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Original Article

The Yi-Qi-Bu-Shen recipe attenuates high glucose-induced podocyte injury via the inhibition of IKK-IκBα-NFκB and ERK/P38 MAPK signaling

Rui-Ting Hu[1,2], Chang-Ling Li[1], Xi Zhang[1], Chun-Yao An[3], Chun-Mei Ma[1], Xiu Li[3], Xiao-Hong Liu[3], Wen-Jing Li[3], Yun Qiao[1], De-Shan Liu[1]

[1]Department of Traditional Chinese Medicine, Qilu Hospital of Shandong University, Jinan 250012, P.R. China; [2]Hospital of Traditional Chinese Medicine of Linyi City, Linyi 276002, P.R. China; [3]Shandong University of Traditional Chinese Medicine, Jinan 250355, P.R. China

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Abstract: The exact mechanisms underlying diabetic nephropathy (DN) remain unknown, but some studies suggest that structural and compositional changes of the slit diaphragms (SD) between podocytes may play an important role in this course. The Yi-Qi-Bu-Shen recipe (YB) is a traditional Chinese herbal formula that is commonly used for the treatment of diabetes mellitus and especially DN. This study investigated the effect of high glucose (HG) on expression of nephrin, podocin, and CD2AP in podocytes and the treatment effect of YB on these molecules. Conditionally immortalized human podocytes were exposed to medium containing normal glucose (NG) or HG for 24 hours. The podocytes cultured in a HG environment had relatively lower expression of nephrin, podocin, and CD2AP both in mRNA and protein synthesis levels compared to NG group. Nephrin, podocin, and CD2AP expression in the HG group was effectively restored by the treatment of YB. In addition, inflammatory cytokines such as IL-1β, IL-6, TNF-α, and MCP-1 were significantly decreased in HG+YB group compared with the HG group. Moreover, YB suppressed HG-induced activation of IKK-IκBα-NFκB and ERK/P38 MAPK signaling pathways. NFκB, ERK, and P38 inhibitors attenuated HG-induced podocyte injury and increased nephrin, podocin, and CD2AP expression further revealed the healing mechanisms of YB. This study demonstrates that YB treatment attenuated HG-induced podocyte injury through suppressing activation of IKK-IκBα-NFκB and ERK/P38 MAPK signaling pathways and reducing IL-1β, IL-6, TNF-α, and MCP-1 levels, suggesting that YB is a potential therapeutic drug for DN.

Keywords: The Yi-Qi-Bu-Shen recipe, podocyte, nephrin, podocin, CD2AP

Introduction

Diabetic nephropathy (DN) is one of the most serious complications of diabetes mellitus and the main cause of the end-stage renal disease (ESRD) [1, 2]. The characteristics of DN include mesangial cells proliferation, mesangial matrix accumulation, and glomerulosclerosis [3, 4]. Microalbuminuria and later proteinuria are the most important clinical manifestations in the developing process of DN. The glomerular filtration barrier (GFB), which prevents proteins leaking from capillaries to urine, includes endothelial cells, glomerular basement membrane (GBM) and podocytes. Any injury of these portions may lead to the occurrence of microalbuminuria and later proteinuria [5, 6].

Podocytes are highly differentiated epithelial cells that interface with the GBM surface and play a significant role in maintaining the integrity of the GFB. Increasing evidence suggests that podocytes prevent protein leakage from plasma to primary urine [7, 8]. Slit diaphragms (SD) between podocytes are the essential determinant of podocyte function [9]. Some specific proteins in the SD could affect the structure and function of podocytes, the most important of them are nephrin, podocin, and CD2AP. Nephrin is a transmembrane glycoprotein and the structural backbone of SD, which encoded by the NPSH1 gene. Podocin is a hair-like structured membrane protein expressed specifically in SD, which can enhance the signal transduction induced by nephrin and is involved

in the structure of cytoskeleton. CD2AP is a cohesive protein that connects SD and the cytoskeleton and plays a stabilizing role in podocytes. In DN patients and animal models, the mRNA and protein expressions of these molecules are significantly reduced and closely related to the progression of proteinuria [10-12]. Hence, finding a drug that can protect these molecules from injury is essential for DN treatment.

Chronic inflammation is closely associated with permeability changes in the GFB and proteinuria in DN. Inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are elevated even in the early stage of DN. Subsequently, TNF-α amplifies the activation of inflammatory cytokines, thereby leading to DN progression [13-15]. Among several signaling pathways that regulate chronic inflammation, it has been reported that NFκB and MAPK are associated with podocyte injury in DN [16, 17].

YB has been used in Qi'lu Hospital for more than 20 years [18, 19], previously clinical data demonstrated that YB reduces proteinuria and decreases blood glucose level in DN patients. However, the exact mechanisms of YB in the treatment of proteinuria remain unknown. Since podocyte injury has been found to be the primary determinant of proteinuria, the aim of the present study is to investigate the protective effects of YB on HG-induced podocyte injury.

Materials and methods

Materials

RPMI-1640 culture medium and D-glucose were purchased from Sigma-Aldrich. Fetal bovine serum (FBS), TRIzol reagent, SYBR Green Master Mix and PierceTM fast Western blot kit were purchased from Thermo Fisher Scientific. iScript Advanced cDNA synthesis kit was purchased from Bio-Rad. Cytotoxicity detection kit (LDH) and X-tremeGENE HP DNA transfection reagent were purchased from Roche Applied Science. BCA protein assay kit was purchased from Beyotime Biotechnology. The primary antibodies were purchased from Cell Signaling Technology and Affinity Biosciences. pNFκB-TA-Luc, pRL-TK and a Dual-Luciferase Reporter Assay System were purchased from Promega. IκBα inhibitor BAY 11-7085 was purchased from Santa-Cruz. P38 MAPK inhibitor SB203580 and ERK MAPK inhibitor U0126 were purchased from Cell Signaling Technology.

