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Chinese Journal of Natural Medicines 2019, **17**(9): 0641–0649
Chinese Journal of Natural Medicines

Bupleurum polysaccharides ameliorated renal injury in diabetic mice associated with suppression of HMGB1-TLR4 signaling

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Available online 20 Sep., 2019

[ABSTRACT] *Bupleurum* polysaccharides (BPs) is isolated from *Bupleurum smithii* var. *parvifolium*, a key traditional Chinese medicine. The study was to investigate the effects of BPs on diabetic kidney injury. After two intraperitoneal injections of streptozotozin (STZ) 100 mg·kg^{-1}, renal injury in diabetic mice was induced and BPs was orally administrated at dosages of 30 and 60 mg·kg^{-1}·d^{-1}. The STZ injected mice developed renal function damage, renal inflammation and fibrosis known as diabetic kidney disease (DKD). BPs significantly reduced serum creatinine level and urinary albumin excretion rate, with the attenuated swelling of kidneys. BPs treatment obviously alleviated the pathological damage of renal tissue. The progression of renal injury in BPs treated mice was inhibited with less expression of type IV collagen (Col IV), fibronectin (FN) and α-smooth muscle actin (α-SMA). The inhibition of inflammation in kidney was associated with the reduced level of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). BPs administration suppressed the over-expression of toll like receptor 4 (TLR4) and high-mobility group box 1 (HMGB1) with lowered activity of nuclear factor kappa B (NF-κB) in renal tissue of diabetic mice. Oral administration of BPs effectively prevented the development of renal injury in diabetic mice. This study suggested that the protection provided by BPs might affect through the interruption of HMGB1-TLR4 pathway, leading to the inhibition of renal inflammation and fibrotic process.

[KEY WORDS] Renal injury; *Bupleurum*; High-mobility group box; Toll-like receptor 4; Inflammation; Renal fibrosis; Polysaccharides

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2019)09-0641-09

Introduction

Among U.S., adults aged 20 years or older with diagnosed diabetes, the estimated crude prevalence of chronic kidney disease (stages 1–4) was 36.5% during 2011–2012 [1]. Diabetic kidney disease (DKD) is one of the most serious complications of diabetes [2]. The exact etiology of DKD remained unknown and an increasing amount of data have shown that inflammatory mechanism played an important role in the progression of disease [2-3].

Toll like receptors (TLRs) are innate immune receptors expressed by immune cells and non-immune cells including tubular epithelial cells, endothelial cells and podocytes [4]. TLR4 recognizes pathogen-associated molecular patterns (PAMP) present on microorganisms but also recognizes damage associated molecular patterns (DAMP) that are generated in response to tissue injury [5]. Emerging evidence has indicated that high-mobility group box 1 (HMGB1), a highly conserved nonhistone nuclear protein that serves as a DAMP molecule, is associated with the pathogenesis of diabetes mellitus [6]. By binding to TLR4 and activated the downstream signaling pathway, HMGB1 initiated local inflammation, injure kidneys and result in the diabetic kidney disease [2, 6].

Bupleurum polysaccharides (BPs) isolated from the root of *Bupleurum smithii* var. *parvifolium* has anti-inflammatory [7-8] and anti-oxidative properties [9]. BPs treatment enhanced

[Received on] 14-Jan.-2019
[Research funding] This work was funded by the National Natural Science Foundation of China (Nos. 81673658, 81274165 and 30925042), Ministry of Science and Technology of China (No. 2018ZX09735003-002) and the Science and Technology Commission of Shanghai Municipality (No. 12JC1400800).
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These authors have no conflict of interest to declare.
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phagocytic functions of macrophages and inhibited lipopolysaccharide induced proinflammatory cytokines production [7]. In rodent models, BPs ameliorated acute lung injury [10] and lupus nephritis [11]. The therapeutic efficacy of BPs on suppression of inflammatory diseases could be associated with the modulating of TLR4 signaling pathway [12].

In our previous study, BPs protected pancreatic β cells and liver hepatocytes and ameliorate diabetes, which is associated with its anti-oxidative and anti-inflammatory properties [9]. However, the protective effect of BPs on diabetic kidney injury and its mechanism remained to be proved. In the present study, mice model with diabetic kidney injure was established to further elucidate the mechanism underlying the renal beneficial effect of BPs.

Materials and Methods

Isolation and characterization of Bupleurum polysaccharides

The roots of *Bupleurum smithii* var. *parvifolium* were purchased from Shanghai Hua-Yu Chinese Materia Medica Co. Ltd., which is categorized as DFC-CH-H2003121602 and has been deposited in the Herbarium of Materia Medica (Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China). The extraction and chromatographic studies of crude polysaccharides were performed as previously described [7, 13]. The crude polysaccharides contained one major polysaccharide with several minor ones, determined by high-performance gel permeation chromatography [9]. As we previously reported, BPs contained 74.8% of total carbohydrate and 41.5% of uronic acid. It was mainly consisted of Ara, Gal, Glc and Rha in the ratio of 6.35 : 3.15 : 1.47 : 1, along with trace of Man and Xyl [7, 13].

