Original Article

DOI: 10.5582/bst.2017.01104

Paeoniflorin prevents TLR2/4-mediated inflammation in type 2 diabetic nephropathy

Tingmin Zhang[§], Qijin Zhu[§], Yunxia Shao, Kun Wang, Yonggui Wu[*]

Department of Nephrology, the First Affiliated Hospital, Anhui Medical University Hefei, Anhui, China.

Summary **Paeoniflorin is an effective Chinese traditional medicine with anti-inflammatory and immune-regulatory effects. The aim of this study was to investigate the underlying renoprotective mechanism of Paeoniflorin. *In vivo*, db/db mice were intraperitoneally injected with Paeoniflorin at a dose of 15, 30, or 60 mg/kg respectively. The immunostaining of TLR2, TLR4, CD68, NF-kB p65 and the mRNA level of inflammatory factors, together with the protein expression of TLR2/4 signaling were evaluated. Our data demonstrated that Paeoniflorin could decrease the urinary albumin excretion rate and inhibit macrophage infiltration and activation through blockage of the TLR2/4 signaling pathway compared with the db/db group *in vivo*. *In vitro*, RAW264.7 cells were categorized into control, bovin serum albumin (BSA)-stimulated, advanced glycation end products (AGEs)-stimulated, Paeoniflorin intervention and oxidized phospholipid (OxPAPC)-inhibited groups. The cell viability, the optimal stimulated time and concentration were measured as well as the TLR2/4 signaling activation determined by RT-PCR, Western blot and ELISA. Our data demonstrated that Paeoniflorin reduced the AGEs-induced TLR2/4 activation and inflammatory responses, which was consistent with the TLR2/4 inhibitor group. These findings indicate that Paeoniflorin prevents macrophage activation *via* inhibition of TLR2/4 signaling expression in type 2 diabetic nephropathy.**

Keywords: Paeoniflorin, TLR 2/4, diabetic nephropathy, inflammation, macrophage

1. Introduction

Diabetic nephropathy (DN), as the most common diabetic microvascular complication, accounts for a third of all patients with diabetes mellitus (*1-2*). Data from developed countries show that diabetic nephropathy is the first cause of renal replacement therapy (*3*). However, existing clinical interventions which contain strict control of hyperglycemia and hypertension, and block the renin–angiotensin–aldosterone axis have been demonstrated only to delay the progression of DN, not to cease or reverse the pathological state (*4-5*).

Recent studies have appreciated that the central pathogenesis of DN resulting from diabetes is oxidative injury, as well as inflammatory and immune responses (*6*). Numerous studies have proposed that macrophage infiltration and activation in diabetic kidney initiate inflammation *via* the release of some related factors, which subsequently lead to the development and progression of DN. Toll-like receptors (TLRs) are germline-encoded receptors which have been found on either antigen-presenting cells (including macrophages, and monocytes) or kidney intrinsic cells, especially renal tubular epithelial cells and endothelial cells (*7*). The recognition of TLRs ligands triggers the innate immune response for the activation of TLRs signaling promotes the transcription of NF-κB, which causes an inflammatory cascade with a high release of pro-inflammatory cytokines and chemokines (*8*). Nevertheless, *in vivo* and *in vitro* studies, showed increased expression in TLR2 was observed respectively in a streptozocin (STZ) induced diabetic model and high glucose circumstances (*9*), while it was also noted

Released online in J-STAGE as advance publication June 18, 2017.

[§]These authors contributed equally to this work.

[*]*Address correspondence to:*
Dr. Yonggui Wu, Department of Nephrology, the First Affiliated Hospital, Anhui Medical University, No.218 Jixi Road, Hefei, Anhui, China.
E-mail: wuyonggui@medmail.com.cn

that an attenuation in NF-κB and pro-inflammatory cytokines activation with silenced or inhibited TLR4 expression (*10*), indicates the significance of TLRs in mediating the whole process. Furthermore, TLR2 and TLR4 can be found in macrophages, which are strongly related to its activation. Thus, we assume that the terminal kidney injury called DN resulted from the TLR2/4-mediated inflammatory state triggered by a certain diabetic microenvironment.

Paeoniflorin, a typical Chinese herbal medicine ingredient, is a primary component of total glucosides of paeony, isolated from the dried root of Paeonia lactiflora Pall (Family: Ranunculaceae) which was used in traditional Chinese prescriptions for anti-inflammatory, analgesic and diuretic effects (*11*). The drug has been admitted by US Food and Drug Administration for marketing since 1998 and Paeoniflorin itself has been shown to have not only anti-inflammatory, immune-regulatory, anti-allergic (*12*), and antinociceptive effects (*13*), but also antioxidative (*14*) and antiproliferative (*15*) activities as well by a growing number of scientific research studies. An experiment conducted by Fu *et al*. discovered a lower level of urinary albumin and ameliorated glomerular hypertrophy, together with decreased macrophage infiltration and inflammatory factor production in Paeoniflorin-treated rats (*16*). Also, Huang *et al*. revealed TLR as one of the paeonol target genes relevant to the anti-inflamatory effect (*17*). Therefore, the former evidence implicated that Paeoniflorin might mediate the inflammatory response *via* the TLRs signaling pathway in DN. Our study was designed to investigate the mechanism of how Paeoniflorin prevents TLR2/4-mediated inflammation in type 2 diabetic nephropathy.

2. Materials and Methods

2.1. *Drugs and reagents*

Paeoniflorin [C23H28O11, MW: 480.45, purity: 98.78% (HPLC)], LD50: 9,530 mg/kg] was bought from Nanjing GOREN BIO Technology Co., Ltd (Nanjing, China) as the commercial product. Its molecular structure is shown in Figure 1. OxPAPC was prepared by invivoGen Biotechnology (invivoGen, USA). CCK-8 Cell Counting Kit was obtained from Vazyme Biotech Co. (Nanjing, China). The rabbit anti-TLR4, MyD88, Trif, iNOS antibodies were purchased from Abcam Biotechnology (Abcam, Cambridge, UK) and p-IRF3, NF-κB p65, NF-κB p-p65 were from Cell Signaling Technology (CST Beverly, MA, USA), together with TLR2 from EMD Millipore Corporation (EMD Millipore, USA). The rabbit anti- p-IRAK1 were from Santa Cruz Biotechnology (Santa Cruz, California, USA). TNF-α and IL-1β ELISA kit were from R&D Systems (R&D Systems, USA), while MCP-1 ELISA kit was obtained from RIBIO TECH (RIBIO TECH , Beijing, China).

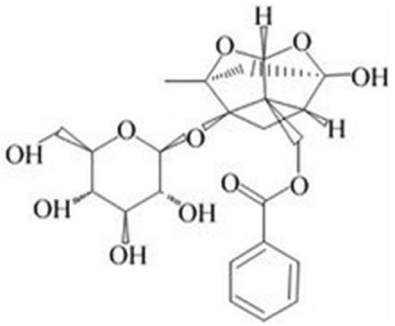

Figure 1. The chemical structures of Paeoniflonin.