Composition and preparation of the Yi-Qi-Bu-Shen recipe (YB)

The composition and preparation of the YB extract are identical with our previous studies [18, 19]. Briefly, the YB was composed of crude Radix Astragali, prepared Rhizome of Rehmannia, Rhizoma Polygonati, Rhizoma Chuanxiong, Herba Epimedii, Fructus Lycii, Rhizoma Atractylodis, Radix Puerariae and Rhizoma Coptidis. All crude drugs were the products of Jinan Jianlian Traditional Chinese Medicine Co. Ltd. They were chopped finely and extracted with 20 times the amount of distilled water at 100°C for 2 hours, a process that was repeated three times. The extract was evaporated under pressure and insoluble substances were removed via filtration and then dried to obtain YB powder in a vacuum drying furnace (yield: 28.06%). The YB powder was prepared by the Manufacturing Laboratory of Qilu Hospital, Shandong University (one gram was equal to 3.564 g of crude drug).

Study subjects

A total of 160 patients suffering from type 2 diabetes and presence of microalbuminuria, aged 51 to 73 years old, were randomly enrolled between January 2015 and December 2016 from the Qi'lu hospital of Shandong University. Glucose cutoff values were used for diagnosis of DM based on instruction of the World health organization. The urine albumin to creatinine ratio (ACR) ranged from 30 to 300 μg/mg creatinine on two of three morning urine collections and was considered as microalbuminuria. Exclusion criteria were history of malignancies, acute or chronic inflammatory and infectious diseases, diabetic ketosis, ketoacidosis, and use of drugs possibly affecting glucose metabolism (eg, glucocorticoid). All the participants received oral hypoglycemic agents metformin and acarbose based on their clinical condition. All the patients received Irbesartan against DN and atorvastatin against hyperlipidemia. Additionally, the treatment group received the Yi-Qi-Bu-Shen Recipe therapy for six months. The study was carried out in compliance with the Ethics Committee of Qi'lu hospital. Written informed consent was obtained from all participants.

Table 1. Primers for qRT-PCR

Primers	Forward: 5'-3'	Reverse: 5'-3'	Product length (bp)
Nephrin	GAGGCTGGTGTGTTTGGCTA	GCCAGGATCGTCACGTTAGT	273
Podocin	TCTGGTTCTGCGTAAAGGTTGT	GCACAAGGAATTGCACAGCTT	294
CD2AP	TGGAGATAACAAAAACAGATACCGA	TTTGGAGCTGGAGCCTTAGC	271
IL-1β	ACAGATGAAGTGCTCCTTCCAG	AAGCCCTTGCTGTAGTGGTG	93
IL-6	AATAACCACCCCTGACCCAAC	ACATTTGCCGAAGAGCCCT	149
TNF-α	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC	91
MCP-1	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT	190
GAPDH	TCGGAGTCAACGGATTTGGT	TTCCCGTTCTCAGCCTTGAC	181

Cell culture

Conditionally immortalized human podocytes were cultured as previously described [20]. Briefly, to induce proliferation, cells were cultured in plates containing RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), D-glucose (5 mM) , Penicillin (100 U/ml) and streptomycin (100 U/ml) at 33°C with 5% CO_2. To induce differentiation, podocytes were cultured with RPMI-1640 medium (supplemented as above) at 37°C with 5% CO_2 for 14 days. All cell experiments were carried out in serum free conditions on well differentiated podocytes between passages 10 and 20 after overnight serum starvation.

Cell viability assays

Cell viability was determined by monitoring the lactate dehydrogenase (LDH) levels in the podocytes as previously described [21]. Briefly, 1×10^5 terminally differentiated podocytes in 500 uL culture medium were seeded in each well of the 24-well plates. After overnight serum starvation, the culture medium was replaced with fresh medium containing different concentrations of glucose or YB. After 24 h, the LDH activity in the cells was determined utilizing a cytotoxicity detection kit. The absorbance was measured using a microplate reader of Thermo Fisher scientific.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an established procedure [22]. Briefly, total RNA was extracted utilizing the TRIzol reagent. cDNA was synthesized utilizing the iScript Advanced cDNA synthesis kit. qRT-PCR was performed with SYBR Green Master Mix. The amplification protocol used for qRT-PCR analysis was 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Expression levels of target genes were normalized by concurrent measurement of GAPDH mRNA levels. All primers used are listed in **Table 1**.