Regents

The reagents included streptozotocin (STZ) (Sigma, MN, USA); Blood glucose test strips (Abbot Diabetes Care Ltd.); Blood glucose assay kits (FengHui, Shanghai, China); Glyburide (Weibian, Shanghai, China); Creatinine assay kits (FengHui, Shanghai, China); Enzyme-linked immunosorbent assay (ELISA) kit (Boatman, Shanghai, China) for albumin and β2-microglobulin (β2-MG); Rabbit anti-HMGB1 IgG (Proteintech Group, Inc.); Rabbit anti-TLR4, rabbit anti-fibronectin (FN) and rabbit anti-type IV collagen (Col IV) (Abcam, Britain); HRP goat anti-rabbit IgG (Bioworld, Nanjing, China); 1%PMSF-NP40 (Beyotime, Shanghai, China); Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) ELISA kits (Boatman, Shanghai, China); Bicinchoninic acid (BCA) Protein Assay Kit, Radioimmunoprecipitation (RIPA) lysis kit and electrochemiluminescence (ECL) kit (Beyotime, Shanghai, China); PVDF membrane (Millipore, USA); Anti α-smooth muscle actin (α-SMA), HMGB1, TLR4, nuclear translocation of the transcription factor (NF-κB p65) and phosphorylation of nuclear translocation of the transcription factor (p-NF-κB p65) antibodies (Abcam, Britain); Anti β-actin antibody (Bioworld, Nanjing, China).

Animals and ethics statements

Male C57BL/6 mice (8 weeks old) (22–24 g) were purchased from Slaccas-Shanghai Lab Animal Ltd. (SPF Ⅱ Certificate; No. SCXK 2012-0005), and kept under specific pathogen free condition in a 12-h light/dark cycle. All mice received humane care in compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. We followed standard animal experimental procedures approved by the Animal Ethical Committee of School of Pharmacy, Fudan University (approval identification: 2013-1).

Induction of diabetes in mice

After one week of being housed in a new environment, the C57BL/6 mice were fasted but free tap of water for 16 h before the induction of diabetes. Streptozotocin (STZ), freshly prepared in buffer solution (0.1 mol·L^{-1} sodium citrate and 0.1 mol·L^{-1} citric acid), was intraperitoneally injected into the mice in a dose of 100 mg·kg^{-1} for two successive days (Day 1 and Day 2). Mice were injected intraperitoneally with the buffer solution (0.1 mL/10 g) and served as normal group. After one week (Day 9), postprandial blood glucose levels were measured by blood glucose test strips. Mice with blood glucose levels higher than 16.8 mmol·L^{-1} were defined diabetic and chosen for experiments [9].

Treatment

Diabetic mice were allocated into 4 groups randomly: vehicle-treated diabetic mice (model group); BPs (30 or 60 mg·kg^{-1}·d^{-1}) treated diabetic mice; glyburide (7.2 mg·kg^{-1}) treated diabetic mice. There were 12 mice in model group and 8–10 mice in each other group.

BPs was ground, suspended in normal saline, and orally administered to the diabetic mice once daily for 35 days. Glyburide was suspended in 0.5% sodium carboxymethyl cellulose (CMC) for administration as a positive control. The treatment last for 35 days, the mice in normal and model group received normal saline orally. All the mice were on common pellet diet (Shanghai Shilin, cat Q/TJCX-2010) during the study. The ingredients of the pellet met China national standards.

Postprandial urine was collected for 5 hours via metabolism cage in which mice could freely access to water at day 36 and 37 (BPs treatment for 27 and 28 days). At the end of experiment (Day 44), serum and kidneys were collected for assessment. The index of kidney was expressed as the ratio of kidney wet weight (mg) versus body weight (g) [9].

Biochemical assays in serum and urine sample

The level of blood glucose at day 44 was tested by biochemical assay kits. Mouse creatinine was estimated in serum samples using assay kits. Urine was collected for 5 hours via a device in a metabolism cage at Days 36 and 37. The concentration of urinary albumin and β2-MG was tested using an ELISA kit. Urinary albumin excretion rate (UAER) = albumin level in urine (μg·mL^{-1}) × volume (mL) of urine collected for 5 h. UAER represented the total excretion of urinary albumin in 5 hours [11].

Histological studies

Right kidneys of mice were fixed in 10% neutral buffer

formalin and embedded in paraffin. The specimens were processed to obtain 4-μm-thick paraffin sections. The tissue sections were stained by hematoxylin and eosin (HE), periodic acid-Schiff's (PAS) or Masson method. Photographs of the specimens were taken under optical microscope.

Immunohistochemistry

The sections were deparaffined and rehydrated overnight. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol (*V*/*V*) for 15 min, and then non-specific binding sites were blocked with 5% bull serum albumin (BSA). After the removal of BSA, the sections were incubated with primary antibody including rabbit anti-HMGB1 IgG, rabbit anti-TLR4 IgG, rabbit anti-FN IgG or rabbit anti-Col IV IgG at 4 °C overnight in a humidified chamber, and then incubated with HRP goat anti-rabbit IgG at 37 °C for 1 h. Slides were visualized using chromogenic substrate solution 3, 3'-diaminobenzidine (DAB) and counterstained with hematoxylin, and then observed under a microscope (Leica, Inc. Switzerland) [11, 14].

