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Yishenhuoxue formula regulates TGF-β/Smad signal transduction to protect rats against Diabetic kidney disease injury

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Abstract: TGF-β signal pathway activation is vital in the pathogenesis of DKD. We aim to investigate the role of Yishenhuoxue formula on TGF-β/Smad signal transduction in DKD rats. 60 male adult Wistar rats were enrolled and randomly allocated into four groups: N group, M group (given STZ 60mg/kg, ip), H group (given Yishenhuoxue formula 1.0g/kg/day, ig) and L group (given Yishenhuoxue formula 0.5g/kg/day, ig). The levels of BW, 24h UV, SCr, UCr, mALB were measured after 8 weeks treatment, while the levels of KW/BW index, CCr and UAER were calculated by relevant formula. The rats' left kidneys were harvested to detect histological changes by PAS staining and right kidneys were harvested to detect the levels of TGF-β, Smad2/3, phosphorylated Smad 2/3, Smad 7 and CTGF by western blot analysis. We found that Yishenhuoxue formula treatment can protect kidneys from DKD injury, which is illustrated with following criteria: 1) a significant decrement in KW/BW index, 24h UV, SCr, mALB and UAER, while a significant increment in BW, UCr, CCr (p<0.05 vs. M group); 2) minor and segmental changes as slight expansion of the glomerular basement membrane compared with M group; 3) an apparent decrease in levels of TGF-β1, phosphorylated Smad 2/3 and CTGF, while an apparent increase in levels of Smad 2/3 and Smad7 compared with M group (p<0.05). The studies confirm that Yishenhuoxue formula has strong inhibitory effect on TGF-β/Smad signal transduction in DKD rats' kidneys by decreasing expression of TGF-β1, weakening of Smad 2/3 phosphorylation and increasing expression of Smad 7.

Keywords: Yishenhuoxue formula, TGF-β/Smad, signal transduction, diabetic kidney disease, diabetes mellitus.

INTRODUCTION

There are approximate 366 million people all around the world will suffer from diabetes mellitus (DM) until 2030 which is estimated by the World Health Organization (Fernandes, 2016). The increment in morbidity and mortality due to DM or its complications is alarming enough to call the world's attention. Microvascular changes caused by hyperglycemia may lead to progressive injury in kidneys, eyes, blood vessel and so on (Forbes *et al*., 2013; Yan *et al*., 2016). Diabetic kidney disease (DKD) induced by DM has been one of the main inducements of end-stage renal failure (ESRF) (Nieto-Ríos *et al*., 2016). Many studies have shown that hyperglycemic microenvironment can break the balance of cell metabolism and induce all structural compartments' functional impairments in kidney at very early stage of the disease (Jindal *et al*., 2013).

Experiments have shown that DKD is progressive and complex disease involved in many mechanisms such as oxidative stress, inflammation response and fibrosis (Jindal *et al*., 2013; Wada *et al*., 2013; Meng *et al*., 2015). And the activation of transforming growth factor-β (TGF-β)/Smad signal transduction is vital to promote DKD occurrence and progression (Zhang *et al*., 2013; Horbelt *et al*., 2012). Many experiments have found that TGF-β is overexpressed in both animal renal fibrosis models and human counterparts (Sun *et al*., 2011). What's more, the transition of renal tubular epithelial cells to myofibroblasts leading to renal fibrosis is modulated by TGF-β/S mad signal pathway (Zeisberg *et al*., 2010). Connective tissue growth factor (CTGF) as one of the main downstream products of TGF-β/Smad signal pathway contributes to renal fibrosis.

