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Research Article
Renoprotective Effect of Berberine on Streptozotocin-induced Diabetic Nephropathy Rats

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Abstract

Background and Objective: Berberine (BBR), an isoquinoline alkaloid has been used in treating many diseases. However, its effects on diabetic nephropathy inflammation have not been investigated. The purpose of this study was designed to evaluate the anti-inflammation effect of BBR on diabetic nephropathy. **Methodology:** The diabetic rat model was generated by a single intraperitoneal injection of streptozotocin (STZ). Diabetic rats were randomly assigned into the following five groups: Control, Diabetic Nephropathy (DN), losartan (30 mg kg^{-1}, daily), BBR (100, 200 mg kg^{-1}, daily). The BBR and losartan were given intragastricly for 8 weeks. At the end of the experiment, the urine of all rats in each group was collected in a 24 h period. The weight of each rat was recorded. Plasma and kidneys were collected. Kidneys was examined by Hematoxylin Eosin (HE) staining. Renal function parameters such as blood urea nitrogen, plasma creatinine and albuminuria excretion examination were also determined. The levels of blood glucose, malondialdehyde (MDA), triglycerides (TG) and total cholesterol (TCH) in serum were determined using commercial kits according to the manufacturer's instructions. Lastly, real-time PCR and immunofluorescence were performed for measuring TNF-α and IL-6 expression in renal. **Results:** The BBR could improve renal function in diabetic rats (evidenced by mitigation of diabetes-induced changes in kidney index, albuminuria, urea nitrogen and creatinine clearance). The HE examination revealed that BBR-treated groups could ameliorate histological changes of diabetic nephropathy. Furthermore, macrophage infiltration was inhibited and inflammatory molecules were down-regulated by BBR. **Conclusion:** The BBR has renoprotective effects through anti-inflammatory action in diabetic nephropathy. The results indicated that BBR may function as an effective therapeutic agent for diabetic nephropathy and attenuate the progression of renal injury.

Key words: Berberine, creatinine, malondialdehyde, diabetic nephropathy, inflammation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes population is predicted to rise to 552 million by 2030 all over the world according to the International Diabetes Federation[1]. Besides, accompanied with the high prevalence of diabetes mellitus, the incidence of Diabetic Nephropathy (DN) will increase proportionally. The pathogeny of DN is a complex and chronic process. In the early time, glomeruli abnormalities could lead to microalbuminuria, glomerular hypertrophy, glomerular basement membrane thickness and glomerulosclerosis[2]. In the later stage, glomerulosclerosis will appear[2]. At the end stage of DN, the renal function would gradually decline, accompanying tubular atrophy and interstitial fibrosis[2]. Albuminuria that usually recognized as the initial clinical phenotype of DN is generally applied as a marker for kidney damage indiabetic patients[3]. Moreover, higher urinary inflammatory markers could result in the elevation of urinary albuminuria excretion.

Recently, numerous studies have proved that inflammation play a critical role in the pathogenesis of DN[4,5]. Accordingly, inflammatory cytokines can be a target for therapeutic interventions for DN[2,6,7]. The most well-known pro-inflammatory cytokines include interleukin (IL-1, IL-6 and IL-18) and tumornecrosis factor (TNF-α)[5], among which IL-6 and TNF-α are related with the glomerular basement membrane thickening that occurs in DN rats[8-10]. Many studies have reported that increased circulating of TNF-α concentration in patients with diabetic nephropathy were correlated with albumin excretion[11,12]. The inhibition of TNF-α could reduce albuminuria level and improve histological lesions in animals with nephrotoxicity[13]. The IL-6 is another important immune regulatory cytokine that can activate cell surface signaling[14]. The amount of TNF-α and IL-6 is associated with its gene expression. Besides, DN could lead to histopathology changes in kidney. Thereby, concomitant measurement of his to pathology changes, expression of cytokines and its gene expression in kidney tissues are more helpful to depict the effect of a potential drug on intrarenal inflammation.

Berberine (BBR) is an extract from herbal medicine *Coptis chinensis*[15]. A number of pharmacological activities have been reported for berberine, including anti-hyperlipidemic, anti-inflammatory, neuroprotective and some other activities[16-19]. Therefore, BBR was widely used to cure many diseases in clinic[20]. Of note, BBR possess curative effect for diabetes via effectively promoting regeneration in islet cells and contributing to recovery of islet cells function[21,22]. Besides, berberine has been applied to treat diabetes for more than 1400 years in China[21].

Adequate glycemic control is considered as a method to decreased risk of diabetic complications, including DN[19]. However, the currently available drugs, including insulin and the other modern hypoglycemic drugs such as sulphonyl ureas, thiazolidinediones and biguanides would produce lots of undesirable effects to renal[23,24]. Hence, it is of great value to find neuroprotective constituent of herbal medicine, which could alleviate and prevent the progression of DN.