Western blot analysis

Western blot analysis was performed as previously described [23]. The podocytes were washed three times with a precooled PBS containing sodium vanadate then RIPA lysis buffer containing protease inhibitors and phosphatase inhibitors was added to cells. After 30 minutes, the cell lysates collected into 1.5 ml eppendorf tubes and centrifuged at 12000 g for 15 minutes at 4°C. Total proteins were extracted and determined using a BCA protein assay kit. Protein samples were separated by electrophoresis and transferred to PVDF membranes. After being blocked and incubated with primary antibodies, the PVDF membranes were washed with TBST and subsequently incubated with secondary antibody, then measured by PierceTM fast Western blot kit. The primary antibodies included: anti-Nephrin (AF7951, Affinity); anti-Podocin (DF8593, Affinity); anti-CD2AP (DF2298, Affinity); anti-IKKα (AF6012, Affinity Biosciences); anti-IKKβ (AF6009, Affinity Biosciences); anti-p-IKKα/β (AF3014, Affinity Biosciences); anti-IκBα (AF5002, Affinity Biosciences); anti-p-IκBα (AF2002, Affinity Biosciences); anti-ERK1/2 (8867, Cell signaling); anti-p-ERK1/2 (13148, Cell signaling); anti-P38 (14-451, Cell signaling); anti-p-P38 (4092, Cell signaling); anti-JNK (4671, Cell signaling); anti-p-JNK (3708, Cell signaling); and anti-GAPDH (D16H11, Cell signaling).

Transfection and transcription reporter assay

Podocytes were seeded in 24-well plates (1×10^5 cells per well) overnight and changed to

Table 2. Baseline characteristics of Control and YB treatment groups

Index	Groups		T value/x^2	*P* value
	Control	YB treatment		
Gender (M/F)	42/38	39/41		☆
Age (years)	61.91±5.64	61.18±5.47	0.840	0.402
BMI (kg/m^2)	22.38±2.31	22.90±2.21	-1.464	0.145
HbA1c (%)	8.35±0.71	8.44±0.83	-0.759	0.449
FPG (mmol/l)	9.93±0.88	10.12±0.98	-1.248	0.214
2hPG (mmol/l)	13.81±1.02	14.08±1.09	-1.572	0.118
SBP (mmHg)	133.33±9.01	135.55±12.21	-1.304	0.194
DBP (mmHg)	82.16±7.78	83.65±8.24	-1.174	0.242
TC (mmol/l)	4.49±0.59	4.32±0.58	-0.534	0.594
TG (mmol/l)	1.48±0.36	1.51±0.24	1.273	0.205
LDL (mmol/l)	3.24±0.64	3.12±0.60	1.813	0.072
HDL (mmol/l)	1.20±0.16	1.18±0.13	1.152	0.251
Sodium (mmol/l)	140.75±3.75	140.85±2.83	-0.205	0.838
Potassium (mmol/l)	4.33±0.57	4.45±0.48	-1.401	0.163
AST (U/l)	27.34±6.59	28.37±7.03	-0.955	0.341
ALT (U/l)	20.14±7.01	21.19±8.11	-0.882	0.379
Urea (mmol/l)	6.37±2.42	6.89±1.99	-1.484	0.140
Creatinine (μmol/l)	79.22±18.76	77.37±16.43	0.666	0.506
ACR (μg/mg)	185.20±56.65	180.87±56.75	0.483	0.629

☆Chi square test, others were t test.

Table 3. Parameters of the Control group at the baseline and at end of conventional therapy

Index	Control		T value	*P* value
	Pre treatment	Pro treatment		
HbA1c (%)	8.35±0.71	6.40±0.35	22.037	<0.001*
FPG (mmol/l)	9.93±0.88	6.48±0.41	31.941	<0.001*
2hPG (mmol/l)	13.81±1.02	8.74±0.48	40.303	<0.001*
SBP (mmHg)	133.33±9.01	124.80±7.61	6.474	<0.001*
DBP (mmHg)	82.16±7.78	76.20±5.78	5.502	<0.001*
TC (mmol/l)	4.49±0.59	4.16±0.44	4.072	<0.001*
TG (mmol/l)	1.48±0.36	1.29±0.23	3.849	<0.001*
LDL (mmol/l)	3.24±0.64	2.85±0.31	4.932	<0.001*
HDL (mmol/l)	1.20±0.16	1.17±0.13	1.681	0.095
Sodium (mmol/l)	140.75±3.75	139.88±3.89	1.436	0.153
Potassium (mmol/l)	4.33±0.57	4.34±0.54	-0.103	0.918
AST (U/l)	27.34±6.59	28.18±6.51	-0.811	0.419
ALT (U/l)	20.14±7.01	18.79±5.61	1.342	0.182
Urea (mmol/l)	6.37±2.42	6.32±1.75	0.153	0.878
Creatinine (μmol/l)	79.22±18.76	77.97±17.84	0.435	0.664
ACR (μg/mg)	185.20±56.65	118.59±36.42	8.846	<0.001*

*P<0.05 between pre-treatment and pro-treatment in the Control group.

serum free medium the next morning. Cells were transfected with pNF-κB-TA-Luc (100 ng/well) and pRL-TK-Luc (10 ng/well) plasmids using ROCHE X-tremeGENE HP DNA transfection reagent for 18 hours. After treatment with drugs, the cells were lysed and transcriptional activities were calculated using a dual luciferase assay kit and a TD-20/20 luminometer according to the manufacturer's instructions.

Statistics

For data analysis, the results are expressed as mean and standard deviation in the tables and figures. The Kolmogorov-Smirnov test was used to check the normality of the data. Differences between 2 groups were analyzed using the Student's t test and differences ≥3 groups were compared by one ways analysis of variance (ANOVA). Wilcoxon test was used to evaluate dependent groups. Mann-Whitney test was used for comparison of the mean differences between the groups. SPSS Statistics Version 22 (IBM Corp, Armonk, NY) was used for all analyses, and the level of significant difference was set at P<0.05.

