

# Liuwei Dihuang pill treats diabetic nephropathy in rats by inhibiting of TGF- $\beta$ /SMADS, MAPK, and NF- $\kappa$ B and upregulating expression of cytoglobin in renal tissues

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## Abstract

Liuwei Dihuang pill (LDP) was assessed for its effects on renal deficiency.

90 STZ induced DN rats were divided into groups (n=22) without treatment (STZ) and LDP treated (STZ-L) (n=23), Zhenwu decoction treated (STZ-Z) (n=22), and valsartan treated (STZ-V) (n=23) groups, with 16 normal control rats. Total urine protein (TP), blood urea nitrogen (BUN), and serum creatinine (Cr) were measured. Superoxide dismutase (SOD), nitric oxide synthase (NOS), and malondialdehyde (MDA) concentrations as well as expression/phosphorylation of SMAD3, SMAD2, and  $\alpha$ -SMA, TGF- $\beta$ , RI /II, P38, ERK, and NF- $\kappa$ B in renal tissues were determined. In vitro experiments analyzed the effect of enhanced TGF- $\beta$  containing rat serums of the STZ groups on mesangial cells with and without transient transfection with a cytoglobin-containing plasmid.

LDP treatment reduced the kidney coefficient, serum creatinine, blood urea nitrogen, and urine protein and prevented pathological changes. Expression of SOD and NOS in kidney tissue was increased but MDA expression reduced. LDP modulated multiple pathways, and its administration inhibited the phosphorylation of SMADS, ERK, p38, and the expression of NF- $\kappa$ B,  $\alpha$ -SMA, and TGF- $\beta$  RI/II, and upregulated the expression of cytoglobin. In vitro studies revealed that overexpression of cytoglobin suppressed phosphorylation of Smad2, ERK, and p38 induced by TGF- $\beta$  and expression of NF- $\kappa$ B,  $\alpha$ -SMA, and TGF- $\beta$  RI.

LDP prevented renal fibrosis and protected glomerular mesangial cells by upregulation of cytoglobin and suppression of multiple pathways involving TGF- $\beta$ /SMADS, MAPK, NF- $\kappa$ B signaling.

**Abbreviations:**  $\alpha$ -SMA = alpha-smooth muscle actin, BUN = blood urea nitrogen, Cr = creatinine, CYGB = cytoglobin, DN = diabetic nephropathy, ECM = extracellular matrix, ERK = extracellular signal regulated kinase, FN = fibronectin, H&E = hematoxylin and eosin, HCA = hydrocortisone acetate, LDP = Liuwei Dihuang pill, LN = laminin, MAPK = p38 mitogen-activated protein kinases, MDA = malondialdehyde, NF- $\kappa$ B = nuclear factor kappa light chain enhancer of activated B cells, NOS = nitric oxide synthase, SMAD = mothers against decapentaplegic homolog, SOD = superoxide dismutase, STZ = streptozotocin, TCM = traditional Chinese medicine, TGF- $\beta$  = transforming growth factor beta, TGF- $\beta$ R = TGF- $\beta$ -receptor, TP = total urine protein, UUO = unilateral ureteral obstruction.

**Keywords:** cytoglobin, diabetic nephropathy, Liuwei Dihuang pill, MAPK, SMADS, TGF- $\beta$

Editor: Sanket Patel.

**Authorship:** YZ was responsible for the conception and design of the study. ZJX, SS, ZJ L, YML, and RYZ were responsible for acquisition of data. ZJX performed the data analysis. ZJX drafted the manuscript. YZ revised and commented the draft. All authors participated in interpretation of the findings and all authors read and approved the final version of the manuscript.

**Funding:** National Natural Science Foundation of China (Grant No. 81173406). Key subjects of Shanghai Municipal Education Commission (5th) (Grant No. J50301). Training and construction project of traditional Chinese Medicine in Pudong area (Grant No. PDZYXK-4-2014002). Shanghai further accelerate the development of traditional Chinese medicine three years of action (Grant No. ZY3-CCCX-2-1001). Traditional Chinese medicine clinical demonstration subject construction project in Pudong area (Grant No. PDZYXK-3-2013005).

The authors have no conflicts of interest to disclose.

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Medicine (2017) 96:3(e5879)

Received: 5 January 2016 / Received in final form: 13 December 2016 / Accepted: 19 December 2016

<http://dx.doi.org/10.1097/MD.0000000000005879>

## 1. Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes, affecting around 30% of patients with type 1 diabetes and 20% of patients with type 2 diabetes.<sup>[1]</sup> It is also the leading cause of end-stage renal failure worldwide, representing over 50% of patients on renal replacement therapy in some parts of the world.<sup>[2]</sup> Thus, it is of great importance to investigate the pathogenesis of DN and effective therapies to treat this condition.

A number of pathological mechanisms have been identified during the development of DN. In the kidney of DN patients, many oxygen radicals are generated in a variety of biochemical reactions, such as nonenzymatic glycosylation of proteins, self-oxidation of glucose, and by activation of the polyol (sorbitol-aldose reductase) pathway.<sup>[3,4]</sup> The activity of anti-oxidases, such as super oxide dismutase (SOD), is largely suppressed due to hyperglycemia-induced metabolic disturbance. Consequently, the uncontrolled oxidative stress response leads to severe renal damage in a direct or indirect way.<sup>[5]</sup> Also, because of the continuing oxidative stress response, degradation of the extracellular matrix (ECM) is impeded, which promotes renal tubular epithelial cells to differentiate into myofibroblasts leading to renal fibrosis.<sup>[6]</sup> In mesangial cells, the activation of a variety of signaling pathways has been linked to the presence of hyperglycemia and uncontrolled ROS, including the NF- $\kappa$ B, mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways. This activation results in the prolific secretion of inflammatory factors, which may also promote the progression of DN.<sup>[5,7,8]</sup> TGF- $\beta$  binds to its receptors on the cell membrane, TGF- $\beta$ R I and RII. The SMAD pathway is activated in a similar manner to stimulate the ECM synthesis of glomerular cells. This initiates the phenotypic switch of mesangial cells, which starts to express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Then, excessive synthesis of collagens (types I, III, and IV), fibronectin (FN) and laminin (LN) by mesangial cells further promotes the development of renal fibrosis.<sup>[7]</sup> It has also been shown in a recent study that expression of renal cytoglobin was increased following the activation of myofibroblasts. However, the detailed connection between cytoglobin and renal fibrosis remains to be elucidated.<sup>[9]</sup>