TGF-β is a polypeptides with multifarious biological activities like cell proliferation, apoptosis and tumor cell metastasis in a wide range of biological systems (Kubiczkova *et al*., 2012; Wu *et al*., 2017; Liu *et al*., 2016). TGF-β family have three isoforms as TGF-β1, TGF-β2 and TGF-β3 in mammals. There are highly conserved regions in their essential structure and same receptor signal pathway to function activity. Nevertheless, the distribution of TGF-β three isoforms is quite different. TGF-β1 is widely existed and abundant expressed isoform which can be produced by all kinds of renal resident cells (Meng *et al*., 2015). TGF-β2, firstly found in human glioblastoma, is mainly expressed in nervous system. And TGF-β3 is isolated from rhabdomyosarcoma cell line. The bioactive TGF-β1 binds to its receptors which are some cell surface proteins and makes them phosphorylate, ubiquitylate and sumoylate. Through Smad pathway the signal transduce from cytoplasm into nucleus regulating the target genes expression.

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We have found Yishenhuoxue formula, a traditional Chinese herbal compound, has protective effects on kidney injury induced by STZ. Herein, we investigated the role of Yishenhuoxue formula on TGF-β/Smad signal transduction in rats with DKD to reveal its action mechanism.

MATERIALS AND METHODS

Chemicals

Yishenhuoxue formula is a traditional Chinese herbal compound as clinical prescription and composed of ginsenoside, fructus schisandrae chinensis, astragali radix,, radix rehmanniae praeparata, ophiopogonis radix, alismatis rhizoma and their amount ratio is 1:1:2.5:5:1.5:1. The astragali radix, radix rehmanniae praeparata, ophiopogonis radix and alismatis rhizome are mixed and extracted by water. After filtrating and concentrating, extractum A is made. The fructus schisandrae chinensis is extracted by alcohol and extractum B is made by same method. Then extractum A, extractum B and ginsenoside are blended, dried, and crush into fine powder to prepare granules. The character identification of deoxyschizandrin, verbascoside and 23-O-acetylalisol B are detected by TLC method. The content assay of ginsenoside Rg1, ginsenoside Re and astragaloside IV are performed by HPLC.

Rat serum creatinine (SCr), urinary creatinine (UCr) and microalbuminuria (mALB) ELISA kits were purchased from Jiancheng Bioengineering Institute, Nanjing, China. STZ was purchased from Sigma and AB-PAS kit was purchased from Solarbio life sciences. And polyclonal antibodies used in Western blot analysis were bought include anti-TGF-β (Santa Cruz Biotechnology), anti-Smad2/3 (Cell Signaling), anti-phosphorylated Smad2/3 (Cell Signaling), anti-Smad7 (Bioss), anti-CTGF (Bioss) and anti-GAPDH (Proteintech) for rats. A cocktail of proteases inhibitors was purchased from Roche. The other reagents were in analytical grade.

Animals

60 male adult Wistar rats (180-220g) were bought from Laboratory Animal Center of Jilin University. The experiments were allowed by the Animal Care Committee of Jilin University Pharmaceutical College. The animals were kept in a room temperature of 22±2°C on a 12h light/dark cycle and fed on a normal laboratory diet and water ad libitum.

In vivo experiments

The rats were marked and randomly allocated into four groups (n=8): 1) N group (vehicle injection), 2) M group (rats were given 60mg/kg of STZ, ip), 3) H group (STZ-injection rats were given 1.0g/kg/day of Yishenhuoxue formula, ig) and 4) L group (STZ-injection rats were given 0.5g/kg/day of Yishenhuoxue formula, ig). Rats' blood glucose levels were measured at 72h after STZ-injection except control group. Only the ones with blood glucose concentration higher than 13.8mmol/L had been employed in our study.

After 8 weeks' treatment, the rats were housed in individual metabolic cages to collect 24h urine samples. The body weight (BW) and 24h urine volume (24h UV) were measured. The blood plasma and urine samples of the four groups were employed to detect SCr, UCr, mALB levels by assay kits according to the manufacturer's instructions. The creatinine clearance (CCr) was calculated by the formula of $CCr = \frac{UCr \times urine\ flow\ in\ 24h}{SCr \times BW}$, and urine albumin excretion rate (UAER) was calculated by mALB × 24h UV. After euthanized, kidneys (left) were harvested and weighed. The KW/BW index was calculated by kidney weight ÷ body weight × 100%. After rinsed free from blood with PBS, the kidneys were fixed in 10% neutral formalin and embedded in paraffin. And then the kidney tissue slides were stained with AB-PAS kit. Histological judgment using a light microscope (Nikon Ti) was performed in a blind assessment by pathologists.