Although anti-inflammatory properties of BBR have been reported in previous studies[25-27], the effect of BBR on kidney inflammation in subjects with DN have not been investigated. Therefore, the aim of this study was to verify the hypothesis that BBR could prevent pro-inflammatory cytokines express in STZ-induced DN rats. Losartan was used as a positive agent in the present study[28,29] due to its strong renoprotective effect. The blood glucose level and renal function were measured for each rat. Besides, renal histological changes, serum biochemical properties, pro-inflammatory mediator gene expression and inflammatory responses were also determined to explore mechanism of renoprotective effect of berberine. The results firstly suggested that BBR possessed a renoprotective effect for DN.

MATERIALS AND METHODS

Materials: The BBR chloride (98% pure) was obtained from Northeast General Pharmaceutical. Losartan was bought from Meilun biology technology (Dalian, China). The reagent strips with a glucometer of glucose was obtained from Johnson and Johnson Services, Inc. (NJ, USA). Kits to measure total cholesterol, triglyceride and malondialdehyde were obtained from Beyotime Institute of Biotech (Shanghai, China). Other reagents including streptozotocin (STZ) were bought from Sigma-Aldrich (St., Louis, MO, USA) unless indicated elsewhere. The trizol reagent was obtained from Invitrogen (CA, USA). The IL-6 and TNF-α monoclonal antibody were bought from Bio-rad Laboratories (Hercules, CA, USA). Alex-fluorconjugated second antibodies and FITC-labeled dextran were obtained from Promega (Madison, USA). Immunofluorescence staining and image analysis system were purchased from Biocomp Inc. (South San Francisco, USA).

Induction and treatment of diabetes in rats: The experimental protocol was reviewed and approved by the Animal Ethics Committee of Jinan University (Ethical approval number was SCXK2013-0002). Sprague-Dawley (SD, male) rats were bought from Guangdong Laboratory Animal Commission (Foshan, China). The rats weighing approximately 180-200 g were placed in the standard animal room

(12 h light/dark cycle). A single intraperitoneal injection (i.p.) of STZ (dissolved in 0.1 M citrate buffer, 45 mg kg^{-1}) was applied to the diabetic rat model generation. There and 5 days after STZ administration, fasting blood glucose level was determined from blood sampling intail vein through the hand-held glucometer (Johnson Services, NJ, USA). Rats with blood glucose levels $\geq$11.1 mM were defined as diabetic rats and then, they were divided into four groups at random: DN, losartan (30 mg kg^{-1}, daily), BBR-100 (100 mg kg^{-1}, daily) and BBR-200 (200 mg kg^{-1}, daily). Diabetic rats that were administrated losartan (30 mg kg^{-1}, daily) were set as a positive group. The BBR and losartan were administrated for 8 weeks. Besides, normal rats were set as control group. At the end of the experiment, rats were euthanized by sodium pentobarbital (50 mg kg^{-1}, i.p.) after 8 weeks of this dietary regimen. Urine samples for each rat were collected using metabolic cages for a 24 h period. Rats in five groups were also weighed, followed by plasma and kidneys collecting. Right kidneys were frozen and stored for biochemical analyses. Meanwhile, left kidneys were fixed in 4% paraformaldehyde and embedded in paraffin for histological detection.

Collection of blood samples: Blood samples were collected after 12 h overnight fasting. Samples were immediately transferred into centrifuge tubes containing heparin and centrifuged (4°C, 3000×g, 15 min). Serum was collected (-20°C). Biochemical analysis was performed according to the manufacturer's illustration.

Histological examination: The samples of renal tissues were harvested, fixed and embedded in paraffin as previously described. The paraffin sections (5 μm) were stained by hematoxylin and eosin for histological examination. The percentage of positive staining areas in the glomeruli and tubules was evaluated by a semiquantitative analysis method using the image J version 1.46 m software (NIH image). The tissue sections were randomly selected for the analysis using light microscopy.

