The Qi-Bang-Yi-Shen formula ameliorates renal dysfunction and fibrosis in rats with diabetic kidney disease *via* regulating PI3K/AKT, ERK and PPARγ signaling pathways

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ABSTRACT

Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease (CKD) and a growing public health problem worldwide. Losartan potassium (Los), an angiotensin II receptor blocker, has been used to treat DKD clinically. Recently, multi-herbal formula has been shown to exhibit therapeutic activities in DKD in China. Thus, we aimed to explore the protective effects of combination of Los and Qi-Bang-Yi-Shen formula (QBF) on DKD rats. Streptozotocin (STZ) injection was used to establish a rat model of DKD. Next, the blood urea nitrogen (BUN), creatinine (CRE) and uric acid (UA) levels were detected in serum samples from DKD rats. Hematoxylin and eosin (H&E), periodic Acid Schiff (PAS) and Masson staining were performed to observe glomerular injury and glomerular fibrosis in DKD rats. In this study, we found that QBF or Los treatment could decrease serum BUN, CRE, UA levels and reduce urine albumin-to-creatinine ratio (ACR) in DKD rats. Additionally, QBF or Los treatment obviously inhibited glomerular mesangial expansion and glomerular fibrosis, attenuated glomerular injury in kidney tissues of DKD rats. Moreover, QBF or Los treatment significantly reduced PI3K, AKT and ERK1/2 protein expressions, but increased PPARγ level in kidney tissues of DKD rats. As expected, combined treatment of QBF and Los could exert enhanced reno-protective effects compared with the single treatment. Collectively, combination of QBF and Los could ameliorate renal injury and fibrosis in DKD rats *via* regulating PI3K/AKT, ERK and PPARγ signaling pathways. These findings highlight the therapeutic potential of QBF to prevent DKD progression.

Key words: Diabetes mellitus; diabetic kidney disease; traditional Chinese medicine; losartan potassium.

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Introduction

Diabetes mellitus (DM) is a common endocrine and metabolic disorder also known as wasting-thirst disorder.[1-5] Meanwhile, hyperglycemia and insulin resistance are hallmarks of DM.[1-5] Diabetes, as it is well known, frequently causes severe clinical complications such as diabetic kidney disease (DKD).[6] DKD is a major cause of morbidity and mortality in diabetic patients, as well as the leading cause of kidney failure worldwide.[7] Unfortunately, about 30-40% of patients with DM will develop kidney complications. [8,9] Recently, it has been shown that the blockade of the renin-angiotensin-aldosterone system (RAAS) is effective to control glycemia and blood pressure, and then prevents the development of DKD.[10-12] The angiotensin II receptor blockers (ARBs), a kind of RAAS inhibitors, are commonly used to treat DKD.[13,14] Losartan potassium (Los), a classic ARB, has been shown to exhibit renal protective effects in DKD.[15,16] Moreover, Los was found to reduce blood pressure and attenuate kidney fibrosis in mice with CKD.[17] However, the clinical application of ARBs is limited, because of their side effects including dry cough and elevated serum potassium level.[18]

Combination of traditional Chinese medicine (TCM) and ARBs has been considered as effective treatments for DKD.[19] The Qi-Bang-Yi-Shen formula (QBF), composed of *Astragalus membranaceus* (Fisch.) Bge (Huang qi, HQ), *Rehmannia glutinosa Libosch* (Di huang, DH), *Panax notoginseng* (Burk.) F. H. Chen (San qi, SQ), *Arctium lappa* L. (Niu bang zi, NBZ), *Paeonia lactiflora* Pall. (Bai shao, BS), and *Glycine max (L.)* Merr. (black beans), is drawn up by Prof. Chen and Prof. Jian according to the TCM syndrome differentiation. The QBF has the functions of replenishing blood and tonifying kidney, and dispelling dampness and stasis, which was used to treat wasting-thirst disorder. HQ, DH, and SQ have been shown to exhibit reno-protective effects in kidney diseases.[20-22] Meanwhile, the main active component of BS (total glucosides of paeony) and NBZ (arctigenin) have been shown to have therapeutic effects in CKD.[23,24]

However, the mechanism by which QBF improves renal dysfunction in DKD remains unclear. Thus, in this study, we explored the underlying mechanism of QBF in DKD rats and investigated the reno-protective role of combination of QBF with Los on DKD rats. These findings may highlight the therapeutic potential of QBF and Los to prevent DKD progression.