Determination of inflammatory cytokines

Kidneys tissues were homogenized in 1% PMSF-NP40 and centrifuged at 16 000 *g* for 15 min at 4 °C. The levels of TNF-*α* and IL-6 in kidneys supernatants were measured using the ELISA kits. Protein concentration was determined by BCA Protein Assay Kit [9].

Western blot assay

The kidney proteins were prepared using assay RIPA lysis kit. The protein concentration was examined by the BCA method. Electrophoresis was done on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electroblotted on to a PVDF membrane. The membranes were blocked in Tris-buffered saline and Tween 20 (TBST) containing 5% skim milk for 1 h at room temperature, and incubated with the primary antibodies: *α*-SMA, HMGB1, TLR4, p-NF-*κ*B p65, NF-*κ*B p65 and *β*-actin. After overnight incubation at 4 °C, HRP-labeled goat anti-rabbit IgG was added and the incubation lasted for 2 h at room temperature. The signals were visualized using an enhanced ECL kit and captured with a camera-based imaging system [15].

Statistical analysis

Quantitative variables were expressed as means ± SD. One-way analysis of variance (ANOVA) was used to analyze the difference between groups. If any significant changes were found, post hoc comparisons were performed using Fisher's PLSD. *P*-value < 0.05 was considered significant.

Results

Effects of BPs on renal function in diabetic mice

STZ-induced diabetic mice were found to have a body weight loss compared with non-diabetic mice and showed polydipsia, polyphagia and polyuria which were typical symptoms of diabetes. After the diabetic mice were given BPs, the blood glucose dropped significantly and the typical symptoms of diabetes improved (Fig. 1) [9].

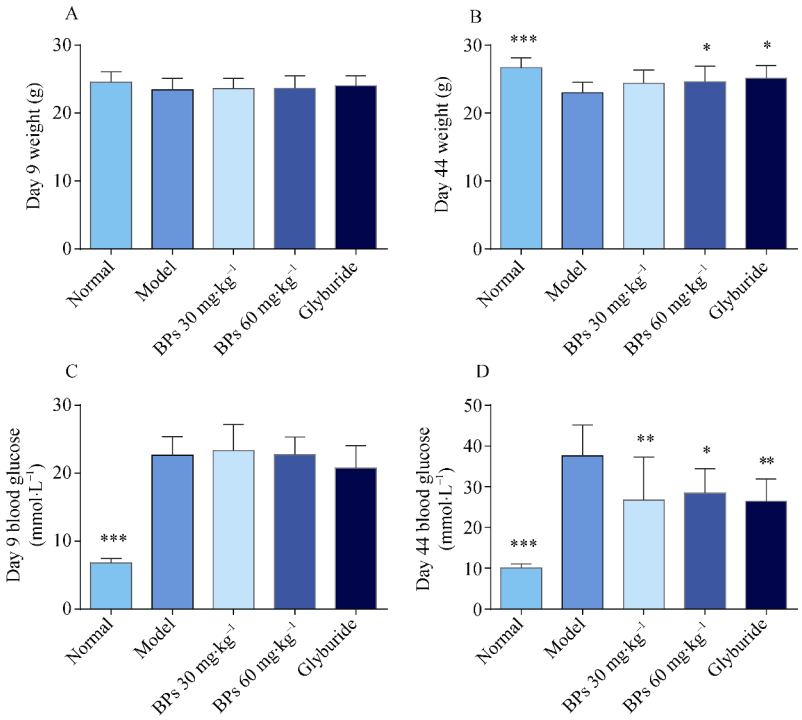


Fig. 1 Effects of BPs on body weight and the level of blood glucose. Mice were treated with BPs (30 or 60 mg·kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}), or vehicle from day 9 to day 44. The weight of mice showed at day 9 (A) and day 44 (B). The level of blood glucose at day 9 was determined by blood glucose test strips (C), while the level of blood glucose at day 44 was tested by biochemical assay kits (D). Data expressed as means ± SD. *n* = 8–12. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* vehicle treated model group, tested by one-way ANOVA and the Fisher's PLSD

Compared with normal group, model group showed a remarkable increase in kidney index, serum creatinine level, UAER, and β2-MG level.

After 5 weeks of treatment, BPs (60 mg·kg^{-1}) decreased renal swollen ($P < 0.05$), lowered serum creatinine level ($P < 0.05$) and reduced UAER ($P < 0.05$). The level of β2-MG decreased mildly (Fig. 2).

Effects of BPs on kidney pathology in diabetic mice

Pathological changes of kidney and expression of matrix were observed using H&E, PAS and MASSON staining methods. In normal group, histological examination of the kidneys through light microscope showed no abnormality in the glomeruli and renal tubules and no any overt increase or sclerosis in the mesangial matrix. In the model group, glomerular hypertrophy coexisted with atrophy and mesangial matrix incrassation; some tubules vacuolated. Stained with Masson, some glomeruli and tubulointerstitial showed slight fibrosis. After 5 weeks of treatment with BPs (60 mg·kg^{-1}) or Glyburide, the pathological progresses mentioned above were alleviated (Fig. 3).

