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Original Article

The aqueous extract of Lycopus lucidus Turcz exerts protective effects on podocytes injury of diabetic nephropathy via inhibiting TGF-β1 signal pathway

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Abstract: Diabetic nephropathy (DN) is known as a major microvascular complication leading cause of end-stage renal disease, it generally followed by the process of podocyte fragmentation and detachment. Transforming growth factor β1 (TGF-β1) signaling pathway plays a pivotal role in the initiation and progression of DN. In present study, we aim to investigate the effect of lycopus extracts on podocytes injury and TGF-β signaling. In present study, lycopus extracts treatment abolished the gain in blood glucose and body weight in a dose dependent manner and possessed protective effect on the renal damage, which was indicated by the decreased concentration of Scr, BUN and urine creatinine of serum. Histopathological examination also demonstrated lycopus extracts exert protective effect on renal damage. Western blotting and immunohistochemical results revealed lycopus extracts treatment upregulated the expression of nephrin and down-regulated the expression levels of TGF-β1 and Smad4. Moreover, lycopus extracts treatment suppressed TGF-β1-induced phosphorylation of Smad2/3, ERK1/2 and p38 both in vivo and vitro. In conclusion, lycopus extracts is a novel agent that ameliorate podocytes injury by inhibiting TGF-β signaling pathway and possess potential therapeutic effect on renal damage of DN rats.

Keywords: Diabetic nephropathy, podocytes, Lycopus lucidus Turcz, TGF-β1, Smads

Introduction

Diabetic nephropathy (DN) is known as s a major microvascular complication leading cause of end-stage renal disease (ESRD) occurring in diabetes mellitus (DM) [1]. It is reported that about 30% DN patients may suffer from ESRD [2]. Previous studies have indicated that DN are the vital player in increasing mortality and morbidity in developed countries because of its severe consequences and high prevalence [3]. For this reason, it is urgent to study the pathophysiologic mechanism underlying the progression of DN induced by hyper glycaemia.

Proteinuria, a risk factor for progression of DN, is caused by podocyte pathology including the abnormalities of morphology and function in podocytes [4]. Podocytes, found lining the Bowman's capsules in the kidneys, are identified as highly specialized cells possessed multiple metabolic and structural functions. Cell hypertrophy, induced by accumulation of advanced glycation end-products (AGEs), is often preceded by diabetic podocytopathy [5]. Slit diaphragm injuries were induced the podocyte hyperactivity, followed by a decreased volume and the amounts of organelles. The pedicels were effaced completely followed by the thinner primary processes of podocytes. In addition, the fragmentation and detachment of podocytes were also occurred sequentially. All these actions denote the slit diaphragm degradation and the development of proteinuria [6]. After injury, the podo-

cytes undergo the process of epithelial-mesenchymal transition (EMT) through downregulation of expression levels of epithelial markers, including nephrin [7]. It is well established that high glucose conditions can promote podocyte EMT [8]. Changes of nephrin consistent with the epithelial feature expression [9].

Previous studies have demonstrated that TGF-β receptor type I (TGF-βRI) kinase is activated when TGF-β bind to TGF-βRII, which result in the phosphorylation of Smad2/3. Subsequently, Smad complex, composed by phosphorylated Smad2/3 and normal Smad4, was formed to regulate its target gene transcription when the complex translocated into the nucleus [10]. External stimuli, such as cell injury, may activate some pathways to affect TGF-beta signaling. It has been shown that the stress-activated protein kinases p38 and ERK play fundamental roles in TGF-beta signaling in a variety of systems [11]. TGF-β1 is up-regulated with high glucose condition and the TGF-β1 overexpression is also detected in DN glomeruli tissue. High glucose condition also accelerates the response of podocytes to environmental levels of TGF-β1 [12]. Hence, the molecular mechanisms of pathogenesis of DN podocyte injury need further to be revealed.

Lycopus lucidus Turczis, an oriental traditional Chinese medicine, was reported to possess therapeutic effect on blood circulation and blood stasis [13]. This crude drug has the suppression of high glucose-induced vascular inflammatory process [14]. our previous study demonstrated that lycopus extracts treatment notably reduced the urine protein and volume in STZ-induced DN rats. Lycopus extracts possess a protective capability in renal damage in DN via inhibit the TGF-β signaling pathway [13]. However, the underlying mechanism of lycopus extracts effect on podocytes is still not clear. Because of podocytes injury is key links in the progression of diabetic proteinuria, we hypothesized that lycopus extracts possess a protective effect on podocytes injury through inactivation of TGF-β signaling pathway.

In the present study, we aimed to further investigate the underlying mechanism of Lycopus lucidus Turcz protective effect on podocyte injury in STZ-induced DN rats and provide more evidences to confirm that Lycopus lucidus Turcz could be an effective agent for DN clinical therapy.

Materials and methods

Lycopus extract preparation

The lycopus extract was provided by Jiangsu Research Institute of Traditional Chinese Medicine. Dried lycopus (100 g) was immersed in distilled water (1 L), then boiled for 20 min at 100°C and filtered with 325-mesh sieve. Finally, the water extract concentrated to 100 ml at 40°C and further lyophilized. The power was stored at -80°C and diluted for the experiment using.

Animals

Male Sprague-Dawley (SD) rats (160-180 g) were procured from Slaccas-Shanghai Lab Animal Ltd. All rats were kept in standard cages on a 12-hour light/dark cycle under 22-24°C with humidity (55%-65%) and given ad libitum access to food and tap water. All rats were given humane care in accordance with the Guidelines established by the Animals Care and Ethical Committee of Jiangsu Province Institute of Traditional Chinese Medicine.

