The effects of the water-extraction of *Astragali Radix* and *Lycopi herba* on the Pathway of TGF-smads-UPP in a rat model of Diabetic Nephropathy

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ABSTRACT

Background: Astragali Radix and Lycopi Herba were widely used in clinical practice for treating the diabetic nephropathy (DN), but their therapeutic mechanisms were not clear. **Objective:** To observe the effects of the water-extraction of Astragali Radix and Lycopi Herba on the signaling pathway of TGF-Smads-UPP in streptozotocin (STZ)-induced DN. **Materials and Methods:** Sprague-Dawley (SD) rats were randomly divided into the normal control (NC) group and the model group. The NC group was fed with a standard diet and the other five diabetic groups received a high-fat diet. After 4 weeks, five diabetic groups were treated with STZ (30mg/kg i.p.). The NC group rats were treated with citrate buffer. Tail random blood glucose (RBG) was measured 72h later using a strip-operated blood glucose sensor and monitored every 2 weeks until drug intervention. Rats with RBG levels less than 16.7mmol/L were excluded from the diabetic groups. At the end of 4 weeks after STZ injection, 24h microalbuminuria was collected and detected. The microalbuminuria was measured by radioimmunoassay (RIA). The blood glucose was tested using a blood glucose meter. The kidney was dissected from each SD rat. Proteins and mRNA of TGF-β1, Smads and Smurf were tested by western-blot and real-time PCR analysis, and 26S proteasome activity was measured by an ELISA kit. **Results:** The water-extraction of Astragali Radix and Lycopi Herba significantly lowered fasting glucose and urine albumin in diabetic rats through inhibition of TGF-β1 mRNA and protein expression in the STZ-induced diabetic rats, and regulation of the Smad3, Smad7, Smurf1, Smurf2 mRNA and protein expression, as well as elevated 26S proteasome activity to play control effect in DN. **Conclusion:** 0.9 g/ml water-extraction of Astragali Radix and Lycopi Herba group has significant therapeutic effects on the STZ-induced diabetic rats, and this regulation depends on TGF-Smads-UPP signaling pathway.

Key words: Astragali Radix, diabetic nephropathy, lycopi herba, TGF-Smads-UPP

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INTRODUCTION

Diabetic nephropathy (DN) is a microangiopathy in kidney which is caused by diabetes. It is a serious systemic complication of diabetes. Damage of kidney leads to glomerular hyperperfusion and hyperfiltration in the early step of the DN, followed by intermittent and persistent microalbuminuria, resulting in overt proteinuria, which was the expression of DN clinically. It eventually leads to kidney failure.[1] Currently, kidney disease of type 2 diabetes has a similar process, but the time of onset is less precise.

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The high blood glucose may be the initial factor, and through a series of intracellular signal transduction pathways it regulates transforming growth factor-β1 (TGF-β1)-induced extracellular matrix accumulation, and leads to glomerular sclerosis, thus mediates the occurrence and development of DN.[2] Ubiquitin-protein ligase E3 includes the Smad ubiquitination regulatory factor 1 (Smurf1), Smad ubiquitination regulatory factor 2 (Smurf2). The Smurf1 has effects on the Smad1 and Smad5, and inhibits the TGF-β signaling pathway by Smad1, Smad5, Smad 6. Effecting on the Smad2 and Smad3, the target of Smurf2 may be TGF-β/Smad2 signaling pathway, and block the TGF-β signaling pathway significantly.[3,4]

A recent study showed that, among other changes, the expression of genes related to the ubiquitin-proteasome system was consistently altered in type 2 diabetic islets. The ubiquitin-proteasome system is a major intracellular

pathway for degradation of proteins.[5] The ubiquitinated protein recognized by the proteasome is degraded. Polyubiquitinated proteins is referred to as the 26S proteasome, which has a 20S catalytic core and two 19S regulatory caps.[6]

For thousands of years, traditional Chinese medicine (TCM) has played an indispensable role in the fight against disease in China. Investigations of the effects of TCM are now attracting increasing attention around the world. Astragali Radix and Lycopi Herba were traditional medicine used to treat the DN clinically. The water-extraction of Astragali Radix and Lycopi Herba had significant curative effect to reduce blood glucose and alleviate DN clinically, but the mechanism remained unclear. In this research, the water-extraction of Astragali Radix and Lycopi Herba might play a controlling effect by the TGF-β1 to interfere the specific signaling proteins of TGF-Smads. The study on the water-extraction of Astragali Radix and Lycopi Herba to treat the DN was carried on, and the results revealed the pathological mechanisms of DN were closely related with the TGF-β1 and TGF-Smads. With all this in mind, hereby we provide evidence that changes in the TGF-Smads-UPP pathway may be involved in DN.

