herbal prescription (162.5 mg/kg-650 mg/kg bw/d) composed of tea and lycium bararum polysaccharides and *Eucommia ulmoides* on diabetic nephropathy. Following parameters were measured: (1) body weights, 24 h's urine volume, urine protein, serum glucose and insulin levels, blood urea nitrogen, serum creatinine, advanced glycosylation end products (AGEs); (2) kidney weight, aldose reductase and protein kinase C in kidney tissue; (3) histopathology of kidney. Body weight and albuminuria of diabetic nephropathy rats were improved and blood glucose, blood urea nitrogen and serum creatinine were decreased. Excretion and sensitivity to insulin was activated. Moreover, the prescription evoked normalisation of diabetes-stimulated kidney NADPH oxidase and reduced levers of advanced glycosylation end products and percentages of cell membrane protein kinase C activity accounted for gross activity. It also restrained thickening of glomerular mesangial area and basement membrane and degeneration of tubular. The prescription seems to be benefit for preventing development of diabetic nephropathy.

Key Words: NIDDM, Diabetic nephropathy, Tea polysaccharides, Lycium bararum polysaccharides, *Eucommia ulmoides*.

INTRODUCTION

Diabetic nephropathy (DN) is one of the most serious complications in diabetes mellitus. Once diabetic nephropathy happens, diabetic will have persistent proteinuria and declining renal function. The morbidity and mortality due to diabetic nephropathy is constantly increasing in industrialized nations[1].

Chinese medicines have been confirmed to have curative effects of prevention and cure of diabetic nephropathy at some extent in recent years[2,3]. The prescription used in this paper is a multi-herb mixture of extractives from *Lycium bararum*, green tea and *Eucommia ulmoides* leaves. *Lycium bararum* and *Eucommia ulmoides* are common medicines for tonifying kidney in Chinese medical. *Lycium bararum* has function of nourishing yin and tonifying kidney. *Lycium bararum* polysaccharides (LBP) as one of the important active ingredients in lycium bararum, has been confirmed to have nephroprotective function[4]. *Eucommia ulmoides* is a rare tonic herb and has function of nourishing liver and kidney and promoting urination. Research has been reported that polyphytocompound of *Eucommia ulmoides* and panax pseudoginseng has protective effect on early stage nephropathy secondary to experimentally-induced diabetes[5]. Green tea is a traditional health beneficial drink in many Asian countries. The important and effective ingredient of green tea, tea polysaccharide (TPS), has been reported to have hypoglycemic effect in many studies[6-8]. Therefore, LBP, TPS and *Eucommia ulmoides* are effective active ingredients for curing diabetes and diabetic nephropathy in traditional medicines.

In present study, the prescription was made from TPS, LBP and water extracts of *Eucommia ulmoides* leaves (WEEL). Animal experiment was designed to investigate the prescription potential effects on preventing the development of diabetic nephropathy of diabetic rats.

EXPERIMENTAL

Sample preparation and HPLC analysis: Base on the previous report[9], lipids, monosaccharides and oligosaccharides were removed from ground dried fruit of *Lycium barbarum*. After concentration, sedimentation and lyophilization, the crude LBP was obtained. Purity of LBP is 48.75 % by Sephadex G 200.

Crude TPS was extracted according to our lab method reported before[10]. 10 g leaf of *Eucommia ulmoides* was exposed to 100 mL hot water (60-70 ºC, 1 h) in a cone flask which put in shaking water-bath apparatus. Then the decoction was

obtained after filtration. WEEL was obtained after lyophilisation of the liquid. Purity of TPS is 37.63 % by HPLC. The WEEL was standardized to contain 3.58 % pinoresinol diglucoside, 63.3 % crude flavonoids, 1.2 % iridoids and 1.445 % chlorogenic acid.

The prescription was prepared by mixing extracts of the three herbs. The proportion of crude LBP, TBS and WEEL were 1.5:3:1 in prescription.