RNA extraction and RT-PCR: The expression of TNF-α and IL-6 were determined by real-time reverse transcription polymerase chain reaction (RT-PCR). As previously reported[30], the extraction of total RNA was performed using Trizol reagent. Quantification of target genes (TNF-α and IL-6) was implemented on ABI PRISM 7500 real-time PCR system (Applied Biosystems, USA) with SYBR green fluorophore (TOYOBO). The threshold cycle (Ct) scores of target is normalized to reference and relative to calibrator sample. The amount of PCR products was normalized with β-actin. Relative expression = $2^{-\Delta\Delta Ct}$ method[31]. The primers for mRNA detecting were designed according to rat sequences available in the data banks:

Gene	GenBank	Forward	Reverse
TNF-α	NM_012675.3	ATTGTGGCTCTGGGTCCAAC	AGCGTCTCGTGTGTTTCTGA
IL-6	NM_012589.2	CTGGTCTTCTGGAGTTCCGT	TGGTCCTTAGCCACTCCTTCT
β-actin	NM_031144.2	CACACCCGCCACCAGTTC	CCCATACCCACCATCACACC

Immunofluorescence (IF) staining: Paraffin-embedded sections (5 μm) of renal, followed by deparaffinizing in xylene and rehydrating to distilled water in alcohols. An 1100-W microwave was applied to antigen retrieval at high power for 3 min at 10 mmol L^{-1} sodium citrate buffer (pH 6.0) and 12 min at 50% power. Tissue sections were cooled at 25°C. The endogenous peroxidase activities of the renal tissues were eliminated by incubating in 0.3% hydrogen peroxide for 10 min. The paraffin slices were cleaned using PBS solution and then incubated in 5% goat serum for 1 h and treated with 3% (v/v) H_2O_2 for 30 min. Next, the incubation of renal tissues and primary antibodies was performed at 4°C overnight. Besides, the slides were incubated with fluorescein-conjugated secondary antibody for 1.5 h (20-25°C). Zeiss axiophot fluorescence microscope was used for the detection of the paraffin sections. The positive staining was quantified by using an imaging densitometer and the image analysis software.

Statistical analysis: Data in this study were presented as Mean±Standard Deviation (SD). The unpaired student's t-test was applied to the statistical analysis. Graph PadPrism 6 software was used to the post hoc pair wise comparisons. The prior level of significance was set at $p<0.05$.

RESULTS

Effects of BBR on Fasting Blood Glucose (FBG) level: The FBG value for each group was recorded and listed at Table 1. As shown in Table 1, before the treatment, DN, lorsatan, BBR-100 and BBR-200 groups possessed significantly higher

Table 1: Fasting Blood Glucose (FBG) level in each group (n = 8)

		FBG (mmol L^{-1})	
Groups		1st week	8th week
Control		4.57±1.10	4.57±1.10
DN		20.04±1.60##	22.17±1.18##
Losartan		20.11±1.58##	21.89±1.24##
BBR	100 mg kg^{-1}	20.42±1.53##	16.90±1.10*
	200 mg kg^{-1}	20.46±1.42##	15.49±1.47**

Data were displayed as Mean±SD, *p<0.05, **p<0.01 (compared with DN group), ##p<0.01 (compared with control group)

glucose level compared with the control group ($p<0.01$), indicating that the diabetic rat model was successfully established. After 8 weeks of BBR treatment, the FBG level in BBR-100 and BBR-200 groups was significantly decreased to 16.90 and 15.49 mmol L^{-1} compared to the control, respectively (Table 1).

BBR treatment improves serials of indexes about renal function for diabetic rats: Average value of body and kidney weight, Blood Urea Nitrogen (BUN) and creatinine and urine albuminuria were determined for all five groups (Fig. 1). Clearly, the rats in DN group had significant higher kidney/body weight ratio (kidney index), plasma BUN and creatinine level and urine albuminuria level compared with the control group ($p<0.01$). It was interesting to find that berberine treatment (100 and 200 mg kg^{-1}) significantly decreased kidney index, plasma BUN, creatinine and urine albuminuria levels compared with untreated diabetic group (DN). Taken together, the results demonstrated that BBR could improve renal functions in diabetic nephropathy.

Effects of BBR on renal histological changes: Hematoxylin and eosin (HE) staining of renal tissues was performed for five groups (Fig. 2). The results showed clearly that both the control and losartan groups had normal histopathological appearance (Fig. 2a, c). On the contrary, both tubular degeneration and thickening of the glomerular membrane existed in renal tissues of DN group (Fig. 2c). Interestingly, the renal histology was improved in BBR-100 group (Fig. 2d). In addition, the high dose BBR (200 mg kg^{-1}) could decreased tubular degeneration and vacuolation of endothelium area to near positive control group (Fig. 2e). Renoprotective effect of berberine (100 and 200 mg kg^{-1}) on diabetic rats was evident with a moderate decrease in tubular degeneration and fibroblasts proliferation of cells in renal. The further details of morphology changes were displayed in Table 2.

Effects of BBR on serum biochemicalpara meters: Malondialdehyde (MDA), triglycerides (TG) and total cholesterol (TCH) levels in serum were determined for five groups (Fig. 3). Three serum biochemical parameters in BBR-100 and BBR-200 group were significantly lowered ($p<0.01$) compared to the DN group. Total cholesterol (TCH) levels were slightly decreased after BBR-100 treatment ($p<0.05$), indicating that BBR could improve serum biochemical properties (including lipid metabolic parameters) for diabetic nephropathy (Fig. 3).