Diabetes rat induction

After three-day acclimatization, the rats were allocated into two groups consisting of 5 and 25 rats by feeding either NPD or HFD (60% fat, as a percentage of total kcal) ad libitum, respectively [15]. After 4 weeks (W), the rats were fast for overnight, subsequently, these rats were intraperitoneally injected freshly prepared 35 $mg \cdot kg^{-1}$ streptozotocin (STZ) dissolved in cold citrate buffer (0.1 $mol \cdot L^{-1}$) to induce diabetes rats. One week later, postprandial blood glucose and insulin levels were detected. The rats with decreased insulin sensitivity index and blood glucose levels higher than 16.7 $mmol \cdot L^{-1}$ were identified as diabetic rats and selected for the next experiments. Normal rats, which were age-matched, were given an equal volume of vehicle as the control group. All rats used in the study were weighted and recorded water intake once a week since feeding HFD or NPD, 24 h-urine samples were collected and recorded weekly and blood was obtained from the tail vein to measure non-fasting blood glucose.

Treatment with Lycopus lucidus Turcz

The DN rats were randomly divided into 5 groups (n=5) composed of one non-treated dia-

betic group (model group), one losartan potassium treated DN group (positive group) and three lycopus extracts treated DN groups (3, 6, 12 g/kg). The lycopus extract was orally administered to the DN rats once daily for 5 weeks. Normal non-diabetic rats were given normal saline orally as control.

Cell culture studies

Rat podocyte cells were procured from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI medium (Gibco, Karlsruhe, Germany) supplemented with 15% fetal bovine serum (FBS). All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. To determine the protective effect of lycopus on the podocyte injury, podocytes were randomly allocated to each group: control (50 mL glucose-free RPMI medium + 45 mg D-glucose dry powder), model (45 mL high glucose RPMI medium (30 mmol/L) + 5 ml normal serum), positive (45 mL high glucose RPMI medium (30 mmol/L) + 5 ml losartan potassium-containing serum), High (45 mL high glucose RPMI medium (30 mmol/L) + 5 ml lycopus-containing serum), Middle (45 mL high glucose RPMI medium (30 mmol/L) + 2.5 ml lycopus-containing serum + 2.5 ml normal serum), Low (45 mL high glucose RPMI medium (30 mmol/L) + 1.25 ml lycopus-containing serum + 3.75 ml normal serum) and incubated for 48 h.

Preparation of lycopus-containing serum

A total of fifteen male SD rats (180±20 g) divided into three groups: normal serum (disinfectant drinking water of same volume), losartan potassium-containing serum (5.2 mg/kg, i.g.), and lycopus-containing serum (12 g/kg, i.g.). The drug was administered at intervals of 12 hours twice daily for 3 days. After the last feeding, blood was aseptically drawn from the abdominal aortas of the rats in each group during the first hour and centrifuged at 3000 r/min immediately to harvest the lycopus-containing serum. The drug-containing serum was stored at -80°C and inactivated at 56°C for 40 min before use.

Biochemical assays

To assess renal function, the levels of serum glucose, insulin, TG, TC, BUN and Scr were measured by an automatic biochemistry analyzer. Urine creatinine and Urine mAlb were detected with commercial enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jian Cheng Co., Nanjing, China), following the manufacturer's protocols.

Histological analysis

Rat kidney tissue was fixed with 4% paraformaldehyde in PBS at room temperature for 24 h, then dehydrated in graded alcohols and embedded in paraffin. Later, the paraffin-embedded kidneys tissues were sectioned into 2-μm-thick sections, periodic acid-silver metheramine (PASM) staining and hematoxylin and eosin (H&E) staining was performed to evaluat the pathological changes. Kidney tissues were examined using a confocal microscope at the 200× magnification.

Immunohistochemistry

Slides were immunostained by the streptavidin-biotin-peroxidase complex (SABC) method on paraffin sections. Slides were deparaffinized, rehydrated and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. After being blocked with 5% Bovine Serum Albumin (BSA), slides were incubated primary antibody overnight. Rabbit anti-nephrin antibody (1:500, cat.no ABT319) were purchased from Millipore (Billerica, MA), rabbit anti-p-Smad2/3 (1:500, cat.no sc-11769) were purchased from Abcam (Cambridge, UK), rabbit anti-mad4 (1:50, cat.no PA5-11695) were purchased from Invitrogen (Carlsbad, California), rabbit anti-p-ERK1/2 (1:500, cat.no 4370T) and rabbit anti-p-p38 (1:500, cat.no 4551T) were purchased from Cell Signaling Technology. All slides were incubated with biotinylated secondary antibodies and SABC reagent. The detection was visualized using chromogen and counterstaining with 3, 3'-diaminobenzidine-tetrahydrochloride followed and analyzed with a light microscope.

Western blot analysis

Total protein (25 μg) was separated by SDS-PAGE and transferred to membranes. The membranes were blocked with 5% skimmed milk powder and incubated with appropriate primary antibodies at 4°C overnight. Rabbit anti-nephrin antibody (1:1000, cat.no ABT319), were purchased from Millipore (Billerica, MA), rabbit

anti-p-Smad2/3 (1:1000, cat.no 8828S,) rabbit anti-Smad2/3 (1:1000, cat.no 8685S), rabbit anti-Smad4 (1:1000, cat.no 46535T), rabbit anti-p-ERK1/2 (1:1000, cat.no 4370T), rabbit anti-ERK (1:1000, cat.no 9194S), rabbit anti-p-p38 (1:1000, cat.no 4551T) and rabbit anti-P38 (1:1000, cat.no 14451s) were purchased from Cell Signaling Technology (Danvers, USA). Subsequently the membranes incubated with appropriate secondary antibody on ice for 2 h. Protein expression levels were visualized with Tanon-5200 Chemiluminescence Imager with ECL western blotting substrate (Millipore).

Insulin and glucose tolerance tests

All rats were fast 6 h and then injected insulin (1 U/kg) to test insulin tolerance. Blood glucose levels were tested at baseline and 40, 90, and 150 minutes after injection. Glucose tolerance testing (GTT) was performed according to the same protocol, glucose (1.5 g/kg) was injected intraperitoneally. All rats were tested both in ITT and GTT. Area-under-curve (AUC) were calculated by the trapezoid rule, as previously described [16].

Statistical analysis

Statistical data analysis was presented as means ± SEM with SPSS 19.0 and GraphPad Prism 5.0. The one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to analyze the differences between multiple groups. $P<0.05$ was considered statistically significant.