Experimental protocols: 50 male adults Sprague-Dawley (SD) rats aged 8 weeks (200 ± 10 g) were supplied by Shanghai animal experimental center of Chinese academy of sciences. They were housed single per cage with a 12/12 h light/dark cycle and an ambient temperature of 22-25 ºC. 10 rats were randomly selected as normal control (group C, n = 10) with a normal diet which was eligible to the standard of China for rats; the others administered as diabetic nephropathy model groups. The model rats were induced based on reference[11] and our lab works before. Animals in model group were fed for six weeks with a high-fat diet (65 % common diet + 10 % plant oil + 10 % lard + 10 % sucrose + 5 % cholesterol), then injected with streptozotocin (STZ, 30 mg/kg body weight (BW), Sigma, USA) by celiac injection, followed by more two weeks feeding with high-fat feed. Glucose (Glu) in serum and serum creatinine (Scr) were measured and clearance rate of creatinine (Ccr) was computed. A fasting blood glucose level of 16.7 mmol/L was used as a minimum value to define diabetes. The diabetic nephropathy model rats were defined mainly in terms of albuminuria and levels of Scr and Ccr. Renal hypertrophy was also considered. Ccr was calculated as follows[12].

$$\text{Ccr (mL/min/kg)} = \text{Urine creatinine (mmol/L)} \times \text{urine volume produced per minute (mL/min)} / \text{Serum creatine (mmol/L)} \times \text{body weight (kg)}$$

Then the model rats were divided into four different groups: diabetic nephropathy model control group (given with distilled water like group C and group DM), low dose of the optimized formula prescription (162.5 mg/kg.bw/d, DM + L), middle dose (325 mg/kg.bw/d, group DM + M) and high dose (650 mg/kg.bw/d, group DM + H). The prescription was given every day intragastrically. During the intervention period of 30 days both normal control rats and diabetic nephropathy model rats were continued on their original diets (normal diet or high-fat diet).

All investigations were carried out in accordance with the 'Principles of laboratory animal care' of NIH and the protocol for animal study of Animal Management Committee of Jiangsu Province, China.

Collection of blood and bioassay: At the end of the intervention period, the 24 h's urine was collected and urine protein (UPr) was determined. The blood samples were withdrawn from femoral. One kidney from executed animal was fixed in 10 % formalin for histopathological examination. Tissue homogenate was prepared by the other kidney of rats to assay aldose reductase (AR) and protein kinase C (PKC).

Laboratory analyses: Glu in serum was measured by glucose oxidase method with assay kits (Rongsheng Biotech Inc., Shanghai, China). Insulin was determined by radioimmunoassay (Kits from Chemclin Biotech Inc., Beijing, China). Urea nitrogen (BUN) in serum was performed by Urease Bertholer method. 24 h Pr and Scr were performed by Coomassie brilliant blue method and carbazotic acid method, respectively. All kits purchased from Jiancheng Bioengineering Institute, Nanjing, China. Serum AGEs-*p* was analyzed with flow-injection analysis method[13]. Kidney AR was measured according to YANG[14]. PKC measurement was accorded to the instruction of kit that was provided by Gibco Company. Kidneys were processed for light microscopic examination by using haematoxylin-eosin (HE) stain sections. Transforming growth factor-β (TGF-β) was determined by immunohistochemistry staining.

Statistical analysis: Data were shown as means ± standard deviation ($\bar{x} \pm s$). Statistical significance was estimated by one-way analysis of variance (ANOVA) among the multiple groups and SNK test among comparison of different prescription groups. Repeated-measures ANOVA were used to analyze the differences of bodyweight or Glu. A *p*-value of less than 0.05 was regarded to be significant. All statistical analyses were performed using the software of SPSS (v. 13) program.

RESULTS AND DISCUSSION

Effects of the prescription on physical and blood indicates of diabetic nephropathy rats: After success of making the model of diabetic nephropathy rat, model rats showed polydipsia and hydrouria with hair tangle and acted slowly, while group C didn't behave disorderly. There were two rats died in group DM and one rat in groups DM + L and DM + M during the experiment. At the first week of intervention, the model rats had no significant differences between group DM and prescription groups (group DM + L, DM + M, DM + H) in body weight (ANOVA: $F = 1.713$, $p = 0.166$). After the experiment, repeated-measures ANOVA showed that body weights increased in prescription groups compared with group DM; although they were still lower than group C (Fig. 1A). The prescription decreased the fasting and postprandial serum glucose (Fig. 1B) and the areas under the curve of prescription groups were significantly different with group DM after prescription treatment (Table-1). These indicated that treatment with the prescription resulted in a significant reduction of serum glucose of the diabetic nephropathy rats.