Materials and methods

Drugs

The Qi-Bang-Yi-Shen Formula (QBF) is composed of HQ, DH, SQ, NBZ, BS and black beans at a rate of 10:5:1:5:5:5 (w/w/w/w). These herbs were provided by the Jiangyin Tianjiang Pharmaceutical Co., Ltd. The Losartan potassium tablets was obtained from the Merck Sharp & Dohme Limited (Approval Number: National medicine standard word J20180054; Hangzhou).

Animal study

Male Sprague-Dawley (SD) rats were obtained from the Department of Laboratory Animal Operations in Shanghai Institute of Planned Parenthood Research. This study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and conducted according to the international rules and guidelines. After adaptive feeding for 2 weeks, rats were randomly divided into control (n=5) and DKD (n=35) groups. In the DKD groups, streptozotocin (STZ; 55 mg/kg) in 1% Na-citrate buffer (pH=4.5) was intraperitoneally (i.p.) injected to each rat. Seven days after STZ induction, the blood glucose levels of rats were determined and rats with blood glucose levels ≥16.7 mM were selected as DKD rats in the following experiments. Next, DKD rats were randomly divided five groups: (I) DKD + Vehicle (DKD + Veh) group, (II) DKD + low-dosage of QBF (L-QBF, 10 mL/kg) group, (III) DKD + high-dosage of QBF (H-QBF, 20 mL/kg) group, (IV) DKD + Losartan (Los) group and (V) DKD + Los + H-QBF group. Rats in the control and DKD + Veh groups were given deionized water by gastric gavage per day for 12 weeks. Rats in the DKD + L-QBF, DKD + H-QBF, DKD + Los and DKD + Los + H-QBF groups were given L-QBF (10 mL/kg), H-QBF (20 mL/kg), Los or H-QBF (20 mL/kg) + Los per day by gastric gavage for 12 weeks.

The body weight of each rat was monitored every week. For urinary collection, all rats were housed in metabolic cages, and then urine samples within 24 h were collected every 4 weeks. Next, blood samples were collected through tail vein every 4 weeks, and then blood glucose levels were monitored with a glucometer. After 12 weeks of treatment, all rats were deeply anaesthetized by pentobarbital sodium (3%, 40 mg/kg, i.p.). The blood in inferior vena cava was then collected, centrifuged at 3000 g for 15 min to collect serum samples. Finally, the bilateral kidneys of rats were removed and weighted.

Metabolic measurements

The urine albumin (UA) and urine creatinine (CRE) were measured by the rat albumin ELISA kit (Crystal Chem, Elk Grove Village, IL, USA) and the creatinine (Cr) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. The urine albumin to creatinine ratio (ACR) was calculated (ACR = urine albumin/creatinine). The levels of blood urea nitrogen (BUN), CRE, uric acid (UA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum samples of rats were assessed by biochemical test kits (Nanjing Jiancheng Bioengineering Institute).

Histopathological study

The kidney tissues were fixed with 4% formaldehyde overnight and then embedded in paraffin. After that, paraffin-embedded tissues were cut into 3 μm-thick slices, and then deparaffinized in xylene and rehydrated. Next, the specimens were stained with Hematoxylin and eosin (H&E), periodic Acid Schiff (PAS) and Masson's trichrome for histopathological research. Subsequently, images were observed and photographed by a light microscope. The Image-Pro Plus software was used to quantify mesangial matrix and interstitial fibrosis areas in kidney tissues within 3 random fields.

Immunohistochemical and immunofluorescence staining assays

Slices were deparaffinized, hydrated and then the antigen retrieved in the EDTA antigen-repair buffer using microwave heating for 10 min, followed by blocking with 5% BSA for 30 min. After that, slices were incubated with anti-PI3K (1:250, cat. no. ab191606; Abcam, Cambridge, UK) antibody, anti-ERK1/2 (1:250, cat. no. 11257-1-AP; Proteintech, Rosemont, IL, USA) antibody, anti-PPARγ (1:500, cat. no. 16643-1-AP; Proteintech) antibody and anti-AKT (1:250, cat. no. 10176-2-AP; Proteintech) antibody overnight at 4°C. Next, a secondary anti-rabbit antibody (1:200, ASPEN) in 5% BSA was used for immunohistochemical (IHC) staining, and then images were observed under a light microscope. Additionally, a secondary anti-rabbit antibody was used for immunofluorescence (IF) staining. Subsequently, cell

nuclei were stained with DAPI (10 μg/mL) in darkness for 10 min, and then images were observed under a fluorescence microscope (Eclipse Ci-L, objective: 40x, Nikon, Tokyo, Japan). The Image-Pro Plus software was used to conduct the quantitative analysis within 3 random fields. Sections lacking primary antibody were used as negative control.