Results

Effects of lycopus on body weight and insulin-mediated glucose tolerance

Metabolic parameters were measured at beginning and end of the study. As expected, body weight and blood glucose have no significant difference at baseline. Model group induced by combination of HFD and STZ demonstrated significant weight gain and blood glucose, compared with control mice. Notably, losartan potassium and lycopus blocked the increment in body weight and blood glucose significantly. At end of the study, the trend for lower ad libitum and fasting glucose concentrations was observed in losartan potassium and lycopus-treated animals (**Figure 1A** and **1B**).

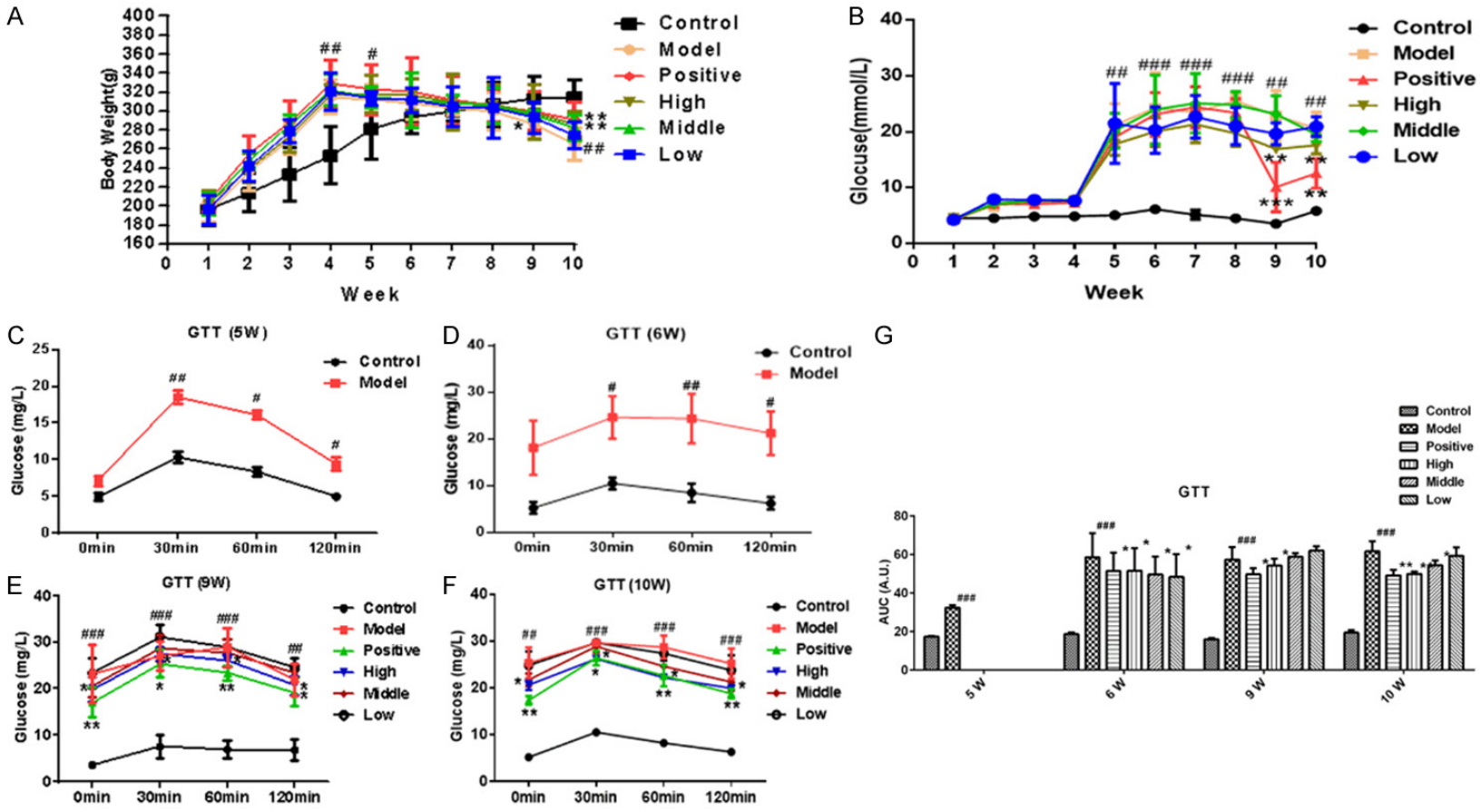
Glucose tolerance tests (GTT) (**Figure 1C-F**) and insulin tolerance tests (ITT) (**Figure 1H-K**) were conducted at 5, 6, 9, 10 weeks. The area under the curve (AUC) of glucose levels of each groups was analyzed. At the endpoint, a significant interaction effect of time and treatment was showed compared with GTT (**Figure 1G**) and ITT AUC (**Figure 1L**) of rats treated with lycopus extracts and the normal rats. Losartan potassium and lycopus-treated rats had a dramatically less AUC during the GTT and ITT at endpoint compared with DN rats.

Effects of lycopus on renal functions

At the end of 5-week treatment, serum creatinine (Scr), blood urea nitrogen (BUN), known as the markers of renal functions, were measured. The results show TGF-β1, Scr, BUN, TG, TC and LDL-C levels strikingly elevated in serum of diabetic nephropathy model rats. In comparation with model group, losartan potassium and lycopus extract had suppression on this increment in a dose dependent manner. Conversely, insulin and HDL-C levels reduced in diabetic nephropathy model rats. Interestingly, those changed were revised by lycopus treatment (**Figure 2A-H**). Furthermore, Urine creatinine and mAlb concentration were increased in model group. As expected, Urine creatinine and mAlb concentration was decreased with losartan potassium and lycopus extract treatment (**Figure 2I** and **2J**). The above results indicated that lycopus extract treatment notably reduced proteinuria and protected podocytes injury in the DN rats.