The 24 h's urine and 24 h UPr was significantly increased in model rats compared with group C. However after administering with the prescription, 24 h urine and 24 h UPr reduced in prescription group than in group DM ($p < 0.05$) (Table-1). These showed that the prescription could ameliorate the symptom of reduction in bodyweight and polyuria caused by diabetes.

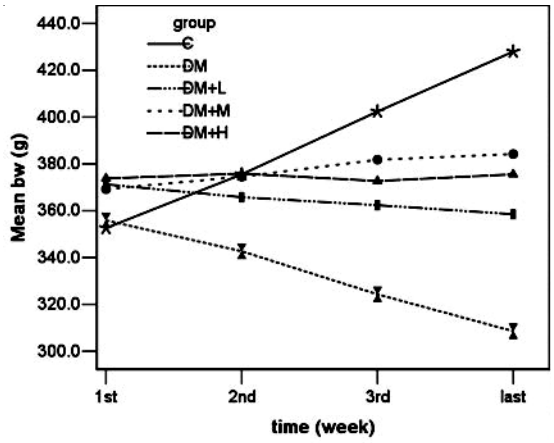
The insulin in group DM + M and DM + H were higher compared with group DM and the differences were significant ($p < 0.05$). Meanwhile, ISI was significantly increased in three prescription groups ($p < 0.05$) (Table-1), showed that the prescription could enhance to excrete and elevate the sensitivity to INS, especially the middle and high dose.

The levels of BUN, Scr and Ccr increased obviously in group DM and had significant difference with group C ($p < 0.05$). Contrast with group DM, these parameters decreased significantly in prescription groups. There were significant differences between group DM and prescription groups ($p < 0.05$), these indicated that the prescription had effect of

TABLE-1
EFFECTS OF THE PRESCRIPTION ON URINEANALYSIS AND BLOOD TEST OF DIABETIC NEPHROPATHY MODEL RATS ($\bar{x} \pm s$)

Group (n)	C(10)	DM(8)	DM + L(9)	DM + M(9)	DM + H(10)
Urine analysis					
24 h's urine (mL)	17.6 ± 3.4	113.6 ± 8.9[a]	74.2 ± 12.2[ab]	63.8 ± 15.7[ab]	70.1 ± 11.9[ab]
24 hUPr(mg)	12.37 ± 1.51	41.74 ± 3.70[a]	21.82 ± 2.78[ab]	22.64 ± 2.46[ab]	23.78 ± 2.03[ab]
Blood test					
Glu AUC	14.16 ± 1.14	66.31 ± 6.55[a]	41.80 ± 7.78[ab]	38.58 ± 10.24[ab]	37.49 ± 8.62[ab]
Ins (mIU/L)	32.65 ± 4.74	29.85 ± 3.99	35.04 ± 4.39	37.60 ± 6.39[b]	40.83 ± 4.64[ab]
ISI	-5.08 ± 0.14	-6.71 ± 0.18[a]	-6.24 ± 0.23[ab]	-6.01 ± 0.28[ab]	-6.18 ± 0.17[ab]
BUN (mM)	7.68 ± 0.69	17.49 ± 1.99[a]	10.54 ± 1.71[abc]	11.29 ± 1.55[abc]	8.75 ± 1.27[b]
Scr (?M)	57.42 ± 4.10	82.89 ± 3.75[a]	71.50 ± 3.16[ab]	62.55 ± 4.57[abd]	66.57 ± 5.96[abd]
Ccr (mL/min/kg)	0.54 ± 0.18	2.78 ± 0.37[a]	1.51 ± 0.42[ab]	1.35 ± 0.38[ab]	1.46 ± 0.35[ab]
AGEs (U/mL)	0.87 ± 0.12	3.36 ± 0.74[a]	2.14 ± 0.25[ab]	1.85 ± 0.33[ab]	1.93 ± 0.22[ab]

[a] $p < 0.05$, ANOVA, *versus* values for normal control group (group C); [b] $p < 0.05$, ANOVA, *versus* values for model control group (group DM); [c] $p < 0.05$, SNK test, as compared with group DM + H; [d] $p < 0.05$, SNK test, as compared with group DM + L.



