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Original Article

Effect of pioglitazone on the expression of renal tissue nephrin in STZ-induced diabetic rats

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Abstract: Objective: This study aims to evaluate the effects of pioglitazone hydrochloride (PIO) on the expression of renal nephrin and explore its possible reno-protective mechanism in streptozotocin (STZ)-induced diabetic rats. Methods: Peripheral blood glucose (BG), urine albumin/creatinine ratio (UACR), urinary nephrin/creatinine ratio (UNER) and glycated hemoglobin (HbA1c) were measured before and/or after PIO treatment. Renal tissues were obtained for observing pathologic change and examining nephrin expression in diabetic rats (without treatment group, 10 and 30 $mg.kg^{-1}.d^{-1}$ PIO-treated diabetic group, normal control group) at the 8^{th} week. Results: BG and HbA1c levels were significantly increased in diabetic rats when compared to normal control group, but there was no significant difference among diabetic groups. UACR and UNER decreased significantly in PIO-treated groups when compared to without treatment group. Renal tissue nephrin mRNA was down-regulated while nephrin protein was increased in PIO-treated groups compared to without treatment group. UNER was positively correlated with UACR ($r = 0.881$, $P < 0.01$). Conclusions: PIO can alleviate kidney injury of diabetic rats, which may be mediated partly through regulating the expression of podocyte nephrin as well as restraining the excretion of urinary nephrin in a dose-dependent manner.

Keywords: Diabetic nephropathy, nephrin, pioglitazone, podocyte, proteinuria

Introduction

Over the past few years, studies have demonstrated that thiazolidinediones (TZDs), including rosiglitazone and pioglitazone hydrochloride (PIO), can improve insulin resistance, dyslipidaemia and glucose metabolism. However, there are several reports regarding the direct protection effects of TZDs on the kidney such as anti-proteinuria, vascular protective, anti-inflammatory, anti-oxidative stress and podocyte protection recently [1, 2]. Nephrin, which was identified as the products of the gene mutated in a patient with the Finnish type of congential nephrotic syndrome, has been considered to be one of podocyte markers and the essential molecules maintaining the barrier function of glomerular basal membrane (GBM). In this study, we investigated the reno-protective effect of different dosages of PIO and its effects on the expression of renal tissue nephrin in the STZ-induced diabetic rats in order to provide some evidences for PIO against podocyte injury.

Material and methods

Animal model

Thirty-eight 2-month-old male Sprague-Dawley rats (weights of 195±20 g) were obtained from the Experimental Animal Center of Anhui Medical University, Hefei, China. The animals were housed at a room temperature of 23±1°C, humidity of 50%-75%, a 12-h light/dark cycle. The rats were randomly divided into 4 groups and 8 in each group. Diabetic models were fasted overnight and induced by a single i.p. injection of streptozotocin (65 mg/kg; Sigma Chemical, St. Louis, MO). Peripheral blood was harvested from vena caudalis 72 hr post-injection to assess BG level, rats with BG more than 16.7 mmol/L indicated the successful induction of diabetes. One week after the STZ injection, 30 diabetic rats were randomly divided into the following three groups: vehicle (0.9% sodium chloride) treatment group, 10 $mg.kg^{-1}.d^{-1}$ PIO treatment group and 30 $mg.kg^{-1}.d^{-1}$ PIO

treatment group (n = 10, respectively) for 8 weeks. Week 0 was defined as the start day of drug administration. 10 ml morning urine sample was collected at the week 0th and 8th and preserved at -80°C for testing urinary albumin (UAlb), creatinine (Ucr) and sediment nephrin. Peripheral BG was tested weekly. To prevent death induced by emergency such as diabetic ketoacidosis, the rats with BG > 33.3 mmol/L were injected with 0.5 unit insulin (Glargine) 2-3 times per week to keep the BG levels range from 20 to 30 mmol/L. After 8 weeks, all animals were anaesthetized (after 14 h of starvation) by using i.p. injection of chloral hydrate 300 mg/kg, then blood sample was collected for the measurements of HbA1c, lipid profile, blood urea nitrogen (BUN), serum creatinine (Scr). The kidneys were surgically removed and weighed separately, then divided into two parts. One part was stored in 10% neutral buffered formalin for immunohistochemical and pathological analysis, and the other was quickly frozen in liquid nitrogen and stored at -80°C for mRNA extraction. This study was audited and approved by Animal Ethics Committee of Anhui Medical University. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China.