Results

The parameters of study subjects

Baseline parameters of all the participants in this study are presented in **Table 2**. There were no significant differences at baseline in two groups. The parameters after treatment are presented in **Tables 3**-**5**. It is notable that the patients' HbA1c, FPG, 2hPG, SBP, DBP, TC, TG, LDL and ACR levels were significantly decreased in both groups after six months' therapy (P<0.05). Additionally, the HbA1c, FPG, 2hPG, and ACR levels of the YB treatment group were lower than the control group (P<0.05).

Table 4. The parameters of YB therapy group at the baseline and at end of YB therapy

Index	YB treatment		T value	*P* value
	Pre treatment	Pro treatment		
HbA1c (%)	8.44±0.83	6.05±0.41	23.083	<0.001*
FPG (mmol/l)	10.12±0.98	5.88±0.39	35.773	<0.001*
2hPG (mmol/l)	14.08±1.09	8.17±0.64	41.623	<0.001*
SBP (mmHg)	135.55±12.21	125.80±9.67	5.599	<0.001*
DBP (mmHg)	83.65±8.24	77.60±7.08	4.981	<0.001*
TC (mmol/l)	4.32±0.58	4.10±0.48	2.725	0.007*
TG (mmol/l)	1.51±0.24	1.30±0.16	6.481	<0.001*
LDL (mmol/l)	3.12±0.60	2.82±0.35	3.913	<0.001*
HDL (mmol/l)	1.18±0.13	1.15±0.11	1.354	0.178
Sodium (mmol/l)	140.85±2.83	139.96±3.34	1.808	0.072
Potassium (mmol/l)	4.45±0.48	4.41±0.43	0.524	0.601
AST (U/l)	28.37±7.03	27.55±7.17	0.733	0.465
ALT (U/l)	21.19±8.11	19.42±5.75	1.603	0.111
Urea (mmol/l)	6.89±1.99	6.52±1.64	1.256	0.211
Creatinine (μmol/l)	77.37±16.43	75.48±15.71	0.740	0.460
ACR (μg/mg)	180.87±56.75	75.42±24.68	15.243	<0.001*

*P<0.05 between pre-treatment and pro-treatment in the YB treatment group.

Table 5. The clinical characteristics after conventional therapy and YB treatment

Index	Groups		T value	*P* value
	Control	YB treatment		
HbA1c (%)	6.40±0.35	6.05±0.41	5.839	<0.001*
FPG (mmol/l)	6.48±0.41	5.88±0.39	9.269	<0.001*
2hPG (mmol/l)	8.74±0.48	8.17±0.64	6.380	<0.001*
SBP (mmHg)	124.80±7.61	125.80±9.67	-0.727	0.468
DBP (mmHg)	76.20±5.78	77.60±7.08	-1.370	0.173
TC (mmol/l)	4.16±0.44	4.10±0.48	0.802	0.424
TG (mmol/l)	1.29±0.23	1.30±0.16	-0.092	0.927
LDL (mmol/l)	2.85±0.31	2.82±0.35	0.496	0.620
HDL (mmol/l)	1.17±0.13	1.15±0.11	0.837	0.404
Sodium (mmol/l)	139.88±3.89	139.96±3.34	-0.157	0.875
Potassium (mmol/l)	4.34±0.54	4.41±0.43	-0.909	0.365
AST (U/l)	28.18±6.51	27.55±7.17	0.585	0.559
ALT (U/l)	18.79±5.61	19.42±5.75	-0.693	0.489
Urea (mmol/l)	6.32±1.75	6.52±1.64	-0.784	0.434
Creatinine (μmol/l)	77.97±17.84	75.48±15.71	0.933	0.352
ACR (μg/mg)	118.59±36.42	75.42±24.68	8.778	<0.001*

*P<0.05 between Control and YB treatment groups.

The influence of HG and YB on cell viability in podocytes

The viability of podocytes was determined by the LDH assay. Podocytes treated with HG (up to 40 mmol/L for 24 hours) not reveal an effect of HG upon cell viability. To preclude the role of osmosis, we also observed the effect of mannitol (40 mmol/L) at the same time and did not find mannitol to affect the cell viability (**Figure 1A**). Cells were also treated with different concentrations of YB in normal glucose (5.5 mmol/L) and HG (40 mmol/L) medium for 24 hours, respectively. As shown in **Figure 1B**, YB did not affect cell viability in either condition below the 200 ug/mL YB dose. Thus, subsequent experiments employed a 100 ug/mL dose of YB which was demonstrated to have no effect on cell death.

The effects of HG and YB on nephrin, podocin and CD2AP expression

Nephrin, podocin, and CD2AP are all important components of SD. The mRNA and protein expression of these three markers was analyzed to explore the variation of SD under HG conditions. First, HG remarkably decreased the expression of nephrin, podocin, and CD2AP at both mRNA and protein levels. However, the same dose of mannitol did not affect their expression (**Figure 2**). Interestingly, co-treatment with YB attenuated the effect of HG evidenced through significantly restored mRNA and protein expression of the three markers (**Figure 2**), suggesting a therapeutic action of YB in HG-induce podocyte injury.