Liuwei Dihuang pill (LDP) is an ancient traditional Chinese medicine (TCM), which effects on chronic inflammation, oxidative stress, and diabetes-related renal diseases have been demonstrated in previous studies.<sup>[10,11]</sup>

In animal studies, LDP has been shown to suppress lipid peroxidation through multiple mechanisms in the brain and protect against brain damage as a result of lipid peroxidative reactions.<sup>[12]</sup> In animal models of Yin deficiency induced by corticosterone treatment, which manifested in low grade fever, dry fur, enhanced aggression, rough skin as well as frequent water uptake and urination with low amounts of urine, Ma et al<sup>[13]</sup> reported that LDP helped with the clearance of free radicals in the immune organs and greatly reduced the production of lipid peroxide. Moreover, Li et al<sup>[14]</sup> treated rats with DN with LDP and found that renal functions were significantly improved, shown by the measurements of 24 h total protein, blood urea nitrogen (BUN), and serum creatinine (Cr). Therefore, more evidence needs to be garnered in order to support the use of LDP in the treatment of patients with DN.

In the present study, the therapeutic effects of LDP and its potential molecular mechanisms *in vitro* and *in vivo* were investigated and we used Zhenwu Decoction and valsartan to

compare efficacies. We demonstrated an improvement in both renal functions and pathological changes after the administration of LDP in a rat model of DN. Multiple pathways were involved as shown by our results. We demonstrated that LDP was able to suppress the oxidative stress response, inhibit TGF- $\beta$ /SMAD, MAPK, and NF- $\kappa$ B pathways, and upregulate the expression of cytoglobin, thereby preventing renal fibrosis and protecting glomerular mesangial cells.

## 2. Methods and materials

### 2.1. Diabetic rats

A total of 110 healthy SD rats of a specific pathogen free grade were selected and used after 1 week of acclimatization. To generate a diabetic model, 94 rats were fasted for 12 hours and then injected with a single intraperitoneal dose of STZ (STZ, Cat. 85882, Sigma, 60 mg/kg in 0.1 M citrate buffer, pH 4.5). One week after STZ administration, the fasting tail vein blood glucose was measured, and rats with persistent hyperglycemia over 16.7 mmol/L ( $n=90$ ) were considered to be diabetic and selected for further experiments, whereas 16 untreated rats served as controls. The diabetic rats were further divided into a STZ group ( $n=22$ ) without treatment, a group treated with LDP (STZ-L) ( $n=23$ ), a group treated with Zhenwu Decoction (STZ-Z) ( $n=22$ ), and a group treated with valsartan (STZ-V) ( $n=23$ ) (Fig. 1A). Our study was approved by the Animal Ethics Committee of Shanghai Pudan Hospital and Shanghai University of traditional Chinese medicine.

### 2.2. Drug preparation

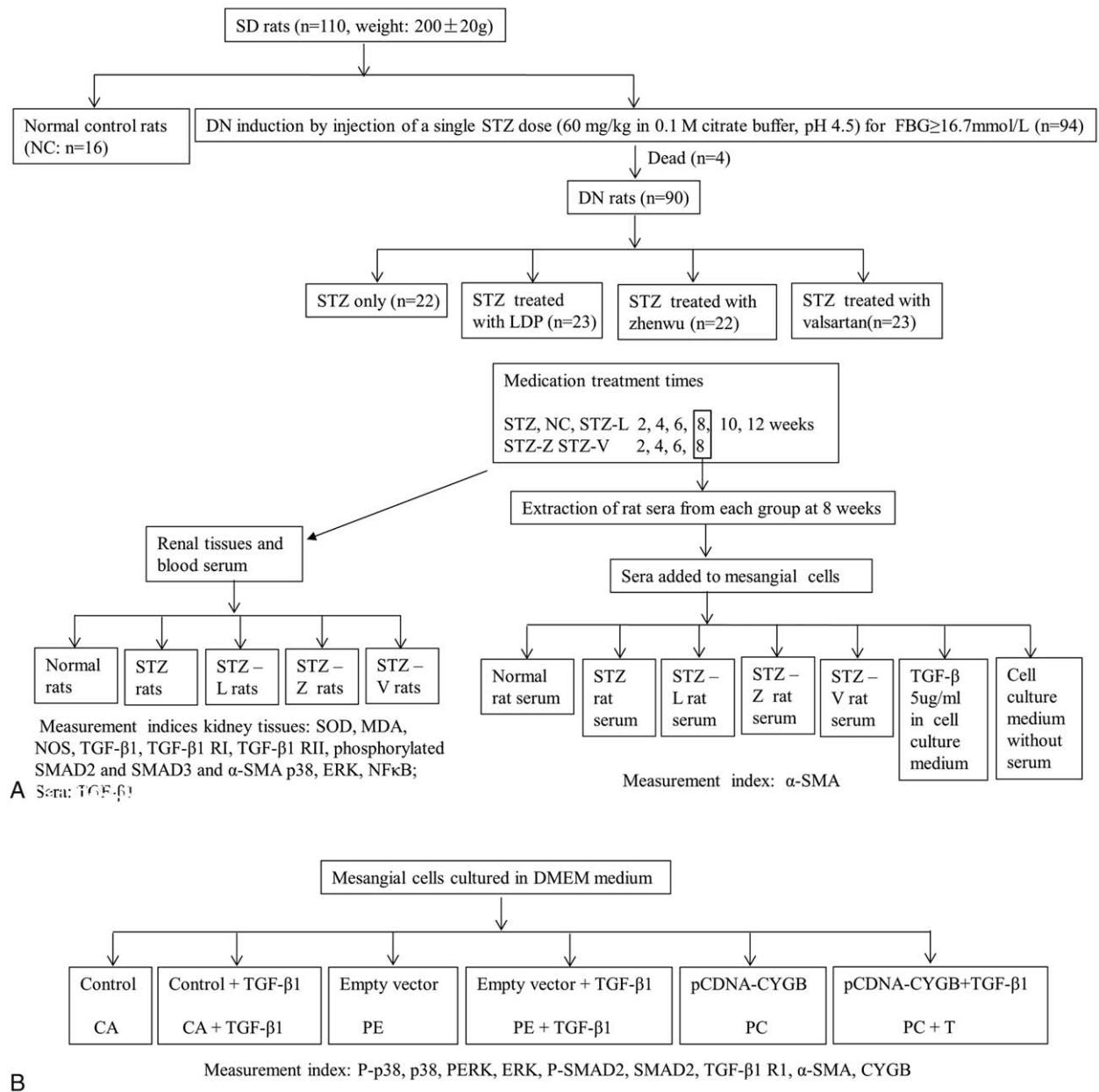
LDP (0.67 g/mL, 75 g as an example) was prepared as follows: 24 g prepared rehmannia root, 12 g dogwood, 12 g dried yam, 9 g alisma, 9 g cortex moutan, and 9 g white poria were dissolved in 111 mL of distilled water. The treatment duration was 12 weeks. Zhenwu decoction (0.3 g/mL, 42 g as an example) was prepared with 9 g white poria, 9 g peony, 6 g white atractylodes rhizome, 9 g fresh ginger, and 9 g monkshood, and valsartan (Novartis) was dissolved to make an aqueous solution (3 mg/mL). The treatment durations for the Zhenwu decoction and valsartan were 8 weeks. All drugs were administered as 1 mL/per 100 g body-weight by the intragastric route.