MATERIALS AND METHODS

Animals and drugs

Animals Adult male and female Sprague–Dawley (SD) rats (6 weeks of age, 200 ± 20g) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (certificate No. 0194036). Rats were housed in a 12h light–dark cycles and had free access to chow and water. All animal experiments were conducted in accordance with the NIH Principles of Laboratory Animal Care and the institutional guidelines for the care and use of laboratory animals at Liaoning University of TCM.

The dosage ratio of Astragali Radix and Lycopi Herba as 1: 1 and preparation into the decoction which contains the original herbs 0.1, 0.3, 0.9/ml.

Model and grouping

Animals were divided into the following groups: Normal control (NC), positive control (PC), diabetes, and diabetic rats were treated with the water-extraction of Astragali Radix and Lycopi Herba (0.1, 0.3, 0.9 g/ml). The NC group was fed with a standard diet and the other groups received a high-fat diet. After 4 weeks, PC, diabetic and treat groups were treated with streptozotocin (STZ) (30 mg/kg i.p.) that had been freshly dissolved in citrate buffer (Sigma, St Louis, MO, USA). The NC group was administered vehicle citrate buffer. Tail random blood glucose (RBG) was measured 72h later with a strip-operated blood glucose sensor (J & J, New Jersey, USA) and monitored every 2 weeks until drug intervention. Rats with RBG levels less than 16.7 mmol/L were excluded from the diabetic group. After two weeks of the model mode, the PC group was treated with benazepril hydrochloride, the treat groups were treated with the water-extraction of Astragali Radix and Lycopi Herba (0.1, 0.3, 0.9 g/ml), NC and diabetic groups were fed with water, and kept for 4 weeks. During the experiment with free water, insulin and other hypoglycemic agents were not used.

Western blot analysis

The proteins of renal cortex extracts were separated by cell lysates buffer (20 mmol/L Tris-HCL, PH 7.5, 0.1 mmol/L Na3VO4, 25 mmol/L NaF, 25 mmol/L β-glycerophosphate, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 2 μg/ml leupeptin) followed by electrotransfer to Nitrocellulose membrane and probed with anti- TGF-β1(Santa Cruza, USA), anti- Smad3 and Smad7 (Santa Cruza, USA), anti-mouse/rabbit-Smurf1 and -Smurf2(Santa Cruza, USA). Secondary antibodies added a marker on alkaline phosphatase. The quantity of the results was analysed by FlourChem V 2.0 (American).

RT-PCR analysis

Total RNA was extracted from rat kidney cortices with TRIzol reagent (Invitrogen, USA).A standard reverse transcriptase reaction kit (Toyobo, Japan) was used to synthesize cDNA. The primer pairs were designed from the published cDNA sequences encoding rat TGF-β1, Smad3, Smad7, Smurf1 and Smur2. The primer sequences for TGF-β1 were 5'-GGTGGACCGCAACAACG TGAGCACTGAAGCGAAAGC-3' (sense) and 5'-CGTGCGTGACATTAAAGAG TTGCCGATAGTGATGACCT-3' (antisense); for Smad3 were 5'-AGGGCTTTGAGGCTGTCTACC GTCCAC GCTGGCATCTTCTG-3' (sense) and 5'-CCGTAAAGACCTCTATGCCAACA CGGACTCATCGTACTCC TGCT-3' (antisense); for Smad7 were 5'-CCCCTTTGGATCAGCATT TGTGGATAGGCCCGTGT-3' (sense) and 5'-CGTGCGTGACATTAAAGAG TTGCCGATAGTGATGACCT-3' (antisense); for Smurf1 were 5'- GGTGGCACTGCACTCCTAGAAC GCGCGGACCCAAAGTAGAAC-3 (sense) and 5'-GCCAACACAGTGCTGTCT AGGAGCAATGATCTTGATCTT-3' (antisense); for Smurf2 were 5'-GGGAACGCCCAACAAGAC ATTGCGGATCTCCCACCC-3 (sense) and 5'-CTACCAGCGTTTGGATCTAT TGTCTCGGGTCTGTAAACT-3' (antisense). PCR performed with TProfessional standard PCR (Biometra, Germany); thermal cycling conditions were 90°C for 30s and 95°C for 5s, follow by 40 cycles of 95°C for 15s, 60°C for 1 min, and finally, 95°C for 15s. The relative amount

of mRNA was calculated with the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

ELISA analysis

The activity of 26s proteasome of renal tissue was detected by ELISA kit (R and D, USA) according to the manufacturer's instructions.