Western blot assay

Total protein was extracted from kidney tissues using the RIPA lysis buffer, and then the BCA assay kit was used for evaluating protein concentration. After that, proteins were extracted by 10% SDS-PAGE and then transferred onto PVDF membranes. Next, anti-PI3K (cat. no. ab191606; Abcam), anti-AKT (cat. no. 10176-2-AP; Proteintech), anti-p-AKT (cat. no. AF0016; Affinity Biosciences, Cincinnati, OH, USA), anti-ERK1/2 (cat. no. ab184699; Abcam), anti-p-ERK1/2 (Abcam, cat. no. ab201015), anti-PPARγ (cat. no. 16643-1-AP; Proteintech) and anti-β-actin (cat. no. 66009-1-Ig; Proteintech) antibodies were used to probe the proteins. Later on, membranes were probed with the corresponding secondary antibody for 2 h. Subsequently, blots were visualized by an ECL detection system, and then quantified by the AlphaEaseFC software.

RT-qPCR assay

The TRIzol reagent was used to isolate RNA. After that, the M-MLV reverse transcriptase kit was used for synthesizing cDNA. Next, qPCR process was performed using the SYBR Green/Flourescein qPCR Master Mix kit. Gene expression was normalized against GAPDH and calculated using $2^{-\Delta\Delta ct}$ method.

The primers were as follows: AKT: forward, 5'-TTTCAAGC-CCCAGGTCAC-3' and reverse, 5'-CCGTTCACTGTCCACA-CACT-3'; PI3K: forward, 5'-CATCAA TGGCAACACTCTAAG-3' and reverse, 5'-AGGACAGGTGGATACGAAAT-3'; ERK1/2: forward, 5'-GCAAGACCAGAGTGGCTATCA-3' and reverse,