### 2.3. Assessment of renal function

Renal functions were measured with an automatic biochemical analyzer (HITACHI, Model 7080), including 24 hours TP, BUN, and serum Cr concentrations.

### 2.4. Renal pathology

Rats were anesthetized by a single intraperitoneal dose of pentobarbital sodium (3%, 0.2 mL/100 g body weight), and their bodies fixed in the supine position. After the abdominal cavity was exposed, we evaluated the kidneys regarding their color, texture, shape, volume, and adhesion. The kidneys were then removed and weighed. For each rat, one-third of a single kidney was harvested and fixed for 24 to 48 hours in 10% neutral buffered formalin solution. After being thoroughly dehydrated with ethanol and embedded with paraffin, tissues were sliced to a thickness of 3  $\mu$ m, and H&E or Masson staining was performed before the samples were examined under light microscopy. We also harvested tissues from the renal cortex and corticomedullary



**Figure 1.** Flowchart of the present study. (A) Rat kidney tissue and serum analyses as well as incubation of mesangial cells with indicated rat sera. (B) CYGB transfection experiment of mesangial cells. CYGB = cytoglobin.

border, which were made into cubes (1 mm × 1 mm × 1 mm). These tissues were then fixed for 2 hours at low temperatures in 2% glutaraldehyde, and thoroughly washed with 0.1M phosphate buffer. After further fixation in 1% osmic acid for 2 hours and rinsing with phosphate buffer for 0.5 hours, the samples were dehydrated, embedded, and sectioned and subsequently examined with an electron microscope (Model TECNAI-12, Philips-FEI Co., Ltd., Netherlands).

**2.5. Extraction of renal total protein and measurement of oxidative stress indexes**

Around 100 mg of renal tissue, previously preserved at -80°C, were incubated in 1 mL cold lysis buffer, homogenated

with ultrasound 3 times, and rested on ice for 20 minutes. Then, we performed centrifugation (12,000 r/min for 15 min at 4°C) to obtain the supernatant. The supernatant was stored at -80°C for 2 days before another round of centrifugation was performed (12,000 r/min for 15 min at 4°C) to obtain the protein solutions. The total protein samples from the renal tissues were measured with a kit (Nanjing Jiancheng Biological Technology co., LTD.) to determine the concentration of SOD, MDA, and NOS, which reflected the intensity of the oxidative stress in these tissues as well as to analyze the TGF-β/SMAD signaling pathways in the renal tissues via TGF-β, TGF-β RI, TGF-β RII, phosphorylated SMAD2 and SMAD3 and α-SMA expression rate measurements.

### 2.6. Serum preparation and TGF-β1 measurement

Eight weeks after drug administration, blood was drawn from the abdominal aorta of the rats and stored at 4°C overnight. Then, we performed centrifugation (3000 r/min for 15 min) to obtain the serum. After destructing serum complements (56°C water bath for 30 min) and sterilization via ultrafiltration, the concentration of TGF-β1 was measured by ELISA as instructed (TSZ Biological Technology co., Ltd.).

### 2.7. Construction and transfection of the cytoglobin plasmid into rat mesangial cells

We used 2 primers (5'-TCCACCGGTATGGAGAAAGTGCCG GG-3' and 5'-CATACCGGTCGGCCCTGAAGAGGGCAGA-3') to obtain the gene of cytoglobin from the cDNA library of adult rat brain. The gene was then integrated in the vector (pCMV-2A-EGFP), and the sequence was further validated by sequencing. Then, the plasmid was transfected into rat mesangial cells (purchased from China Center for Type Culture Collection) via Lipofectamine 2000 as instructed. The transfected rat mesangial cells (obtained from ATCC) were cultured in the DMEM medium with 10% fetal bovine serum (Fig. 1B).

### 2.8. Western blot

The amount of protein and the extent of phosphorylation were evaluated with western blotting for both in vivo and in vitro experiments. Antibodies used in the assays included anti-α-SMA antibody (Cat. 100M4795, Sigma), anti-CYBG antibody (Cat. ab57713, Abcam), rabbit monoclonal anti-NF-κB antibody (Cat. 4764, Cell Signaling), anti-p38 antibody (Cat. 9212S, Cell Signaling), anti-P-p38 antibody (Cat. 4511S, Cell Signaling), anti-TGF-β RI antibody (Cat. 2310427, Millipore), anti-TGF-β RII antibody (Cat. 11888, Cell Signaling), anti-ERK antibody (Cat. 4695, Cell Signaling), anti-TGF-β antibody (Cat. #3711 Cell Signaling), anti-P-ERK antibody (Cat. 4370, Cell Signaling), goat anti-rabbit HRP IgG monoclonal antibody (Cat. MR-R100, Shanghai Mingrui Biological Technology Co., Ltd.). The optical densities of the bands were measured and calculated using a gel image analysis system (FR-200, Shanghai Furi Science & Technology Co., Ltd.).

### 2.9. Statistical analysis

All data are presented as the mean ± SD. Two-way ANOVA for comparison of 2 different treatment groups at different time

courses and unpaired Student's *t* test for comparison between 2 groups were performed using SPSS Windows (Version 16.0. Chicago, SPSS Inc.). *P* values <0.05 were considered to be statistically significant.