Measurement of blood glucose

Blood glucose was measured by a blood glucose meter (J & J, USA).

24h urine albumin

After being fed for 4 weeks, rats were fasted, but water was placed in metabolic cages to collect 24-hour urine and detect rats' urine albumin. Urinary albumin was detected by radioimmunoassay (RIA).

Statistical analysis

All data were expressed as mean ± S.D. Different groups were assessed by ANOVA, and the difference between each two groups was analyzed with Student's *t*-test by SPSS 18.0.

RESULTS

General conditions for rats

Except for the NC group, the rats from other groups all showed less ingestion in different degrees, low spirit, sluggish action, much more drinking of water, increased urine output and weight loss. The treatment groups which were treated with the water-extraction of Astragali Radix and Lycopi Herba had the best general condition.

Plasma glucose

Tested blood glucose at the end of the 4th week, diabetic rats had a significant increase in blood glucose, while a decrease in the treatment groups. Compared with the diabetes groups, the drug intervention groups significantly decreased (**$P < 0.01$) [Figure 1].

24h urine protein of rats

For the rats of the treatment group, the excretion of the urine protein was tested at the end of the 4th week. STZ-induced DN rats exhibited significantly higher urine protein levels compared with the NC rats, although the urine protein in STZ-DN rats treated with the water-extraction of Astragali Radix and Lycopi Herba was lower than that of vehicle treated diabetic group at the end of the 4th week after STZ-injection. Compared with the diabetic group, urine albumin had significantly decreased after drugs intervention (**$P < 0.01$) [Figure 2].

The expression of TGF-ß1 protein and its mRNA in DN rats

Compared with the NC group, the expression of TGF-β1 protein and its mRNA levels increased significantly in the diabetic group but decreased in the treatment groups, at the end of the 4th week after STZ- injection. The drug intervention groups significantly decreased compared with the diabetes group (**$P < 0.01$, *$P < 0.05$), and the 0.9 g/ml water-extraction can improve this phenomenon significantly [Figure 3].

The expression of Smad3 and Smad7 protein and its mRNA in DN rats

Smad3 and Smad7 protein expression existed in the normal rat kidney tissues, and there was a slight expression of Smad3 and Smad7 mRNA in the renal cortex. Compared with the control group, the Smad3 protein and mRNA expression of the diabetes and treatment groups increased significantly, but the Smad7 protein and mRNA expression declined significantly. Compared with the diabetes group, the Smad3 protein and mRNA expression of the treatment groups declined significantly, and the Smad7 protein and mRNA expression increased significantly. While, compared with the diabetes group, Smad3 protein and mRNA of the treatment groups declined significantly(**$P < 0.01$), but the Smad7 protein and mRNA increased significantly(**$P < 0.01$, *$P < 0.05$). In the treatment groups, the 0.9g/ml treatment group