Laboratory measurement

Peripheral BG was tested by Accu-chek Active System *(Roche Diagnostics GmbH, Germany)*. Affinity chromatography was used to measure HbA1c *(Primus, USA)*. Urinary was measured by using radioimmunoassay *(Northern Biotechnology Research Institute, Beijing, China)*. Urinary nephrin were measured by enzyme-linked immunosorbent assay *(R&D Systems, Minneapolis, MN, USA)*. Ucr and Scr were detected by picric kinetic analysis *(Rongsheng, Shanghai, China)*. Serum BUN was analyzed by urease method *(Rongsheng, Shanghai, China)*. Lipid profile, including total cholesterol (TC), triglyceride (TG), HDL-C and LDL-C, were analyzed with an automatic biochemistry analyzer *(HITACHI 7600-020, HITACHI Ltd. Tokyo, Japan)*. All of the operations were performed according to the manufacturer's instruction.

Immunohistochemistry for nephrin protein expression of renal tissue

Paraffin-embedded tissues were cut into 4 μm sections. After deparaffinization and rehydration in an ethanol series, antigen retrieval was performed by heating in a microwave oven. After cooling, endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide (H_2O_2) solution for 10 min, followed by three times wash in a phosphate-buffered solution (PBS) (pH 7.2) for 15 min. Nonspecific protein binding was blocked with 10% normal goat serum in Tris-buffered saline (*Beijing Zhongshan Biotechological Ltd., P.R. China*) at room temperature for 1 h. Rabbit polyclonal anti-nephrin *(Beijing Biosynthesis Biotechnology Co., LTD)* was added in a 1:200 dilution at 4°C overnight. The next day by three rinses in PBS (pH 7.2), the staining was performed by Elivision TM plus two-step System, then rinsed three times in PBS and stained firstly in a fresh mixture of diaminobenzidine (DAB) for 10 min, and again in hematoxylin for 2-3 min, and then washed in distilled water for 10 min, dehydrated in ethanol, and coverslips were applied. To keep the technical variables constant, samples were processed in a batch. Forty high-power (400×) fields were randomly selected for each renal section. Images were captured morphometrically by the Micropublisher 5.0 *(Qimaging, Canada)* coupled to an Olympus BX-41 microscope, then were morphometrically assessed by the Image Pro Plus image analysis system *(Version 5.0, Media Cybernetics, Silver Spring, MD)*. The measurement parameters included area sum and integrated optical density (IOD). In this study, we defined IOD as nephrin protein expression in each slide.

RNA extraction, cDNA synthesis and qPCR

The total RNA was extracted from the renal tissue using Trizol reagent *(Invitrogen Carlsbad, CA, USA)*. For each sample, approximately 1 μg of total RNA was treated with DNase I (Sigma) to remove any residual DNA and converted to cDNA using the ImProm-II reverse transcription system (Promega) according to the manufacturer's instructions. Reactions were carried out in 20 μl volumes and all cDNA samples were diluted 1:5 in DNAse-free water prior to real-time PCR. The primers were 5'-TACCACAGCATTTCCACG-3' (forward primer) and 5'-GGGCTCGGCTGTATGTATT-3' (reverse primer) for nephrin; 5'-AAGGTCATCCCAGAGCTGAA-3' (forward primer) and 5'-CTGCTTCACCACCTTCTTGA-3' (reverse primer) for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). qPCR was performed with the following condi-

Table 1. Comparison of the levels of UACR, UNE, URCR, BG, HbA1c, SCr, BUN, TG, TC, HDL-C, LDL-C, FDFR and GBMT among five groups