2.2. *Animals*

The wild db/m littermates and db/db mice were purchased from the Model Animal Research Center of Nanjing University and housed individually in cages under standard conditions with 12-hour light-dark cycle, free access to food and water, room temperature of 24°C and humidity of 60%. The experimental protocols were approved by Ethical Committee of Animal Research of Anhui Medical University and executed according to the recommendations of Laboratory Animal Care and Use.

2.3. *Experimental design*

The db/m mice were recognized as the non-diabetic control group (n = 12). The db/db mice whose blood glucose levels were over 16.7 mmol/L, were regarded as diabetic and were randomly divided into four groups (diabetic control group and Paeoniflorin intervention groups) among which there existed no differences, number of rats in each group was 12. Paeoniflorin intervention groups were intraperitoneally injected with Paeoniflorin daily at the dose of 15, 30, or 60 mg/kg respectively at 10-weeks of age, while the non-diabetic control group and diabetic control group were injected with an equivalent amount of saline. All the mice were sacrificed at 12-weeks-of-age and the kidneys were harvested for further analyses. The weight of the body and kidney, as well as blood glucose of each mouse were recorded using an electronic scale and glucose analyzer, while the blood samples were obtained by jugular artery catheterization.

2.4. *Metabolic data analysis*

Prior to sacrifice, samples for 24 h urine were gathered from the mice for the calculation of urinary albumin excretion. Creatinine clearance rates were calculated by urinary creatinine concentration/serum creatinine × urine flow per minute.

2.5. *Cell culture*

RAW264.7 cells were purchased from Shanghai cell bank of Chinese Academy of Science, China and seeded in high glucose DMEM medium with an osmotic pressure of 250 mmol/L, which was supplemented with 10% fatal bovine serum (FBS) and 1% penicillin G, streptomycin. The serum free medium was used to culture the cells for 24 h in order to synchronize the cells. Prior to the experiment, the cells were inoculated in 96-well plates, at a density of 5,000-10,000 cells per plate. Different concentrations of Paeoniflorin were added in cell culture for a day and a CCK-8 assay kit was used to determine cell viability. Under 200 μg/mL AGEs stimulation, the cells were collected at different time points, and the total protein was extracted to observe the expression of TLR2, TLR4 and iNOS protein in order to determine the optimal time of stimulation. In addition, different concentrations of Paeoniflorin were also added half an hour ahead of AGEs stimulation and TLR2, TLR4 and iNOS expression was assessed to determine the best concentration for Paeoniflorin treatment. Then the synchronized RAW264.7 cells were divided into the control group, BSA-stimulated group, AGEs-stimulated group, Paeoniflorin intervention group and oxidized phospholipid (OxPAPC)-inhibited group. The BSA-stimulated group was cultured with 200 μg/mL BSA and the AGEs-stimulated group, Paeoniflorin intervention group as well as the OxPAPC-inhibited group were all cultured with 200 μg/mL AGEs respectively for 24 hours. The Paeoniflorin intervention group was treated with various concentrations of Paeoniflorin half an hour before AGEs. Furthermore, an hour before the AGEs stimulation, the OxPAPC-inhibited group was added in OxPAPC to block the TLR2/4 signaling pathway.

2.6. *Immunohistochemistry*

Fresh renal tissues were fixed with 4% paraformaldehyde and embedded with paraffin, which were later cut into 2 μm sections. After deparaffinization, the tissue slices were treated with 3% hydrogen peroxide and then heated in a microwave in order to enclose the endogenous peroxidase and retrieve the antigen. Primary antibody (CD68, TLR2, TLR4, NF-κB p65) was incubated at 37°C with polyperoxidase-anti-mouse/rabbit IgG, followed by 3,3-diaminobenzidine (DAB, Sigma) and hematoxylin staining. Quantitation for CD68 positive cells, representing the recruitment of macrophages in tissue sections were counted randomly in 20 high-power (×400) interstitial fields. However, the staining of TLR2, TLR4 and NF-κB p65 was analyzed by a computer system.

2.7. *Western blot*

Animal protein of homogeneous kidney samples and cell protein were lysed and combined with loading buffer, boiled, then separated using 10-12% SDS-PAGE. Proteins were electro blotted onto a nitrocellulose membrane, incubated with primary antibody TLR2, TLR4, MyD88, p-IRAK1,Trif, p-IRF3, NF-κB p-p65, or NF-κB p65 overnight at 4°C after blocking with skimmed milk for 1 hour. The combination of secondary antibody (the goat anti-rabbit IgG) was applied once the membrane was washed. Finally the observation of the image using enhanced chemiluminescence and the protein content was quantitated using the documentation system.

2.8. *RT-PCR*

The RNA extracted from the kidney and the cells *via* Trizol reagent (Invitrogen, California, USA) was used to reverse transcribe to cDNA with the help of a Reverse transcription Kit (Promega, USA), which was in turn augmented by RT-PCR using Power SYBR Green PCR Master Mix (Bio-Rad, USA) and GAPDH primers (Sangon Biotech, Shanghai, China). The forward and reverse primer for the detected RNA sequence were as follows: TNF-α: 5'-CCCTCCTGGCCAACGGCATG-3' and 5'-TCGGGGCAGCCTTGTCCCTT-3'; the TLR2 (MQP030650), TLR4 (MQP032465), MCP-1 (MQP027672), IL-1β (MQP027422), iNOS (MQP029793). Primers were bought from GeneCopoeia, USA. Finally, the relative expression of genes was analyzed by using 2_ΔΔCt.

2.9. *ELISA*

At the end of the experiment, the culture medium of the RWA 264.7 cells was collected and the content of TNF-α, IL-1β, and MCP-1 in it was determined by ELISA kits.

2.10. *Statistical analyses*

Data were analyzed with the help of SPSS 16.0 and continuous variables were expressed as mean ± SD. All the data were compared by ANOVA analysis. The difference between groups was tested using the LSD and Levene method for a homogeneity test of variance, in which a *p* value under 0.05 was considered significant.

3. Results

3.1. *Clinical parameters*

The mice in the control group were responsive with active behavior, bright body hair, normal diet, water and a good mental state. Diabetes mellitus model group mice gradually showed obvious symptoms of diabetes: eating and drinking more, increased urine output,

Table 1. Changes of clinical and metabolic parameters in five groups of mice

Group	Dose (mg/kg)	Blood glucose (mmol/L)	Body weight (g)	Kidney weight (g)	Albumin excretion rate (μg/24 h)	Creatinine clearance rate (mL/min)
db/m	–	7.06 ± 1.22	24.64 ± 1.14	0.18 ± 0.02	16.91 ± 4.89	0.97 ± 0.29
db/db	–	33.46 ± 3.28**	45.35 ± 2.38**	0.25 ± 0.03**	1144.5 ± 81.3**	2.47 ± 0.64**
db/db+ paeoniflorin	15	34.08 ± 2.40	43.46 ± 2.69	0.25 ± 0.05	589.17 ± 69.42##	1.89 ± 0.57##
	30	32.67 ± 3.59	45.61 ± 2.97	0.23 ± 0.02#	497.32 ± 51.22##	1.71 ± 0.49##
	60	31.08 ± 2.56	43.39 ± 2.36	0.22 ± 0.03##	456.98 ± 55.46##	1.59 ± 0.53##

**$p < 0.01$, compared with the db/m group; #$p < 0.05$; ##$p < 0.01$, compared with the db/db group. Data are presented as Mean ± SD. Number of rats in each group was 12.

weight loss, bleak coat, messy, dirty, delayed behavior and were apathetic. Diabetic mice given paeoniflorin intervention and TLR2/4 knockout showed a mild performance compared to diabetes mellitus model group mice.

