Original Article

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Renal protective effect of Paeoniflorin by inhibition of JAK2/STAT3 signaling pathway in diabetic mice

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Summary **Paeoniflorin is the main bioactive components of the root of *P.lactiflora* Pall., and has been widely used as an anti-inflammation and immunomodulatory agent. However, the effect and mechanisms of Paeoniflorin in diabetic nephropathy (DN) remains to be elucidated. In the present study, streptozotocin (STZ)-induced type 1 diabetic mice model was used to investigate the protective effect of Paeoniflorin and the role of the Janus kinase (JAK) 2/signal transducer (STAT) 3 signaling pathway on DN. After treatment with Paeoniflorin at a dose of 25, 50 and 100 mg/kg once a day for 12 weeks, both the functional and histological damage to diabetic mice kidney had been attenuated significantly. Additionally, these reno-protective effects were associated with alleviating macrophage infiltration and inflammatory factors expression as well as suppression of the JAK2/STAT3 signaling pathway. These data reveal that Paeoniflorin attenuates renal lesions in diabetic mice and these protective effects may be associated with the prevention of macrophage infiltration and inhibition of the JAK2/STAT3 signaling pathway.**

Keywords: Paeoniflorin, diabetic nephropathy, JAK2/STAT3, inflammation, macrophage

1. Introduction

As a major severe complication of diabetes mellitus (DM), diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) and imposes high social and economic burden worldwide (*1*). A study from Zhang *et al*. (*2*) indicated that with the increasing prevalence of DM, chronic kidney disease related to diabetes had become more common than chronic kidney disease related to glomerulonephritis in China. Increasing evidence showed that the pathogenesis of DN was associated with the interactions between metabolic and hemodynamic alteration, oxidative stress, inflammation, activation of the renin-angiotensin-aldosterone system (RAAS) and other factors (*3*). Studies have indicated that chronic low grade inflammatory reactions play critical roles in the initiation and progression of DN (*4*). Presently, available therapies such as intensive hyperglycemia, blood pressure control, and inhibition of the RAAS have been shown merely to slow but insufficiently prevent the progression of DN (*5*). Thus, it is of pressing need to identify novel therapeutic targets that might lead to the prevention of DN.

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway regulates a broad range of biological effects such as cell proliferation, differentiation, inflammation, and apoptosis (*6*). The JAK/STAT pathway transmits extracellular ligand signals directly to the nucleus to induce a variety of cellular responses. Currently, there are four members of the JAK family (JAK1, JAK2, JAK3, and Tyrosine Kinase 2) and seven STATs (STAT1, 2, 3, 4, 5a, 5b and 6) identified in mammals (*7*). Recent studies have shown that the JAK/STAT pathway is activated in numerous acute and chronic renal diseases, moreover, JAK2 and STAT3 are the JAK and STAT forms that have been most clearly identified in the onset and progression of DN (*8*). The accumulated evidence for JAK2/STAT3 activation in the pathogenesis of DN implied a novel therapeutic target for potential

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intervention in this disease.

Paeoniflorin is a monoterpene glycoside extracted from the dried root of *P.lactiflora* Pall. As the principle bioactive component (> 90 %) of the Chinese traditional medicine total glucosides of paeony (TGP) (*9*), Paeoniflorin exerts numerous pharmacological effects including anti inflammation, immunomodulation, neuroprotective and antitumor effects (*10-13*). Fu *et al*. (*14*) reported that Paeoniflorin prevented DN by inhibiting activation of nuclear factor-κB (NF-κB) and renal macrophage infiltration in streptozotocin (STZ)-induced diabetic model rats. Furthermore, our previous studies demonstrated that Paeoniflorin inhibited toll-like receptor (TLR) 2/4-mediated inflammation and exerted a renoprotective role on type 2 diabetic nephropathy in both *in vitro* and *in vivo* studies (*15*). However, the exact mechanisms of Paeoniflorin in DN still remains unclear. Additionally, whether the renoprotective effects of Paeoniflorin in DN are induced *via* the JAK2/STAT3 signaling pathway requires further exploration.

Therefore, the aim of the present study was to evaluate whether Paeoniflorin could alleviate renal damage in STZ-induced type 1 diabetes mellitus using C57BL/6J mice. Moreover, we hypothesized that Paeoniflorin might exert renoprotective effects by inhibiting the JAK2/STAT3 signaling pathway.