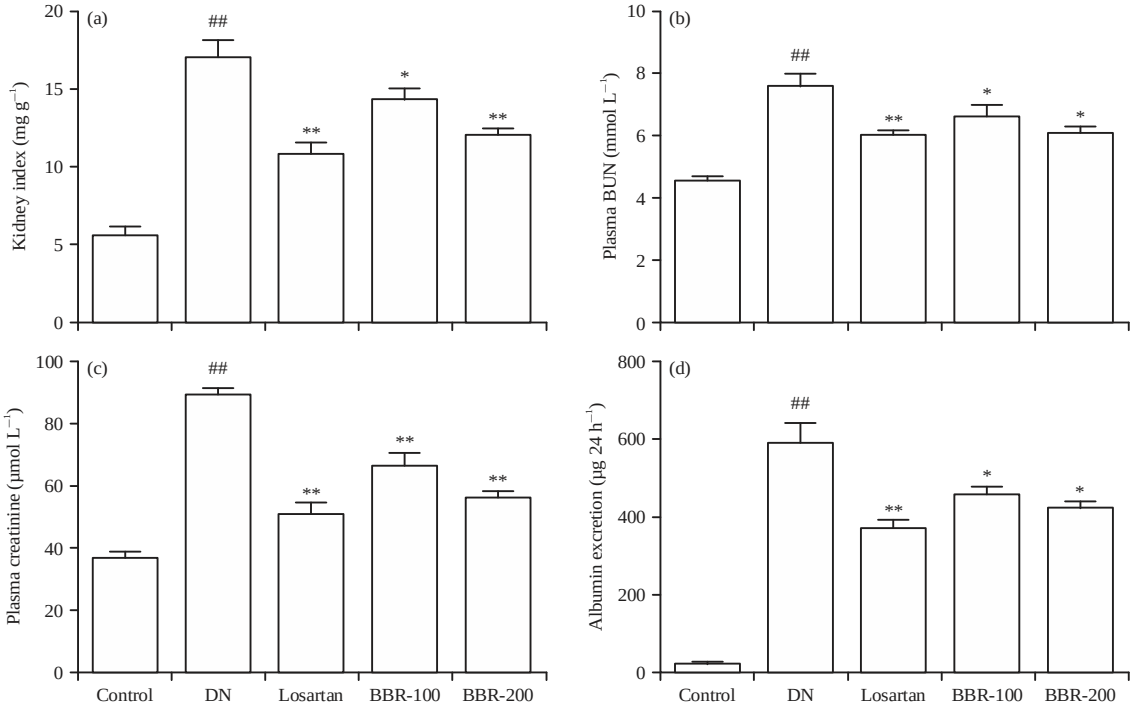

Fig. 1(a-d): Serials of indexes about renal function for diabetic rats (n = 8), (a) Kidney index, (b) Blood urea nitrogen (BUN), (c) Creatinine content in plasma and (d) Albuminuria excretion for 24 h. *$p<0.05$, **$p<0.01$ (compared with DN group), ##$p<0.01$ (compared with control group)