The metabolic data and the monitored clinical parameters of the five groups of mice are demonstrated in Table 1. On average, the enhanced blood glucose level and body weight were observed in the db/db group compared to db/m mice, but differences with Paeoniflorin treatment were not noticed. Despite the similar blood glucose level and body weight, mice given Paeoniflorin treatment exhibited a considerable dose-dependent decrease in albuminuria compared to the db/db group, which was still higher than that of db/m group.

3.2. *Immunohistochemical analysis for distribution of TLR2, TLR4, CD-68 and NF-κB p65 in mice kidneys*

Immunohistochemistry showed that staining of TLR2 was mainly observed in the tubulointerstitium, while the expression of TLR4 was found on glomeruli in addition to tubulointerstitium. In db/m mice kidneys, the positive result of IHC staining for TLR2 and TLR4 was barely noted, however the db/db mice illustrated typical manifestation of TLR2 and TLR4, which was recognized as overexpression. Compared with the db/db, the intensity of TLR2 and TLR4 immunostaining was significantly decreased parallel to the concentration of the Paeoniflorin given to the db/db mice, proving that the treatment with Paeoniflorin can decrease the expression of TLR2 and TLR4 in diabetic models. In order to gain better insight into the inflammatory level, we introduced macrophage recruitment into our investigation. CD68, as a parameter representing renal macrophage accumulation, was occasionally noticed in db/m mice kidneys, yet, the CD68 positive macrophage infiltration was found markedly increased in db/db mice. Immunohistochemical staining indicated a statistical reduction in CD68 positive expression in kidneys from Paeoniflorin treated mice with diabetes as compared to db/db mice, which verifies the effects of Paeoniflorin on macrophage accumulation and infiltration. NF-κB p65 was highly expressed in the nucleus and cytoplasm of glomerular mesangial cells and renal tubular epithelial cells from db/db mice compared to the rare expression in db/m mice, however, NF-κB p65-posive expression was decreased with the Paeoniflorin treatment in a dose-dependent manner (Figures 2 and 3, Table 2).

3.3. *The mRNA results of iNOS, TNF-α, IL-1β and MCP-1 in mice kidneys*

As shown in Figure 4, low level expression of iNOS, TNF-α, IL-1β and MCP-1 was discovered in conformity with transcriptional regulation of mRNA levels in db/m mice. In contrast, the mRNA levels of iNOS, TNF-α, IL-1β and MCP-1 was significantly elevated in db/db mice and fell remarkably with Paeoniflorin treatment, which was highly consistent with the Western blot results.

3.4. *Western blot analysis of TLR2,TLR4, MyD88, p-IRAK1,Trif, p-IRF3, NF-κB p-p65, NF-κB p65, and IL-1β expression in mice kidneys*

TLR2 and TLR4 together with the downstream signaling molecule protein expression in the experimental subjects was further confirmed by Western blot analysis, which showed a significant upregulation of TLR2,TLR4, MyD88, p-IRAK1,Trif, p-IRF3, NF-κB p-p65, and NF-κB p65 in db/db mice compared to db/m mice. The same goes with the inflammatory cytokines-IL-1β. By comparison, the expression of the above protein was significantly attenuated by 15, 30 and 60 mg/kg Paeoniflorin treatment (Figure 5).

3.5. *Effect of Paeoniflorin on AGEs-stimulated production of TLR2, TLR4 and iNOS*

To determine the stimulated time of AGEs and concentration of Paeoniflorin intervention, we evaluated the viability of RAW 264.7 cells under different concentrations of Paeoniflorin and TLR2, TLR4, iNOS expressions when stimulated with AGEs for various lengths of time. In Figure 6, the Cytotoxicity assay showed that the viability of RAW 264.7 cells declined at 10^{-4} mol/L Paeoniflorin intervention while there was no influence at concentrations between 10^{-4} and 10^{-8}. Figure 7 also demonstrated the overexpression of TLR2 and iNOS 4 hours after AGEs stimulation in RAW 264.7 cells, while TLR4 was remarkably elevated from

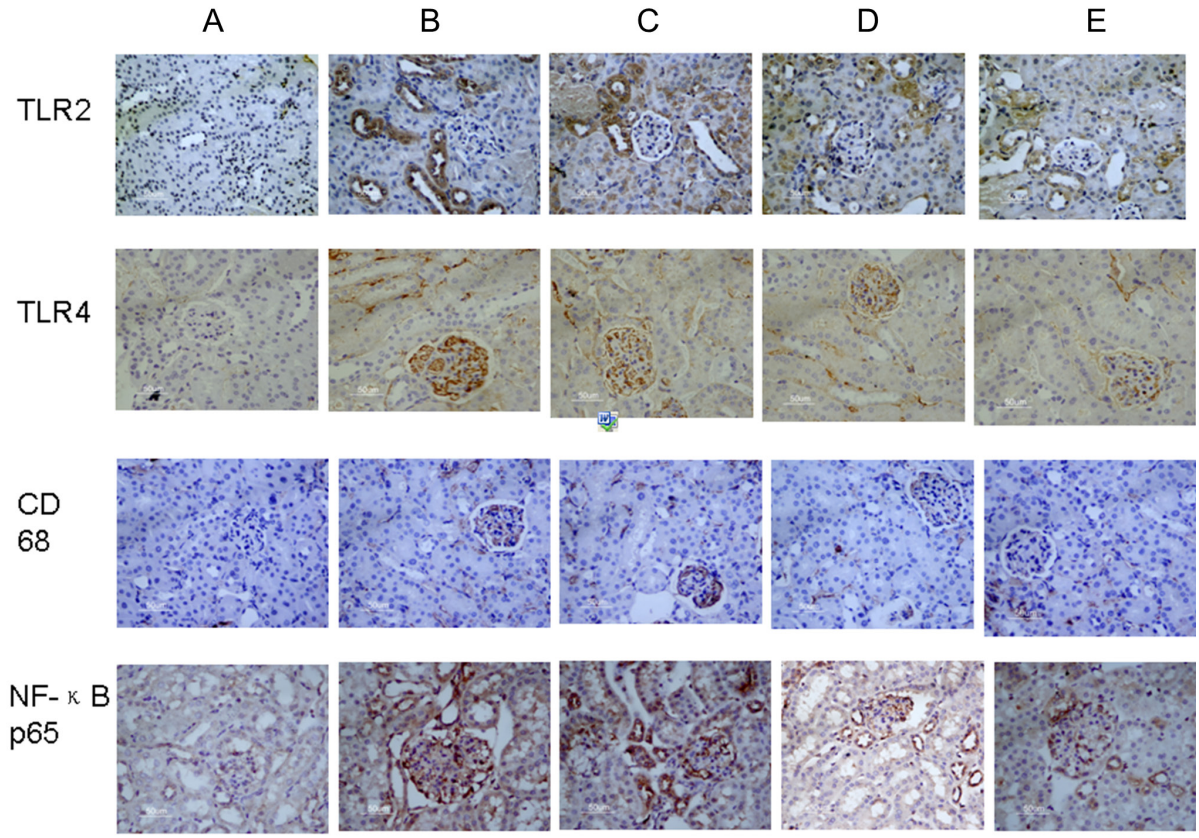

Figure 2. Effects of Paeoniflonin on TLR2, TLR4, CD 68 and NF-κB p65 immunostaining in mice kidney. (A), db/m group; **(B)**, db/db group; **(C)**, db/db+PF 15 mg/kg group; **(D)**, db/db+PF 30 mg/kg group; **(E)**, db/db+PF 60 mg/kg group. Original magnification 400×.