Parameters	Time (week)	Normal control group (NC)	No treatment diabetic group (DM)	10 mg.kg^{-1}.d^{-1} Diabetic group (DR1)	20 mg.kg^{-1}.d^{-1} Diabetic group (DR2)	30 mg.kg^{-1}.d^{-1} Diabetic group (DR3)
UACR (mg/g)	0	32.65±8.04	36.93±7.98	37.67±9.40	37.05±8.34	35.362±6.99
	8	35.05±6.75	138.51±18.87*	107.53±18.36$^{*,\#}$	89.04±16.12$^{*,\#,\triangle}$	89.93±16.46$^{*,\#,\triangle}$
UNE (ng/g)	0	95.25±13.24	96.29±7.69	95.64±9.98	96.06±8.39	99.69±9.46
	8	97.00±10.39	683.25±8.74*	414.94±10.51$^{*,\#}$	310.50±12.17$^{*,\#,\triangle}$	303.31±9.41$^{*,\#,\triangle}$
URCR (μg/g)	0	13.96±3.84	12.95±2.95	14.54±1.97	14.21±2.32	15.11±3.03
	8	15.78±4.41	52.99±7.54*	44.75±6.59$^{*,\#}$	37.55±7.81$^{*,\#,\triangle}$	35.58±4.55$^{*,\#,\triangle}$
BG (mmol/L)	0	3.95±0.64	19.79±1.75*	20.03±2.33*	20.14±2.24*	19.91±2.11*
	8	4.00±0.77	21.76±1.57*	22.26±2.02*	21.96±2.10*	21.83±1.89*
HbA1c (%)	8	3.80±0.57	11.07±1.55*	10.55±1.24*	10.54±1.43*	10.49±1.10*
KI (×10^{-3})	8	3.07±0.43	6.11±0.62*	5.29±0.73$^{*,\#}$	4.74±0.36$^{*,\#,\triangle}$	4.72±0.36$^{*,\#,\triangle}$
SCr (μmol/L)	8	55.10±8.04	106.50±16.20*	93.18±13.06*	93.67±17.28*	98.30±13.64*
BUN (mmol/L)	8	2.96±0.49	10.01±1.52*	8.12±1.02$^{*,\#}$	7.80±1.28$^{*,\#}$	7.16±0.88$^{*,\#}$
TG (mmol/L)	8	1.11±0.21	1.74±0.24*	1.43±0.35$^{*,\#}$	1.43±0.10$^{*,\#}$	1.41±0.39$^{*,\#}$
TC (mmol/L)	8	1.25±0.27	1.57±0.35	1.55±0.29	1.53±0.39	1.48±0.35
HDL-C (mmolL)	8	1.24±0.22	0.71±0.11*	0.85±0.14*	0.93±0.23$^{*,\#}$	0.93±0.27$^{*,\#}$
LDL-C (mmol/L)	8	0.73±0.12	1.28±0.23*	1.12±0.25*	1.16±0.17*	1.09±0.18*
GBMT (nm)	8	101.79±15.70	294.07±29.31*	210.43±16.83$^{*,\#}$	132.03±17.98$^{*,\#,\triangle}$	129.66±18.3$^{*,\#,\triangle}$
FPFR	8	0.03±0.02	0.87±0.04*	0.73±0.04$^{*,\#}$	0.51±0.05$^{*,\#,\triangle}$	0.47±0.04$^{*,\#,\triangle}$

Note: UACR, Urinary albumin; UNE, urinary sediment nephrin; URCR, urinary retinol-binding protein; BG, blood glucose; HbA1C, Glycated hemoglobin; KI, kidney index; SCr, serum creatinine; BUN, blood urea nitrogen; TG, Triglycerides; TC, Total Cholesterol; HDL-C, High-Density Lipoprotein Cholesterol; LDL-C, Low-Density Lipoprotein Cholesterol; FDFR, foot process fusion ratio; GBMT, glomerular basement membrane thickness. Data are shown as mean ± SD. *P < 0.05 compared with Normal Control Group at the same time point; $^{\#}$P < 0.05 compared with No Treatment Diabetic Group at the same time point; $^{\triangle}$P < 0.05 compared with 10 mg.kg^{-1}.d^{-1} PIO treatment Group at the same time point.

tions: 50°C for 2 min, predenaturation at 94°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 10 min on ABI Prism 7000 Sequence Detection System (Applied Biosystems). The mRNA expressions of nephrin were normalized to GAPDH.