YB suppresses the pro-inflammatory cytokines in podocytes

Because chronic inflammation is the main cause of diabetic nephropathy and podocyte injury, the mRNA levels of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, and MCP-1 were first detected in HG-induced podo-

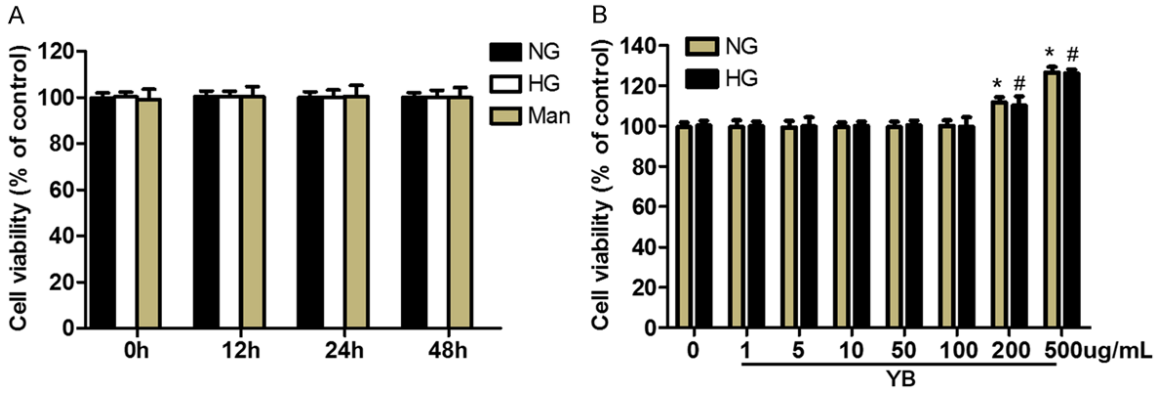

Figure 1. Cell viability assays. A. Podocytes were cultured in serum-free medium with normal glucose (NG, 5.5 mmol/L), high glucose (HG, 40 mmol/L) or mannitol (Man, 40 mmol/L) for 24 hours. Cell viability was assessed by a cell death kit as described in method and materials. B. Podocytes were cultured in serum-free medium with or without the Yi-Qi-Bu-Shen recipe (YB) in NG or HG medium for 24 hours. Cell viability was assessed by a cell death kit as described in method and materials. (*P<0.05 vs NG control; #P<0.05 vs HG control).

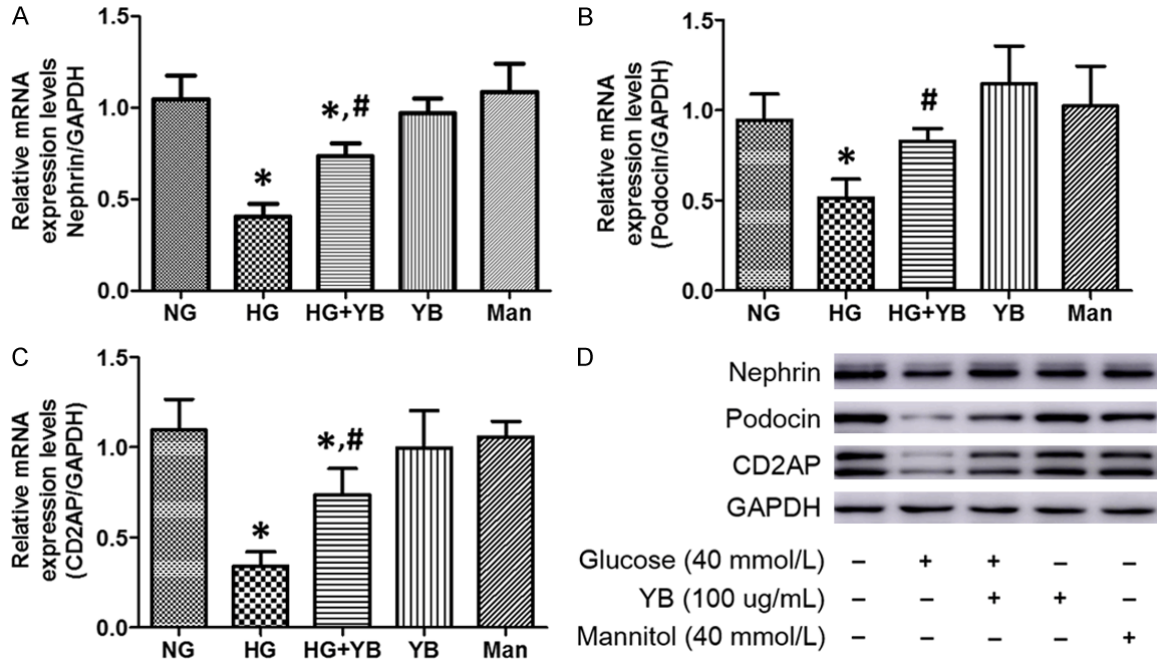

Figure 2. Effect of YB on HG-induced expression of nephrin, podocin and CD2AP in podocytes. A-C. Podocytes were treated with HG and YB for 24 hours and then mRNA levels of nephrin, podocin and CD2AP were determined by qRT-PCR. (*P<0.05 vs control; #P<0.05 vs HG group). D. Podocytes were treated with HG and YB for 24 hours and then protein levels of nephrin, podocin and CD2AP were determined by Western blot.

cytes. Interestingly, qRT-PCR revealed that the mRNA expressions of nephrin, podocin and CD2AP were all up-regulated in the HG group compared with the control group. Treatment with YB could significantly inhibit the transcriptional activity of these inflammatory cytokines induced by HG (**Figure 3**). These results revealed a potent anti-inflammatory profile of YB in podocytes.

Molecular mechanism by which YB attenuated HG-induced inflammation

Because these pro-inflammatory cytokines are mainly regulated at the transcriptional level by the activation of NFκB and MAPKs, the effect of YB on HG-induced activation of these signaling pathways in podocytes was analyzed.