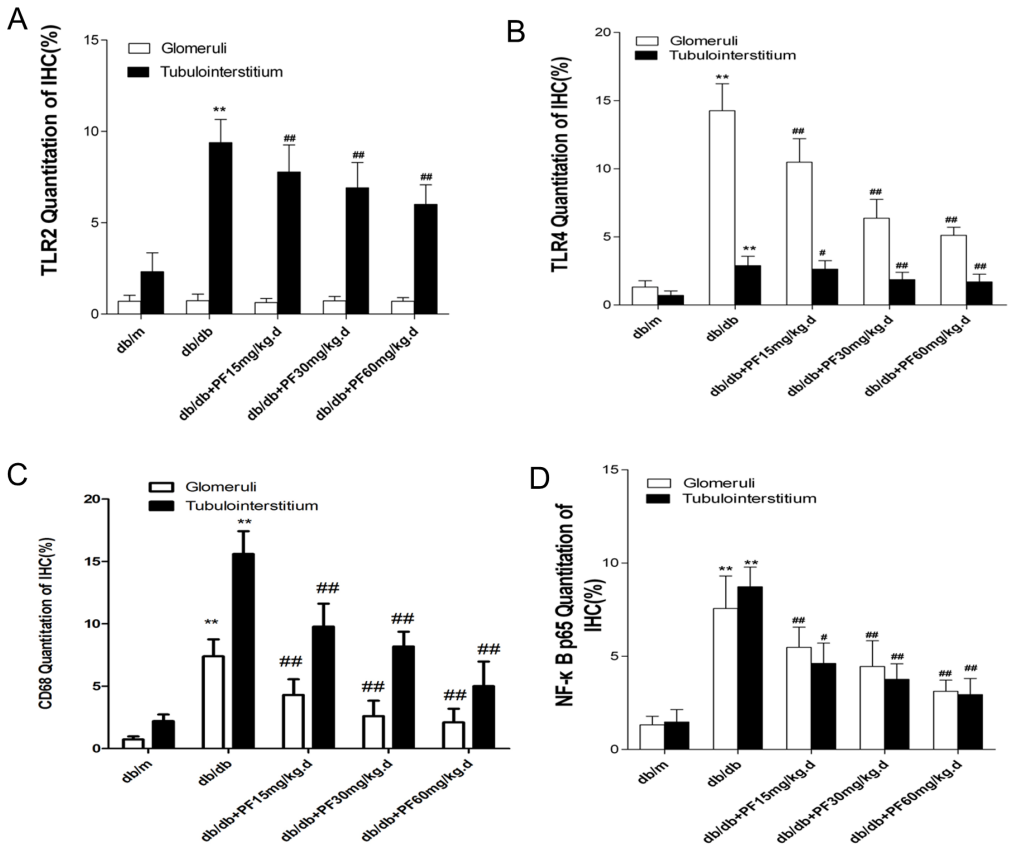

Figure 3. (A-D), Quantitative analyses of the TLR2, TLR4, CD 68 and NF-κB p65 immunohistochemical staining results. The results are presented as Mean ± SD. $^{**}p < 0.01$ *vs*. db/m group, $^{\#}p < 0.05$ *vs*. db/db group, $^{\#\#}p < 0.01$ *vs*. db/db group.

Table 2. Quantition of IHC in glomeruli and tubelointerstitium(cells/mm^2)

Group	Dose (mg/kg)	TLR2		TLR4		CD68		NF-κB P65	
		glomeruli	tubelointerstitium	glomeruli	tubelointerstitium	glomeruli	tubelointerstitium	glomeruli	tubelointerstitium
db/m	–	0.700 ± 0.330	2.320 ± 1.040	1.320 ± 0.460	0.700 ± 0.330	0.730 ± 0.250	2.200 ± 0.540	1.320 ± 0.460	1.470 ± 0.670
db/db	–	0.730 ± 0.360	9.390 ± 1.260**	14.260 ± 1.980**	2.890 ± 0.680**	7.400 ± 1.360**	15.600 ± 1.830**	7.560 ± 1.750**	8.730 ± 1.050**
db/db+ paeoniflorin	15	0.630 ± 0.220	7.780 ± 1.480##	10.480 ± 1.730##	2.630 ± 0.620#	4.300 ± 1.260##	9.780 ± 1.840##	5.480 ± 1.080##	4.620 ± 1.090#
	30	0.720 ± 0.240	6.910 ± 1.390##	6.370 ± 1.380##	1.860 ± 0.540##	2.600 ± 1.240##	8.180 ± 1.190##	4.450 ± 1.380##	3.760 ± 0.840##
	60	0.700 ± 0.200	6.000 ± 1.080##	5.120 ± 0.590##	1.700 ± 0.560##	2.1000 ± 1.100##	5.000 ± 1.980##	3.120 ± 0.600##	2.950 ± 0.860##

**$p < 0.01$, compared with the db/m group; #$p < 0.05$; ##$p < 0.01$, compared with the db/db group. Data are presented as Mean ± SD. Number of rats in each group was 12.

Figure 4. The mRNA results of iNOS, TNF-α, IL-1β and MCP-1 in mice kidneys. The results are presented as Mean ± SD of at least three repeated experiments. **$p < 0.01$ *vs*. db/m group, ##$p < 0.01$ *vs*. db/db group.

Figure 5. Effects of Paeoniflonin on TLRs signaling activation and IL-1β expression in db/db mice. (A), The db/db mice were treated with different Paeoniflonin concentrations. **(B)**, The protein levels of TLR2, TLR4, MyD88, p-IRAK1, Trif, p-IRF3, IRF3, NF-κB p-p65, NF-κB p65 and IL-β were assessed by Western blot analyses. The values are presented as Mean ± SD of at least three repeated experiments. **$p < 0.01$ *vs*. db/m group, ##$p < 0.01$ *vs*. db/db group.