Histologic examination for renal tissues

Part of renal cortex was fixed in 2.5% glutaraldehyde, and then 1% osmic acid, dehydrated, and embedded in Epoxy resin. Ultrathin sections were prepared and stained with lead citrate for transmission electron microscopy (*JEM 1200EX, Jeol, Tokyo, Japan*). Five micrographs at a magnification of 6,000 were randomly selected from each specimen at different views. The total length of GBM and the fused foot processes (FP) was measured as X and Y. Foot process fusion ratio (FPFR) was expressed as Y/X. GBM was divided into several parts with 1 cm as minimum unit and the thickness of each part was measured under the electron microscope. A sum of GBM thickness was calculated as A and the quantity of all parts was calculated as B. The average thickness of GBM (GBMT) was expressed as A/B. All the parameters were measured by the Image Pro Plus image analysis system (*version 6.0, Media Cybernetics, Silver Spring, MD*).

Statistical analysis

Data were presented as mean ± SD and analyzed using Statistical Package (SPSS 13.0). Statistical differences among multiple groups were assessed by LSD test. Correlations between UNER and UACR, KI were examined by Pearson correlation analysis. A p value < 0.05 was considered to be statistically significant.

Results

Animal characteristics

Diabetic rats from group DM to PIO-treated groups had marked hyperglycaemia at any time point (P < 0.01, versus group NC). HbA1c levels elevated markedly at the 8th week in group DM (P < 0.01, versus group NC) with no significant difference between group DM and PIO-treated groups. Serum BUN and TG levels were decreased in PIO treated-groups (P < 0.05, versus group DM). The level of HDL-C in groups