2. Materials and Methods

2.1. *Drugs and reagents*

Paeoniflorin [(C23H28O11, MW: 480.45, purity = 98.78% (high-performance liquid chromatography), lethal dose = 9,530 mg/kg, Figure 1)] was purchased from Nanjing Goren Biotechnology Co., Ltd (Nanjing, China). STZ was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The rabbit anti-JAK2, STAT3, phosphorylated JAK2(p-JAK2), phosphorylated STAT3(p-STAT3) antibodies were bought from Cell Signaling Technology (Beverly, MA, USA) and CD68 was bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin antibodies, anti-mouse IgG and anti-rabbit IgG horseradish-peroxidase (HRP) were brought from Wuhan Sanying Biotechnology (Wuhan, China). GAPDH and tumor necrotic factor-α (TNF-α) primers were obtained from the Shanghai Sangon Biotech Co., Ltd (Shanghai, China). Primers for inducible nitric oxide synthase (iNOS) (MQP029793), interleukin-1β (IL-1β) (MQP027422), and monocyte chemoattractant protein-1 (MCP-1) (MQP027672) were purchased commercially from GeneCopoeia, Inc. (Rockville, MD, USA).

2.2. *Animals and experimental design*

Male C57BL/6J mice (*n* = 60; 8-10 weeks old; weight between 18-20 g) were purchased from the Model Animal Research Centre of Nanjing University. All mice were housed under standard raising condition with room temperature (20-24℃), humidity (50-55%) with a 12/12 h light/dark cycle and free access to food and water in the Experimental Animal Center of Anhui Medical University.

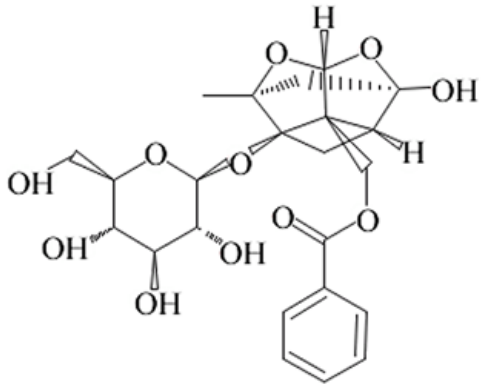

Figure 1. The chemical structure of Paeoniflorin.

After 1 week for acclimation, mice were randomly divided into five groups (*n* = 12 per group): (1) normal control group (Con group); (2) diabetic model group (DM group); (3) Paeoniflorin 25mg/kg treatment group (DM+PF 25 mg/kg); (4) Paeoniflorin 50 mg/kg treatment group (DM+PF 50mg/kg); (5) Paeoniflorin 100 mg/kg treatment group (DM+PF 100 mg/kg). The type 1 diabetic models were produced by intraperitoneal injection of STZ (50 mg/kg in 0.1 M citrate buffer, pH 4.5) daily for 5 consecutive days (16). Mice in the control group received an equal volume of the citrate buffer. Blood glucose was measured 1 week after STZ injection, and mice with blood glucose over 16.7 mmol/L were considered diabetic and selected for the following study. Paeoniflorin gavage was initiated once a day at a dose of 25, 50, and 100 mg/kg for 12 weeks while the Con group and the DM group received an equivalent volume of distilled water (*17*). After 12 weeks, blood and 24 h urine samples were collected. After that, all the mice were sacrificed and body weights were recorded. Kidney samples of mice were dissected, weighed and processed for further analyses. All animal investigation was approved by the Ethical Committee of Animal Research of Anhui Medical University, and the mice were sacrificed according to the Guide for the Care and Use of Laboratory Animals recommendations.

2.3. *Measurement of general and metabolic parameters*

Body weight (BW), kidney weight (KW) and blood glucose was recorded by an electronic scale and glucose analyzer. All Mice were housed individually in metabolic cages for 24 h to collect urinary samples before being sacrificed. The concentration of 24 h

urine albumin was determined by using a mouse microalbumin ELISA kit (Abcam Biotechnology, Cambridge, UK).

2.4. *Histological examination*

For histological and immunohistochemistry analysis, 4% paraformaldehyde-fixed kidney tissues were dehydrated through a graded series of ethanol, routine paraffin embedding, and then cut into 3 μm sections to make tissue slides. Periodic acid-schiff (PAS) staining was performed under a light microscope. PAS staining was used for histological grading and the glomerular mesangial expansion index and tubulointerstitial damage index as previously described was measured randomly in 10 visual fields (*18*).