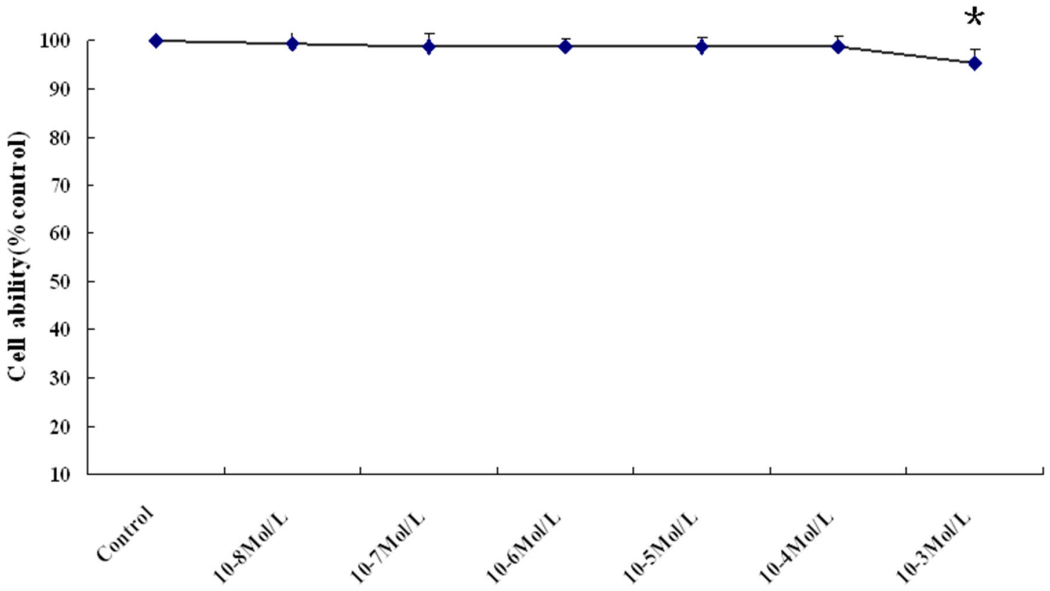

Figure 6. Cell viability analysis of Paeoniflonin treatment on RAW 264.7 cells. 10^{-8}-10^{-3} mol/L Paeoniflonin was respectively treated on RAW 267.4 cells and cell viability was detected by using a CCK-8 assay. The results are expressed as Mean ± SD of at least three repeated experiments. $^{*}p < 0.05$ *vs*. control group.

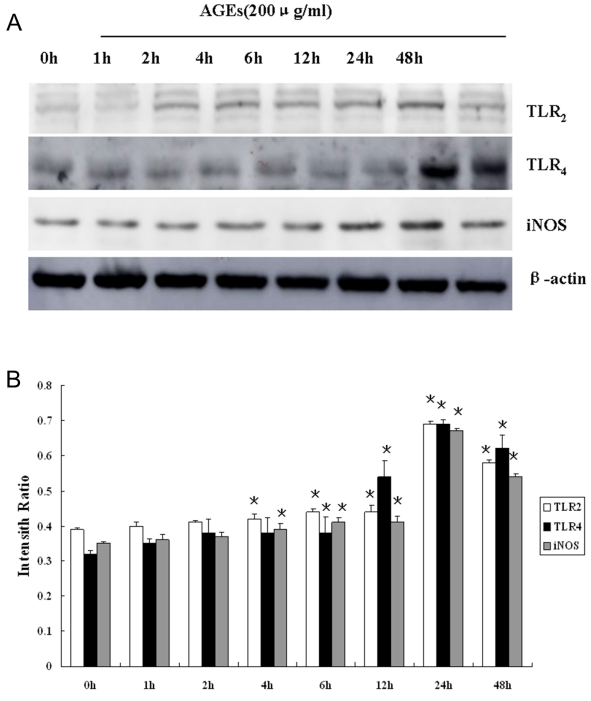

Figure 7. Effects of AGEs on TLRs and macrophage activation at different time points. (A), RAW 264.7 cells were cultured with 200 ug/mL AGEs for time of 0, 1, 2, 4, 6, 12, 24 and 48 h. **(B)**, The protein levels of TLR2, TLR4 and iNOS were determined by Western blot. Values are presented as Mean ± SD of at least three repeated experiments. $^{**}p < 0.01$ *vs*. 0 h group.

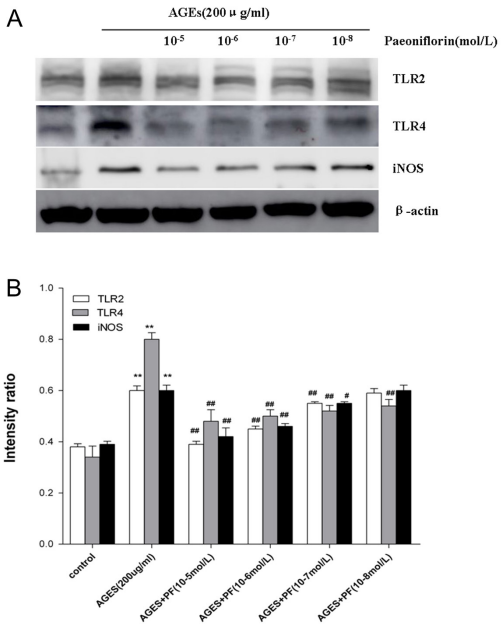

Figure 8. Involvement of TLRs and macrophage activation under different Paeoniflonin concentrations on AGEs-stimulated cells. (A), AGEs-stimulated RAW 264.7 cells were treated respectively with 10-8-10-3 mol/L Paeoniflonin. **(B)**, The protein levels of TLR2, TLR4 and iNOS were determined by Western blot. Values are presented as Mean ± SD of at least three repeated experiments. $^{**}p < 0.01$ *vs*. 0 h group.

6 h, which all peaked at 24 h. Thus, we cultured cells with AGEs for 24 h in the subsequent experiments. In the selected four doses (10^{-5} to 10^{-8} mol/L), TLR2, TLR4, and iNOS expression could be inhibited in a concentration dependent manner by Paeoniflorin when stimulated by AGEs, compared to the AGEs stimulated group. Therefore, we chose 10^{-5} mol/L as the intervention dose (Figure 8).

3.6. *Advanced glycation end products (AGEs) activated the expression of TLRs signaling and macrophage activation on RAW 264.7*

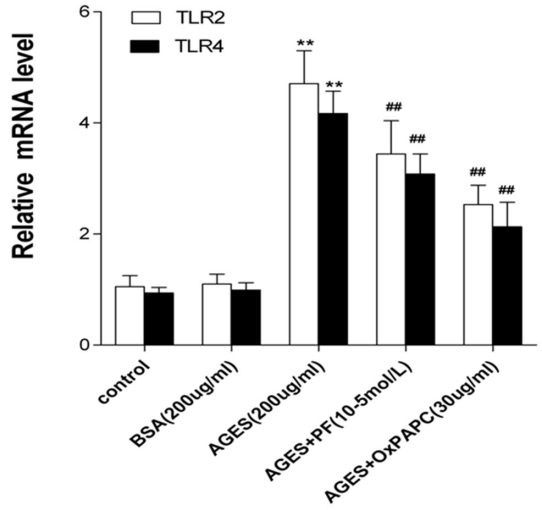

Figure 9. Effects of Paeoniflonin and OxPAPC on TLR2 and TLR4 mRNA level in AGEs-stimulated *RAW 264.7* cells. Values are presented as Mean ± SD of at least three repeated experiments. $^{**}p < 0.01$ *vs*. control group, $^{\#\#}p < 0.01$ *vs*. AGEs group.