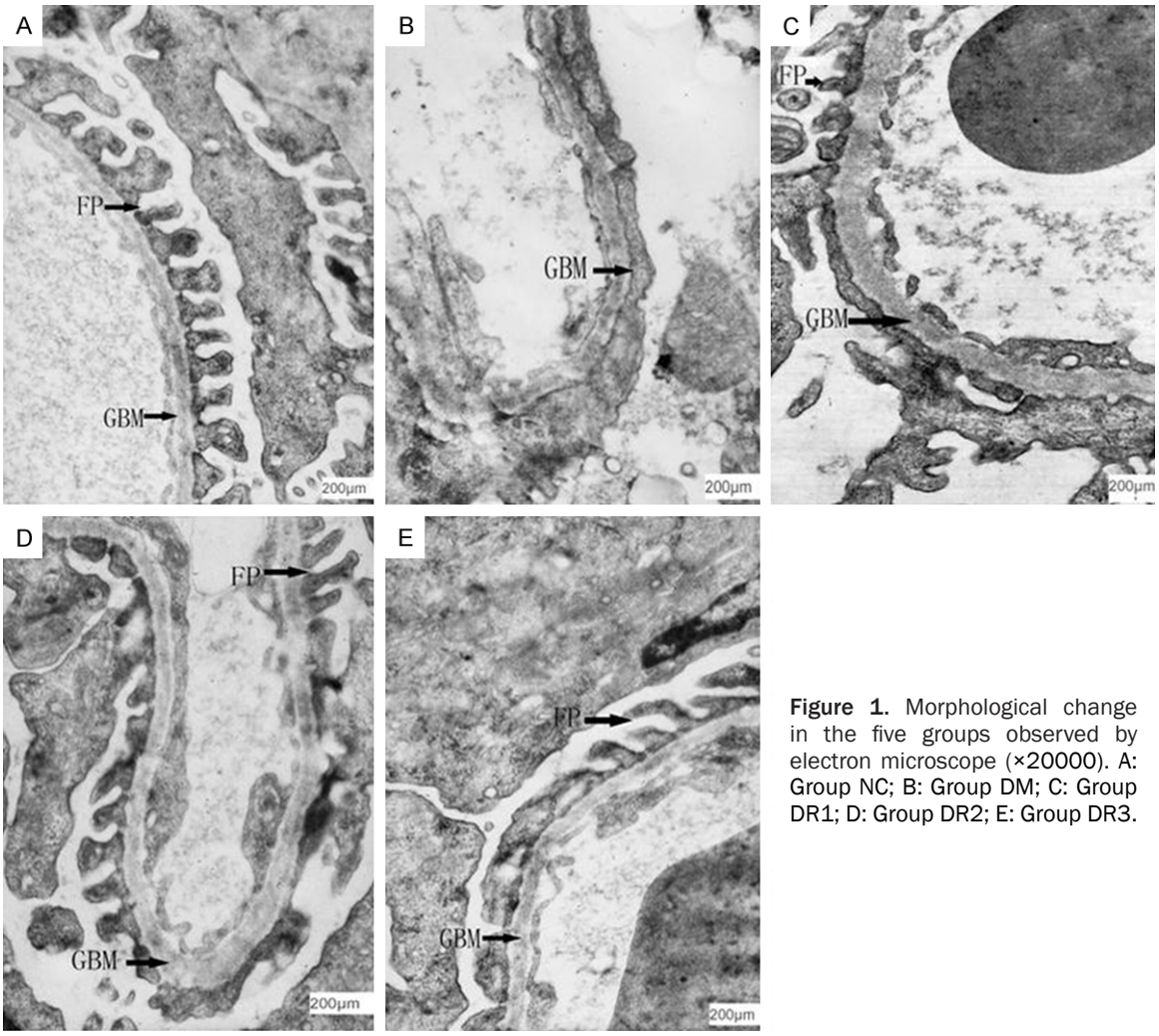

Figure 1. Morphological change in the five groups observed by electron microscope (×20000). A: Group NC; B: Group DM; C: Group DR1; D: Group DR2; E: Group DR3.

DR2 and DR3 increased significantly ($P < 0.05$, versus group DM), yet the level of HDL-C in group DR1 increased slightly ($P > 0.05$, versus group DM) (**Table 1**).

At the 0^{th} week, there was no difference in UACR among all five groups. But at the 8th week, the level of UACR increased significantly in group DM ($P < 0.05$, versus group NC), UACR in PIO-treatment groups decreased significantly ($P < 0.05$, versus DM group), which in group DR2 and DR3 were lower than that in group DR1 ($P < 0.05$), whereas no significant differences were found between group DR2 and DR3 (**Table 1**).

Histopathologic changes in experimental animals

As shown in **Figure 1**, the structure and width of GBM and epithelial foot processes were mostly normal in group NC at the 8th week. In group DM, the ultrastructure of GBM became ambiguous, the FPFR increased significantly. In addition, it was noted that some epithelial FP were destroyed, even vanished, but GBMT and FPFR decreased markedly in PIO-treated groups compared with group DM ($P < 0.05$), but still increased compared with group NC ($P < 0.05$). Furthermore, the changes mentioned above in group DR2 and DR3 were superior to those in group DR1 ($P < 0.05$).

UNER changes among five groups

At the 0th week, free nephrin was found in the urine of both normal control rats and diabetic rats. At the 8th week of the study, the UNERs increased significantly both in group DM and PIO-treated groups. The UNERs in PIO-treated groups were decreased significantly as compared to group DM, in addition, UNERs in group