Effects of BPs on kidney fibrosis in diabetic mice

The extracellular matrix protein Col IV and FN in renal tissue was detected immunohistochemically. Brown stained area of these sections showed that basement membrane of most glomeruli and some tubules contained a small amount of Col IV in normal group while a large amount of Col IV in model group. Glomeruli of mice in normal group expressed trace amount of FN. In model group, FN was highly expressed on glomerular mesangium. BPs and Glyburide treatment reduced the high expression of Col IV and FN (Fig. 4).

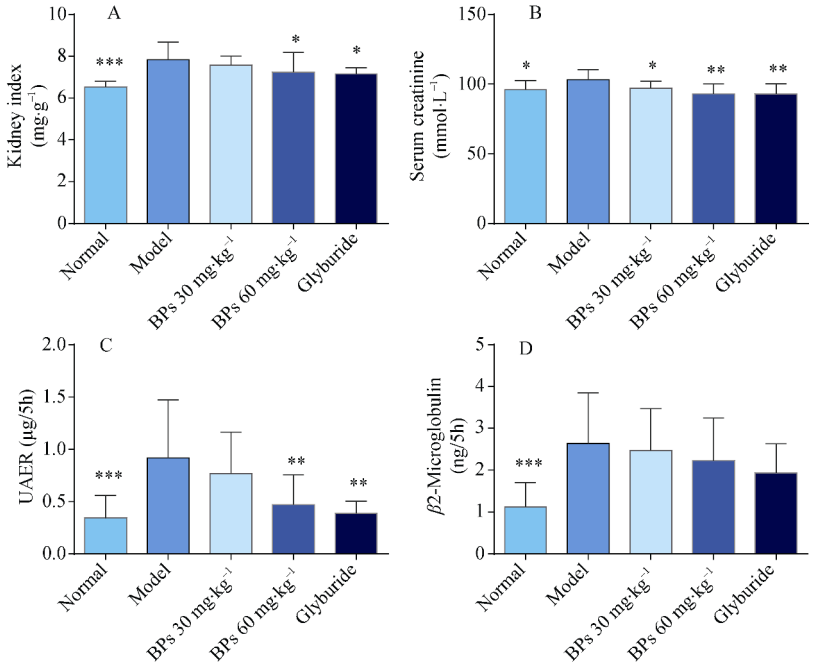
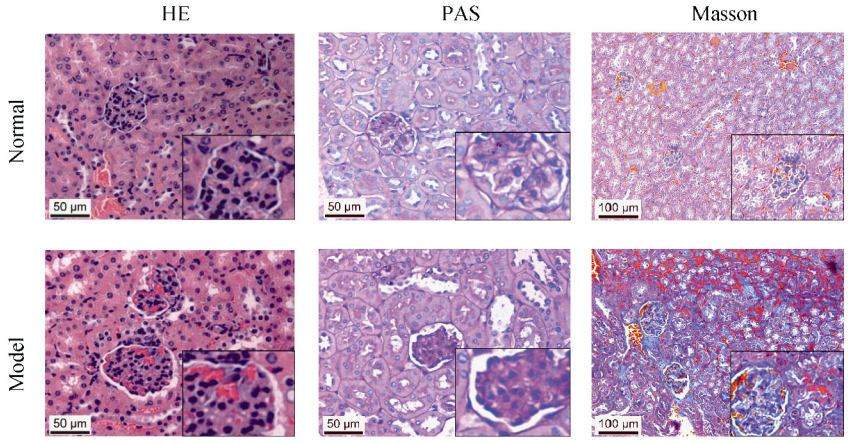


Fig. 2 Effects of BPs on kidney injure. Mice were treated with BPs (30 or 60 mg kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}), or vehicle from day 9 to day 44. Kidney index = kidney weight (mg)/body weight (g) (A). The levels of serum creatinine were tested by biochemical methods (B). Urinary albumin excretion rate (UAER) = albumin level in urine (μg·mL^{-1}) × volume (mL) of urine collected for 5 h (C). Levels of β2-MG were tested in urine collected for 5 h (D). Data expressed as means ± SD. n = 8–12. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* vehicle treated model group, tested by one-way ANOVA and the Fisher's PLSD