To identify whether the activated inflammatory effect of hyperglycemia on macrophages is mediated by TLRs, we cultured *RAW 264.7* with AGEs *in vitro*. As we can see in Figure 9, the mRNA level of TLR2 and TLR4 was elevated in the AGEs group, but decreased in both the Paeoniflorin and OxPAPC group. Western blot analysis in Figures 10A and 10B showed that proteins of TLRs signaling pathway including TLR2,TLR4, MyD88, p-IRAK1,Trif, p-IRF3, NF-κB p-p65, NF-κB p65 and iNOS were prominently increased on RAW 264.7 under AGEs condition, while the Paeoniflorin treatment and OxPAPC intervention both exhibited a significant reduction in the expression of TLR2,TLR4, MyD88, p-IRAK1,Trif, p-IRF3, NF-κB p65, NF-κB p-p65 and iNOS.

3.7. *TLRs mediated pro-inflammatory responses in RAW 264.7 exposed to AGEs*

To visualize the effects of hyperglycemia on inflammation initiated by macrophages, the following events were carried out *in vitro* with RAW 264.7 cells. The secretion of TNF-α, IL-1β and MCP-1 were determined *via* ELISA, finding that RAW 264.7 exposed to AGEs demonstrated a higher concentration of TNF-α, IL-1β and MCP-1, whereas TNF-α, IL-1β and MCP-1 in Paeoniflorin treated cells were profoundly diminished. In order to further clarify the pivotal role of TLRs in inflammatory responses and Paeoniflorin treatment, the inhibitor of the TLRs was introduced. The results of RAW 264.7 cultured in OxPAPC were similar to those seeded in Paeoniflorin after exposure to AGEs, which corresponded to what was obtained from Western blots (Figure 11).

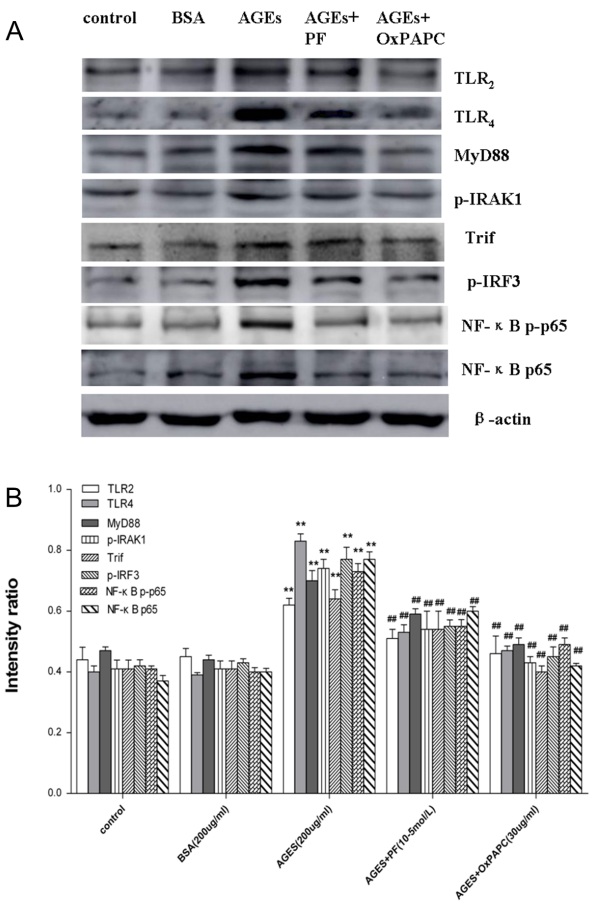

Figure 10. Effects of Paeoniflonin and OxPAPC on the cell signaling activation in AGEs-stimulated RAW 264.7 cells. (A), Cells were pretreated with Paeoniflonin for 0.5 h or OxPAPC for 1 h and then stimulated with AGEs. **(B)**, The protein levels of TLR2, TLR4, MyD88, p-IRAK1,Trif, p-IRF3, IRF3, NF-κB p-p65, NF-κB p65 were assessed by Western blot analyses. Values are presented as Mean ± SD of at least three repeated experiments. $^{**}p < 0.01$ *vs*. control group, $^{\#\#}p < 0.01$ *vs*. AGEs group.

4. Discussion

DN is a long-term complication, and the prevention of its progression remains to be a challenge. Increasing evidence reviewed lately, indicates that TLR2/4-mediated inflammation might be a promising factor leading to an effective approach to DN blockage and treatment. In renal biopsy of DN patients, macrophages accumulated and turned out to play a pathogenic role in DN progression (*18*). In addition, research on type 1 diabetic patients showed the increasing expression of TLR2, pro-inflammatory cytokines in blood leukocytes (*19*) and upregulation of TLR4 in human renal tubules (*10*). Studies performed by Alaa S.*et al*. demonstrated that the absence of macrophage TNF-α stimulated by TLRs could prevent diabetic mice from having complications like DN and chronic renal injuries, which could be mainly expresed in albuminuria decrease and hypertrophy reduction (*4*). Also, the introduction of Chinese traditional medicine – Paeoniflorin, which