50mg fresh kidneys (right) were treated with 1 mL lysis buffer and 10μL (100×) proteases inhibitors cocktail. Then the tissue homogenates were boiled for 10min and clarified by centrifugation. After determined by the BCA kit, equivalent protein amounts were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P polyvinylidene fluoride membranes. The blots were then hybridized with anti-TGF-β, anti-Smad2/3, anti-phosphorylated Smad2/3, anti-Smad7, anti-CTGF and anti-GAPDH antibodies respectively and antigen-specific signals were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Gel analyses were performed to test for the gray value of the western blot bands using Image J.

STATISTICAL ANALYSIS

Statistical analyses were performed by SPSS 20.0 programs. Results were expressed as means ± SDs and analyzed utilizing One-way ANOVA and LSD, post hoc. And statistical significance was set at $p<0.05$.

RESULTS

Yishenhuoxue formula protects kidney from DKD injury

As shown in fig. 1, DKD rats (M group) induced by STZ-injection presented a significant increase in KW/BW index, 24h UV, SCr, mALB and UAER, while a significant decrease in BW, UCr, CCr ($p<0.05$ vs. N group).

Shown in fig. 1A and 1B, rats in H group and L group had higher levels of BW (H group vs. M group, $p<0.05$; L group vs. M group, $p<0.05$) and lower levels of KW/BW

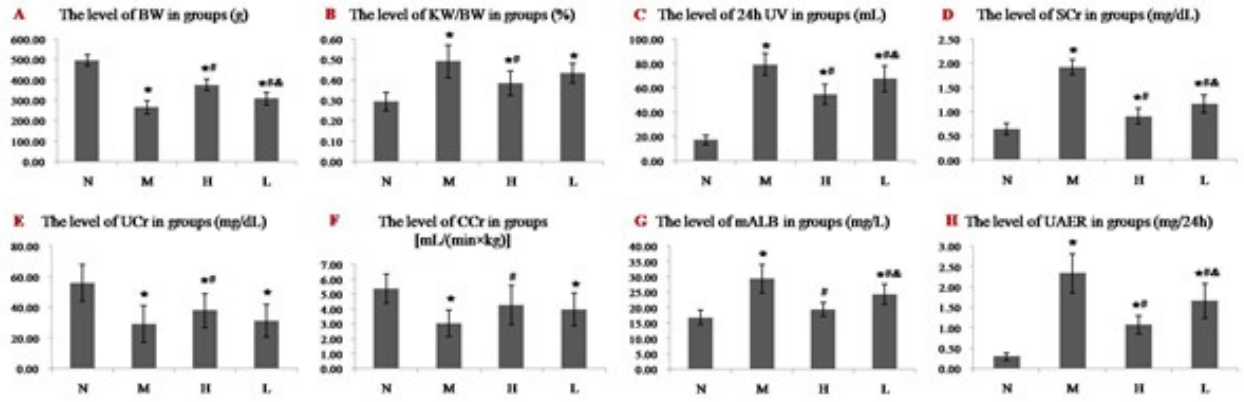

Fig. 1: The levels of BW, KW/BW index, 24h UV, SCr, UCr, CCr, mALB and UAER in groups. A: The level of BW; B: The level of KW/BW index; C: The level of 24h UV; D: The plasma level of SCr; E: The urine level of UCr; F: The level of CCr; G: The urine level of mALB; H: The level of UAER. *p<0.05 vs. N group; #p<0.05 vs. M group; &p<0.05 vs. H group.