2.5. *Immunohistochemistry (IHC) analysis*

After being deparaffinized with xylene and hydrated with graded alcohol, the kidney tissue slides were placed in 3% hydrogen peroxide to block endogenous peroxidase activity. For antigen retrieval, kidney slides were transferred to 10 mmol/L citrate buffer solution (pH 6.0) at 100℃ for 15 min. Then the slides were incubated at 37℃ for 30 min with goat serum to prevent nonspecific binding and incubated with primary antibodies: anti-CD68 (1:50), anti-p-JAK2 (1:100), and anti-p-STAT3 (1:100) at the given dilutions overnight at 4℃. After washing, the slides were incubated with polyperoxidase-anti-mouse/rabbit IgG for 30 min at 37℃ followed by color development with 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) and hematoxylin staining was used. The numbers of CD68-positive cells and the percentage of the p-JAK2 and the p-STAT3 brown positive staining areas in glomeruli and tubulointerstitium areas were calculated respectively in 10 random high-power (×400) fields. Quantitative analysis was performed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

2.6. *Western blot analysis*

Protein samples used for Western blot analysis were extracted from mice renal samples and the protein concentration was determined with a Bio-Rad protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of proteins were separated by 8-12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline and Tween 20 (TBST) buffer for 2 h and then incubated overnight at 4℃ with different primary polyclonal antibodies against p-JAK2 (1:1,000), JAK2 (1:1,000), p-STAT3 (1:1,000), and STAT3 (1:1,000). After washing, the membranes were incubated with HRP-labeled secondary antibody (1:35,000) at room temperature for 1 h. The blots were revealed using an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Little Chalfont, UK) and the protein content was quantified and analyzed using a Leica Q500IW image analysis system (Leica Ltd., Cambridge, UK). β-actin served as a loading control. The band density was measured using Image J software.

2.7. *Real-time PCR (RT-PCR)*

Total RNA was extracted from kidney tissues using TRIzol reagent (Invitrogen, California, USA). The transcription of cDNA was synthesized with a standard reverse transcription reaction kit (Promega, Madison, WI, USA) and RT-PCR was performed using the SYBR Green PCR Master Mix kit (Bio-Rad Laboratories, Hercules, CA, USA). The amplification conditions were set as follows: initial hold steps (denaturation at 95°C for 10min) and 35 cycles of a 2-step PCR (95°C for 15s and 60℃ for 30s). The primers used in RT-PCR were GAPDH (forward, 5′-GGTGAAGGTCGGTGTGAACG-3′; reverse, 5′-CTCGCTCCTGGAAGATGGTG-3′), TNF-α (forward, 5′-GCTGAGCTCAAACCCTGGTA-3′; reverse, 5′-CGGACTCCG CAAAGTCTAAG-3′). iNOS (MQP029793), IL-1β (MQP027422), and MCP-1 (MQP027672). Primers were purchased from GeneCopoeia, Inc. The relative expression levels of the target PCR product was calculated after adjusting for GAPDH by using the 2-ΔΔCt method (*19*).

2.8. *Statistical analysis*

SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation (SD) and the results were analyzed by one-way analysis of variance (ANOVA). The difference between groups was tested by least significant difference (LSD) and the Levene method was used for homogeneity test of variance. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Paeoniflorin attenuates renal damage in diabetic kidneys*

Initially, to investigate the effects of Paeoniflorin on diabetic kidney, we evaluated renal function by measuring general and metabolic parameters including blood glucose, KW/BW ratio and 24 h urinary albumin excretion rate (UAER). The DM group demonstrated elevated blood glucose, increased KW/BW ratio and the differences were significant compared with that of the control group. Paeoniflorin treatment for 12 weeks had

Table1. General and metabolic parameters from five groups of mice models

Parameter	Con	DM	DM+PF 25 mg/ kg	DM+PF 50 mg/ kg	DM+PF 100 mg/ kg
Blood glucose (mmol/L)	5.78 ± 3.16	30.46 ± 2.45**	26.52 ± 3.62	26.33 ± 5.64	30.84 ± 5.07
KW/BW ratio (g/100g BW)	0.70 ± 0.07	0.84 ± 0.11*	0.79 ± 0.11#	0.77 ± 0.05#	0.77 ± 0.13#
UAER (mg/24 h)	0.09 ± 0.02	1.69 ± 0.13**	1.25 ± 0.16##	0.84 ± 0.15##	0.32 ± 0.09##

All the values are represented as mean ± SD (n = 12 per group). *P < 0.05, **P < 0.01 *vs*. Con; #P < 0.05, ##P < 0.01 *vs*. DM. Con, normal control group; DM, diabetic model group; PF, Paeoniflorin; KW/BW, kidney weight/body weight; UAER, urinary albumin excretion rate.