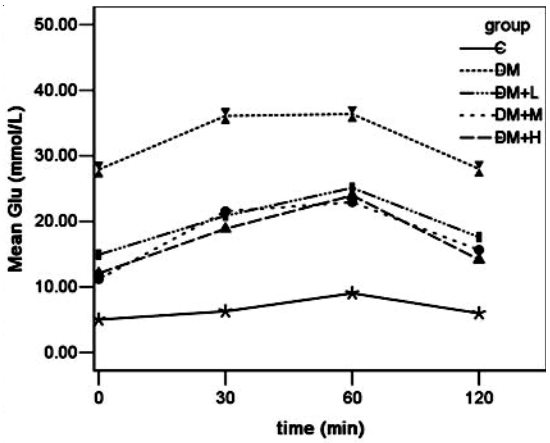
repeated-measures ANOVA:
BW*group (F=5.117,P<0.001);
group (*F*=26.090,*P*<0.001)

Student-Newman-Keuls[a,b]

group	N	Subset 1	Subset 2	Subset 3
DM	8	332.850		
DM•L	9		364.478	
DM•H	10		374.470	
DM•M	9		377.439	
C	10			389.605
Sig.		1.000	.075	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 151.622.
a. Uses Harmonic Mean Sample Size = 9.137.
b. Alpha = .05.

A. Trend of body weight



repeated-measures ANOVA:
Glu*group (F=3.072,P<0.01);
group (*F*=67.744,*P*<0.001)

Student-Newman-Keuls[a,b,c]

group	N	Subset 1	Subset 2	Subset 3
C	10	6.5790		
DM•H	10		17.2596	
DM•M	9		17.8262	
DM•L	9		19.6275	
DM	8			32.0947
Sig.		1.000	.283	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 10.786.
a. Uses Harmonic Mean Sample Size = 9.137.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = .05.

B. Trend of blood glucose

Fig. 1. Trends of body weight and blood glucose after the prescription administrated

improving the renal function in diabetic nephropathy rats. Using SNK test, there were significant differences in groups DM + L and DM + M *versus* DM + H for BUN ($p < 0.05$) as well as group DM + L *versus* DM + M and DM + H for Scr ($p < 0.05$). But there was no difference in 24 h Pr and Ccr. These indicated that giving high dose of prescription was more benefit than giving low or middle dose in increasing BUN scavenging, while no difference in Scr scavenging during three prescription groups (Table-1).

In this study, we observed that AGEs significantly increased in the group DM ($p < 0.05$). The levels of serum AGEs in the prescription supplement groups were significantly lower than group DM ($p < 0.05$) (Table-1), indicating the prescription reduced the level of AGEs in diabetic nephropathy rats, thus preventing the occurrence of complications such as DN. There were no significant differences for AGEs among prescription groups using SNK test.

Effects of the prescription on the renal functional indexes of diabetic nephropathy rats

Effects on kidney index: Kidney weight and kidney index of group DM were greater than group C and the differences were significant ($p < 0.05$). But they decreased after administered by the prescription. Although kidney indexes were still

higher than group C in group DM + M and DM + H, there was no statistical difference during them ($p > 0.05$) (Table-2). This provided evidence for the prescription nephroprotective effect.

Effects on activities of AR: Polyol pathway is one of the glucose metabolism pathways and has an extremely close relationship with the pathological process of DN. AR is the key rate-limiting enzyme for polyol pathway. Thus AR activity was determined by NADPH and NADH reaction systems. The results showed that NADPH reaction rate in group DM was significantly higher than group C ($p < 0.05$), indicating that AR activity of group DM was significantly activated. NADPH reaction rate in prescription groups were significantly lower than group DM ($p < 0.05$) and no difference with group C ($p > 0.05$) (Table-3). NADH reaction rate in prescription groups is also lower than group DM, although it had no difference among each group, indicating that the prescription could partly inhibit AR activity. Among three prescription groups, there is no significant difference for NADPH and NADP levels.

Effects on activities of PKC: The study found that the gross activity of PKC in each group has no significant difference ($p > 0.05$). Compared with group C, cytoplasm PKC activity was significantly decreased and membrane PKC activity increased inversely in group DM ($p < 0.05$). However cytoplasm PKC activity in prescription groups were significantly higher than group DM ($p < 0.05$), while as both membrane PKC activity and percentage of membrane counted for gross activity were significantly lower than group DM ($p < 0.05$) (Table-3). Compared three prescription groups, there were no significant differences for percentage of membrane counted for gross activity using SNK test.