Effects of lycopus on renal histological changes

To further confirmed the efficacy of lycopus extracts in protecting renal function in STZ-exposed rats, the histological changes in each group were analyzed with light microscopy, as shown in **Figure 3**. Compared with control group, the rats in the model group were observed significant renal damage, including glomerular atrophy, mesangial expansion and inflammatory cell infiltration. However, the treatment of losartan potassium and lycopus extracts improved the renal histopathological injuries (**Figure 3**). The histological results revealed that lycopus extracts treatment ameliorates the pathological changes in the diabetic nephropathy renal tissue.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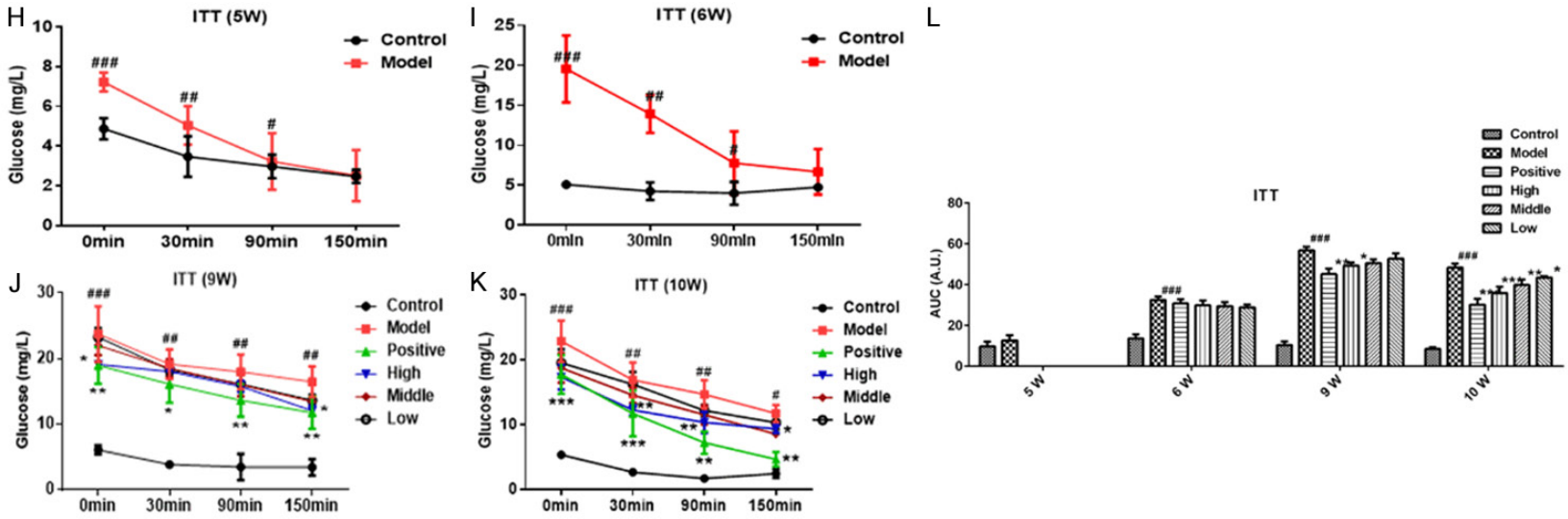


Figure 1. Influence of lycopus on body weight and insulin-mediated glucose tolerance in rats with DN (n=5 for each group). (A, B) Weight and blood glucose gain after combination of high-fat diet and STZ treatment, respectively. (C-G) Glucose tolerance test, GTT assessed at 5 weeks (C), 6 weeks (D), 9 weeks (E) and 10 weeks (F) after high-fat diet. (H-L) Insulin tolerance test, ITT assessed at 5 weeks (H), 6 weeks (I), 9 weeks (J) and 10 weeks (K) after high-fat diet. $^{\#\#}P<0.05$, $^{\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* Control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Model.