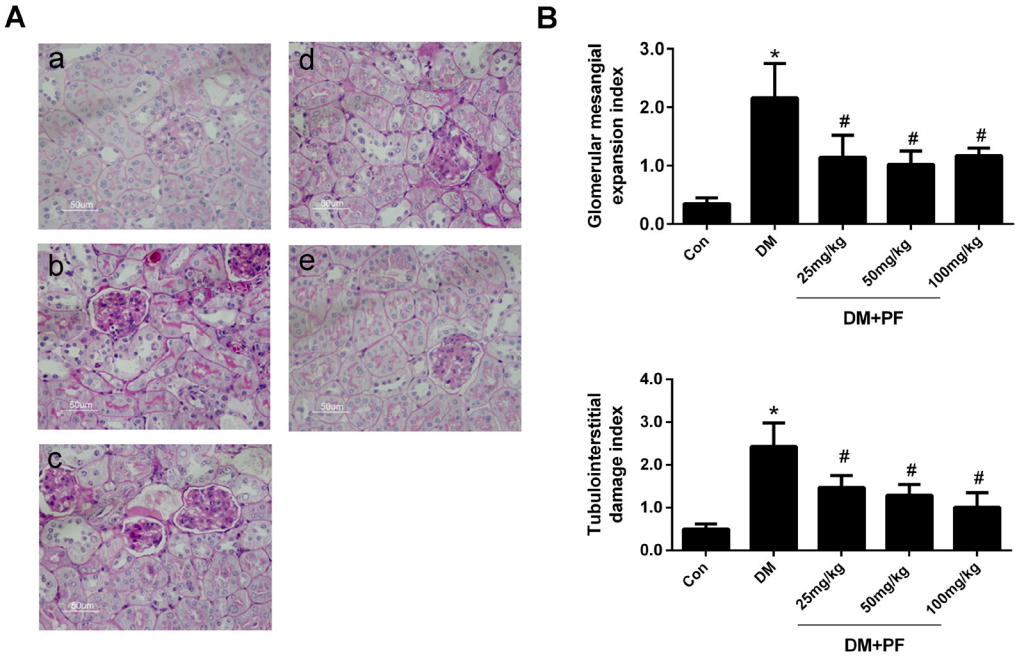

Figure 2. Histology features of renal tissues. (A) Representative images of kidney samples stained with PAS in the **(A-a)** Con, **(A-b)** DM, **(A-c)** DM+PF 25 mg/kg, **(A-d)** DM + PF 50 mg/kg and **(A-e)** DM + PF 100mg/kg groups. **(B)** Quantification of PAS staining. All the values are represented as mean ± SD of at least three independent experiments (n = 12 per group). *p < 0.05 *vs*. Con; #p < 0.05 *vs*. DM. Con, normal control group; DM, diabetic model group; PF, Paeoniflorin; PAS, periodic acid-schiff. Original magnification, ×400 in A.

no significant effect on the hyperglycemia in diabetic mice. However, the KW/BW ratio of the DM group was significantly reduced after Paeoniflorin treatment in diabetic mice. Similarly, the diabetic mice had a remarkably higher 24 h UAER than those in the control group. After Paeoniflorin treatment for 12 weeks, the raised 24 h UAER of the diabetic group was attenuated significantly in a dose-dependent manner although their levels were still higher than those of the control group. These results showed that Paeoniflorin could preserve STZ-caused renal lesions (Table 1).

In addition to clinical renal function results, we further evaluated renal histopathologial alterations in kidney samples. Results from PAS staining indicated that both of the glomerular mesangial expansion index and tubulointerstitial damage index of the DM group were particularly higher when compared with the control group (Figure 2B). By contrast, Paeoniflorin treatment for 12 weeks decreased pathological lesions index remarkably compared to the DM group (Figure 2A and B). Thus histological assessment further confirmed that Paeoniflorin treatment could ameliorate progression of DN.

3.2. *Paeoniflorin suppresses macrophage infiltration and macrophage-mediated inflammatory factors in diabetic kidneys*

The induction of diabetes was associated with the recruitment, retention, and activation of macrophages in mice kidneys, as evidenced by increased expression of macrophage markers and proinflammatory factors. In this study, CD68 as a macrophage surface marker is used to investigate the role of Paeoniflorin in inflammatory responses related to DN in renal tissues. No obvious infiltration of CD68-positive macrophages was detected in the glomerular and tubulointerstitial areas of the normal control group. In contrast, we observed abnormal macrophage infiltration in the DM group. Paeoniflorin treatment markedly reduced such abnormal macrophage infiltration in the diabetic kidneys in a dose-dependent manner (Figure 3A and 3B).