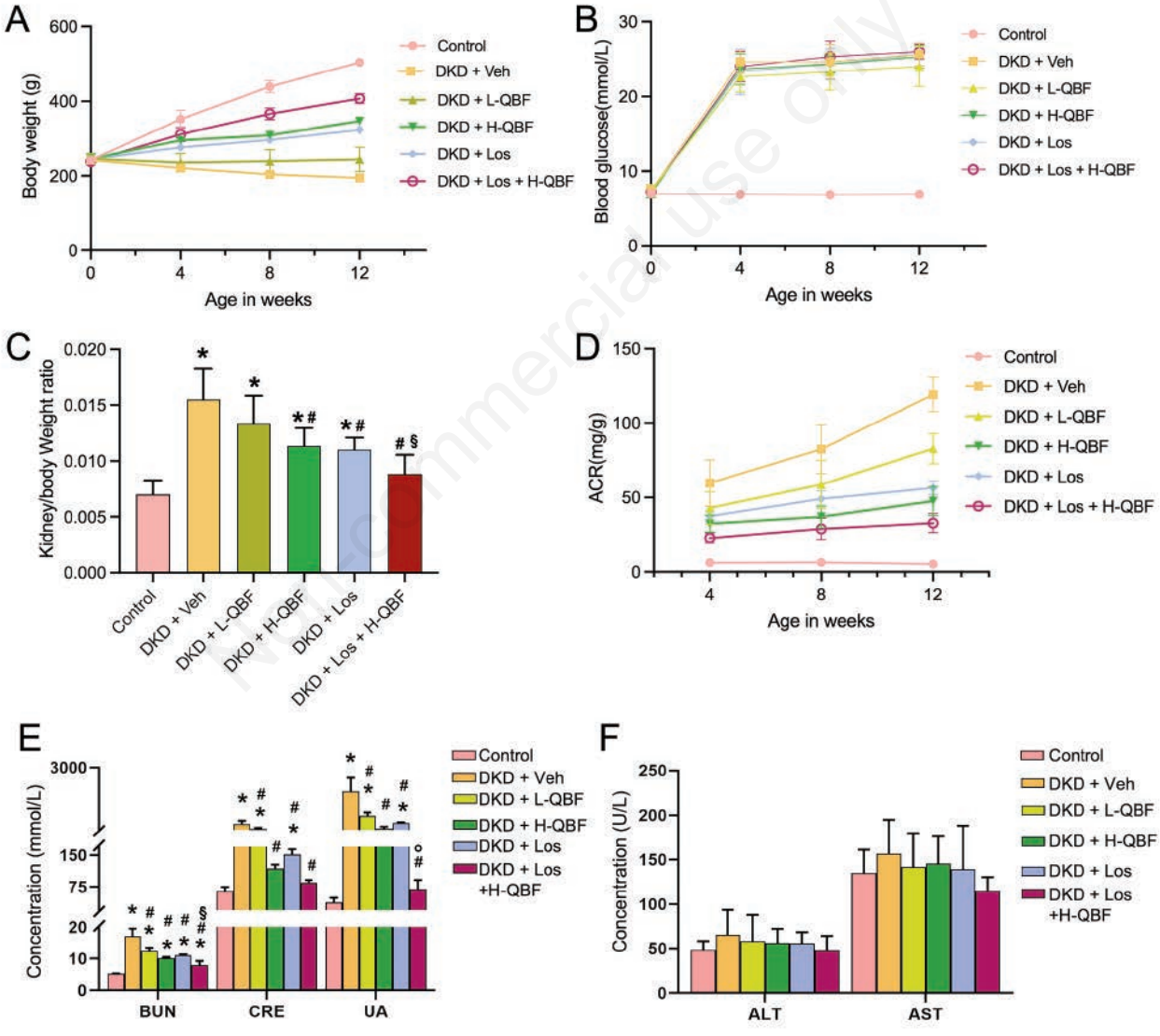

Figure 1. QBF treatment improves renal dysfunction in DKD rats. A) The body weight of each rat was recorded at 0, 4, 8 and 12 weeks of treatment. B) The blood glucose levels were detected at 0, 4, 8 and 12 weeks of treatment. C) The ratio of kidney weight to body weight of each rat was evaluated at 12 weeks of treatment. D) ACR level was determined at 4, 8 and 12 weeks of treatment. E) Serum BUN, CRE and UA levels of each rat was detected. F) Serum ALT and AST levels of each rat was detected. *p<0.05 *vs* control group; #p<0.05 *vs* DKD + Veh group; §p<0.05 *vs* H-QBF group; °p<0.05 *vs* Los group.

5'-TCG GATGCCTATGACATTCTC-3'; PPARγ: forward, 5'-CCTCTCTGTGATGGATGACCACT-3' and reverse, 5'-GCTCTTGTGAACGGGATGTCTT-3'; β-actin: forward, 5'-CCTCTATGCCAACACAGT-3' and reverse, 5'-AGCCAC-CAATCC ACACAG-3'.

Statistical analysis

Each experiment was repeated independently at least three times. Data are expressed as the mean ± SD. Multiple group comparisons were examined by one-way analysis of variance (ANOVA) and Tukey's tests. A p-value <0.05 was considered as significant.

Results

QBF treatment improves renal dysfunction in DKD rats

To explore the effect of QBF on diabetic kidney injury, a DKD rat model was established by STZ injection. As shown in Figure 1A, compared to the control group, the body weight of the rats in the DKD + Veh group reduced gradually over time. However, QBF or Los treatment obviously reduced the weight loss in DKD rats compared to the DKD + Veh group (Figure 1A). Additionally, the blood glucose levels of rats in the DKD + Veh group were sharply increased after 4 weeks of diabetes induction, compared with the control group (Figure 1B). Meanwhile, compared with the DKD + Veh group, QBF or Los treatment did not affect the blood glucose levels of DKD rats, suggesting that a successful and stable diabetic animal model was established (Figure 1B). Moreover, a significant increase in kidney/body weight ratio and ACR was seen in DKD rats; however, QBF or Los treatment significantly reversed these