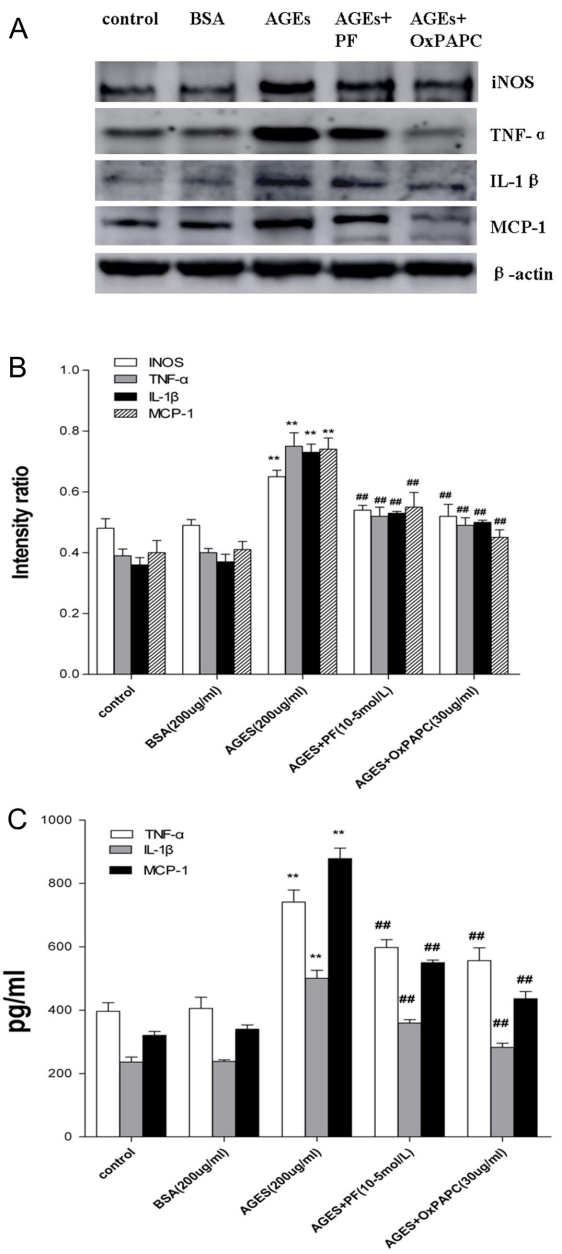

Figure 11. Effects of Paeoniflonin and OxPAPC on macrophage activation and inflammatory cytokines expression in AGEs-stimulated RAW 264.7 cells. (A), Cells were pretreated with Paeoniflonin for 0.5 h or OxPAPC for 1 h and then stimulated with AGEs. **(B)**, The protein levels of TNF-α, IL-1β, MCP-1 and iNOS were assessed by Western blot analyses. **(C)**, Expression of secreted TNF-α, IL-1β, MCP-1 were determined by ELISA kit. Values are presented as Mean ± SD of at least three repeated experiments. $^{**}p < 0.01$ *vs*. control group, $^{\#\#}p < 0.01$ *vs*. AGEs group.

was reported to have anti-inflammatory and immune-regulatory effects, was found to be helpful for DN treatment and prevention (*14,16*), though the underlying protective mechanism of its action was largely unclear. In the present study, we demonstrated that the mechanism of Paeoniflorin treatment in DN might be through the TLR-involved anti-inflammatory process.

Paeoniflorin as a typical main principal bioactive component of Paeoniae Radix (*20*), is one of the major constituents that form total glucosides of paeony (TGP) (*21*) and has been put into clinical treatment for rheumatoid arthritis (*22*), hepatitis (*21*), systemic lupus erythematosus, and mesenteric hyperplastic nephritis (*23-25*). Xu *et al*. has done research on rats, which suggested that the protective affect of TGP on DN was associated with the blockage of TLR2 and TLR4 activation (*26*). In our study, we illustrated that Paeoniflorin therapy decreased proteinuria and ameliorated creatinine clearance rate in db/db mice. We also presented the blocking of NF-kB activation and macrophage recruitment, together with the suppression of the inflammatory cytokines and chemokines (TNF-α, IL-1β, MCP-1, and iNOS) in the Paeoniflorin group, which was highly consistent with that reported by Fu *et al*. (*16*). The reduction of selective TLRs signaling molecule expression was also observed and the above results are compatible with those *in vitro* experiments on AGEs-induced RAW 264.7 cells when treated with Paeoniflorin suggesting the renoprotective role Paeoniflorin played in TLRs-mediated DN progression.

Previous essays have reported that macrophage infiltration and activation play an essential role in the pathogenesis of DN. In recent studies, increasing evidence demonstrated that the phenotype of the infiltrated macrophage was the major character that ultimately decided the sequelae of DN (*27*). M1 macrophages promote the inflammatory response and tissue injury, while the M2 macrophages provide the anti-inflammatory and tissue protective effects (*28*). The transformation of M1 phenotype could be initiated by pro-inflammatory cytokines such as IFN-y, and TNF-α, which in turn enhanced the secretion of these pro-inflammatory cytokines (*27*). In our experiment, iNOS, as the key M1 macrophage marker, was shown to estimate the population of activated macrophages and the severity of inflammation in DN models. Our data revealed the similar results to Devaraj *et al*, finding the increased expression of iNOS in db/db mice and the M1 phenotype in RAW264.7cells when exposed to AGEs (*8*). Furthermore, we exhibited that Paeoniflorin treatment not only reduced macrophage recruitment but also the M1 phenotype in a dose-dependent manner, resulting in a decrease of proinflammatory cytokine production, which corresponded to our *in vitro* experiments. Thus, we have reason to speculate that the renoprotective effect of Paeoniflorin on DN is likely related to the blockage of M1 macrophage phenotype activation.

Insight into the full mechanism of TLR2/4 singnal pathway has caused increasing attention. A great quantity of research executed in DN models exhibited that once ligand binding occurred, the downstream signaling pathway could be categorized as the MyD88-dependent and the MyD88-independent pathway. The former, was characterized by both TLR2 and TLR4, signals through IRAKs, while the latter pathway only

implemented in TLR4 not TLR2, signals through TRIF and then IRF3. Despite the different pathways, they both reach to activation of NF-κB which can ultimately up-regulate the transcription of factors related to inflammatory responses and thereby initiate inflammation. NF-κB p65 as a family member of NF-κB transcription factors, was monitored as a parameter in our experiment (*29*). Data presented in our study further verified the previous TLRs signal pathway, showing the synergistically enhanced downstream signaling proteins in db/db and AGEs-induced RAW 264.7 models. Therefore, termination of inflammation in diabetes taking TLRs as a target seems to be a suitable treatment project to ameliorate inflammation and progressive DN. In addition, *in vivo* and *in vitro* experiments, these proteins were found synergistically decreased in Paeoniflorin treatment groups, which indicated that Paeoniflorin might inhibit macrophages from activition through the blockage of TLRs expression in DN.

Our strategies designed to further state that the TLRs-inhibited renoprotection of Paeoniflorin theory using TLR2 and TLR 4 inhibitor was carried out in RAW264.7 macrophages. We and others have already displayed that the hyperglycemic environment can activate TLRs and M1 macrophages which strikingly increased in mice kidneys with diabetes. In our subsequent experiment, we incubated the AGEs- induced RAW264.7 macrophages with OxPAPC- an inhibitor of TLR signaling restricted to TLR2/4 (*30*). The results of the statistic reduction of TLR2, TLR4, MyD88, p-IRAK1, p-IRF3, and NF-κB p65, together with TNF-α, IL-1β, MCP-1 and iNOS, which is similar to the Paeoniflorin treatment, making our TLR-mediated macrophage activation and TLR-inhibited Paeoniflorin treatment sensible.

In summary, our findings suggest that the activation of TLR2/4 initiate M1 macrophage polarization and infiltration, resulting in the release of inflammatory cytokines and chemokines, which in turn exacerbate inflammation and ultimately aggravate DN. The research we did on Paeoniflorin treatment for the first time demonstrate that Paeoniflorin prevents macrophage activation *via* inhibition of TLR2/4 expression in type 2 diabetic nephropathy and provides supportive evidence for paeoniflorin therapeutic strategies in DN patients.

Acknowledgements

The project was financially granted by Natural Science Foundation of China (No. 81374034, 81470965).