To further evaluate whether Paeoniflorin modulates

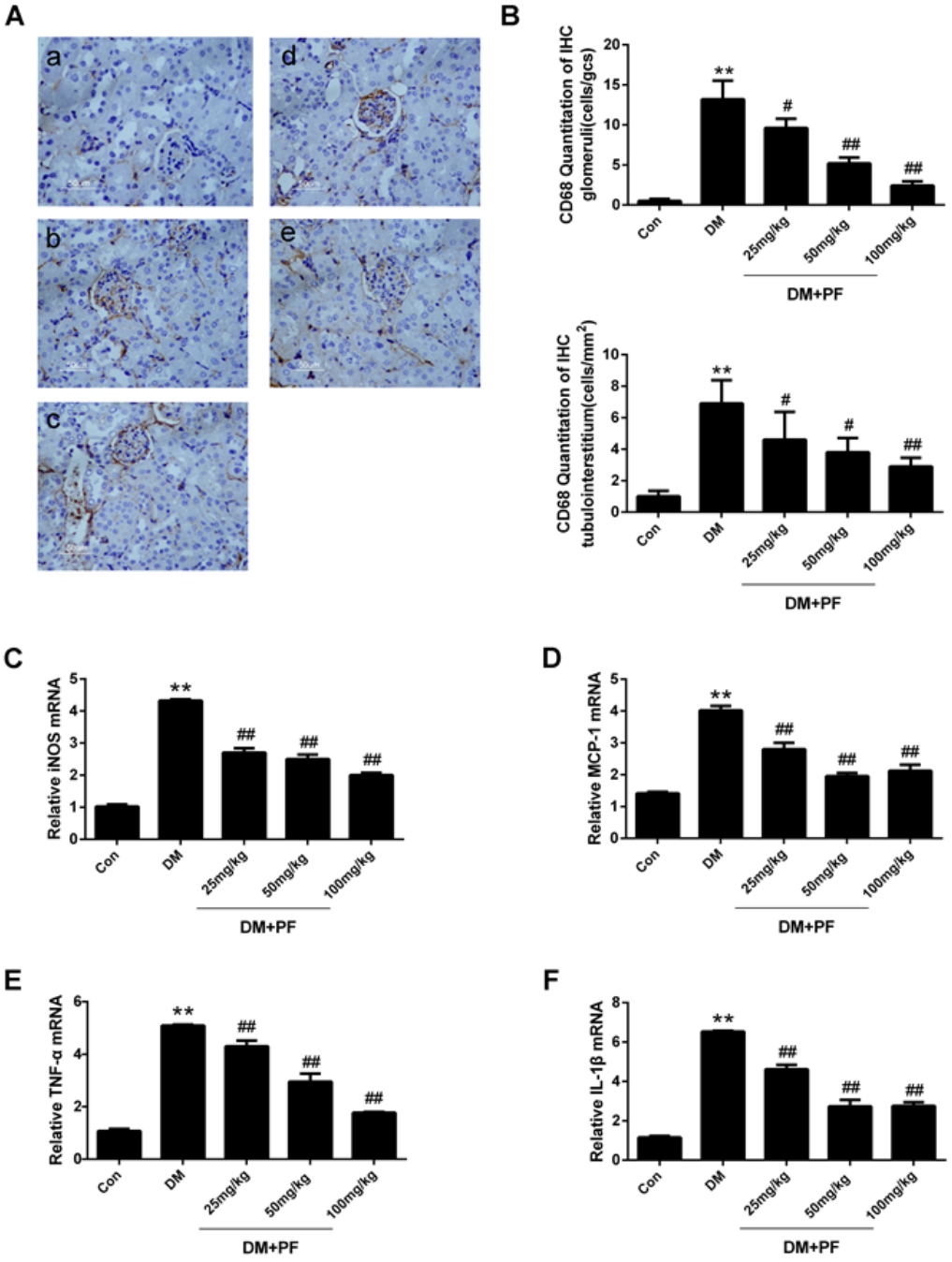

Figure 3. Paeoniflorin inhibits STZ-induced macrophage infiltration and inflammatory cytokine expression in renal tissues. **(A)** Representative images of CD68 IHC staining of kidney samples in the **(A-a)** Con, **(A-b)** DM, **(A-c)** DM+PF 25 mg/kg, **(A-d)** DM+PF 50 mg/kg and **(A-e)** DM+PF 100 mg/kg groups. **(B)** Quantitative analyses of CD68 IHC staining. **(C-F)** mRNA expression of iNOS, MCP-1, TNF-α and IL1-β in renal tissues, as determined by real-time PCR. All values are represented as mean ± SD of at least three independent experiments (n = 12 per group). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. Con; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. DM. STZ, strepozocin; Con, normal control group; DM, diabetic model group; PF, Paeoniflorin; IHC, immunohistochemical; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrotic factor-α; IL1-β, interleukin-1β. Original magnification, ×400 in A.