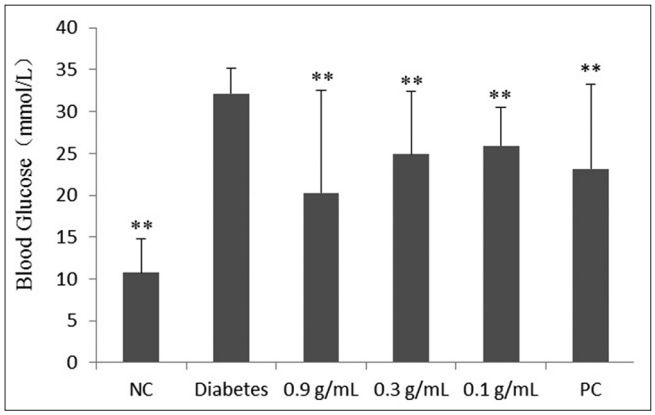

Figure 1: The test results of the fasting plasma glucose

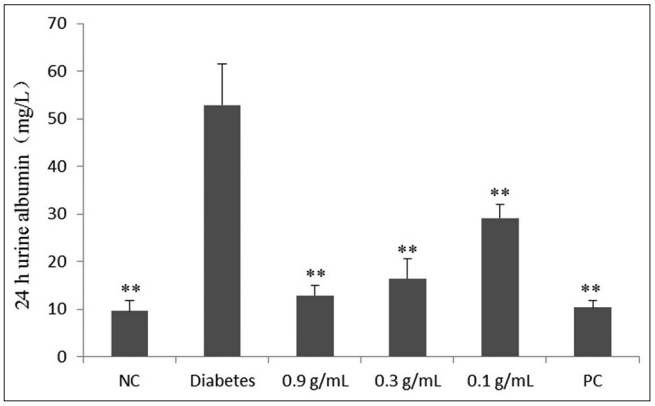

Figure 2: The results of 24 h urine protein of DN rats

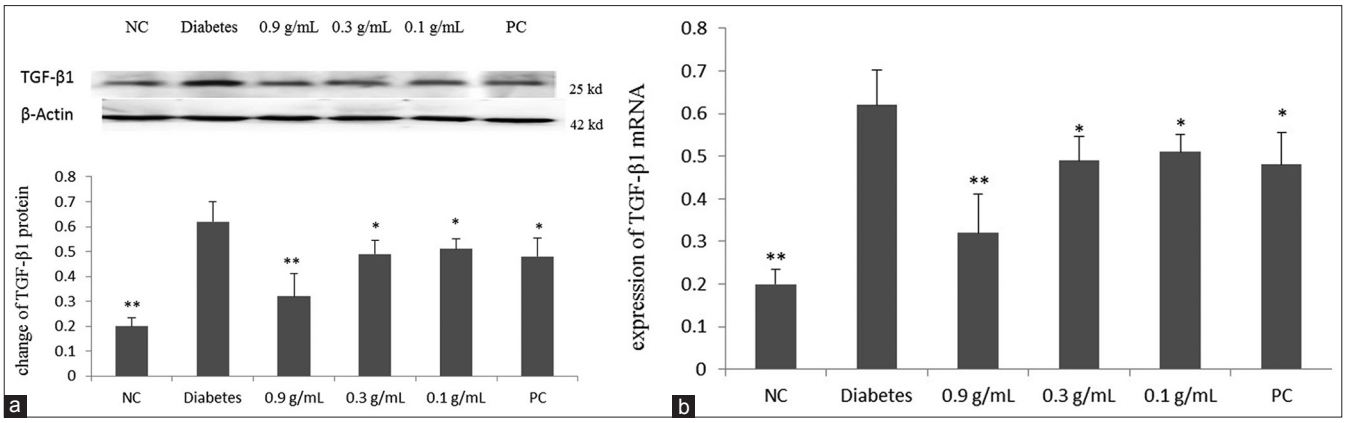

Figure 3: Compared with the control group of the expression of TGF-β1 (a) protein (b) mRNA