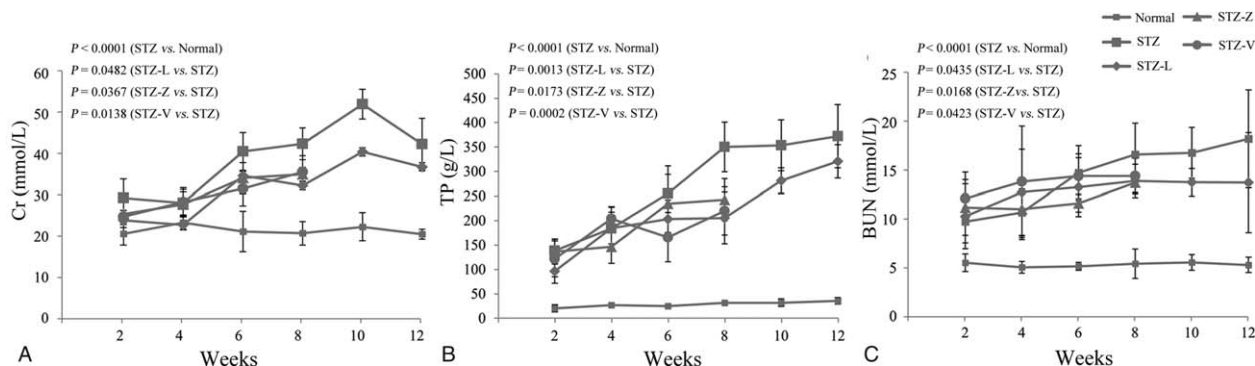
## 3. Results

### 3.1. LDP improved the renal functions in DN rats

According to the measurements of blood glucose levels, we successfully constructed 90 diabetic rats via intraperitoneal injection of STZ. Moreover, compared with normal rats, the measurements of 24 hours total protein (TP), BUN, and Cr were significantly increased in the diabetic rats (STZ group) (*P* < 0.0001), proving the successful induction of DN (Fig. 2A–C). Then, we tried to evaluate the therapeutic effects of LDP on DN rats. Zhenwu decoction, as another traditional Chinese medicine, has been reported to clear efficiently free radicals and improve renal functions in a rat model of Yang deficiency induced by hydrocortisone acetate (HCA), which led to hair loss, humpback, weight loss, cold limbs, diarrhea, scrotal moisture or scrotum swelling and lag of responses.<sup>[15]</sup> We also included Zhenwu decoction and valsartan in our study as controls. After the administration of each drug, neither body weight nor blood glucose levels were significantly affected (data not shown). However, in the STZ-L DN rats treated with LDP, 24 hours total protein, BUN and Cr were significantly reduced (Fig. 1A–C); a similar effect was observed in the STZ-Z rats treated with Zhenwu decoction and STZ-V rats treated with valsartan, but the effects were not as fast and persistent as in the LDP group (Fig. 2).

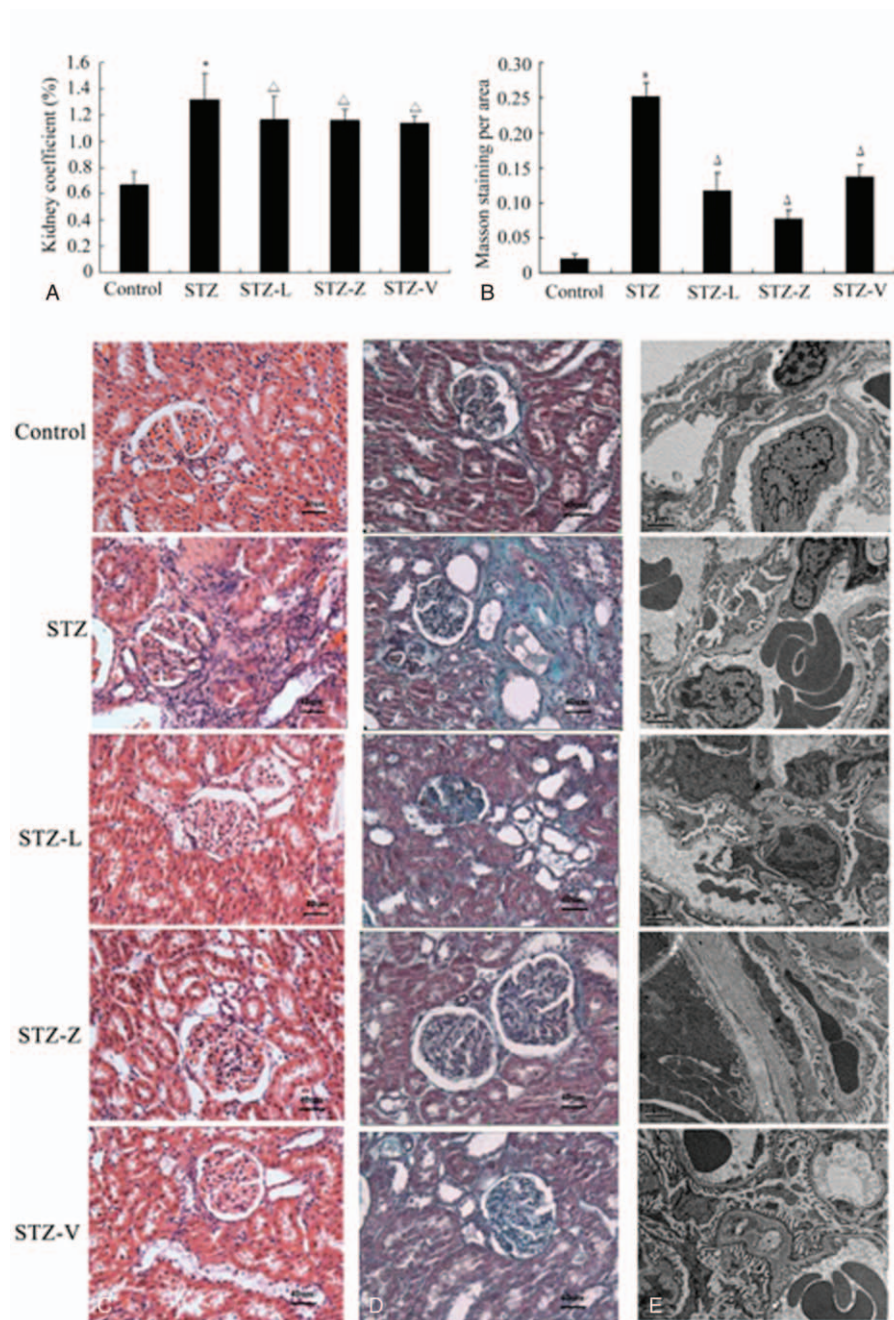
### 3.2. LDP prevented the renal pathological changes in DN rats

The typical pathological changes observed in a DN patient are glomerular hypertrophy, thickening of capillary basement membranes and hyperplasia of the mesangial matrix. In our study, we evaluated the effects of LDP treatment on renal pathology. Compared with the kidneys of normal rats, the kidneys of DN rats were heavier, with increased kidney coefficients; remarkable reductions in kidney coefficients were shown in DN rats treated with LDP, Zhenwu decoction, or valsartan (Fig. 3A). Moreover, when examined under the microscope after hematoxylin and eosin (H&E) staining, the typical pathological changes described above were observed in



**Figure 2.** Treatments with LDP, Zhenwu decoction, and valsartan all improved the renal functions of DN rats as evidenced by the decreased Cr (A), TP (B), and BUN (C). For each time point, 8 rats were randomly selected from each group for the measurement of serum BUN, CR, and urine TP. BUN = blood urea nitrogen, Cr = creatinine, DN = diabetic nephropathy, L=LDP, LDP = Liuwei Dihuang pill, TP = total urine protein, V=valsartan, Z=Zhenwu decoction.