the functional stage of kidney macrophages, expression levels of iNOS were examined to distinguish between pro-inflammatory M1 and anti-inflammatory M2 phenotypes. The mRNA expression level of iNOS in the DM group increased remarkably compared to the control group while Paeoniflorin treatment significantly repressed this overexpression (Figure 3C). Moreover, we also tested the expression levels of proinflammatory cytokines including MCP-1, TNF-α and IL-1β. Similarly, results indicated that Paeoniflorin treatment inhibited the up-regulation of all of these cytokines in the STZ-induced vehicle group to a different extent (Figure 3D-F). These present results indicated that Paeoniflorin could mitigate renal inflammation in

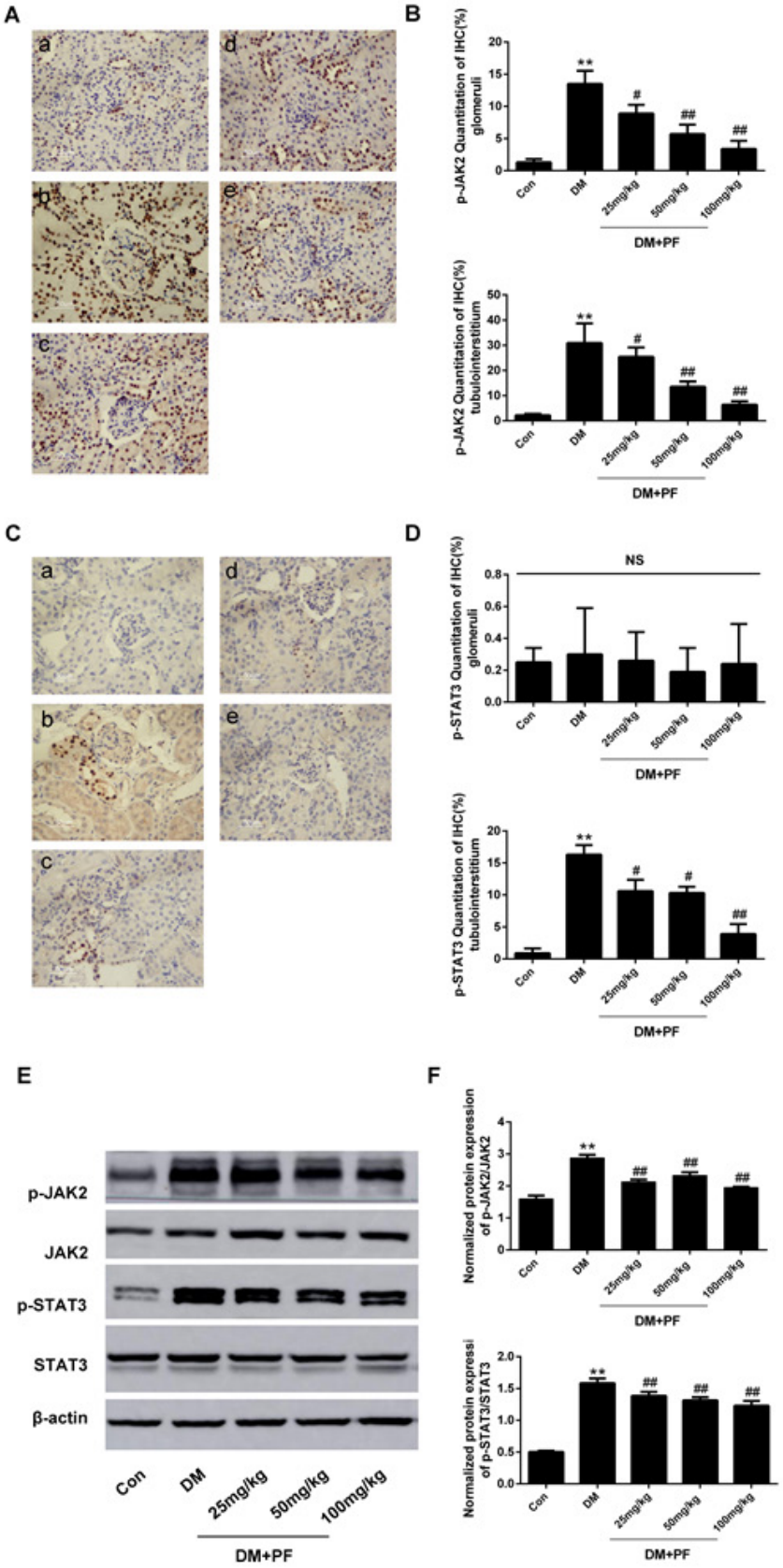

Figure 4. Effects of Paeoniflorin on the expression of p-JAK2, p-STAT3, JAK2 and STAT3 in renal tissues. (A-B) Representative images and quantitative analyses of IHC staining for p-JAK2 expression in the **(A-a)** Con, **(A-b)** DM, **(A-c)** DM+PF 25 mg/kg, **(A-d)** DM+PF 50 mg/kg and **(A-e)** DM+PF 100 mg/kg groups. **(C-D)** Representative images and quantitative analyses of IHC staining for p-STAT3 expression in the **(C-a)** Con, **(C-b)** DM, **(C-c)** DM+PF 25 mg/kg, **(C-d)** DM+PF 50 mg/kg and **(C-e)** DM+PF 100 mg/kg groups. **(E)** Representative Western blots for p-JAK2, JAK2, p-STAT3 and STAT3. **(F)** Normalized protein expression of p-JAK2/JAK2 and p-STAT3/STAT3 by Western blotting. β-actin was detected as a loading control. All the values are represented as mean ± SD of at least three independent experiments (n = 12 per group). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. Con; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. DM. Con, normal control group; DM, diabetic model group; PF, Paeoniflorin; IHC, immunohistochemical; NS, not significant. Original magnification, ×400 in **A** and **C**.

diabetic kidneys by inhibiting macrophage infiltration and further suppress macrophage-mediated expression of inflammatory factors.

3.3. *Paeoniflorin inhibits the activation of JAK2/STAT3 signaling in diabetic kidneys*

As described above, Paeoniflorin exhibited an anti-inflammation effect in the diabetic kidney. For this reason, certain molecules and signal pathways associated with inflammation were examined. We initially measured the expression level of p-JAK2 and p-STAT3 proteins in the glomeruli and tubulointerstitium by IHC staining. The present results indicated that the DM group expressed an intense staining of p-JAK2 in the glomeruli and tubulointerstitium area. Furthermore, Paeoniflorin treatment significantly reduced the stained section of p-JAK2 both in glomeruli and tubulointerstitium compared to the DM group in a dose-dependent manner (Figure 4A and 4B). The same results of p-STAT3 expression were detected in the tubulointerstitium area, however, no significant difference was identified for p-STAT3 expression in the glomeruli area among five groups (Figure 4C and 4D).