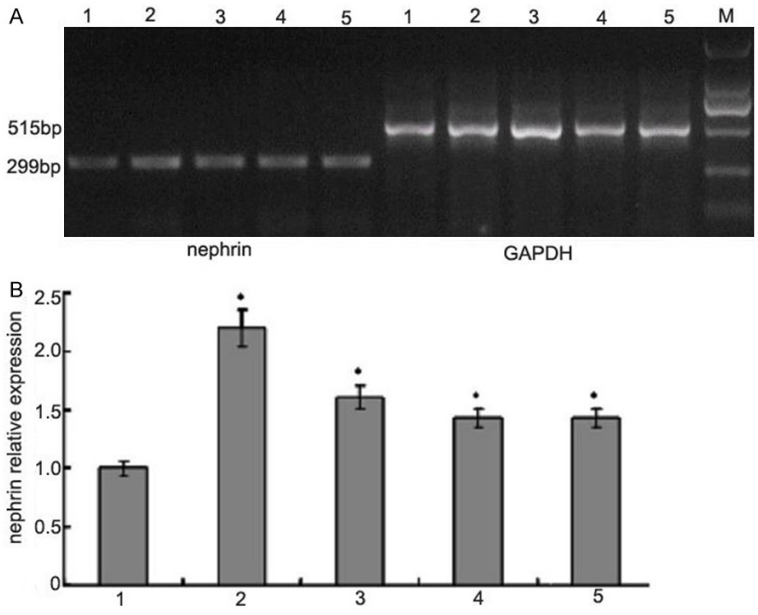

Figure 2. Differences in nephrin mRNA expression in the kidney among different groups. A: Renal nephrin and GAPDH mRNA expression by RT-PCR. B: The ratio of nephrin versus GAPDH. *P < 0.05 compared with NC group. M: DNA marker; 1: group NC, 2: group DM, 3: group DR1, 4: group DR2, 5: group DR3.

DR2 and DR3 was lower than that in group DR1 (P < 0.05), yet no statistical difference was detected between group DR2 and DR3 (**Table 1**).

Nephrin mRNA expression

At the 8th week, nephrin mRNA expression showed an significant increase in group DM as compared to group NC (P < 0.05), moreover the nephrin mRNA expressions were reduced in group DR1, DR2 and DR3 as compared to group DM, yet no differences among different dosages of PIO- treatment groups (**Figure 2A**, **2B**).

Nephrin protein quantitation and localization

At the 8th week, there was a significant decrease in nephrin protein content in group DM as compared to group NC, however there was a marked increase in PIO treated-groups as compared to group DM, whereas there were no significant differences among different doses of PIO-treated groups (**Figure 3**).

Correlation analysis

Pearson correlation analysis showed that UNER was related positively to UACR (r = 0.881, P < 0.01).

Discussion

Peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists or thiazolidinediones (TZDs) were reported by many studies to relieve diabetic nephropathy through promoting insulin sensitization and improving dyslipidaemia and glucose metabolism [3, 4], moreover, accumulating evidences suggested that these agents may have direct renal benefits beyond its effects mentioned above. Pioglitazone complementing insulin in diabetic kidney transplant patients not only improved glycemic control, but also decreased inflammatory markers which may have an impact on overall cardiovascular events and mortalities beyond glycemic control [5]. Some clinical studies [6, 7] revealed that treatment with TZD significantly decreases urinary albumin and protein excretion in patients with diabetes. In animal study, it showed that low dose of pioglitazone (0.6 $mg.kg^{-1}.d^{-1}$) could ameliorate renal fibrosis and preserve renal function in animal model of metabolic syndrome, independently of glycaemic control or effects on body weight [8]. In this study, it was observed that UACR, GBMT and FPFR were ameliorated significantly by an 8-week treatment of PIO, in addition, the parameters mentioned above (including UACR, GBMT and FPFR) in group DR2 and DR3 were lower than those in group DR1, yet no significant difference between group DR2 and DR3. No differences of BG and HbA1c were observed between group DM and PIO-treated groups. These results further demonstrated the renoprotective effects, including the alleviation of podocyte injury, of pioglitazone with a dosage-dependent manner independent of its hypoglycemic effect, consistent with previous reports [8, 9].