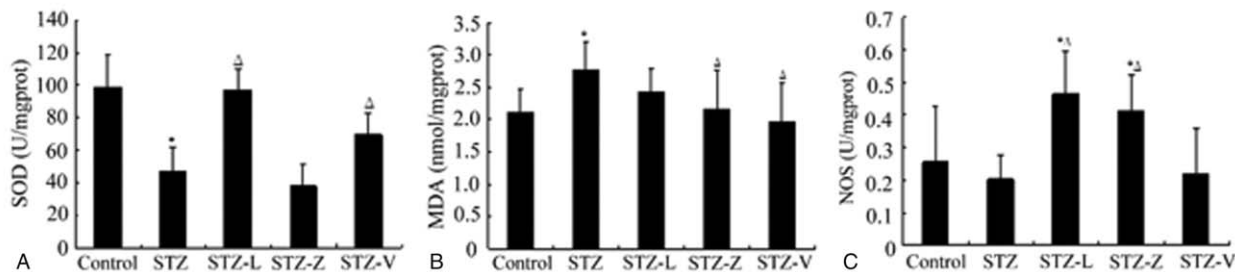




**Figure 3.** Treatment with LDP (n=8), Zhenwu decoction (n=8), and valsartan (n=8) rescued STZ-induced pathological damage in rat kidneys. (A) Decreased kidney coefficient (%) by LDP, Zhenwu decoction, and valsartan treatments in DN rats. (B, D, E) Improved renal morphology after LDP, Zhenwu decoction, and valsartan treatments by HE (B) and Masson collagen (D) staining (blue color) and under the electron microscope (E). (C) Reduction of the calculated signal values per area in Masson collagen staining after LDP, Zhenwu decoction, and valsartan treatments. DN = diabetic nephropathy, HE = hematoxylin and eosin, L = LDP, LDP = Liuwei Dihuang pill, STZ = streptozotocin, Z = Zhenwu decoction, V = valsartan. Kidney coefficient = kidney weight on both sides (g)/body weight (kg) × 100% \**P* < 0.05 compared with normal rats, <sup>Δ</sup>*P* < 0.05 compared with STZ rats.

the DN rats after 8 weeks, and severe renal fibrosis was found at 12 weeks. By contrast, in DN rats treated with LDP (STZ-L), the glomerular structure was generally normal except for subtle vacuolization of renal tubular epithelial cells and occasional protein casts (Fig. 3B). Masson collagen staining revealed large amounts of positive expressions in both the renal tubular cells and renal interstitium in the DN rats, and the glomerular basement membranes and mesangial matrix were green after staining of collagen. In DN rats treated with LDP, Zhenwu

decoction, or valsartan, the calculated signal value per area was significantly reduced in the renal tissues after Masson collagen staining (Fig. 3C and D). Finally, we examined the renal tissues of DN rats with electron microscopy and found thickening of capillary basement membranes and matrix deposition in the mesangial area, which were significantly improved in all the treatment groups (Fig. 3E). Thus, LSP ameliorated the pathological changes in DN rats, consistent with its significant therapeutic effects on DN.



**Figure 4.** Treatment with LDP (n=8), Zhenwu decoction (n=8), and valsartan (n=8) inhibits oxidative stress in renal tissues of DN rats as evidenced by increased SOD (A) and NOS levels (C) but decreased MDA levels (B). All LDP, Zhenwu decoction, and valsartan were administered by the intragastric route to DN rats for 8 weeks. DN = diabetic nephropathy, L = LDP, LDP = Liuwei Dihuang pill, MDA = malondialdehyde, NOS = nitric oxide synthase, SOD = superoxide dismutase, STZ = streptozotocin, X = valsartan, Z = Zhenwu decoction. \*P<0.05 compared with normal rats, <sup>Δ</sup>P<0.05 compared with STZ rats.

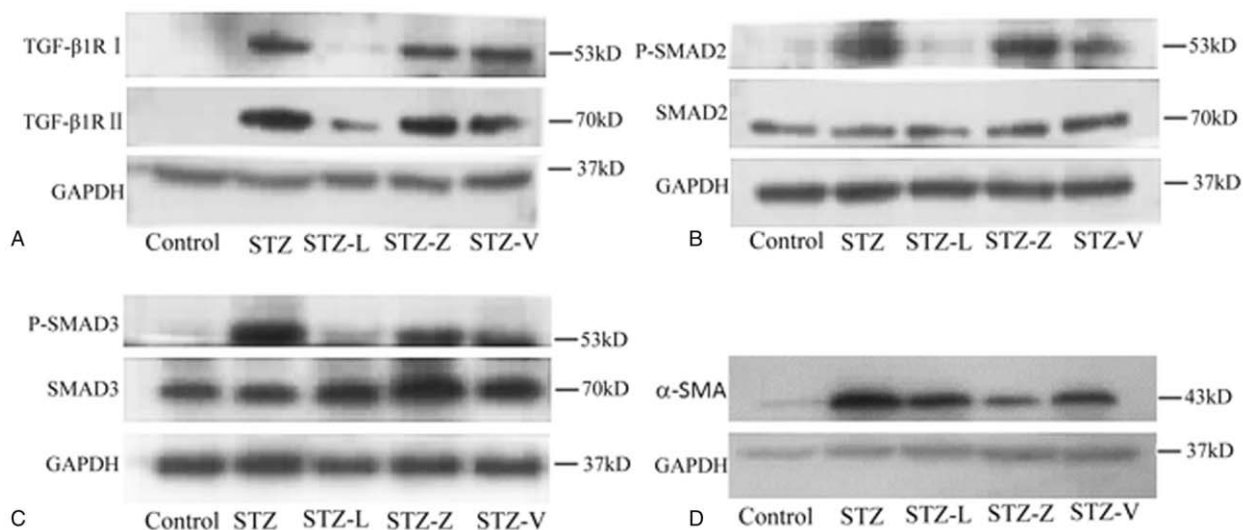
**3.3. LDP protected the renal tissues against oxidative stress in the DN rats**

One of the most well-established mechanism of DN is the massive production of oxygen-free radicals and the impaired antioxidant ability of renal tissues.<sup>[5,16]</sup> We measured the concentration of SOD, MDA, and NOS before and after treatment with LDP. As expected, compared with normal rats, the concentration of SOD was lower and that of MDA was higher in DN rats (Fig. 4A and B). LDP treatment significantly increased the concentration of SOD and NOS, and reduced the concentration of MDA (Fig. 4A–C). Similarly, administration of Zhenwu decoction or valsartan produced the same effects (Fig. 4A–C). Thus, we have proven that in DN rats LDP can protect renal tissues against oxidative stress and ameliorate the damage caused by lipid peroxidation.