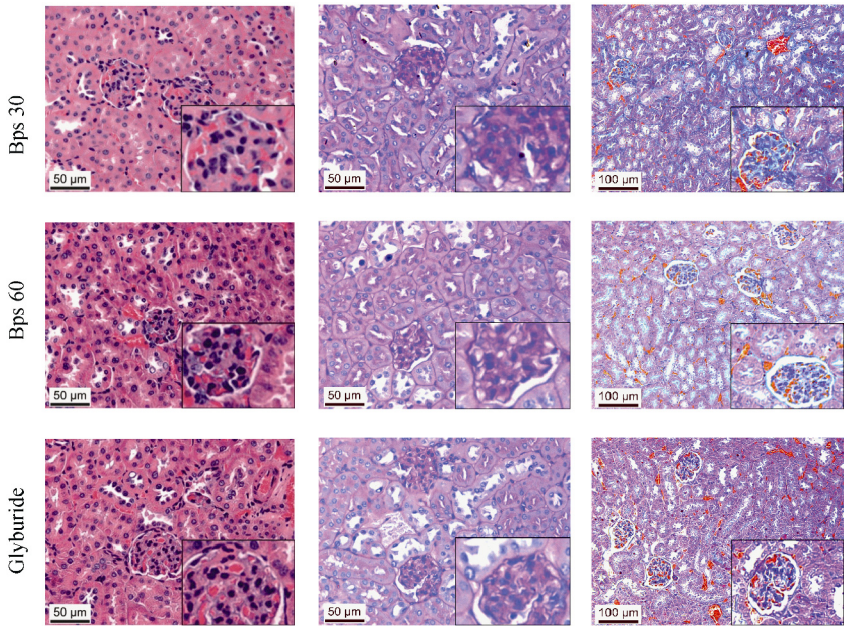


Fig. 3 Effects of BPs on renal pathology of diabetic mice. Diabetic mice were treated with BPs (30 or 60 mg·kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}) or vehicle from day 9 to day 44. For HE and PAS stained, light microscopy 400 × , Bars = 50 μm. For Masson stained, light microscopy 200 ×, Bars = 100 μm

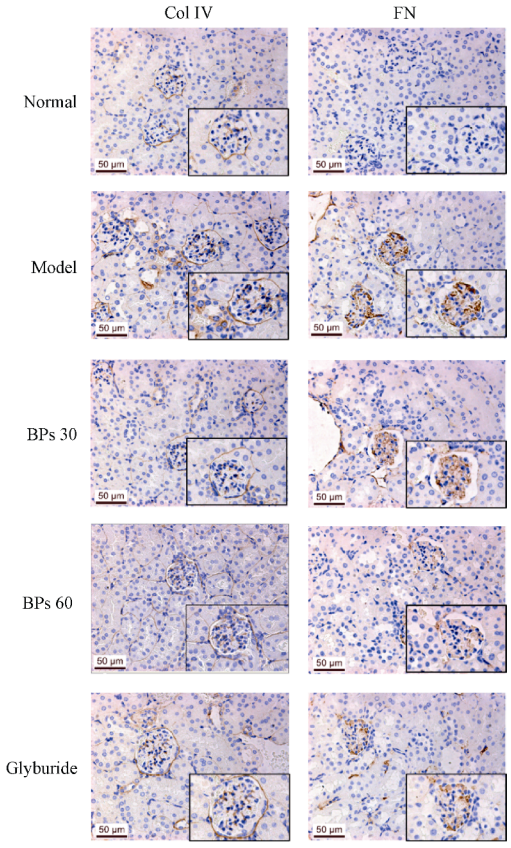


Fig. 4 Effect of BPs on the expression of Col IV and FN in kidney of diabetic mice. Mice were treated with BPs (30 or 60 mg·kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}) or vehicle from day 9 to day 44. The levels of Col IV and FN were determined using immunochemistry. Light microscopy 400 ×, Bars = 50 μm

It was found through Western blot that the level of FN and α-SMA in renal homogenate increased in diabetic mice ($P < 0.01$). Levels of FN decreased significantly in both BPs treatment group ($P < 0.001$) and α-SMA decreased significantly in high dose BPs treatment group ($P < 0.05$) (Fig. 5).

Effects of BPs on renal inflammation in diabetic mice

To evaluate the inflammatory response in mice, the level of TNF-α and IL-6 in renal tissue were determined by ELISA kits. Compared with normal group, the level of TNF-α and IL-6 in model group ascended obviously ($P < 0.001$). BPs (60 mg·kg^{-1}) remarkably descended the levels of TNF-α and IL-6 in renal tissue homogenate ($P < 0.01$) (Fig. 6).

Effects of BPs on the expression of HMGB1, TLR4, p-NF-κB p65 in diabetic mice

As shown in the Fig. 7A, mice in normal group expressed a small quantity TLR4, while the expression increased in model group. The BPs and Glyburide treatment reduced TLR4 expression in renal tissue. Mice in normal group basically expressed HMGB1 in nuclei of renal tissue. Compared with normal group, the expression of HMGB1 in model group increased and a large amount of HMGB1 was found out of nuclei and cells. BPs and Glyburide reduced the over expression and the release of HMGB1.

The levels of TLR4, HMGB1 and p-NF-κB p65 (the ratio of p-NF-κB p65 to NF-κB p65) were detected in renal homogenate. Compared with normal group, the level of HMGB1, TLR4 and p-NF-κB p65 in model group increased obviously ($P < 0.05$). BPs remarkably decreased the level of HMGB1 and p-NF-κB p65 ($P < 0.05$). BPs mildly reduced the level of TLR4 with no significant (Fig. 7B).