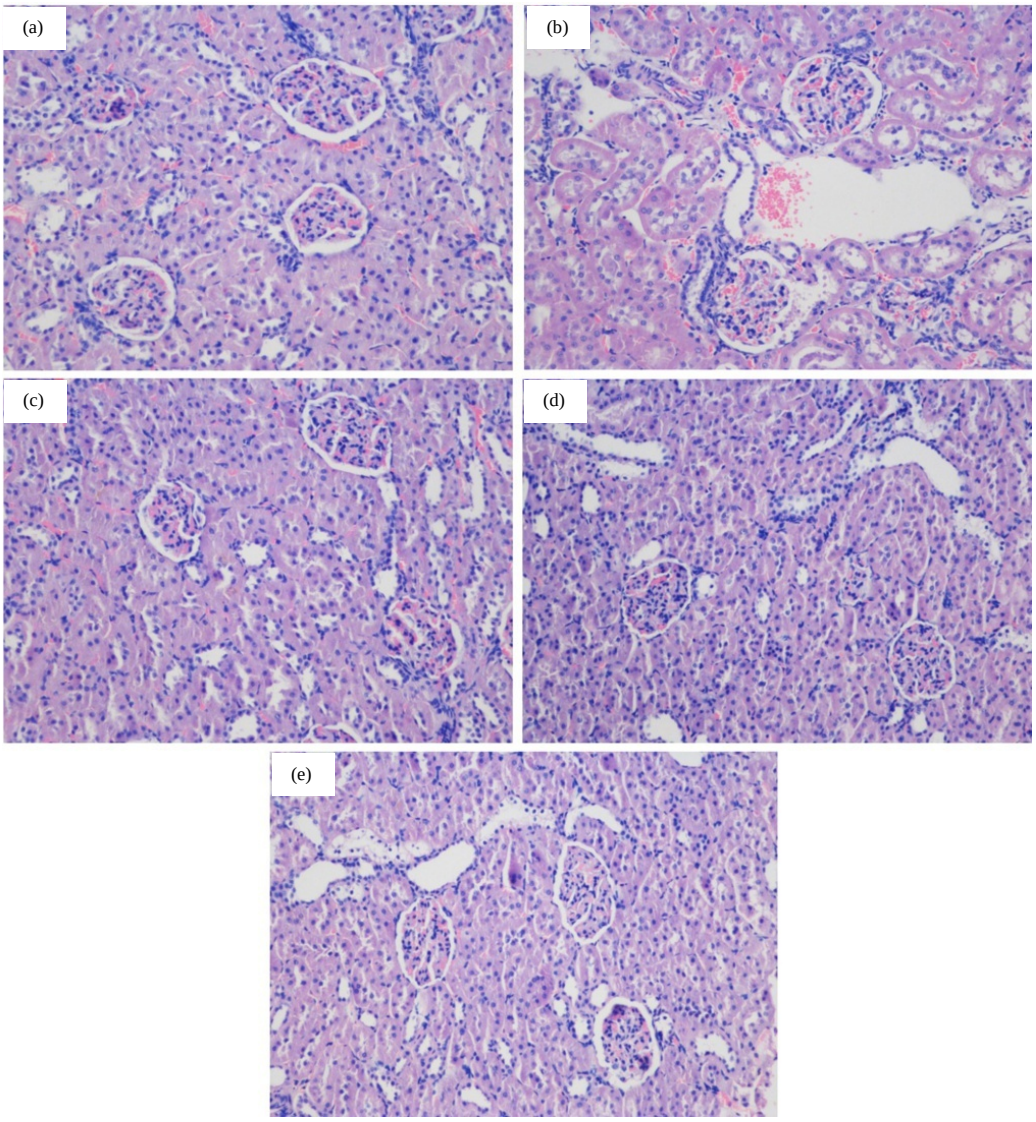

Fig. 2(a-e): Pathological detection was stained by hematoxylin and eosin (HE) in the kidney tissue. All figures magnification was 200×. Microphotograph of the (a) Control group, (b) DN group, (c) Losartan group, (d) BBR-100 group and (e) BBR-200 group

Table 2: Semiquantitative analysis of morphology changes in each group

Morphology changes	Control	DN	Losartan	BBR-100	BBR-200
Tubular degeneration	-	+++	+	++	+
Swelling	-	+++	+	++	++
Congestion of renal blood vessels	-	+++	+	++	+
Thickness of the glomerular basement membrane	-	+++	+	-	+
Vacuolation of endothelium	-	+++	+	++	+
Fibroblasts proliferation	-	+++	+	++	+

-: Normal, +: Mild, ++: Severe levels, revealing no and less than 25, 50, 75% histopathological lesions of total tissues examined, respectively

Effect of BBR on TNF-α and IL-6 mRNA expression: In this study, TNF-α and IL-6 gene expression in renal for five groups were determined through RT-PCR method, normalized to β-actin expression (Fig. 4). Obviously, the expression of IL-6 and TNF-α mRNA in DN rats were significantly increased compared to control ($p<0.01$). Besides, TNF-α and IL-6 levels of mRNA expression in renal from diabetic rats were both significantly decreased in BBR-100 and BBR-200 groups