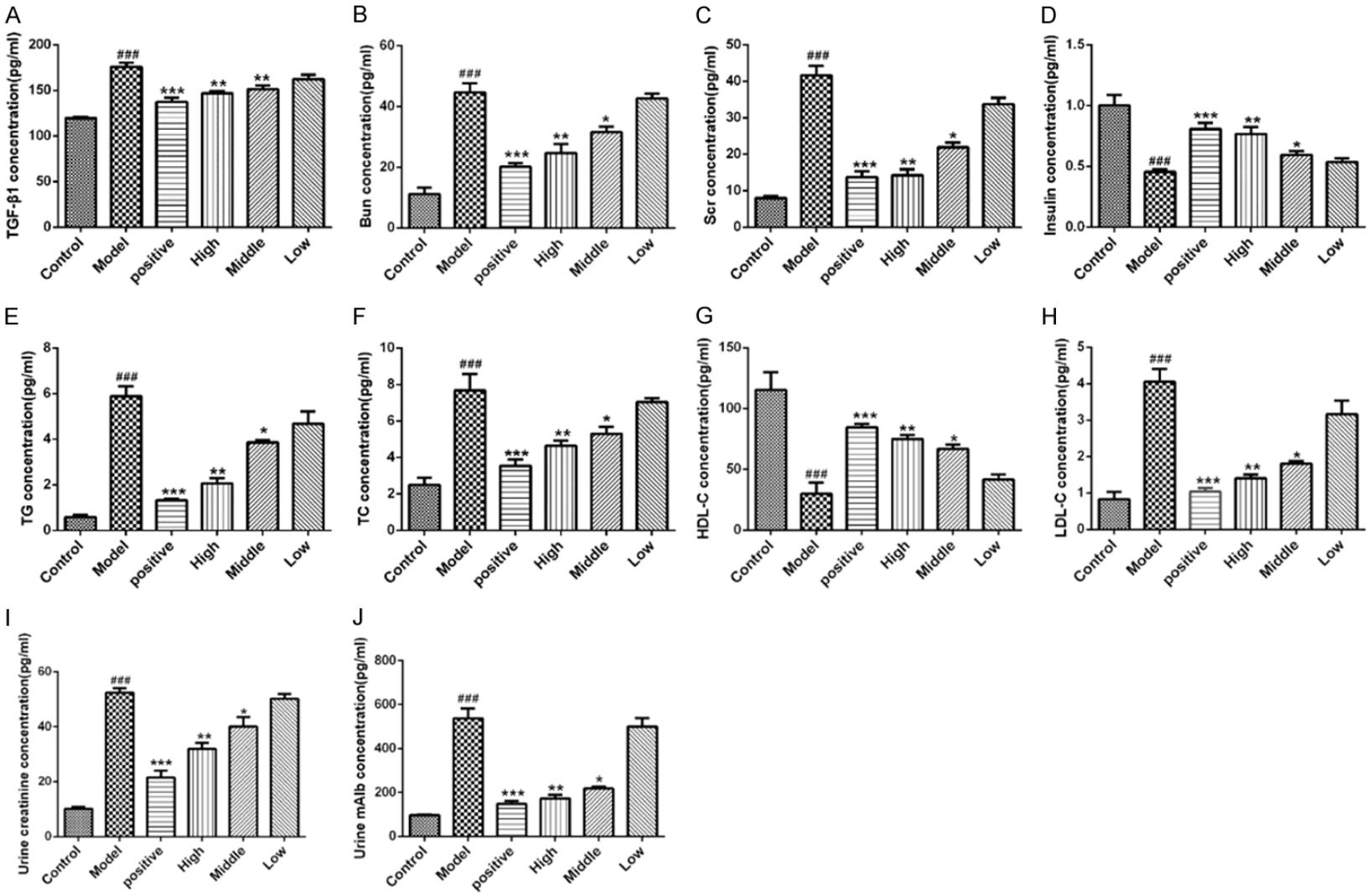


Figure 2. Influence of lycopus on renal functions in DN rats. A. TGF-β1; B. Blood urea nitrogen (BUN); C. Serum creatinine; D. Insulin; E. TG; F. TC; G. HDL-C; H. LDL-C; I. Urine creatinine; J. Urine mAlb. The data were expressed as means ± SD (n=5); $^{###}P<0.001$ *vs.* Control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Model.

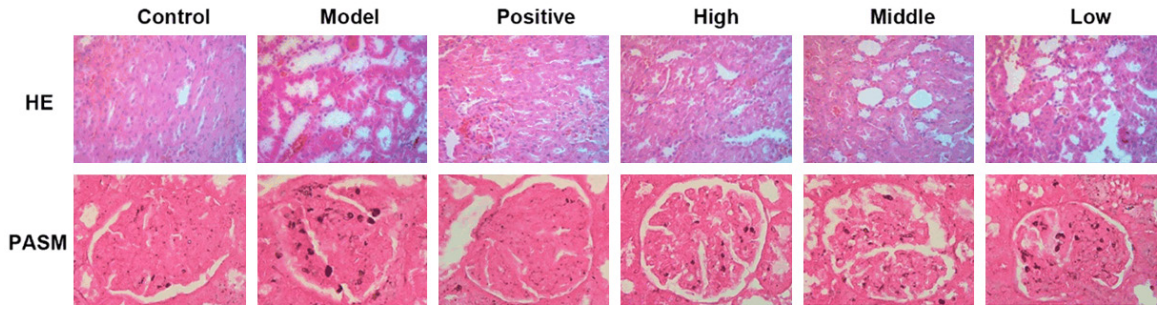


Figure 3. Influence of lycopus on renal histological in DN rats. Representative images of hematoxylin and eosin (H&E) staining (upper panels) and periodic acid-silver metheramine (PASM) staining (lower panels) of kidney tissues from different groups (×400 magnification).