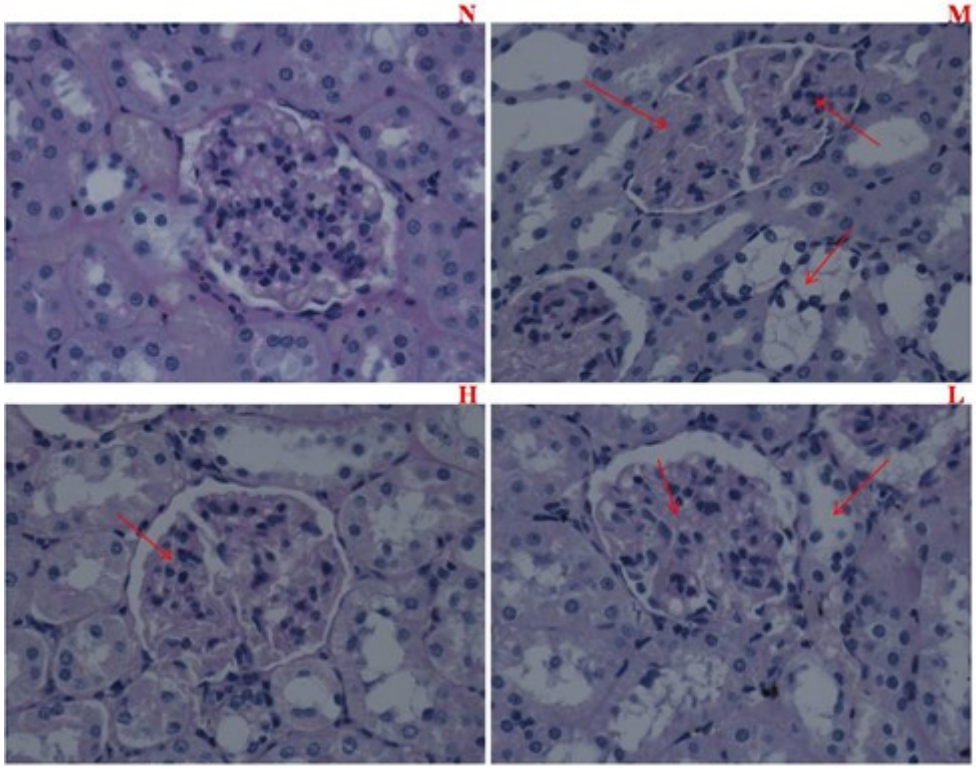

Fig. 2: Photomicrographs of representative AB-PAS staining sections of kidney (400×magnification).

index (H group vs. M group, p<0.05; L group vs. M group, p>0.05) after 8 weeks' Yishenhuoxue formula treatment. Compared with L group, H group had higher BW level (p<0.05) and lower KW/BW index level (p>0.05).

We knew that Yishenhuoxue formula could significantly decrease the level of 24h UV in H group and L group (p<0.05 vs. M group). However, rats in H group had lower 24h UV level than L group (p<0.05) (fig. 1C).

The SCr, UCr and CCr changes of the four groups had presented in fig. 1D, E and F. Yishenhuoxue formula treatment (H group) could significantly attenuate SCr level, while intensify UCr level and CCr level compared with M group (p<0.05). However, there were no significant difference of UCr level and CCr level between L group and M group as p>0.05.

The fig. 1G and 1H indicated that, rats in H group and L group had lower levels of mALB and UAER compared with the ones in M group (p<0.05). And H group had lower levels of mALB and UAER than L group (p<0.05).

Histological changes of renal tissue in groups

The histological results (fig. 2) showed that Yishenhuoxue formula treatment had the ability to protect the kidney tissue from DKD injury. Compared with N group, kidney slides of the rats in M group had significant pathological

changes, including: 1) expansion of glomerular basement membrane, 2) renal tubulointerstitial injury, 3) accumulation of extra cellular matrix and 4) appearance of nodular lesions. We observed that DKD rats treated with Yishenhuoxue formula in H group were very similar to the ones in N group with minor and segmental changes as slight expansion of glomerular basement membrane. The rats in L group exhibited pathological changes such as a moderate expansion of glomerular basement membrane and renal tubulointerstitial damage compared with N group.