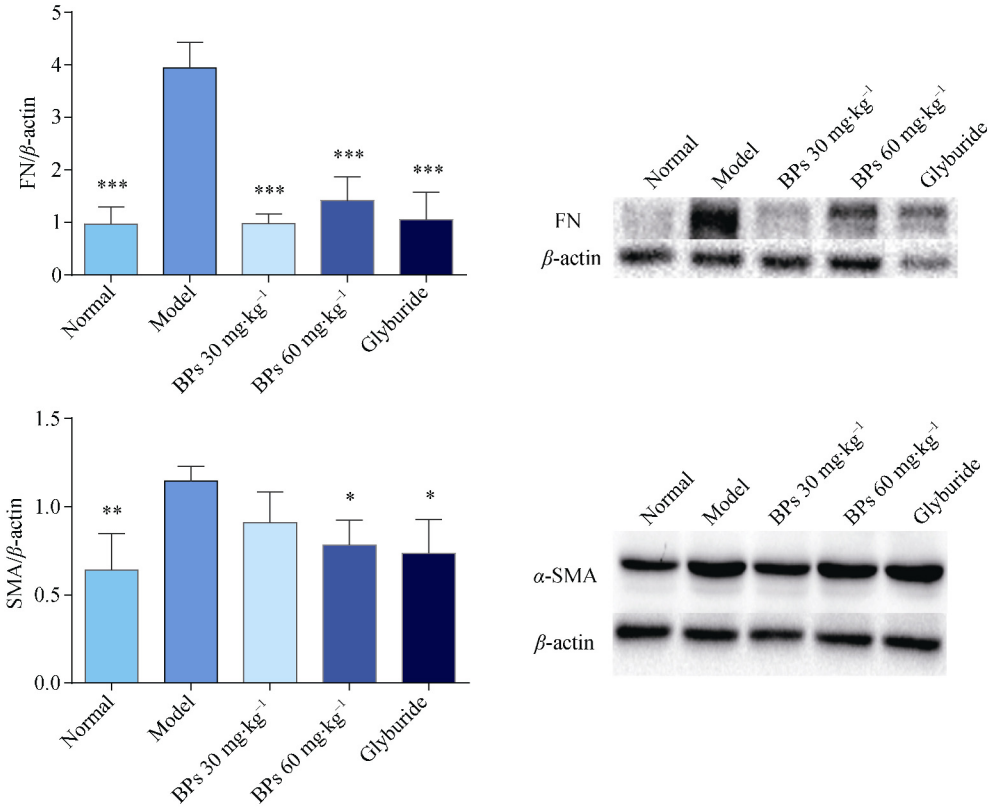


Fig. 5 Effects of BPs on the levels of FN and *α*-SMA in kidney of STZ-induced diabetic mice. Mice were treated with BPs (30 or 60 mg·kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}) or vehicle from day 9 to day 44. The levels of FN and *α*-SMA were detected in kidney using Western blot analysis. Data expressed as means ± SD. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model group, tested by one-way ANOVA and the Fisher's PLSD

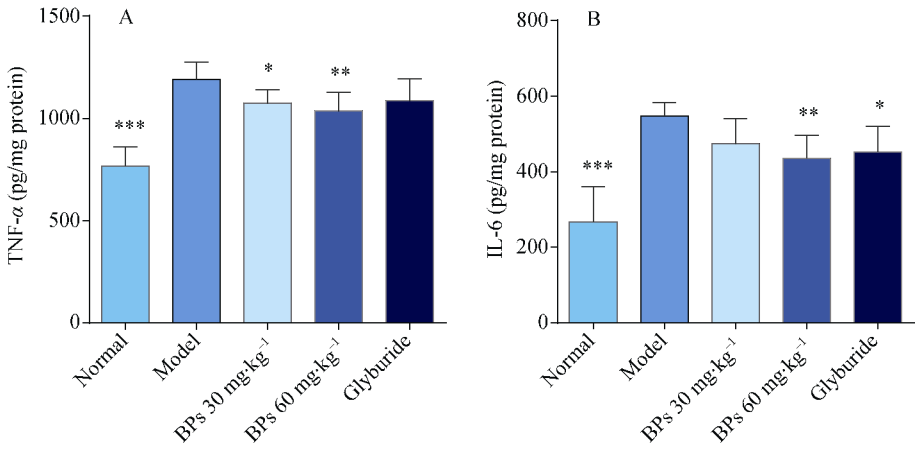


Fig. 6 Effects of BPs on renal inflammation in diabetic mice. Mice were treated with BPs (30 or 60 mg·kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}) or vehicle for 5 weeks. The levels of TNF-*α* (A) and IL-6 (B) expression were detected in kidney using ELISA analysis. Data expressed as means ± SD. n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* model group, tested by one-way ANOVA and the Fisher's PLSD

Discussion

Diabetic kidney disease is one of the leading causes of end-stage renal disease and creates heavy healthcare burdens globally [16]. Serum creatinine level is associated with glomerular filtration rate. The clinical observance of hyper-glomerular filtration rate is a direct indicator of the earliest diabetic renal pathologic alterations and is also associated with urine albumin excretion [17]. After filtration through glomeruli, *β*2-microglobulin is reabsorbed in proximal tubules. Increased urinary *β*2-microglobulin indicates proximal tubule lesion [18]. The renal swelling was reflected by renal index.