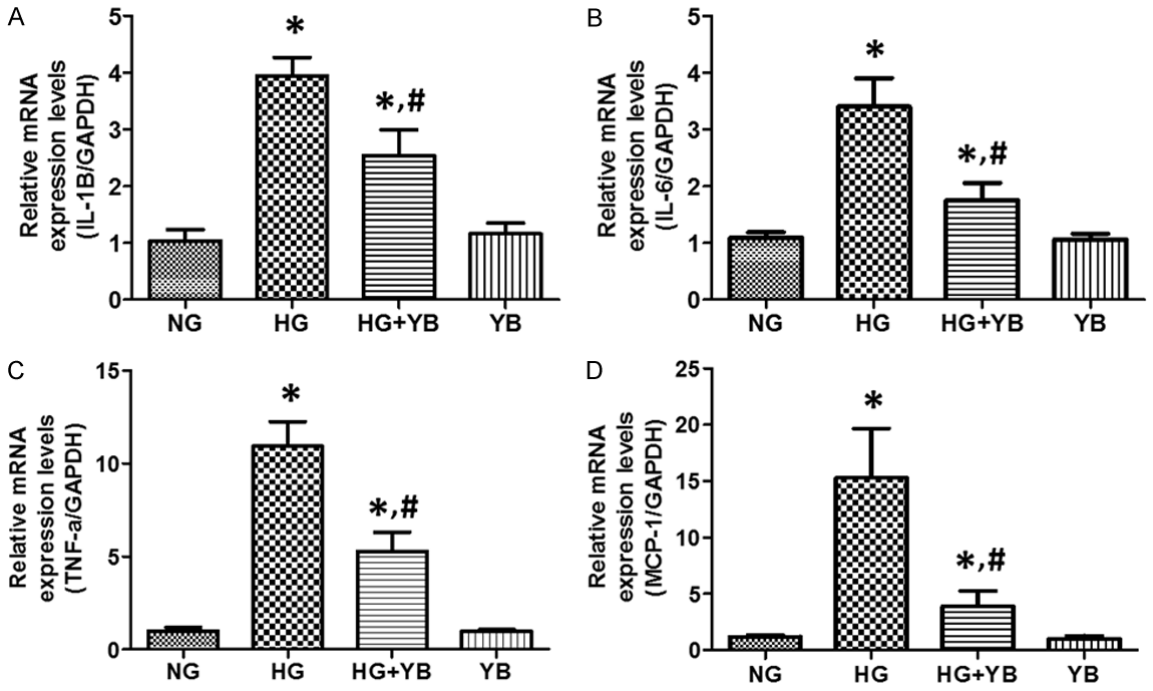

Figure 3. Effect of YB on HG-induced expression of IL-1β, IL-6, TNF-α and MCP-1 in podocytes. A-D. Podocytes were treated with HG and YB for 24 hours and then mRNA levels of IL-1β, IL-6, TNF-α and MCP-1 were determined by qRT-PCR. (*P<0.05 vs control; #P<0.05 vs HG group).

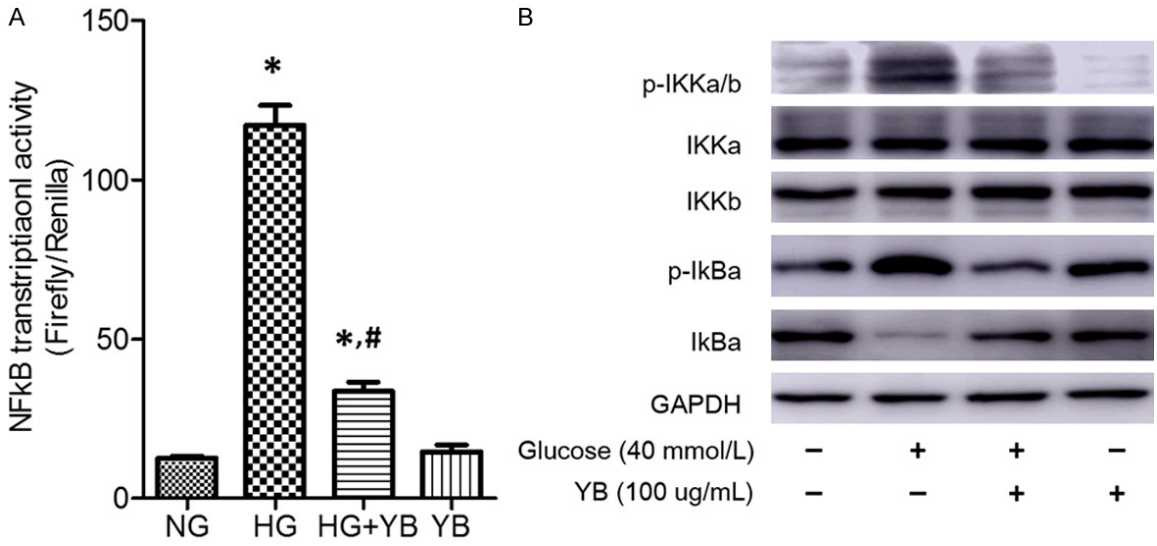

Figure 4. Effect of YB on NFκB signaling pathway in podocytes. A. Podocytes were transfected with NFκB reporter constructs as well as internal control plasmids of pRL-TK, stimulated for 6 hours, and then followed with the assay of NFκB transcriptional activity as described in methods and materials. (*P<0.05 vs control; #P<0.05 vs HG group). B. Podocytes were treated with HG and YB for 24 hours and then protein levels of p-IKKα, IKKα, IKKβ, p-IκBα and IκBα were determined by Western blot.