Consistent with immunohistochemistry analysis, Western blot analysis results further confirmed an upregulation of detectable p-JAK2 and p-STAT3 protein expression levels in the DM group. Compared with the DM group, expression of p-JAK2 and p-STAT3 levels were significantly attenuated by Paeoniflorin treatment for 12 weeks. In contrast, no obvious expression changes of total JAK2 and STAT3 were observed among five groups (Figure 4E and 4F). Thus, these results indicated that the JAK2/STAT3 signaling pathway was activated in DN while Paeoniflorin could exert an inhibition role effect on the JAK2/STAT3 signaling pathway in the STZ-induced diabetic model.

4. Discussion

The global prevalence of DN has been increasing over the last few decades, and satisfactory therapeutic strategies that prevent DN progression still remain a challenge. Renal morphological and functional alterations are two major characterizations of DN. Clinically, microalbuminuria is the earliest detectable biomarker of glomerular damage and is also an independent risk factor for the progression of DN (*20*). In our study, the KW/BW ratio was used as an indicator of renal hypertrophy index in line with former research (*21*). Major pathological changes of DN include thickening of glomerular basement membrane, mesangial matrix accumulation and expansion, glomerulosclerosis, tubular interstitial inflammation and fibrosis which eventually lead to proteinuria and renal failure (*22*).

To date, cumulative studies highlighted beneficial effects of traditional Chinese medicine (TCM) for delaying DN progression and provided new insights into promising drugs for DN prevention (*23*). For instance, Paeoniflorin exhibits a significant anti-inflammatory effect in models of rheumatoid arthritis, hepatic fibrosis, and ulcerative colitis (*24-26*). Notably, research also demonstrated the renoprotective role of Paeoniflorin in kidney disease including renal fibrosis (*27*) and acute renal injury (*28*). Thus in this present study, renal protection from Paeoniflorin was evaluated as a novel therapeutic agent for DN. Our findings showed that Paeoniflorin significantly reduced the albuminuria and attenuated renal hypertrophy in STZ-induced diabetic mice. However, Paeoniflorin did not impact the blood glucose levels of diabetic mice which were not consistent with a Fu *et al*. previous study (*14*). Furthermore, the histology damage in diabetic renal samples was improved a lot after Paeoniflorin treatment. Therefore, our study demonstrated that Paeoniflorin treatment alleviated both the functional and morphological damage of the diabetic mouse model.