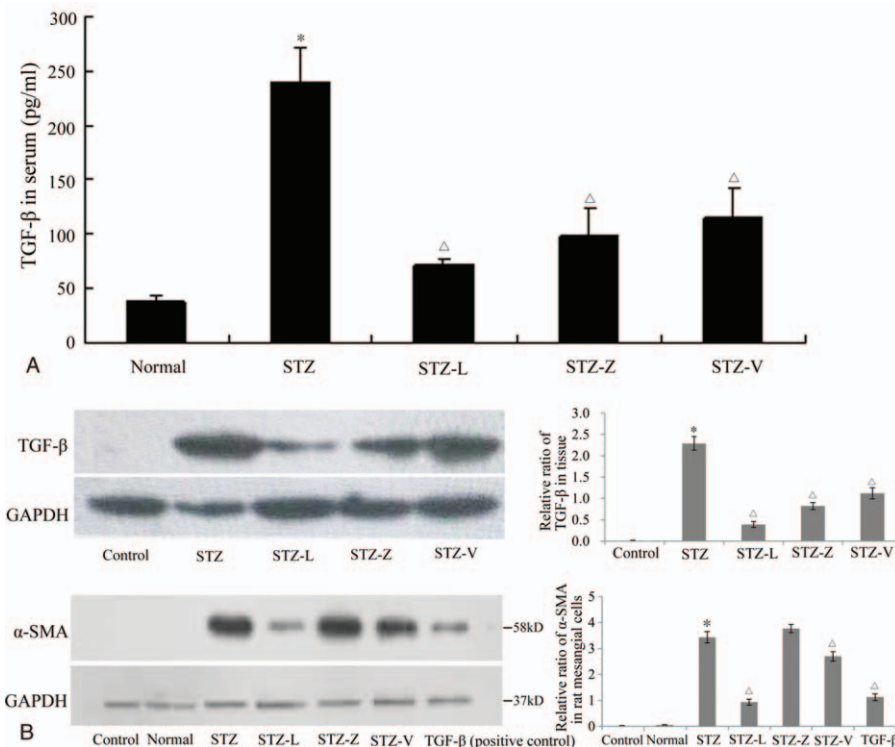
**3.4. LDP inhibited the TGF-β/SMAD signaling pathway in the renal tissues of DN rats**

Previous studies reported that TGF-β could bind to TGF-β RI and RII receptors on the cell membrane and induce

phosphorylation of Smad2/3, thus promoting the synthesis of ECM and expression of α-SMA in renal tubular epithelial cells, both of which are critical for renal fibrosis.<sup>[7,17]</sup> To demonstrate further the mechanisms of the protective role of LDP for DN, we investigated the effects of LDP on the TGF-β/SMAD signaling pathway in DN rats. Compared with normal rats, the renal expression levels of TGF-β RI and RII were significantly increased, and these levels were decreased after treatment with LDP, Zhenwu decoction, or valsartan (Fig. 5A). There was no significant difference in the renal expression of Smad2/3, but the increased level of Smad2/3 phosphorylation was observed in the DN rats, confirming the activation of the TGF-β/SMAD signaling pathway in DN. However, Smad2/3 phosphorylation was remarkably inhibited by treatment with LDP, Zhenwu decoction, or valsartan (Fig. 5B and C). Similarly, we showed that the increased expression of α-SMA in DN rats was also suppressed by our treatment (Fig. 5D). Therefore, we have demonstrated the inhibitory role of LDP in the TGF-β/SMAD signaling pathway, which constitutes 1 important mechanism for its protection against DN.



**Figure 5.** Treatment with LDP (n=8), Zhenwu decoction (n=8), and valsartan (n=8) block the TGF-β/SMAD signaling pathway in the renal tissues of DN rats by decreasing the levels of TGF-β RI, TGF-β RII (A), phosphorylated SMAD2 (B) and SMAD3 (C), and α-SMA (D). LDP, Zhenwu decoction, and valsartan were all administered intragastrically to DN rats for 8 weeks. α-SMA = alpha-smooth muscle actin, DN = diabetic nephropathy, L = LDP, LDP = Liuwei Dihuang pill, SMAD = mothers against decapentaplegic homolog, TGF-β = transforming growth factor beta, TGF-βR = TGF-β-receptor, X = valsartan, Z = Zhenwu decoction.



**Figure 6.** LDP, Zhenwu decoction, and valsartan treatments attenuated the increase of TGF-β concentrations in sera and kidney tissues of DN rats. (A) LDP, Zhenwu decoction, and valsartan treatments diminished the increase of serum TGF-β levels in DN rats as measured with ELISA and (B) in kidney tissue as measured by western blot. (C) Compared with untreated DN rats, the serum of DN rats treated with LDP, Zhenwu decoction and valsartan induced lower levels of α-SMA in in vitro cultured rat mesangial cells. The cells were cultured with rat serum for 24 h, and TGF-β (5 μg/mL) treatment was used as a positive control. Control cells were not treated with any rat serum and normal cells were treated with normal rat serum. α-SMA = alpha-smooth muscle actin, DN = diabetic nephropathy, L=LDP, LDP = Liuwei Dihuang pill, STZ = streptozotocin, TGF-β = transforming growth factor beta, X=valsartan, Z=Zhenwu decoction. \*P < 0.05 compared with normal rats, <sup>Δ</sup>P < 0.05 compared with STZ rats.