Effect of YB on NFκB signaling pathway

As shown in **Figure 4A**, podocytes treated with HG alone show increased transcriptional activity of NFκB compared with the control group. Moreover, the phosphorylation of IKKα/β and IκBα were also up-regulated in the HG group (**Figure 4B**), suggesting activation of NFκB sig-

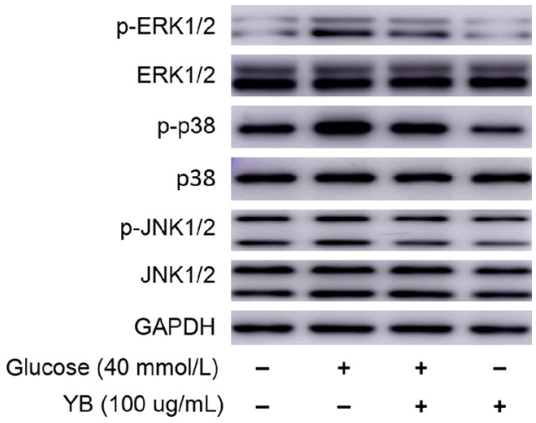

Figure 5. Effect of YB on MAPKs signaling pathways in podocytes. Podocytes were treated with HG and YB for 24 hours and then protein levels of p-ERK1/2, ERK1/2, p-P38, P38, p-JNK1/2 and JNK1/2 were determined by Western blot.

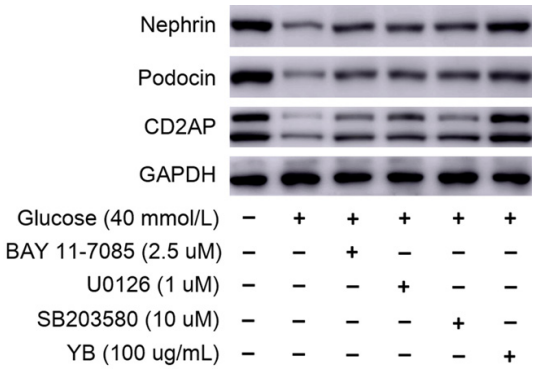

Figure 6. Effect of signaling pathway inhibitors and YB on nephrin, podocin and CD2AP expression in podocytes. Podocytes were treated with HG, Bay 11-7085 (IκBα inhibitor), U0126 (ERK inhibitor), SB203580 (P38 inhibitor) and YB for 24 hours and then protein levels of nephrin, podocin and CD2AP were determined by Western blot.

naling pathway. However, the protein levels of p-IKKα/β and p-IκBα were decreased after YB treatment (**Figure 4B**). Furthermore, the transcriptional level of NFκB was also down-regulated by YB (**Figure 4A**), which demonstrates the inhibitory effects of YB on NFκB signaling pathway.

Effect of YB on MAPK signaling pathway

As shown in **Figure 5**, HG significantly activated the phosphorylation of ERK and P38, but only slightly increased the protein level of p-JNK. After co-treatment of YB, the phosphorylation level of ERK and P38 can be significantly reduced, suggesting that YB may exert its anti-inflammatory effect by inhibiting the ERK and P38 signaling pathway.

Effect of inhibitors on nephrin, podocin and CD2AP expression

Based on the above results, YB may attenuate podocyte injury by inhibiting the IKK-IκBα-NFκB and ERK/P38 MAPK signaling pathways. In order to confirm this hypothesis, the IκBα inhibitor (BAY 11-7085), ERK inhibitor (U0126) and P38 inhibitor (SB203580) were utilized in co-treatment with HG in podocytes. Interestingly, each inhibitor could partly reverse the reduced expression of nephrin, podocin, and CD2AP observed in HG conditions (**Figure 6**), which further suggests that YB attenuated the podocyte injury induced by HG through the IKK-IκBα-NFκB and ERK/P38 MAPK signaling pathways.

Discussion and conclusion

Recent studies have reported that the traditional Chinese medicine prescriptions and constituents exhibit a variety of biological effects such as decreasing blood glucose levels, improving the glomerular filtration rate (GFR) and reducing urine proteins in diabetic patients and animal models [24-27]. The Yi-Qi-Bu-Shen recipe (YB) is a traditional Chinese herbal formula has been used in Qi'lu Hospital of China to treat DN for more than 20 years, the active ingredients of the YB including nine herbs: Astragalus membranaceus (Fisch.) Bge., Rehmannia glutinosa Libosch., Polygonatum sibiricum Red., Ligusticum chuanxiong Hort., Lycium barbarum L., Epimedium brevicornu Maxim., Atractylodes lancea (Thunb.) DC., Pueraria lobata (Willd.) Ohwi, Coptis chinensis Franch. Previous studies have shown that the YB extract can enhance the antioxidant activity of neurons, inhibit hippocampal neuronal apoptosis and promote neuronal survival under HG and hypoxia conditions [18, 19]. In the present study, the function and mechanism of YB in HG-induced podocyte injury were investigated.