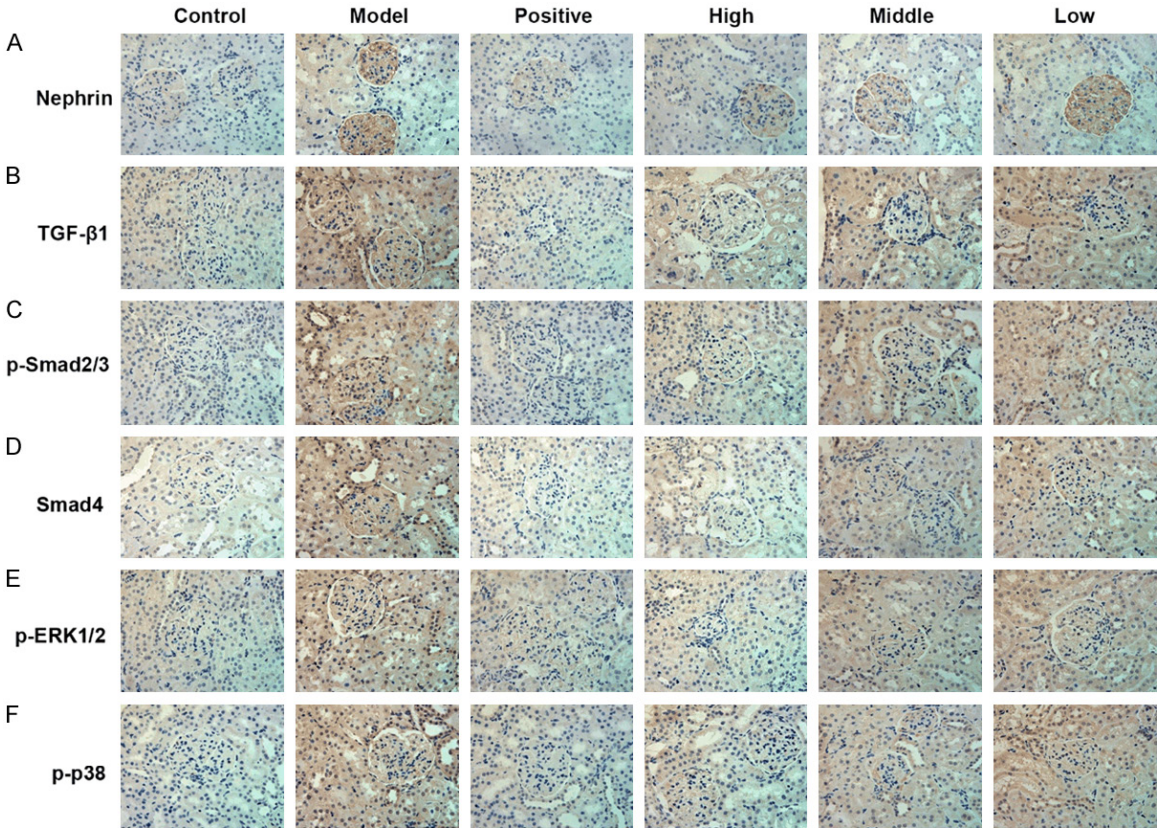
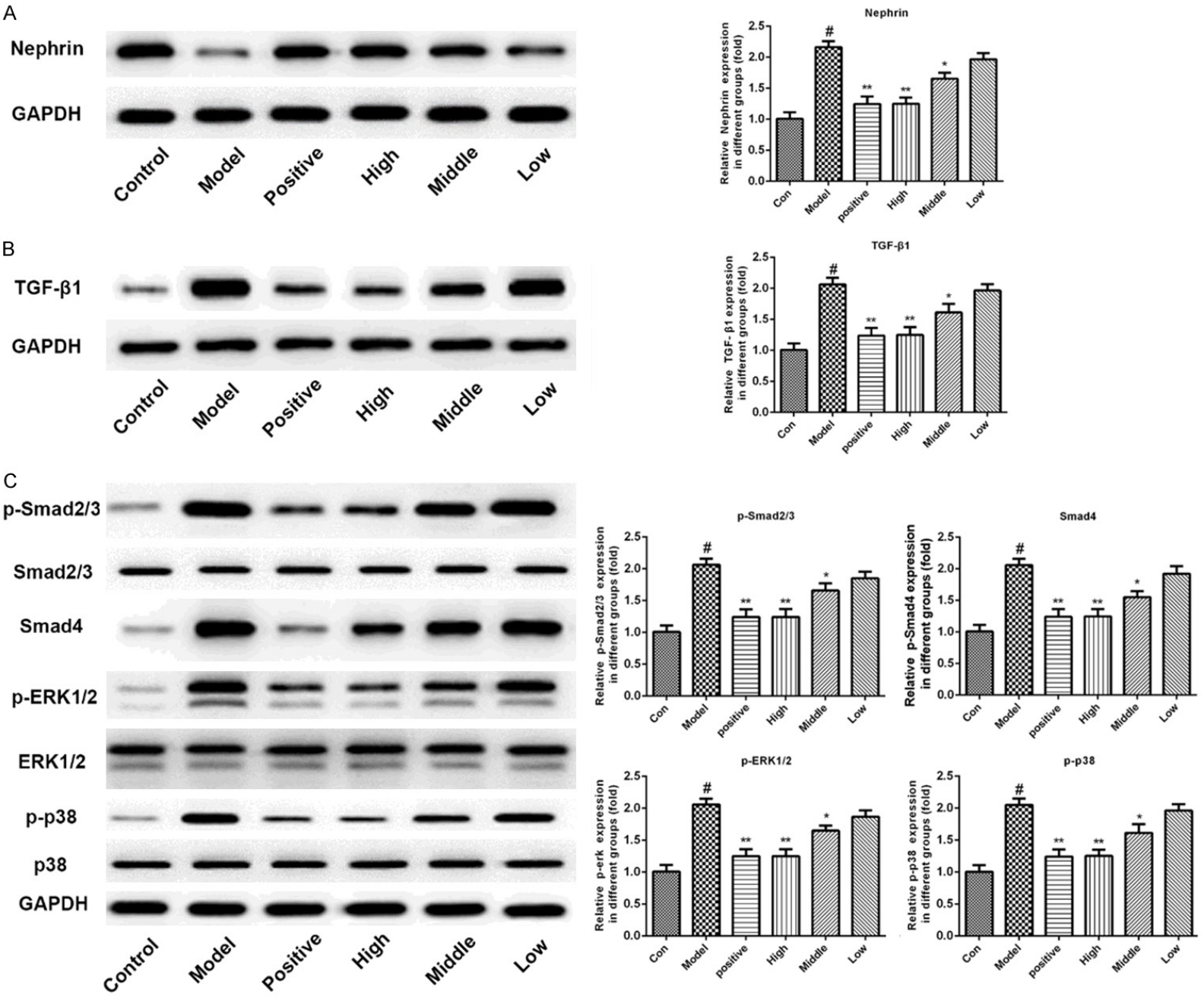


Figure 4. Influence of lycopus on TGF-β1/Smads and MAPK signaling pathway in DN rats by immunohistochemical staining. (A) Nerphin, (B) TGF-β1, (C) P-Smad2/3, (D) Smad4, (E) p-ERK1/2, (F) p-p38 protein expressions of kidney tissues from different groups (×400 magnification).