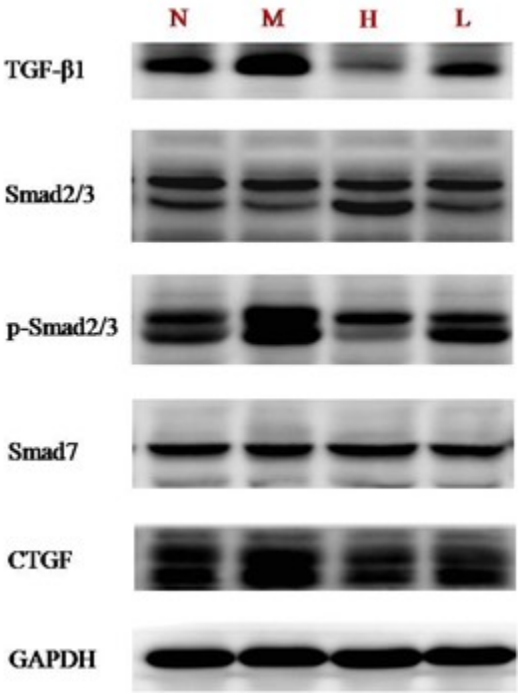

Fig. 3: The western blot analysis of TGF-β/Smad signaling pathway in DKD rats' kidneys.

Yishenhuoxue formula inhibits transforming growth factor β/Smad signal transduction in DKD rats

In order to investigate the effect of Yishenhuoxue formula on TGF-β/Smad signal transduction in rat's kidney, the western blot analysis of TGF-β, Smad2/3, phosphorylated Smad2/3, Smad7 and CTGF were performed. Compared with N group, rats in M group showed aggravated activation of TGF-β/Smad signal transduction by severe expression of TGF-β1, promotion of Smad2/3 phosphorylation and decrease expression of Smad7 (p<0.05) (shown in fig. 3 and fig. 4). And the level of CTGF in M group was apparently increased compared with N group (p<0.05). However, Yishenhuoxue formula treatment in H group could apparently decrease expression of TGF-β, inhibit of Smad2/3 phosphorylation, increase expression of Smad7 and reduce expression of CTGF (p<0.05 vs. M group). Yishenhuoxue formula treatment in L group could also have inhibitory effect on TGF-β/Smad signal transduction, but lower than H group.

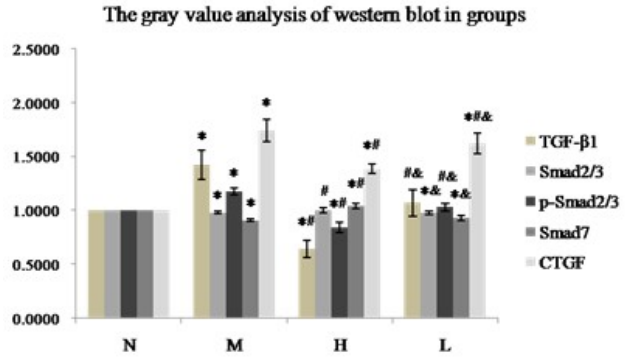

Fig. 4: The gray value analysis of western blot in groups. *p<0.05 vs. N group; #p<0.05 vs. M group; &p<0.05 vs. H group.

DISCUSSION

Hemodynamics changes caused by DM are the contributing factor of micro vascular complications. DKD is the most common and high incidence micro vascular comorbidity of DM with renal cell structural destroy and renal function loss (Sowers, 2007). Although DM patients can get some benefit by strictly controlling levels of blood glucose and pressure, the morbidity of DKD is still high along with aging, lipoprotein metabolic abnormalities and others (Wang *et al*., 2016; Valk *et al*., 2011).