Effects of the prescription on histopathology of rat kidney

Glomerular pathological changes (HE staining): At the end of experiment, renal glomeruli of group DM could be seen clearly enlargement under light microscope. Besides that glomerular capillary basement became thickness, mesangial areas became wide and proximal tubular epithelial cells showed a large number of degeneration. Compared with group DM, the pathological changes of kidney in prescription groups were alleviated, especially the high dose group (group DM + H).

Effect of the prescription on expression of transforming growth factor-β (TGF-β) of rat kidney: Result of immunohistochemistry staining indicates few scattered brown granules which were TGF-β can be seen in renal cortex of group C. However, there were abundant granules in group DM and the colour was strengthened obviously. And image analysis measurement found that kidney tissue stained gray in group DM was significantly higher than group C ($p < 0.05$) (Table-4). However it was alleviated by the prescription administered. Kidney tissue stained gray in the prescription groups were lower than group DM ($p < 0.05$).

TABLE-4
EFFECTS OF THE PRESCRIPTION ON RENAL TGF-β EXPRESSION IN EACH GROUP

Group	n	TGF-β(A)
C	10	0.48 ± 0.02
DM	8	0.76 ± 0.03[a]
DM + L	9	0.65 ± 0.02[ab]
DM + M	9	0.61 ± 0.03[ab]
DM + H	10	0.62 ± 0.02[ab]

[a]$p < 0.05$, ANOVA, *versus* values for normal control group (group C); [b]$p < 0.05$, ANOVA, *versus* values for model control group (group DM).

Many research showed that LBP, TPS and *Eucommia ulmoides* leaves have effects of reducing hyperglycemic and improving hyperlipemia. It is known that hyperglycemic and dyslipidemia are important pathogenic factors for generation of diabetes and its complications. It is an orientation to use LBP, TPS and *Eucommia ulmoides* leaves to cure diabetes and its complications. The nephroprotective effect of LBP is reported in literature[4]. According to research, synergic action of the compound that blended by LBP and TPS is better than single using of them. Here, we use a model of diabetic neph-

TABLE-2
EFFECTS OF THE PRESCRIPTION ON KIDNEY INDEX OF DIABETIC NEPHROPATHY MODEL RATS ($\bar{x} \pm s$)

Group (n)	C(10)	DM(8)	DM+L(9)	DM + M(9)	DM + H(10)
Kidney weight (g)	2.62 ± 0.47	3.10 ± 0.48[a]	2.76 ± 0.46[b]	2.78 ± 0.50[b]	2.75 ± 0.47[b]
Body weight (g)	427 ± 34.3	308 ± 30.9[a]	358 ± 32.1[ab]	384 ± 27.7[ab]	375 ± 21.5[ab]
Kidney index	6.15 ± 0.70	10.08 ± 0.85[a]	7.71 ± 0.47[ab]	7.24 ± 0.35[b]	7.33 ± 0.44[b]

[a]$p < 0.05$, ANOVA, *versus* values for normal control group (group C); [b]$p < 0.05$, ANOVA, *versus* values for model control group (group DM). The kidney index was calculated as (kidney weight× 1000/body weight).

TABLE-3
EFFECTS OF THE PRESCRIPTION ON ACTIVITIES OF KIDNEY AR AND PKC IN DIABETIC NEPHROPATHY MODEL RATS

Group (n)	C(10)	DM(8)	DM + L(9)	DM + M(9)	DM + H(10)
AR (A)					
NADPH	0.089 ± 0.024	0.167 ± 0.019[a]	0.093 ± 0.025[b]	0.095 ± 0.025[b]	0.086 ± 0.023[b]
NADH	0.066 ± 0.025	0.126 ± 0.035[a]	0.105 ± 0.035	0.086 ± 0.048	0.090 ± 0.038
PKC [pmol/(min mg pro)]					
Cytoplasm PKC activity	42.33 ± 5.21	23.68 ± 7.26[a]	31.01 ± 4.83[ab]	34.02 ± 5.65[ab]	31.89 ± 6.08[ab]
Membrane PKC activity	9.21 ± 3.78	25.49 ± 4.70[a]	15.70 ± 6.54[b]	14.16 ± 3.93[b]	14.75 ± 6.73[b]
Gross activity	51.33 ± 5.02	49.17 ± 8.59	46.72 ± 5.73	48.18 ± 6.54	46.64 ± 7.38
Membrane PKC activity accounted for gross activity (%)					
	17.82 ± 7.06	52.33 ± 8.13[a]	33.02 ± 10.84[ab]	29.42 ± 7.34[ab]	31.14 ± 11.44[ab]