Effects of lycopus on TGF-β1/Smads signaling pathway

To further analyze the mechanism of lycopus-induced podocyte protection, the immunohistochemistry, ELISA, western blotting experiments was performed to assess TGF-β1/Smads signaling activation. These data showed that the expressions of TGF-β1, p-Smad2/3, Smad4, p-ERK1/2 and p-p38 (**Figures 4**-**6**) were increased, and nephrin were decreased in the kidneys of rats with high-fat diet and STZ treatment, compared with the control group. The administration of losartan potassium and lycopus extracts reduced the expression levels of TGF-β1 (**Figures 5** and **6**), p-Smad2/3, Smad4,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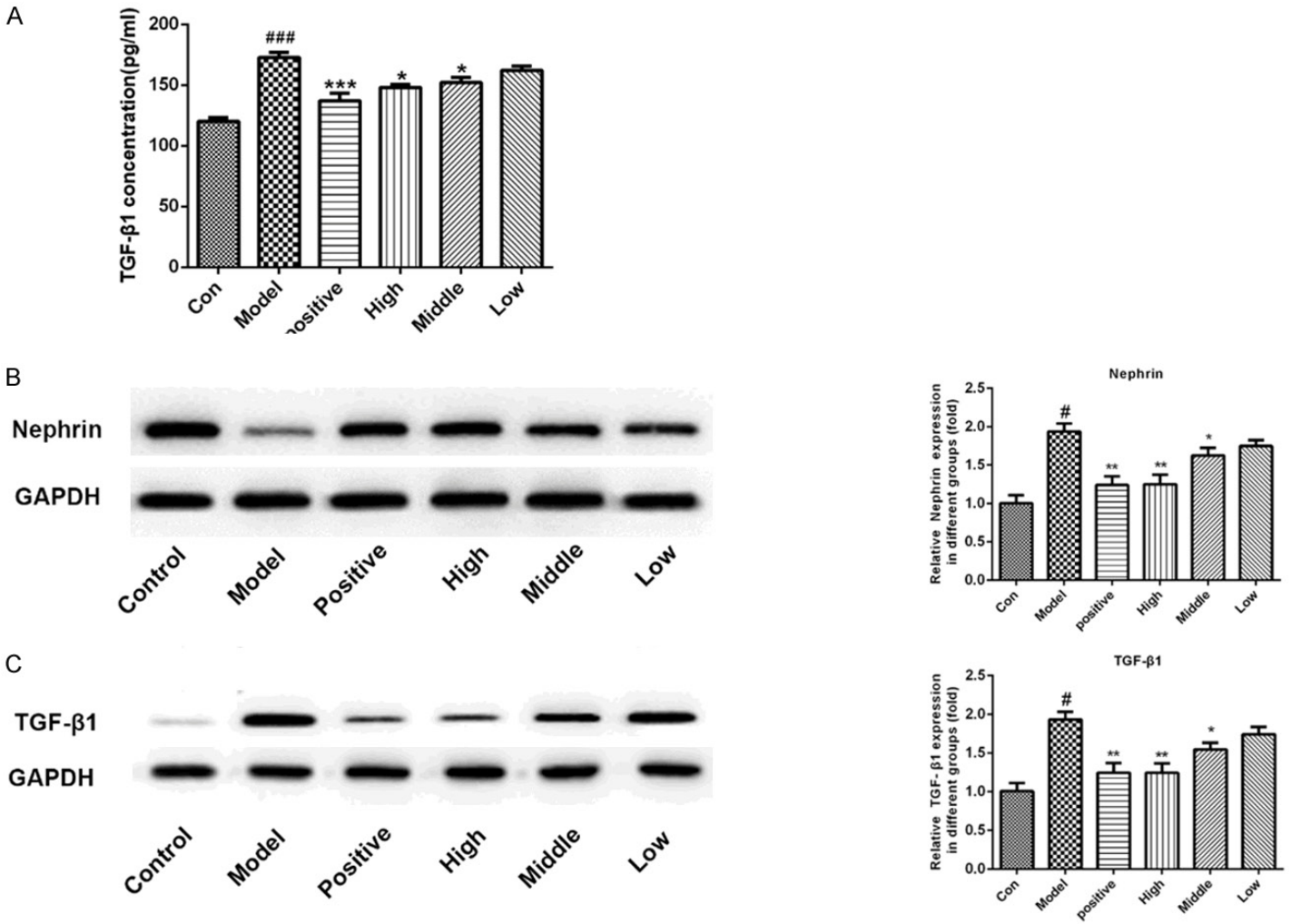
p-ERK1/2
ERK1/2
p-p38
p38
GAPDH
Relative p-Smad2/3 expression in different groups (fold)
Relative p-Smad4 expression in different groups (fold)
Relative p-erk expression in different groups (fold)
Relative p-p38 expression in different groups (fold)

Figure 5. Influence of lycopus on TGF-β1/Smads and MAPK signaling pathway in DN rats. The expressions levels of (A) Nerphin, (B) TGF-β1, (C) P-Smad2/3, Smad4, p-ERK1/2, p-p38 protein of kidney tissues from different groups were detected by Western Blot. Error bars represent the mean ± SEM from three independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Model.