Nephrin, podocin, and CD2AP are all important membrane proteins of podocytes, when their expression down-regulated, the SD and the actin cytoskeleton will be altered. Previous research has revealed that HD could reduce the expression of these membrane proteins through different molecular mechanisms including the MAPK and NFκB signaling pathways

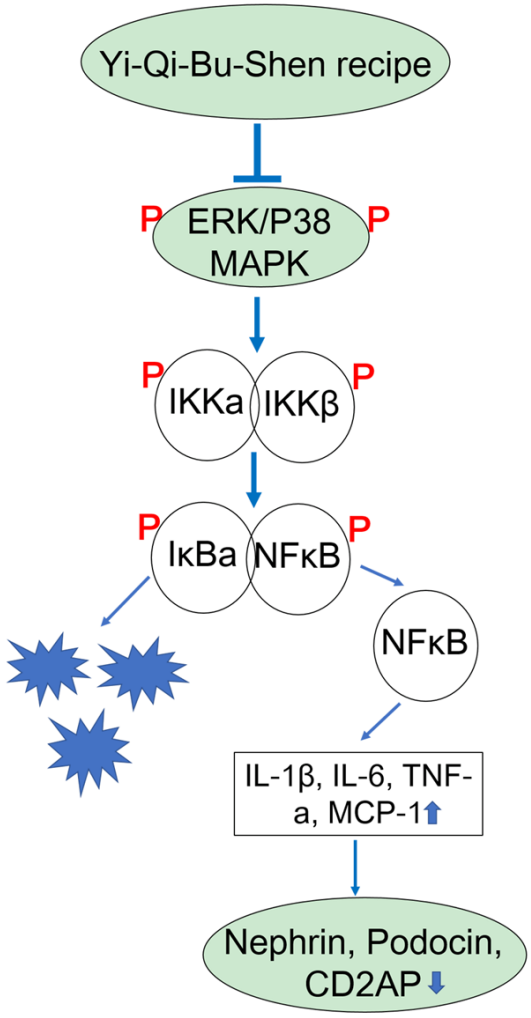

Figure 7. Schematic diagram illustrating the signaling pathways involved in Yi-Qi-Bu-Shen Recipe attenuates HG-induced podocytes injury through the inhibition of ERK/P38 and IKK-IκBa-NFκB signaling cascade. YB acts via direct down-regulation of ERK/P38 MAPK phosphorylation and indirect influence of IKKa/β phosphorylation, which subsequently results in suppression of HG-induced inflammation and podocytes injury.

[28, 29]. However, there are scarcely any drugs able to interfere this process. As shown in **Figure 1**, HG reduced the mRNA and protein expression levels of these molecules, but YB significantly reversed the reduction. These results suggest that YB may be an effective therapeutic drug for the restoration of SD.

Previous studies have indicated that chronic inflammation plays a key role in the pathogenesis of DN. Many inflammatory factors such as pro-inflammatory cytokines, chemokines, and cell adhesion factors are highly expressed in the renal tissue of DN patients. In addition, cytokines and chemokines were also detected in the blood and urine of DN patients [30, 31]. Interleukin is a group of cytokines produced by a variety of cells, which are divided into anti-inflammatory and pro-inflammatory factors according to their physiological functions. Among them, IL-1β and IL-6 are increased in DN patients. As pro-inflammatory factors, they increase cell permeability, enhance extracellular matrix, and promote the production of other inflammatory cytokines, thereby participating in the pathogenesis of DN. TNF-α has a direct cytotoxic effect in podocytes. It can activate the determinants of DN, including various second messengers, transcription factors, growth factors, ICAM and cytokine expression or synthesis. MCP-1 is involved in inducing macrophages moved to the diabetic kidney and the level of MCP-1 in DN patients is significantly increased. The level of urine MCP-1 can, independently or with proteinuria, predict the rate of renal function decline [32, 33]. These results indicate that YB can inhibit the transcription of these inflammatory factors, suggesting that YB may ameliorate podocyte injury by inhibiting chronic inflammatory reactions.

Although several signaling pathways contribute to HG-induced IL-1β, IL-6, TNF-α, and MCP-1 expression as aforementioned, YB might interfere with specific cascades, thereby regulating these molecules' expression in podocytes. Previous studies have shown that MAPK and NFκB signaling pathways could mediate these inflammatory factors expression [34]. Accordingly, first it was explored whether YB could suppress NFκB in podocytes. As shown in **Figure 4**, YB dramatically suppressed HG-induced phosphorylation of IKKα/β and IκBα as well as subsequent NFκB transcriptional activity. In the present study, HG activated the ERK, JNK, and P38 MAPK cascades but YB mainly inhibited ERK and P38 cascades in HG conditions (**Figure 3B**). In addition, it was confirmed that the IκBα inhibitor BAY 11-7085, ERK inhibitor U0126, and P38 inhibitor SB203580 all partly improved nephrin, podocin and CD2AP expression in HG-induced podocytes.

In conclusion, YB significantly ameliorated the decrease of nephrin, podocin, and CD2AP expression induced by HG, reduce the intracellu-

lar mRNA and protein expression of IL-1β, IL-6, TNF-α, and MCP-1 in podocytes, supporting its protective effect in podocytes against injury induced by chronic inflammation. The mechanism underlying the anti-inflammatory effect may be that YB can inhibit the activation of IKK-IκBα-NFκB and ERK/P38 MAPK signaling pathways in podocytes (**Figure 7**). Therefore, this study might offer mechanistic insights concerning a potentially useful therapy for preventing podocyte injury induced by HG.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. De-Shan Liu, Department of Traditional Chinese Medicine, Qilu Hospital of Shandong University, 107#, Wenhua Xi Road, Jinan 250012, P.R. China. Tel: +86 531 82166329; Fax: +86 531 86927544; E-mail: liudeshan@sdu.edu.cn

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