We found that a single intraperitoneal injection of 60mg/kg STZ could sharply raise levels of KW/BW index (p<0.05), 24h UV (p<0.05), SCr (p<0.05), mALB (p<0.05) and UAER (p<0.05), while significantly down levels of BW (p<0.05), UCr (p<0.05), CCr (p<0.05) compared with control group after 8 weeks. We conclude that STZ-injection can reduce renal function by increasing microalbuminuria and decreasing glomerular filtration rates. The renal structural damage as thickening of glomerular basement membrane, tubulointerstitial injury, accumulation of extracellular matrix and nodular lesions in M group have been demonstrated by PAS staining. The results also show that the 1.0g/kg/day Yishenhuoxue formula treatment can apparently decrease the raise levels of KW/BW index, 24h UV, SCr, mALB and UAER induced by STZ (p<0.05). The levels of BW, UCr and CCr are significant increased compared with M group. According to PAS staining, we know that Yishenhuoxue formula can protect renal cell from DKD injury with minor and segmental changes of glomerular basement membrane.

The activation of TGF-β/Smad signal transduction in kidney cells is a pivotal pathogenesis in the development of DKD in both animal models and human counterparts (Giacco *et al*., 2010). There are mainly three types TGF-β receptors on cell surface as TβR I, TβR II and TβR III (Poniatowski *et al*., 2015; Hinck *et al*., 2011). TβR I and TβR II, both transmembrane serine/theronine kinases, are

participated in mediating signal transduction. TGF-β is synthesized as a precursor protein which is cleaved to gain 112 amino acid polypeptide and the latent portion. The precursor proteins are proper folded and dimerized under a variety activating signal of TGF-β and then cleaved from their propeptides to get the mature, dimeric and bioactive form of TGF-β (Kubiczkova *et al*., 2012). The bioactive TGF-β binds to TβR II firstly, and then make a Gly-Ser regulatory region of TβRI phosphorylate. After that, a complex composed of dimeric TGF-β, TβRI and TβR II is formed (Shi *et al*., 2003). The Smad pathway is activated directly after TβRI recognition and phosphorylation. The hetero-tetrameric receptor complex recruits non-activated Smad 2/3 and makes them phosphorylate (Ai *et al*., 2015; Chen *et al*., 2011; Chen *et al*., 2014). Phosphorylated Smad 2/3 turns complexes with Smad4 which translocate from the cytoplasm into the nucleus. CTGF, AP-1 and so on cytokines, the target genes products, disorder the recruitment of Smad2/3 and work on positive feedback loop of Smad signal activation. Smad7 can degrade the activated TβRs via lysosomal and proteasomal to down-regulate TGF-β/Smad signal transduction.

In our study, 1.0g/kg/day Yishenhuoxue formula treatment could apparently inhibit TGF-β/Smad signal transduction by reducing expression of TGF-β1 ($p<0.05$), suppressing phosphorylation of Smad2/3 ($p<0.05$), increasing expression of Smad7 ($p<0.05$) and decreasing expression of CTGF ($p<0.05$) compared with M group. Low dose of Yishenhuoxue formula treatment in L group also has inhibitory effect on TGF-β/Smad signal transduction, but lower than H group.

Yishenhuoxue formula as a Chinese herbal compound can protect kidneys from DKD injury with many advantages of various biological activities and low toxicity. The lack of more scientific acting mechanism will limit its clinical usage as a supplement for DM patients to help stabilize blood glucose homeostasis and improve their complication. And our study may be a specific mechanism of Yishenhuoxue formula on anti-DKD which can expand its application. The other therapeutic mechanisms, long-term effects of Yishenhuoxue formula on DKD and determination of active ingredients will inspire us to do further research.

In conclusion, our experiments provide direct evidence that Yishenhuoxue formula has a kidney protective effect on decrease microalbuminuria, increase glomerular filtration rates and keeping renal cell from injury. Yishenhuoxue formula has strong inhibitory effect on TGF-β/Smad signal transduction by decreasing expression of TGF-β1, weakening of Smad 2/3 phosphorylation and increasing expression of Smad 7. And the inhibitory effect on TGF-β/Smad signal transduction is characterized by dose dependent. But there are still some investigations required to study its mechanism in depth and justify the active constituents among the complex compounds.

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