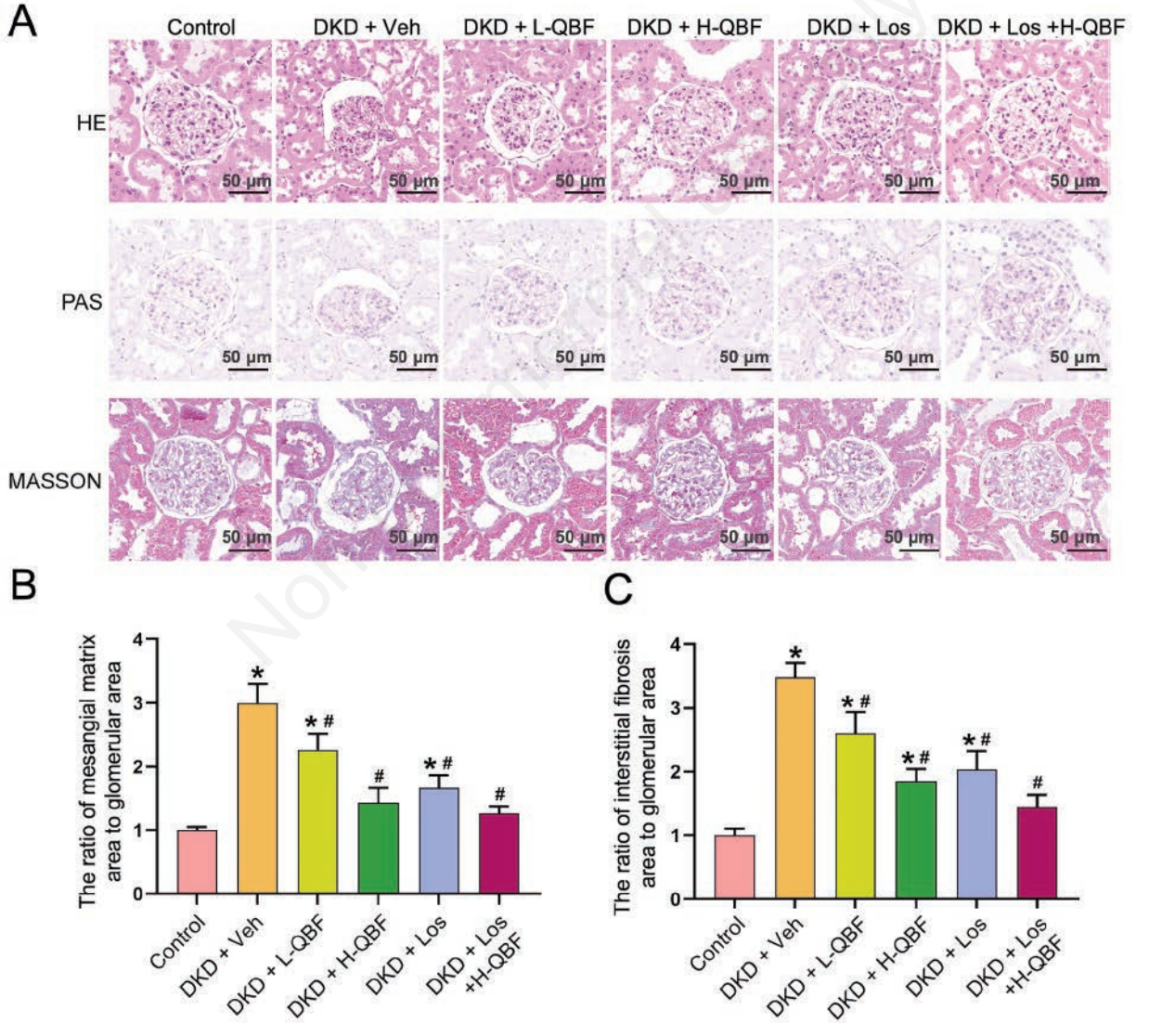

Figure 2. QBF treatment attenuates renal injury and fibrosis in DKD rats. A) Representative images of H&E, PAS, Masson staining in kidney tissues. B) The pathological changes of glomerular mesangial matrix observed by PAS staining. Quantification of mesangial matrix area as percentages of the glomerular area. C) Renal fibrosis area was evaluated by Masson staining. Quantification of interstitial fibrosis area as percentages of the glomerular area. *p<0.05 *vs* control group; #p<0.05 *vs* DKD + Veh group.

changes (Figure 1 C,D). Furthermore, the serum BUN, CRE and UA levels significantly increased in DKD rats, whereas these changes were reversed by QBF or Los treatment (Figure 1E). Meanwhile, compared to the H-QBF or Los treatment group, combination of H-QBF and Los further reduced the serum BUN, CRE and UA levels in DKD rats (Figure 1E). Nevertheless, no differences in the serum ALT and AST levels were observed among the six groups, indicating QBF could not affect the liver function of rats (Figure 1F). Collectively, QBF treatment was able to improve renal dysfunction in DKD rats.