[a]$p < 0.05$, ANOVA, *versus* values for normal control group (group C); [b]$p < 0.05$, ANOVA, *versus* values for model control group (group DM).

ropathy rat that were induced by high-fat diet and injection of STZ to investigate its effects of the prescription that made of LBP, TPS and WEEL. Clinically, DN presents in its earliest stage with microalbuminuria and low levels of Ccr[14]. For present pathophysiological and biochemical results, we are confident that present DN model would correspond to early stage of DN and was thus suitable for assessing effect of the prescription on renal function.

According to the result of study, the prescription that made from TPS, LBP and WEEL reduced blood Glu and elevating excretion and sensitivity of insulin. Body weight and polyuria caused by diabetes in rats were improved, indicating that the prescription ameliorated symptoms of diabetes. Besides that, renal failure caused by diabetes were improved after the prescription treatment, such as reducing kidney weight and kidney index, as well as reducing levels of BUN, 24 h UPr, Scr and Ccr. These indicated that the prescription was also benefit to ameliorate kidney function of DN rats.

In addition to above, other factors in the development of DN, generation of AGEs, acceleration of AR path and activation of PKC[15,16] caused by hyperglycemia were also be improved.

Advanced glycosylation end products can be cross-linked with the glomerular basement membrane components, contributing to the thickening of the membrane and altering signal transduction *via* alteration in the level of soluble signals, such as cytokines, hormones and free radicals[17]. The net effect of tissue accumulation of AGEs leads to the development of DN and other microvascular complications[18]. In this study, AGEs significantly increased in the group DM compared with control. However, it dropped after the prescription treatment. This meant that the prescription was benefit to reduce AGEs.

Acceleration of AR path is another factor for development of DN. Under the action of AR, glucose converts into sorbitol and then it changes into fructose again under the action of sorbitol dehydrogenase. Because sorbitol is not easy to through the cell membrane and the further metabolism failure, leading to accumulation of intracellular sorbitol and fructose, resulting in hyperosmolar state of cell swelling damage[19]. Generally, NADPH and NADH reaction systems were used to determine AR activity. NADPH reaction rate in prescription groups were decreased, despite not in NADH reaction rate in our study. It meant this prescription had the effect of depressing AR path. This may be one of the mechanisms to control DN for the prescription.

Other proposed mechanism that promotes the development of DN including activation of PKC[20]. Activation of PKC leads to increasing secretion of vasodilatory prostanoids, which contributes to glomerular hyperfiltration and production of extracellular matrix by mesangial cells[21]. The phenomenon that PKC transfer to membrane is an important indicator for PKC activation. In present study, the gross activities of PKC in each group were similar. Cytoplasm PKC activity decrease and membrane PKC activity increase indicated over activation of PKC in group DM. Renal function of group DM was significantly abnormal in this experiment. Reduce of membrane PKC activity after administered by the prescription indicated that the prescription was effective to suppress over activation of PKC. Renal functions were improved after administration by the prescription. The mechanism may be related to inhibition of excessive activation of PKC activity.

The levels of TGF-β1 are increased in the glomeruli of rats with streptozotocin-induced diabetes and use of neutralizing antibody to TGF-β1 could prevent renal changes of DN in these animals[22]. According to the results, TGF-β expression increased in group DM, confirming that TGF-β was involved in the pathogenesis of DN. The function how the prescription delayed the occurrence and development of DN and protecting the kidney may be achieved by inhibiting TGF-β production in the environment of high glucose. But the signaling pathway need further research to study how the prescription to resist TGF-β formation.

Function of the prescription made of TPS, LBP and WEEL used in this experiment indicated that the prescription could rectify metabolize turbulence of glucose, simultaneously reducing the injury of glomerular basement membrane and dramatically improving the function of kidney in diabetes rats. It may play a critical role by much pathway on DN especially for high concentration (650 mg/kg bw/d).

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