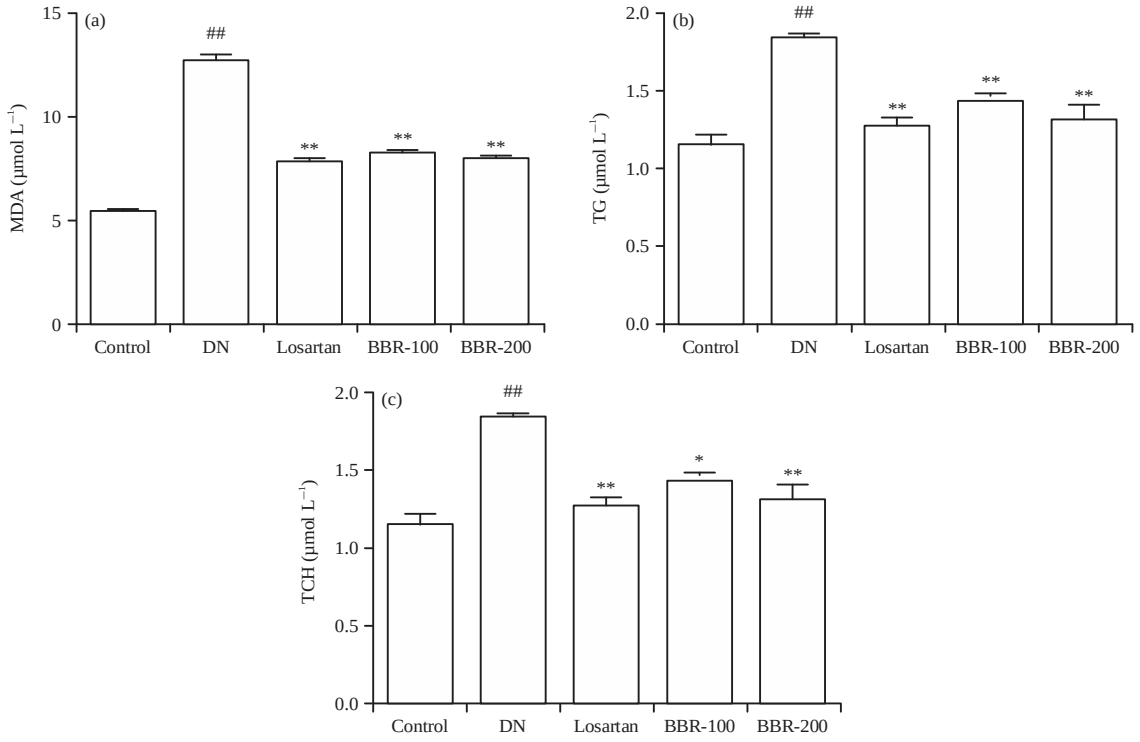

Fig. 3(a-c): Effects of BBR on serum biochemical parameters (MDA, TG and TCH) (n = 8), (a) MDA level, (b) TG level and (c) TCH level in five groups. *p<0.05, **p<0.01 (compared with DN group), ##p<0.01 (compared with control group)

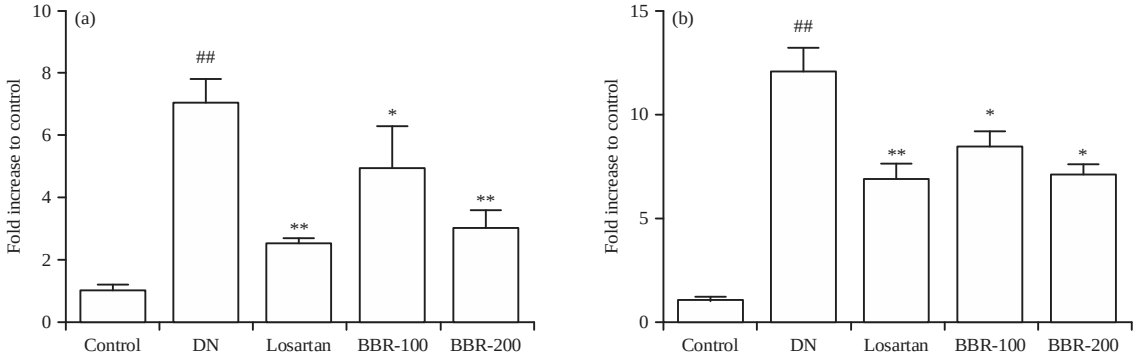

Fig. 4(a-b): mRNA expression of pro-inflammatory mediator (TNF-α and IL-6) in renal for five groups, (a) The mRNA expression of TNF-α and (b) The mRNA expression of IL-6. *p<0.05, **p<0.01 (compared with DN group), ##p<0.01 (compared with control group)

(p<0.05). The result suggesting that BBR has the potential of reducing inflammatory for DN rats.

Effect of BBR on inflammatory responses in diabetic renal: In this study, inhibitory effect of BBR on inflammatory cytokines was confirmed via IF staining (Fig. 5, 6). The results of quantitative analysis for inflammatory expression were displayed at Fig. 7. As compared with control group, DN group exhibited significant increase in fluorescence intensity, while treatment with BBR (100 and 200 mg kg^{-1}) could significantly decrease the fluorescence intensity (p<0.05). Losartan has similar effects on diabetic rats with BBR at a dose of 100 or 200 mg kg^{-1}. These overall data of berberine in STZ induced diabetic rats indicating that BBR could downregulated the