### 3.5. LDP inhibited the TGF-β/SMAD signaling pathway in rat mesangial cells

To investigate further the inhibitory effects of LDP on the TGF-β/SMAD signaling pathway, we treated rat mesangial cells with LDP in vitro. First, we isolated the serum from the DN rats after being treated with drugs for 8 weeks and found that the concentration of TGF-β was significantly higher in DN rats compared with normal rats, and the concentration was reduced after treatment with LDP, Zhenwu decoction, or valsartan (Fig. 6A), and we had similar results in the kidney tissues of all treated rats (Fig. 6B). We added the serum into the culture medium containing rat mesangial cells, and the proliferation of these cells was not affected (data not shown). However, the expression of α-SMA in mesangial cells treated with serum from DN rats was significantly increased, whereas that of cells treated with serum from the treatment group was significantly lower (Fig. 6C), confirming the inhibitory effects of LDP on the TGF-β/SMAD signaling pathway.

### 3.6. LDP inhibited the MAPK and NF-κB signaling pathways in the renal tissues of DN rats

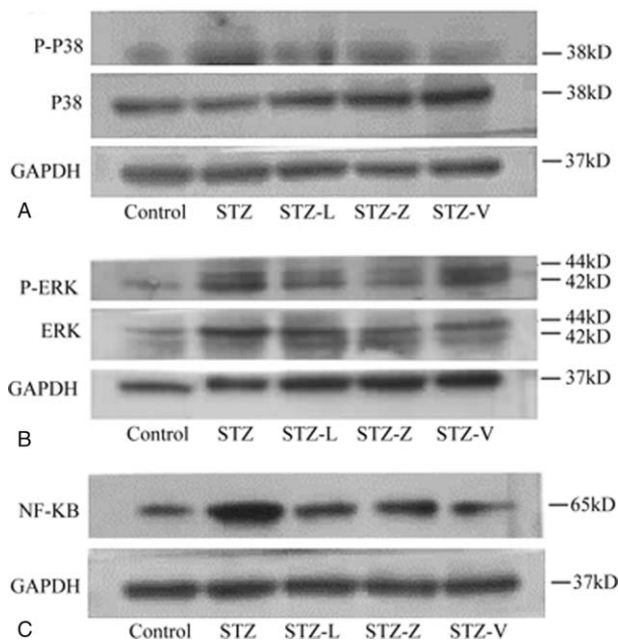
We evaluated the effects of LDP on the MAPK and NF-κB signaling pathways in the renal tissues of DN rats. The level of phosphorylation of p38 and ERK were higher in the kidneys of DN rats compared with normal rats, confirming the activation of MAPK pathway in the DN rats (Fig. 7A and B).

Importantly, LDP, Zhenwu decoction, and valsartan could prevent the phosphorylation of p38 or ERK in the DN rats (Fig. 7A and B). Meanwhile, it has been reported that the additional activation of NF-κB could also induce the expression of inflammatory factors in the kidney, thus accelerating the development and progression of DN. In our study, we showed that, in the DN rats, the expression of NF-κB was significantly higher compared with the normal rats, and treatment of LDP, Zhenwu decoction, or valsartan could prevent the increase of NF-κB (Fig. 7C). Therefore, LDP prevented the activation of the MAPK and NF-κB signaling pathways in the renal tissues of DN rats, which constituted 1 important mechanism for its protection against DN.

### 3.7. LDP prevented the development of DN by upregulating cytoglobin

Recently, cytoglobin (CYGB) has been reported to be closely associated with the pathogenesis of renal fibrosis.<sup>[18,19]</sup> For example, renal interstitial fibrosis was induced by unilateral ureteral obstruction (UUO) in a rat in vivo animal model. The expression of CYGB in the obstructed kidneys was increased remarkably in a time-dependent manner, suggesting that CYGB could be a specific marker for the progression of renal fibrosis.<sup>[19]</sup> We evaluated the expression of CYGB in DN rats, and as shown in Fig. 8A, the expression of CYGB was significantly higher in their renal tissues, confirming the extensive involvement of CYGB in the development of renal fibrosis. Surprisingly, the level of





**Figure 7.** Treatment with LDP (n=8), Zhenwu decoction (n=8), and valsartan (n=8) block MAPK and NF-kB signaling pathways in renal tissues of DN rats by decreasing the levels of phosphorylated p38 (A), ERK (B) and NF-kB (C). LDP, Zhenwu decoction, and valsartan were administered intragastrically to DN rats for 8 weeks. DN = diabetic nephropathy, ERK = extracellular signal regulated kinase, L=LDP, LDP = Liuwei Dihuang pill, MAPK = p38 mitogen-activated protein kinases, NF-kB = nuclear factor kappa light chain enhancer of activated B cells, X=valsartan, Z=Zhenwu decoction.

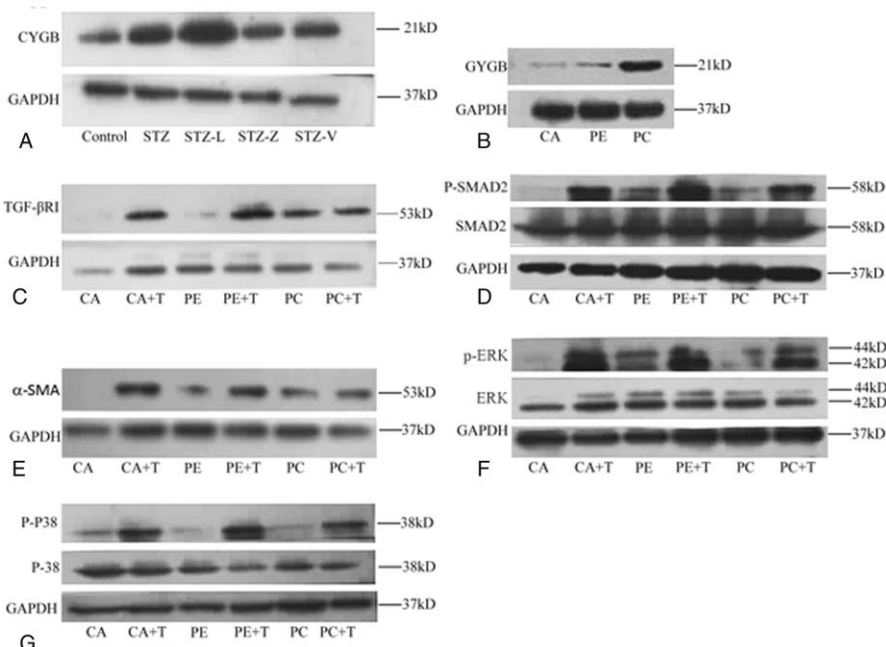
CYGB was only increased in rats treated with LDP, which was not observed in DN rats treated with the other 2 drugs (Fig. 8A).