Accumulated evidence implied the crucial rule of renal inflammation in the development of DN. Recent studies of both animal and human models have showed that macrophage infiltration and the over expression of proinflammatory cytokines lead to renal damage and fibrosis in diabetic kidneys (*29*). The macrophages associated with renal damage can be divided into: classically activated M1 macrophages involved in inflammation and tissue damage or alternatively activated M2 macrophages mediating tissue repair and renal protection (*30*). Currently, iNOS is the most widely used specific marker of M1 macrophages (*31*). In addition, macrophages mediate the over expression levels of proinflammatory cytokines like MCP-1, TNF-α and IL-1β and finally induce renal damage and fibrosis in human and animal DN models (*32*). Consistent with our previous studies (*15*), the renal tissues of the diabetic mice group in our study showed massive CD68-positive macrophage infiltration in the glomerular and tubulointerstitial regions as well as an elevated mRNA expression level of iNOS. Our further investigation showed that expression of pro-inflammatory cytokines including MCP-1, TNF-α and IL-1β also increased dramatically. We noted that Paeoniflorin treatment apparently reduced macrophage infiltration and iNOS expression levels, thus alleviating the pro-inflammatory cytokines in diabetic mice kidneys. Consequently, our findings suggested the potential anti-inflammatory effect of Paeoniflorin in DN and this protective role probably is associated with the suppression of M1 macrophages infiltration in renal tissues.

Activation of the JAK2/STAT3 signaling pathway has been proven to play a pivotal role in the progression of DN in rodent models and human patients (*6*). On the basis of recent studies, inhibition of JAK-STAT signaling, including increased expression

of the suppressors of cytokine signaling proteins and pharmacologic inhibition of JAK and STAT proteins, establish a new therapeutic target for DN (*33*). Baricitinib (ClinicalTrials.gov Identifier NCT01683409), a selective JAK1 and JAK2 inhibitor, has been investigated in phase 2 randomized clinical trials in participants with type 2 diabetic nephropathy (*34*). The study has proven that baricitinib treatment resulted in a reduction in albuminuria after 3 months' treatment (*35*). However, the impact of the JAK2/STAT3 signaling pathway in the renoprotective effect of Paeoniflorin has not been examined. Our experiment showed that the significant upregulation of the protein expression of p-JAK2 and p-STAT3 in diabetic kidney decreased a lot after Paeoniflorin intervention. As the activation of the JAK2/STAT3 pathway can be inhibited by Paeoniflorin treatment, the current study demonstrated that the protection of Paeoniflorin against STZ-caused diabetic damage might be *via* suppressing of JAK2/STAT signaling. Therefore, the inhibition of JAK2/STAT3 signals through Paeoniflorin may be a novel method for DN treatment.

As an important signal in inflammation, the activation of the JAK/ STAT pathway had been shown to promote the polarization of M1 macrophages (*36*). Moreover, Zhai *et al*. (*13*) revealed that Paeoniflorin reduced M1 cells activity through inhibiting the activation of NF-κB signaling pathway while enhancing M2 cells function *via* activating STAT6 phosphorylation in mouse bone marrow derived macrophages. Our study along with previous published results proved the anti-inflammatory role of Paeoniflorin in DN. For this reason, we propose a potential theory that Paeoniflorin attenuates renal inflammation in STZ-induced diabetic mice kidney and these protective effects may be associated with the prevention of macrophage activation through inhibiting the JAK2/STAT3 signaling pathway to some extent. However, it remains unclear whether Paeoniflorin prevents macrophage activation *via* the inhibition of JAK2/STAT3 in DN. Therefore, we need further studies to uncover the exact mechanism of Paeoniflorin on JAK2/STAT3 and M1 macrophage activation in DN.

In conclusion, our study confirms that Paeoniflorin treatment alleviates renal lesions by inhibiting the activation of JAK2/STAT3 signaling pathways and the infiltration of macrophages together with the suppression of pro-inflammatory cytokine expression, thus improving the prognosis of DN. Therefore, our findings suggest that Paeoniflorin as a novel and feasible therapeutic strategy to halt the onset and progression of renal inflammation in DN.

Acknowledgements

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