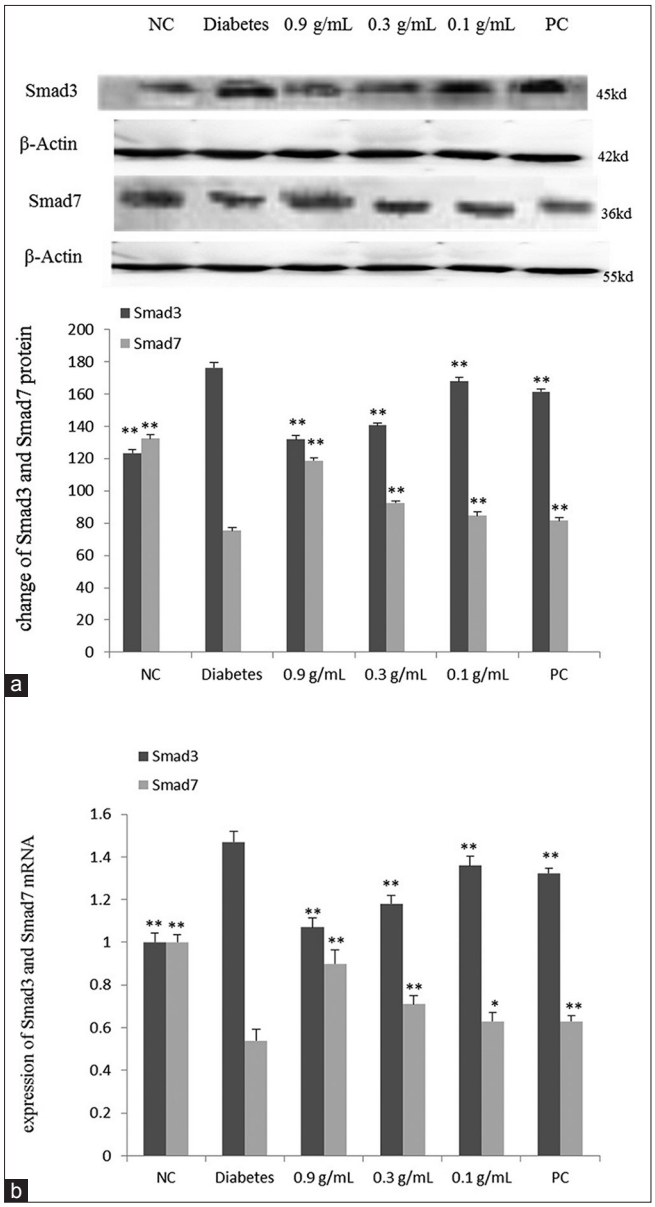

Figure 4: The expression of both Smad3 and smad7 (a) protein, (b)mRNA in DN rats

regulated the Smad3 and Smad7 protein and mRNA significantly(**$P < 0.01$) [Figure 4].

The expression of Smurf1 and Smurf2 protein and its mRNA in DN rats

At the end of the 4th week, the Western-blot results showed that the Smurf1 and Smurf2 protein expression could be detected in the normal rats' kidneys, and both Smurf1 and Smurf2 mRNA had a weakly expression in the rat renal cortex of the NC group. Except the NC group, the expression of protein and mRNA was enhanced significantly in the other groups, but compared with the diabetes group the expression of the protein and mRNA in the treatment group declined markedly(**$P < 0.01$). The expression of both Smurf1 and Smurf2 protein and mRNA declined significantly in the 0.9g/ml treatment group [Figure 5].

The effect of 26s proteasome active on the renal tissue of tats at early DN

At the end of the 4th week, to investigate the effect of STZ-induced DN rats on the proteasome of the rat renal cortex, proteasome activity was measured, and the results showed that 26s proteasome in the rat renal cortex of NC group had high activity, but in the diabetes group it significantly reduced. Compared with the diabetes group, activity of 26s proteasome showed a significant rise in the treatment groups(**$P < 0.01$). The activity of 26s proteasome was the most obvious in the 0.9 g/ml treatment group, and there was no marked difference in the other treatment groups [Figure 6].

DISCUSSION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and action. Diabetic nephropathy (DN), manifested by persistent and clinically evident proteinuria,