Nephrin is a transmembrane protein located at the slit diaphragm complex. Down-regulation of Nephrin in renal tissue can result in proteinuria and podocyte effacement, thus the preservation of nephrin expression may be relevant with

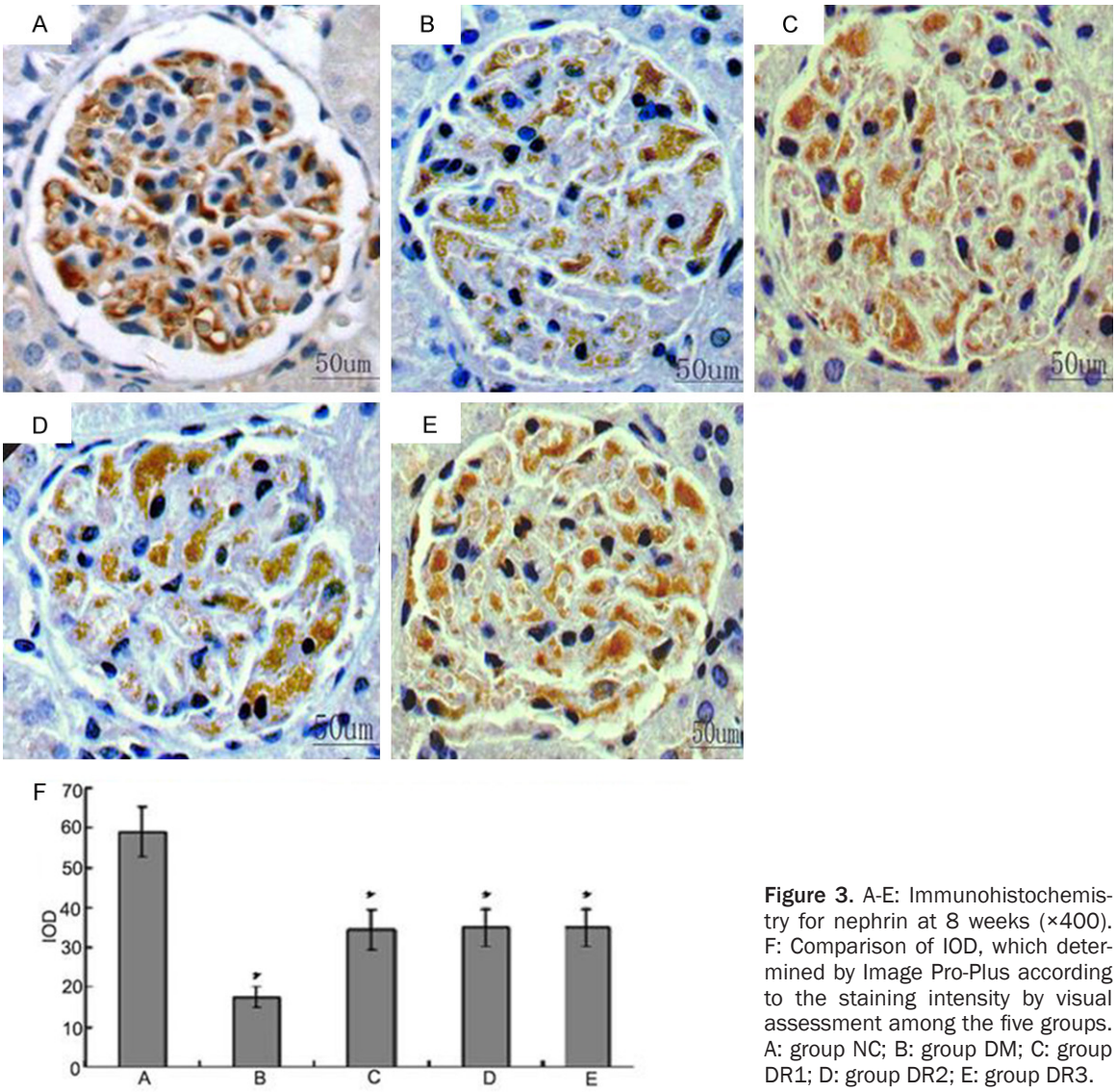

Figure 3. A-E: Immunohistochemistry for nephrin at 8 weeks (×400). F: Comparison of IOD, which determined by Image Pro-Plus according to the staining intensity by visual assessment among the five groups. A: group NC; B: group DM; C: group DR1; D: group DR2; E: group DR3.