After two intraperitoneal injections of STZ (100 mg·kg^{-1}) on mice, the postprandial blood glucose levels were higher than 16.8 mmol·L^{-1} at day 9 and the treatment began. Polydipsia and polyuria in diabetic mice displayed from day 12. A remarkable increase in UAER and β2-MG level was found at day 36 and 37. At the end of experiment (Day 44), the diabetic mice showed significant renal swelling with elevated serum creatinine level.

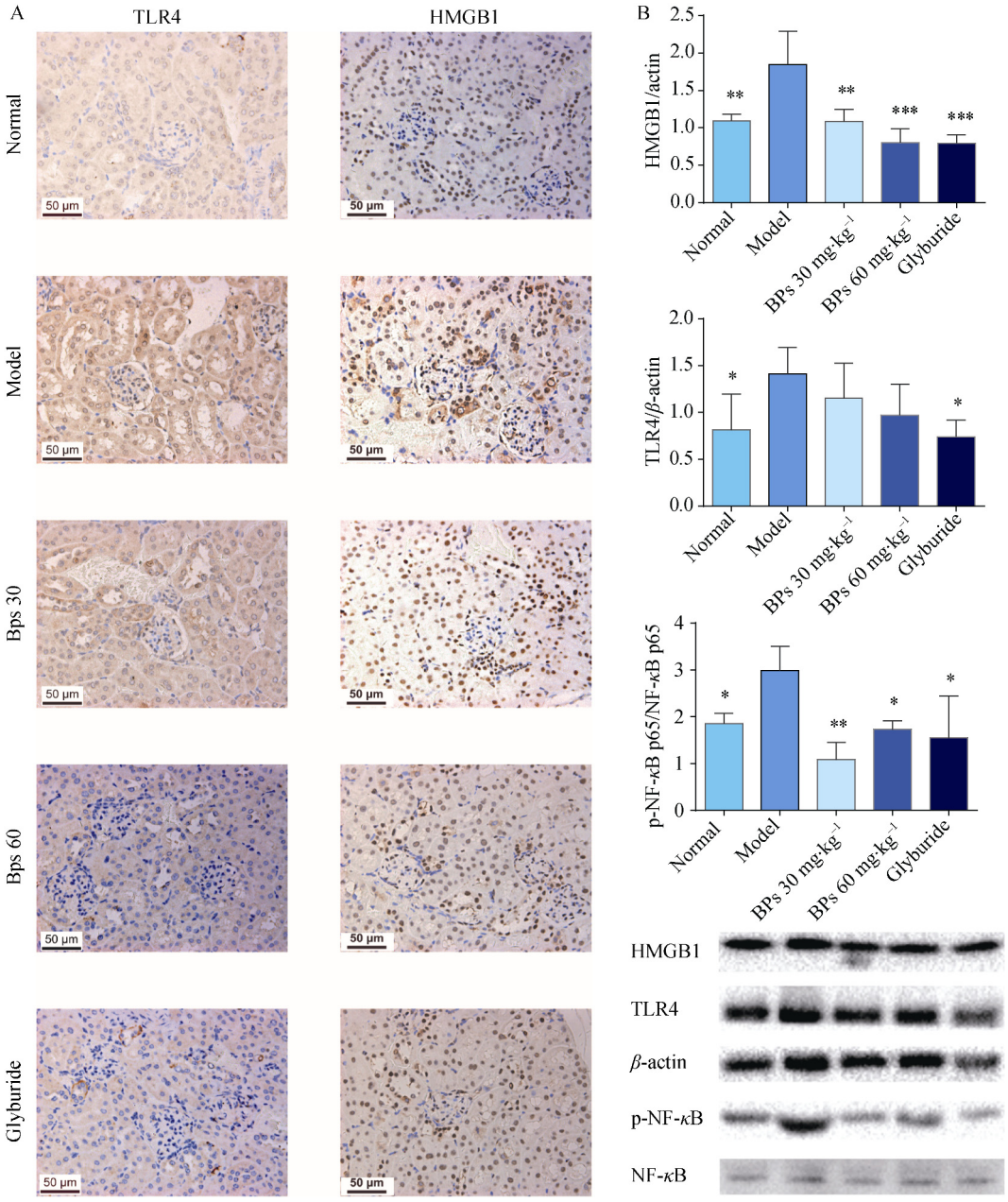


Fig. 7 Effects of BPs on the expression of TLR4, HMGB1 and p-NF-κB p65 in diabetic mice. Diabetic mice were treated with BPs (30 or 60 mg·kg^{-1}·d^{-1}), glyburide (7.2 mg·kg^{-1}·d^{-1}) or vehicle from day 9 to day 44. The expression of TLR4 and HMGB1 were determined using immunohistochemistry (A). Light microscopy 400 ×, Bars = 50 μm. The expression levels of TLR4, HMGB1 and p-NF-κB p65 (the ratio of p-NF-κB p65 to NF-κB p65) were determined using Western blot (B). Data expressed as means ± SD. n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the model group, tested by one-way ANOVA and the Fisher's PLSD

BPs treatment attenuated swelling of kidneys and improved renal function of diabetic mice. BPs administration significantly decreased serum creatinine level and urinary albumin excretion rate with a tendency of decreasing in the level of β2-MG.