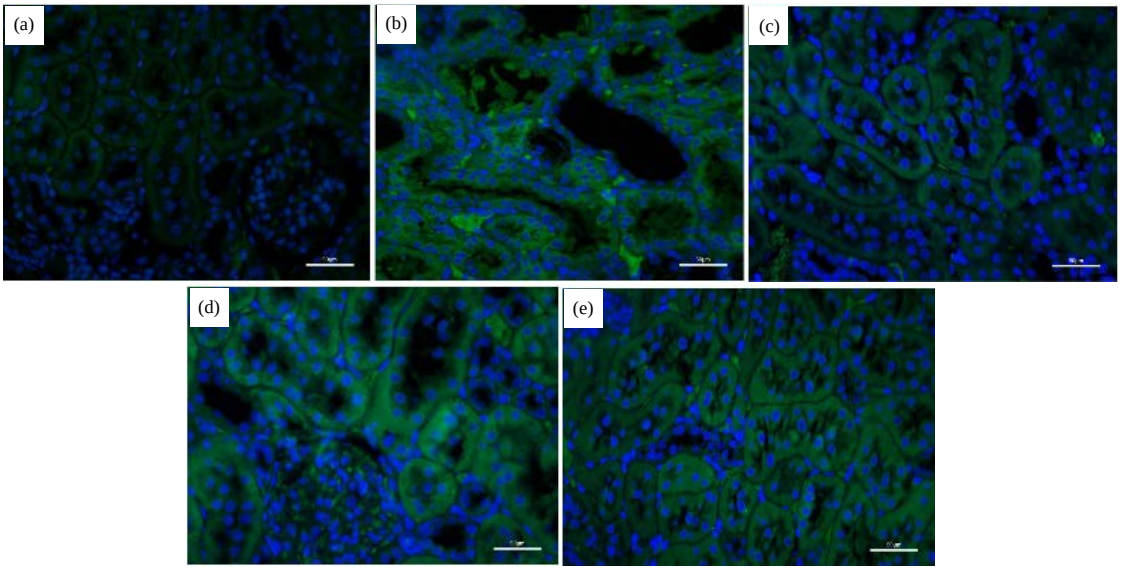

Fig. 5(a-e): Inflammatory responses of TNF-α observed by immunofluorescent micrographs in renal for five groups. The location of inflammatory cytokine (TNF-α, green) were detected by immunofluorescence staining. The nuclei were stained with DAPI (blue) the inflammatory responses in (a) Control group, (b) DN group, (c) Losartan group, (d) BBR-100 group and (e) BBR-200 group

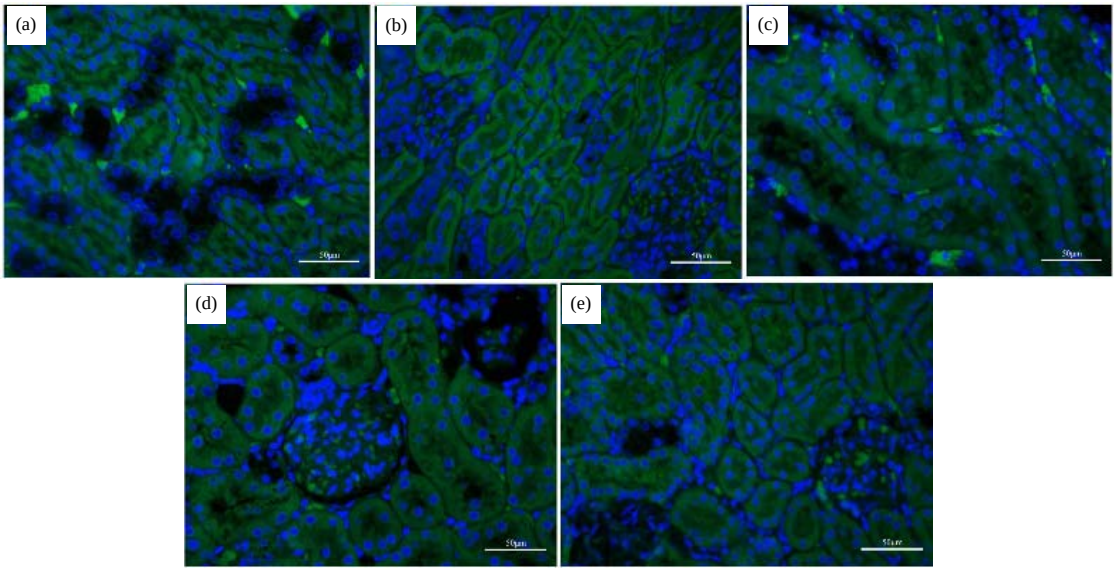

Fig. 6(a-e): Inflammatory responses of IL-6 observed by immunofluorescent micrographs in renal for five groups. The location of inflammatory cytokine (IL-6, green) were detected by immunofluorescence staining. The nuclei were stained with DAPI (blue) the inflammatory responses in (a) Control group, (b) DN group, (c) Losartan group, (d) BBR-100 group and (e) BBR-200 group

inflammatory mediators in diabetic nephropathy. It is consistent with previous assumption that BBR could be a potential protective drug against renal inflammatory mediators.