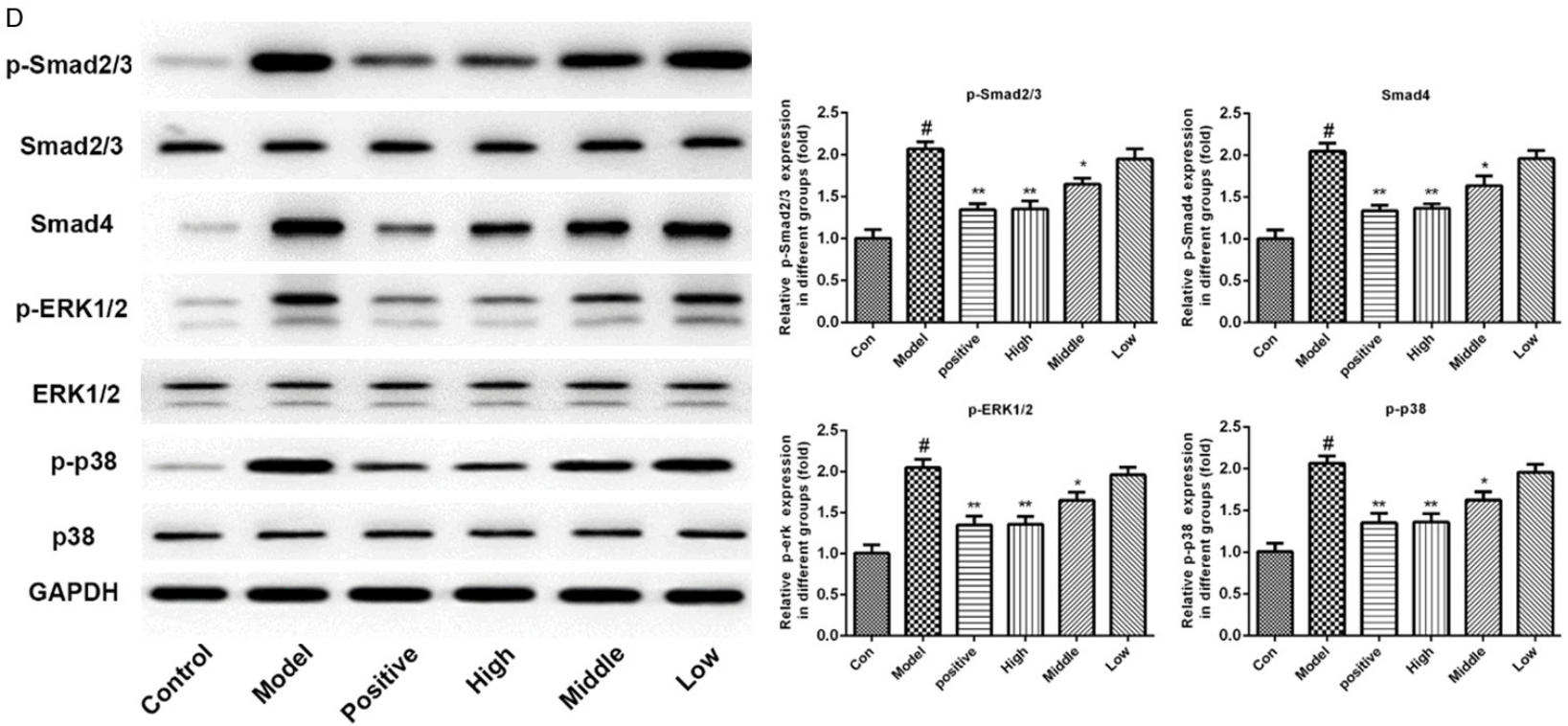


Figure 6. Influence of lycopus on TGF-β1/Smads and MAPK signaling pathway in podocytes. (A) TGF-β1 concentration was detected by ELISA kit. The expressions levels of (B) Nerphin, (C) TGF-β1, (D) P-Smad2/3, Smad4, p-ERK1/2, p-p38 protein of podocytes from different groups were detected by Western Blot. Error bars represent the mean ± SEM from three independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. Control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Model.

p-ERK1/2 and p-p38 dramatically, and enhanced expression level of nephrin compared with model group, both in vivo and vitro. Taken together, above findings suggested that lycopus exert protective effect on podocytes injury by inhibiting the TGF-β1/Smads signaling pathway.

Discussion

Diabetic nephropathy (DN) is a severe complication induced by diabetes characterized by renal failure and tissue fibrosis [17]. Podocyte injury affects the completeness of the glomerular filtration barrier and plays major roles in promoting progress of DN [18, 19]. The diabetic podocytopathy is reported to be the leading cause of microalbuminuria and proteinuria in the DN [6]. In our study, we successfully established the stereotypical pathological changes of the DN kidney in diabetic rats induced streptozotocin (STZ), abnormally increased body weight and insulin resistance were observed in DN rats. In addition, the increased levels of Scr, BUN, urine protein levels, as well as various pathological findings in renal tissues, including glomerular hypertrophy, thickening of capillary basement membranes, and hyperplasia of mesangial matrix were further to demonstrated the DN models was successful produced because of its consistent phenotypes as previous studies [20]. Previous study demonstrated that lycopus extracts treatment improved renal interstitial fibrosis [13]. In this study, all the clinical indexes and pathological findings were improved after lycopus extracts was administered, findings previously reported by our researchers [13]. Hence, more studies were carried out to explore the underlying mechanism that lycopus protects podocytes injury of diabetic nephropathy.

It had been reported that EMT play major roles in podocyte injury in DN, which is closely associated with the onset of proteinuria [21]. The previous study confirmed that TGF-β signaling promotes EMT dramatically via reducing levels of epithelial markers including nephrin [22]. Consistent with our results, TGF-β1 is increased in HFD/STZ-induced DN rats, while lycopus extracts treatment attenuated the TGF-β1 protein levels. Therefore, we presume that TGF-β1 is the potential therapeutic target of DN. Moreover, the TGF-β1/Smad signaling pathway was demonstrated to be elevated in DN [23-25]. Which is consistent with our results, Zhang et al. showed that protective effect of nicousamide against podocyte injury by suppressing the phosphorylation of TβR II and Smad2 in rat kidney [26]. In the present study, Smad2/3 and Smad4 protein were evaluated to further confirmed the effects of Smads on the progress of DN. Our results revealed lycopus extracts attenuated podocyte damage by inhibiting the expression level of Smad4 protein and phosphorylation of Smad2/3 protein as shown in **Figures 5** and **6**. Above results indicted lycopus extracts possessed potential therapeutic effects on podocytes injury in DN rat kidney.

The activation of MAPK pathways has been shown related to TGF-β pathway. In general, ERK1/2 activation is related to glomerular podocytes hypertrophy [27]. The inhibition of MAPK pathways mediated by TNFα can be identified as a strategy for improving podocyte morphology and structural injury [28]. TGF-β possess activation effect on ERK pathway, which affect MAPK phosphatase activity in turn, thereby regulating p38 MAPK pathway. Activation of p38 MAPK signaling pathway may cause podocytes injury in early DN [29]. Inhibition of integrin α6β4/FAK/p38 pathway exert protective effect on podocyte injury-related glomerular diseases [30]. Zhong Ju Xu et al. found Liuwei Dihuang pill ameliorated the renal damage in DN via inhibiting the TGF-β/Smad and MAPK pathways [17]. Consistent with their results, lycopus extracts suppressed the phosphorylation of p38 and ERK1/2 induced by TGF-β1 in vitro and vivo, while total ERK1/2 and p38 have no significant difference by lycopus extract treatment.

In conclusion, the present study clearly demonstrated that lycopus treatment reduces blood glucose, metabolic abnormalities, urinary protein excretion and ameliorate renal damage in high-fat diet combined with STZ-induced DN rats. Moreover, lycopus extract significantly exert protective effect on podocyte injury through regulating TGF-β1/Smad and MAPK signaling pathways. We conclude that lycopus extracts could be used as a candidate agent to ameliorate or treat the development of advanced diabetic renal injury or the clinical therapy of DN.

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Disclosure of conflict of interest

None.

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