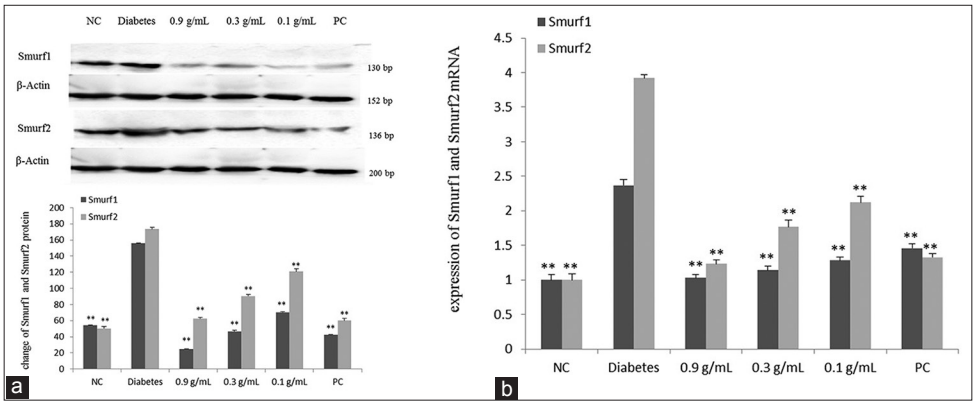

Figure 5: The expression of Smurf1 and Smurf2 (a) protein, (b) mRNA in DN rats

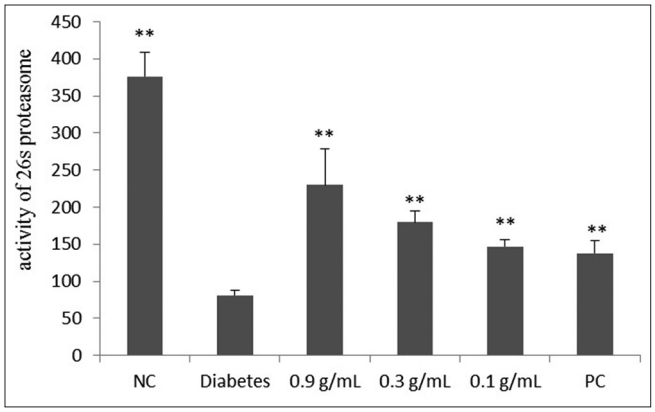

Figure 6: The activity of 26s proteasome

has been considered as an irreversible process for a long time that predicts a rapid decline in renal function.[7] In this research, we demonstrated that the water-extraction of Astragali Radix and Lycopi Herba reversed the damage of diabetic nephropathy by decreasing the levels of plasma glucose and 24h urinary protein as well as kidney weight/body weight.

The TGF-β in the pathogenesis of DN exerts its renal fibrotic action. Hence, developing agents that antagonize fibrogenic signals is a critical issue facing researchers.[8] The TGF-β superfamily is composed of many multifunctional cytokines, including TGF-β, activins, inhibins, anti-mullerian hormone, bone morphogenetic proteins, and myostatin.[9] In normal kidney, there is weak expression of different isoforms of TGF-β mRNA in a few glomerular cells, but remarkable difference is not found in the levels of mRNA expression in the glomeruli and the tubulointerstitium during diseased states.[10] Clinical studies have demonstrated that all three isoforms of TGF-β are elevated in both the glomerular and tubulointerstitial compartments of patients with diabetic nephropathy.[11,12]

In this study, the TGF-β mRNA and protein increased in the STZ-induced diabetic rats, which conformed to the characteristics of clinical diabetes patients. The water-extraction of Astragali Radix and Lycopi Herba reduced expression of the TGF-β1 protein and mRNA compared with diabetic group. The water-extraction of Astragali Radix and Lycopi Herba may be attributed to the reduction of TGF-β1 protein and mRNA synthesis and secretion and the inhibition of a variety of TGF-β1 mediated biological effects.

Smad2 and Smad3 are structurally very similar, with 91% identity in amino acid sequence. Studies on Smad2 and Smad3 knockout mouse phenotypes and comparing expression profiles of the TGF-β target genes in Smad2-deficient cells with those in Smad3-deficient cells, suggested that epithelial and mesenchymal cell exhibit distinct but overlapping Smad2 and Smad3 signaling.[13] TGF-β exerts its renal fibrotic action mainly through downstream signaling molecules Smad2 and Smad3, which, interestingly, are negatively regulated by inhibitory Smad7, which inhibits renal fibrosis and inflammation.[14-18] Both elevated TGF-β signaling and decreased Smad7 protein expression are often present in tissues where an uncontrolled fibrotic response occurs.[19] In kidney disease it has been reported that increased Smad7 mRNA expression is associated with loss of Smad7 protein expression.[20]

The water-extraction of Astragali Radix and Lycopi Herba increased the level of the protein of Smad3, and reduced the protein expression of Smad7. The experimental results illustrate that the water-extraction of Astragali Radix and Lycopi Herba influenced TGF-β to adjust the mRNA and protein of Smad3 and Smad7. The TGF-β/Smad signaling pathways was further confirmed when treatment with anti- TGF-β antibody was shown to significantly reduce renal fibrosis and proteinuria through the inhibition of Smad/TGF-β signaling.

Now we show that the water-extraction of Astragali Radix and Lycopi Herba can also strenghen proteasomal activity. Recently, transcriptome analysis on type 2 diabetes

suggested dysfunction of pancreatic beta cells was at the mercy of ubiquitin-proteasome system.[21] With all this in mind, hereby we provide evidence that the ubiquitin proteasome may be involved in Smad/TGF-β signaling in DN which caused by the type 2 diabetes.

In this research, the water-extraction of Astragali Radix and Lycopi Herba can improve the clinical symptoms for the DN rats which are caused by STZ, and contribute to identifying features of TGF-β-Smad-UPP pathway that may be crucial for the onset and progression of the disease. Whether this concept is applicable to DN will require specifically designed studies.

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