References

1. Middleton RJ, Foley RN, Hegarty J, Cheung CM, McElduff P, Gibson JM, Kalra PA, O'Donoghue DJ, New JP. The unrecognized prevalence of chronic kidney disease in diabets. Nephrol Dial Transplant. 2006; 21:88-92
2. Grace BS, Clayton P, McDonald SP. Increases in renal replacement therapy in Australia and New Zealand: understanding trends in diabetic nephropathy. Nephrology (Carlton). 2012; 17:76-84.
3. Ansell D, Feehally J, Fogarty D, *et al*. UK renal registry 2009: 12th annual report of the renal association. Nephron Clin Pract. 2010;115 suppl 1:c1-c370.
4. Awad AS, You H, Gao T, Cooper TK, Nedospasov SA, Vacher J, Wilkinson PF, Farrell FX, Brian Reeves W. Macrophage-derived tumor necrosis factor-alpha mediates diabetic renal injury. Kidney Int. 2015; 88:722-733.
5. Leoncini G, Viazzi F, Pontremoli R. RAAS inhibition and renal protection. Curr Pharm Des. 2012; 18:971-980.
6. Awad AS, You H, Gao T, Gvritishvili A, Cooper TK, Tombran-Tink J. Delayed treatment with a small pigment epithelium derived factor (PEDF) peptide prevents the progression of diabetic renal injury. PLoS One.2015; 10:e0133777.
7. Ma J, Wu H, Zhao C.Y, Panchapakesan U, Pollock C, Chadban SJ. Requirement for TLR2 in the development of albuminuria, inflammation and fibrosis in experimental diabetic nephropathy. Int J Clin Exp Pathol. 2014; 7:481-495.
8. Devaraj S, Tobias P, Kasinath B.S, Ramsamooj R, Afify A, Jialal I. Knockout of toll-like receptor-2 attenuates both the proinflammatory state of diabetes and incipient diabetic nephropathy. Arterioscler Thromb Vasc Biol. 2011; 31:1796-1804.
9. Mudaliar H, Pollock C, Komala M.G, Chadban S, Wu H, Panchapakesan U. The role of Toll-like receptor proteins (TLR) 2 and 4 in mediating inflammation in proximal tubules. Am J Physiol Renal Physiol. 2013; 305:F143-154.
10. Lin M, Yiu WH, Wu HJ, Chan LY, Leung JC, Au WS,Chan KW, Lai KN, Tang SC. Toll-like receptor 4 promotes tubular inflammation in diabetic nephrophathy. J Am Soc Nephrol. 2012; 23:86-102.
11. Liu HQ, Zhang WY, Luo XT, Ye Y, Zhu XZ. Paeoniflorin attenuates neuroinflammation and dopaminergic neurodegeneration in the MPTP model of Parkinson's disease by activation of adenosine A_1 receptor. Br J Pharmacol. 2006; 148:314-325.
12. Wang C, Yuan J, Wu HX, Chang Y, Wang QT, Wu YJ, Liu LH, Wei W. Paeoniflorin inhibits inflammatory responses in mice with allergic contact dermatitis by regulating the balance between inflammatory and anti-inflammatory cytokines. Inflamm Res. 2013; 62:1035-1044.
13. Yu HY, Liu MG, Liu DN, Shang GW, Wang Y, Qi C, Zhang KP, Song ZJ, Chen J. Antinociceptive effects of systemic various paeoniflorin on bee venom-induced various phenotypes of nociception and hypersensitivity. Pharmacol Biochem Behav. 2007; 88:131-140.
14. Zhang MH, Feng L, Zhu MM, Gu JF, Wu C, Jia XB. Antioxidative and anti-inflammatory activities of paeoniflorin and oxypaeoniflora on AGEs-induced mesangial cell damage. Planta Med. 2013; 79:1319-1323.
15. Hung JY, Yang CJ, Tsai YM, Huang HW, Huang MS. Antiproliferative activity of paeoniflorin is through cell cycle arrest and the Fas/Fas ligand-mediated apoptotic pathway in human non-small cell lung cancer A549 cells. Clin Exp Pharmacol Physiol. 2008; 35:141-147.
16. Fu J, Li Y, Wang L, Gao B, Zhang N, Ji Q. Paeoniflorin prevents diabetic nephropathy in rats. Comp Med. 2009; 59:557-566.
17. Huang H, Chang EJ, Lee Y, Kim JS, Kang SS, Kim

HH. A genome-wide microarray analysis reveals anti-inflammatory target genes of paeonol in macrophages. Inflamm Res. 2008; 57:189-198.
18. Nguyen D, Ping F, Mu W, Hill P, Atkins RC, Chadban SJ. Macrophage accumulation in human progressive diabetic nephropathy. Nephrology (Carlton). 2006; 11:226-231.
19. Ururahy MA, Loureiro MB, Freire-Neto FP, de Souza KS, Zuhl I, Brandao-Neto J, Hirata RD, Doi SQ, Arrais RF, Hirata MH, Almeida Md, de Rezende AA. Increased TLR2 expression in patients with type 1 diabetes: Evidenced risk of microalbuminuria. Pediatr Diabetes. 2012; 13:147-154.
20. Liu DZ, Xie KQ, Ji XQ, Ye Y, Jiang CL, Zhu XZ. Neuroprotective effect of paeoniflorin on cerebral ischemic rat by activating adenosine A1 receptor in a manner different from its classical agonists. Br J Pharmacol. 2005; 146:604-611.
21. He DY, Dai SM. Anti-inflammatory and immunomodulatory effects of paeonia lactiflora pall, a traditional chinese herbal medicine. Front Pharmacol. 2011; 2:10.
22. Zhang W, Dai SM. Mechanisms involved in the therapeutic effects of Paeonia lactiflora Pallas in rheumatoid arthritis. Int Immunopharmacol. 2012; 14:27-31.
23. Ding ZX, Yang SF, Wu QF, Lu Y, Chen YY, Nie XL. Therapeutic effect of total glucosides of paeony on lupus nephritis in MRL/lpr mice. Nan Fang Yi Ke Da Xue Xue Bao. 2011; 31:656-660. (in Chinese)
24. Wu H, Wei W, Song L, Zhang L, Chen Y, Hu X. Paeoniflorin induced immune tolerance of mesenteric lymph node lymphocytes *via* enhancing beta 2-adrenergic receptor desensitization in rats with adjuvant arthritis. Int Immunopharmacol. 2007; 7:662-673.
25. Zhang HF, Xiao WG, Hou P. 2011. Clinical study of total glucosides of paeony in patients with systemic lupus erythematosus. Zhongguo Xi Yi Jie He Za Zhi. 2011; 31:476-479. (in Chinese)
26. Xu XX, Qi XM, Zhang W, Zhang CQ, Wu XX, Wu YG, Wang K, Shen JJ. Effects of total glucosides of paeony on immune regulatory toll-like receptors TLR2 and 4 in the kidney from diabetic rats. Phytomedicine. 2014; 21:815-823
27. Zhang XL, Guo YF, Song ZX, Zhou M. Vitamin D prevents podocyte injury *via* regulation of macrophage M1/M2 phenotype in diabetic nephropathy rats. Endocrinology. 2014; 155:4939-4350.
28. Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. J Clin Invest. 2008; 118: 3522-3530.
29. Mudaliar H, Pollock C, Panchapakesan U. Role of Toll-like receptors in diabetic nephropathy. Clin Sci (Lond). 2014; 126:685-694.
30. Erridge C, Kennedy S, Spickett CM, Webb DJ. Oxidized phospholipid inhibition of Toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4 - Roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. J Biol Chem. 2008; 283:24748-24759.

(Received April 30, 2017; Revised May 30, 2017; Accepted June 1, 2017)