the maintenance of podocyte function and integrality of GBM [10, 11]. Aaltonen *et al* [12] proved that free nephrin could be detected by immunoblotting in the urine of STZ-induced rats at the 4th week and increase further at the 8th weeks. Our results showed that diabetic model rats developed profound urinary nephrin during the 8-week study, which was 7-fold greater than that in normal control rats. PIO-treatment significantly reduced the urinary nephrin excretion in the diabetic rats. Furthermore, the effects of 20 $mg.kg^{-1}.d^{-1}$ and 30 $mg.kg^{-1}.d^{-1}$ dosages were superior to that of 10 $mg.kg^{-1}.d^{-1}$ dosage, which indicated that pioglitazone could restrain the loss of nephrin in the urine with a dose-dependent manner. We also found UNER had a positive correlation with UACR in all diabetic rates, which suggested that the podocyte injury in diabetic rats is associated with proteinuria, and pioglitazone-treatment could protect podocyte through restraining the excretion of urinary nephrin. Further study showed that when compared with normal control rats at the 8th weeks, there was a small but significant increase in nephrin mRNA expression, but a significant decrease in protein expression in renal tissue in diabetic rats. In a recent report, Josephine M *et al* [13] had also demonstrated a significant increase, approximately three-fold in nephrin mRNA expression at 8^{th} week in diabetic rats. The possible explanation is that nephrin protein was being excreted into the urine of these diabetic rats during the early stage of diabetes, the increase in nephrin gene expression may

represent a compensatory increase due to the loss of this protein. In the present study, PIO-treatment was associated with the reduction of nephrin mRNA and increase of nephrin protein expression in renal tissue of diabetic rats, it was also postulated that pioglitazone could protect podocyte through reducing the nephrin protein loss in the glomeruli. However, there were no exact mechanisms available on how TZDs can decrease nephrin protein loss of slit-diaphragm (SD) and protect against podocyte injury in diabetic state. Moreover, it was proved in a rat model of type 2 diabetes that Pioglitazone could attenuate kidney disease progression by down-regulating NF-κB, transforming growth factor (TGF)-β1, plasminogen activator inhibitor type-1 (PAI-1) and vascular endothelial growth factor (VEGF) [1]. Gianluca *et a*l found that PPAR-γ agonists prevented the SD-induced podocyte apoptosis accompanied by the preservation of the Bcl-2 and Bax levels, as well as by the attenuation of caspase 3 activation [14]. TZDs were also found to improve renal microcirculation and endothelial function, to increase renal nitric oxide bioavailability, and decrease renal endothelin 1 expression [15, 16]. Taken together, all these findings suggested that multiple events might mediate the podocyte protective effects provided by PPAR-γ agonists. In addition, our data demonstrated that no significant difference was observed in renal nephrin mRNA and protein expression among different doses of PIO-treatment groups, this may be due to narrow drug dose window, less sample size and shorter research time, thus future studies are needed to characterize the reasons involved.

Clinical study [17] showed that Pioglitazone has effect on improving dyslipidaemia in diabetes. In our study, the level of serum HDL-C decreased, and serum TG as well as LDL-C increased in diabetic rats, Pioglitazone increased serum HDL-C and decreased significantly serum TG and LDL-C level in STZ-induced diabetic rats after 8-weeks treatment. We also found that the effect of improving TG and HDL-C in 20 $mg.kg^{-1}.d^{-1}$ and 30 $mg.kg^{-1}.d^{-1}$ dosage groups was still superior to 10 $mg.kg^{-1}.d^{-1}$ dosage group.

Conclusion

Our results demonstrate that Pioglitazone is effective in alleviating podocyte injury in diabetic rats, which may be mediated in part by reducing podocyte protein-nephrin loss in renal tissue and excretion in urine. The exact mechanisms remain to be investigated further.

Acknowledgements

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Disclosure of conflict of interest

None.

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