DISCUSSION

The transcription of pro-inflammatory chemokines in tubular epithelial cells have been verified as markers of

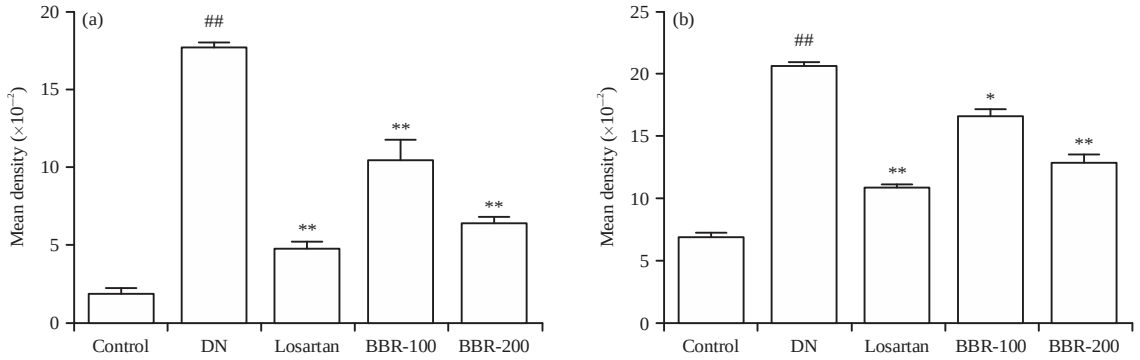

Fig. 7(a-b): Quantitative analysis of TNF-α and IL-6 expression in five groups, (a) The expression of TNF-α and (b) The expression of IL-6. *p<0.05, **p<0.01 (compared with DN group), ##p<0.01 (compared with control group)

progressive DN[32]. In addition, DN was closely relevant to glomerular endothelial injury mediated by inflammation[33]. Taken together, these findings indicated that inflammation was a key factor for DN. Berberine, which is usually used as an antibiotic drug for diarrhea and for diabetic patients in traditional Chinese medicine for many years[34,35]. It have been confirmed in rats that BBR could improve airway inflammation and inhibit NF-κB signaling pathway[25]. However, its anti-inflammatory properties on diabetic nephropathy remain unknown. Accordingly, this study was implemented to research the anti-inflammatory effect of berberine on diabetic nephropathy.

The TNF-α and IL-6 were primary mediators that lead to lots of pathophysiological changes associated with DN[10,36]. In this study, the results indicated that BBR could significantly reduce diabetic-induced increase of TNF-α and IL-6. The level of TNF-α and IL-6 in renal were raised significantly in diabetic rats in previous study, consisting with our findings[37]. Moreover, TNF-α in the kidney was inhibited significantly in losartan-treated nephropathy rats[38]. Numerous evidence have indicated that increased inflammation response was associated with renal disease[39]. The results of serials of indexes about renal function suggest that BBR possessed protective ability for diabetic nephropathy rats. Hence, this interaction raised a possibility that the BBR played its renoprotective effect via the suppression of inflammation response.

The study was also found that BBR suppress the level of IL-6 and TNF-α mRNA indiabetic renal. The previous studies suggested that diabetic nephropathyis mediated by an inflammatory positive feedback loop involving the IL-6 genes[36,40]. Overproduction of IL-6 could enhance inflammatory loop, resulting in inflammation response. In line with this study, similar results were shown in previous studies with respect to the capacity of anti-inflammatory properties to reduce TNF-α and IL-6 level.

The DN development was also associated with dyslipidemia according to epidemiological investigation. Although the application of natural products for the therapy of metabolic diseases have been reported, a potential drug of BBR has not been revealed[41,42]. In this study, BBR induced a positive protective effect distinctly by significantly decreasing the total triglycerides and cholesterol in serum, renal levels of malondialdehyde, indicating that BBR has some ameliorative effect on dyslipidemia in diabetic nephropathy. However, underlying mechanisms remain underexplored.

The *in vivo* study firstly evaluated the renoprotective effect of BBR on diabetic nephropathy disease. The results suggested that BBR could significantly reduce the expression of pro-inflammatory mediator gene expression in diabetic renal, resulting in a decrease of inflammatory responses. Furthermore, BBR could reduce plasma BUN and creatinine level and urine albuminuria level in DN rats, indicating that BBR possessed a protective effect on kidney. Therefore, this study is of great value for providing a potential drug for the therapy of diabetic nephropathy.

CONCLUSION

The results showed that BBR possessed renoprotective effect on STZ-induced diabetic nephropathy and could improve kidney function. Moreover, BBR could significantly reduce pro-inflammatory cytokines (i.e., TNF-α and IL-6) level.

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