HE, PAS and Masson stain showed that mice kidneys in model group had degenerative changes such as glomerular hypertrophy, mesangial expansion and hypertrophy, widening of basement membranes and interstitial fibrosis. These changes coordinated with early stage diabetic kidney disease

nephropathy patients' pathologic changes [19]. BPs treatment obviously alleviated the pathological damage of renal tissue.

Progression of renal injure is manifested by mesangial matrix expansion, followed by a fibrotic process in the tubulointerstitial region and renal insufficiency [20]. The fibrotic reaction is characterized by an increased production of extracellular matrix components, such as FN and collagen [21]. The formation of fibroblasts increased the producing of α-SMA in the renal tissue [20]. FN as the endogenous TLR4 ligands triggers further NF-κB activation leadings to more secretion of proinflammatory cytokines [22].

In diabetic mice of model group, Col IV was highly expressed in glomerular mesangial matrix, in basement membrane of glomeruli and tubules, and FN highly expressed in glomerular mesangial matrix tested by immunohistochemical method. BPs treatment reduced the expression of Col IV and FN, attenuated extracellular matrix deposition in glomerular mesenterium and tubulointerstitium. According to the results of Western blot, levels of FN and α-SMA increased significantly in model group, but decreased in BPs treatment groups. All these results indicated the process of renal fibrosis was prohibited by BPs treatment.

High TNF-α level directly damaged mesangial cells and vascular endothelial cells in glomerular and tubular epithelial cells, followed by lasting inflammation [23]. IL-6 is another important mark of inflammation mediated diabetic kidney disease, which can change dynamics of extracellular matrix and cause the thickening of glomerular basement membrane [24]. The increase of TNF-α and IL-6 production was found in renal tissue in diabetic mice. BPs treatment significantly decreased the expression of TNF-α and IL-6. The results suggested that the decreased expression of renal pro-inflammatory cytokines closely correlate with the reducing renal damage in diabetic mice.

More and more research proved that immune responses and inflammatory reactions have a stimulating role in progression of diabetic kidney disease [2, 25]. TLRs are characterized as one species of pattern recognition receptors to recognize PAMP and DAMP, and thus are involved in innate immune responses against infection and injury [6]. TLR4 has received particular attention because of its activation in metabolic abnormalities in animal models and in human with obesity and diabetes [2, 26]. Under normal conditions, only a small amount of TLR4 expressed in kidneys, but according to recent studies, a large amount of TLR4 expressed in kidneys of mice with diabetic kidney disease, suggesting that TLR4 might engage in the pathological process of diabetic kidney disease [2, 5, 22].

HMGB1 is one of endogenic ligands of TLR4 [5], which is widely expressed in nuclei and can be either actively secreted by stimulated immune cells or passively released by diabetes damaged cells [6]. Subsequent studies demonstrated that HMGB1 might promote inflammation via interacting with TLR4 and activating its downstream signaling pathways [6]. Some recent researches have also found hyperglycemia induced HMGB1 release which leads to tubulointerstitial inflammation during diabetic kidney disease [27]. HMGB1, via interacting with its receptors, ultimately results in the activation of NF-κB and the production of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α, causing persistent progression of diabetic kidney disease [6]. HMGB1-TLR4 signaling appeared to mark a brand new direction in diabetic complication treatment [28].

According to immunohistochemical results, more HMGB1 and TLR4 expressed in mice kidneys in the model group than in the normal group, and BPs treatment decreased both expressions. Western blot results showed that BPs treatment had the trend of inhibiting TLR4 overexpression and obviously repressed the expression of HMGB1. Treatment with BPs markedly reduced the activity of NF-κB, significantly reduced the levels of inflammatory cytokines, such as IL-6 and TNF-α, by inhibiting the activity of HMGB1-TLR4 signaling pathway.

Pro-inflammatory response promoted the release of HMGB1 and the accumulation of FN in fibrosis process [29]. Danger signals such as HMGB1 and FN via TLR4 activation contributed to the pathogenesis progression of renal injury [30-31]. Via BPs treatment, the feedback process was relieved and the development of renal injure in diabetic mice was prevented.

Conclusions

In conclusion, we have demonstrated that BPs attenuated inflammatory reaction and fibrosis process in renal tissue of diabetic mice. The amelioration of BPs on renal injury in diabetic mice and could be accounted for its inhibitory potential on HMGB1-TLR4 signaling. Because of the difficulty in achieving complete euglycemia, a new approach to preventing diabetic complications is required. Therefore, BPs may offer a new therapeutic approach for diabetic renal injury.

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Cite this article as: LIU Zhen-Zhen, WENG Hong-Bo, ZHANG Li-Jie, PAN Ling-Yu, SUN Wei, CHEN Hai-Xia, CHEN Mei-Yu, ZENG Tao, ZHANG Yun-Yi, CHEN Dao-Feng, LI Hong. *Bupleurum* polysaccharides ameliorated renal injury in diabetic mice associated with suppression of HMGB1-TLR4 signaling [J]. *Chin J Nat Med*, 2019, **17**(9): 641-649.