In order to investigate further the role of CYGB in the pathogenesis of renal fibrosis, we investigated the effects of CYGB overexpression on the TGF-β/SMA and MAPK pathways in renal mesangial cells via transient transfection of a CYGB-containing plasmid.

As shown in Fig. 8B, CYGB was successfully overexpressed in rat renal mesangial cells. Normally, increased levels of TGF-β would induce the expression of TGF-β R1, α-SMA, and the phosphorylation of Smad2, which were suppressed in cells with CYGB overexpression (Fig. 8C–E). Similarly, the phosphorylation of p38 and ERK induced by TGF-β activation was also inhibited by CYGB overexpression (Fig. 8F and G). Therefore, CYGB could suppress the activation of renal mesangial cells by suppressing the SMAD and MAPK signaling pathways, thus preventing the development of renal fibrosis and protecting renal functions in DN rats.

#### 4. Discussion

Diabetic nephropathy is a severe renal disease characterized by kidney injury and tissue fibrosis. In this study, we successfully reproduced the stereotypical pathological changes of the DN kidney in diabetic rats. In rats treated with STZ, we observed an abnormally increased kidney coefficient, serum creatinine, blood urea nitrogen, and 24-hour 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. We showed that all the clinical indexes and pathological findings were improved after



**Figure 8.** CYGB suppresses the activation of renal mesangial cells by suppressing the SMAD and MAPK signaling pathways. (A) LDP treatment further increases the expression of CYGB in renal tissues of DN rats. (B) Overexpression of CYGB in in vitro cultured mice mesangial cells after transfection with a CYGB containing plasmid. (C–E) Overexpression of CYGB inhibit TGF-β 1-induced expression of TGF-β R1 (C) and α-SMA (E), and phosphorylation of SMAD2 (D), p38 (G), and ERK (F). The cells are harvested after 24 h of TGF-β 1 stimulation (5 μg/mL). α-SMA = alpha-smooth muscle actin, CA = control without transfection, CYGB = cytoglobin, DN = diabetic nephropathy, ERK = extracellular signal regulated kinase, LDP = Liuwei Dihuang pill, MAPK = p38 mitogen-activated protein kinases, PC = CYGB containing plasmid, PE = empty plasmid, SMAD = mothers against decapentaplegic homolog, T = TGF-β, TGF-β = transforming growth factor beta, TGF-βR = TGF-β-receptor.



LDP was administered, findings previously reported by other researchers.<sup>[13,14]</sup> Therefore, in the rat model of diabetes, we further proved the therapeutic effects of LDP in protecting renal function and preventing renal fibrosis.

The oxidative stress response has been considered as one of the most important underlying mechanisms in DN development and progression.<sup>[8]</sup> This was further proven in the present study in renal tissue of DN rats, with the level of SOD remarkably reduced and the level of MDA increased. On the other hand, the antioxidative effects of LDP was first shown in our study, which could ameliorate the antioxidative damage in DN rats by significantly increasing SOD and NOS while decreasing MDA. Additionally, this finding suggested that suppression of oxidative stress is an effective way to prevent or treat DN.

Moreover, the activation of TGF- $\beta$ /SMAD and MAPK signaling pathway was shown to occur in rats with diabetes and renal oxidative stress. We demonstrated that the activation of these specific pathways was the key to the expression and secretion of various inflammatory factors and growth factors, which further promoted deposition of ECM, myofibroblast proliferation, and ECM secretion. These reactions eventually produced renal fibrosis in the DN rats.<sup>[20,21]</sup> Interestingly, administration of LDP dramatically reduced the level of serum TGF- $\beta$ 1, the expression of TGF- $\beta$ R in renal tissues, and the high phosphorylation of SMAD2/3 in DN rats, indicating that multicomponent and multitarget mechanisms suppressed the TGF- $\beta$ 1/SMADS signaling pathway. The same effects were also observed in the expression of p38, NF- $\kappa$ B, and the phosphorylation of ERK in renal tissues, and LDP also inhibited the MAPK and NF- $\kappa$ B signaling pathways in an efficient way. Hence, we have demonstrated the critical roles of TGF- $\beta$ /SMAD, MAPK, and NF- $\kappa$ B pathways in treating DN with LDP.

The results from the present study also provide novel insights into the role of CYGB in the pathogenesis of renal fibrosis. Similar to previously reported data,<sup>[9]</sup> CYGB levels were increased in DN rats, and this action was further enhanced by the administration of LDP. More importantly, in mesangial cells cultured *in vitro*, overexpression of CYGB was able to promote the phosphorylation of SMAD2, p38 and ERK induced by TGF- $\beta$ , and the expression of TGF- $\beta$  R1 and  $\alpha$ -SMA was also increased. These findings suggest a potential role of CYGB in preventing renal fibrosis and the pathogenesis of DN through the activation of SMAD and MAPK pathways. Furthermore, the elevated expression of CYGB is likely to provide critical support for LDP to suppress the SMAD and MAPK signaling pathways. A future study will be required to investigate the mechanisms involved in LDP upregulation of the expression of CYGB.

Taken together, there was no difference in TP, BUN, and creatinine values or kidney coefficients between the 3 treatment groups, but LDP treatment yielded the highest SOD and NOS concentrations and lowest TGF- $\beta$ , TGF- $\beta$  1RI, TGF- $\beta$  1RII, P-Smad 2 and P-Smad 3 concentrations in kidney tissues compared to Zhenwu decoction and valsartan treatments. Also, in the rat sera, the highest attenuation of TGF- $\beta$  expression was achieved by LDP, which reflected in the lowest  $\alpha$ -SMA expression of mesangial cells after incubation with sera from LDP treated STZ rats.

A limitation of our present study was the relative short duration of drug treatments and like for other studies with multitherbal mixtures, that additional unknown mechanisms than the one we observed cannot be excluded.

## 5. Conclusions

We have demonstrated the protective role of LDP in the renal function of DN rats. LDP was able to preserve the function of mesangial cells and prevent the progression of renal fibrosis by suppressing the oxidative stress response, ameliorating the damage caused by lipid peroxidation, and inhibiting the TGF- $\beta$ /SMAD, MAPK and NF- $\kappa$ B pathways. Furthermore, LDP could upregulate the expression of CYGB, through which the TGF- $\beta$ /SMAD and MAPK signaling pathways were further suppressed. Thus, the detailed molecular mechanism involved in LDP therapy for DN has provided a more reliable theoretical basis for the clinical application of LDP in treating DN patients.

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