QBF treatment attenuates renal injury and fibrosis in DKD rats

To assess renal histological injury in DKD rats, H&E, PAS, Masson staining assays were performed. The results of H&E and PAS staining assays showed that compared with the control group, obvious glomerular atrophy and glomerular mesangial matrix expansion were observed in kidney tissues of DKD rats; however, QBF or Los treatment obviously prevented these histopathological changes in kidney tissues of DKD rats (Figure 2 A,B). In addition, the results of Masson staining assay showed that QBF or Los treatment led to a significant decline in glomerular fibrosis in DKD rats (Figure 2 A,C). To sum up, QBF treatment could attenuate renal injury and fibrosis in DKD rats.

QBF treatment suppresses PI3K and Akt protein expressions in DKD rats

It has been shown that PI3K/AKT and ERK signaling pathways are involved in DKD progression.[25,26] Therefore, the expressions of PI3K, AKT and ERK1/2 in DKD rats were evaluated by IF, IHC, Western blot and RT-qPCR assays. As shown in Figure 3 A-D, the protein and mRNA levels of PI3K were obviously elevated in kidney tissues of DKD rats compared to the control group; however, QBF or Los treatment significantly reduced PI3K levels in kidney tissues of DKD rats. Moreover, the total and phosphorylated AKT and ERK1/2 protein expressions and AKT and ERK1/2 mRNA levels were notably elevated in kidney tissues of DKD rats; however, QBF or Los treatment declined AKT and ERK1/2 levels in kidney tissues of DKD rats (Figures 4 A-D and 5 A-D). Collectively, QBF treatment could improve renal dysfunction in DKD rats *via* regulating PI3K/AKT and ERK signaling.

QBF treatment increases PPARγ protein expression in DKD rats

Evidence has found that upregulation of PPARγ could prevent the progression of DKD.[27] Thus, we explored whether QBF treatment could affect PPARγ level in DKD rats. As indicated in Figure 6 A-D, the protein and mRNA levels of PPARγ were markedly declined in kidney tissues of DKD rats compared to the control group. However, compared to the DKD + Veh group, QBF or Los treatment upregulated PPARγ levels in DKD rats (Figure 6 A-D). Collectively, QBF treatment could improve renal dysfunction in DKD rats *via* upregulating PPARγ.

Discussion

DKD is the leading cause of kidney failure worldwide.[28] It is specifically manifested as hyperglycemia, hypertension, albuminuria, decreasing glomerular filtration rate (GFR).[29,30] Meanwhile, mesangial lesions, glomerular destruction, tubulointerstitial fibrosis and podocyte injury are main manifestations of DKD as well.[31,32] In China, TCM has been widely applied for the treatment of DKD.[33] Zhang *et al.* showed that HQ could decrease albuminuria and serum CRE levels in patients with DKD, and it can be used as adjunctive therapy for CKD treatment.[34] Dai *et al.* reported that Liuwei Dihuang decoction could improve insulin resistance in rats with type 2 DM.[35] Lin *et al.* showed that astragalus mongholicus Bunge and SQ formula could attenuate renal injury and inflammation in DKD mice *via* inhibiting NF-κB signaling pathway.[36] Zhong *et al.* showed that arctigenin, a component of NBZ, was able to prevent podocyte injury and attenuate proteinuria in mice with DM.[37] Paeoniflorin, an active ingredient isolated from BS, could ameliorate adriamycin-induced podocyte injury through activating PPARγ signaling.[38] These Chinese herbs have been shown to exhibit therapeutic activities in diabetes or kidney-related diseases.

QBF is composed of six herbs including HQ, DH, SQ, NBZ, BS and black beans. However, the effect and the underlying mechanism of QBF on renal dysfunction in DKD remain largely unclear. In general, multi-herbal formula exerts a curative role in various diseases through herbal compatibility principle.[39,40] Herbal formulas are composed of two or more herbs to exert the effect-enhancing and toxicity-reducing activity.[41] Zhai *et al.* found that HQ and SQ formula could synergistically attenuate diabetic podocyte injury in diabetic rats *via* upregulation of nephrin.[42] In this study, we found that QBF treatment could decline the serum BUN, CRE and UA levels and reduce urine ACR in DKD rats. Meanwhile, QBF treatment obviously prevented glomerular injury in kidney tissues of DKD rats *via* alleviating renal mesangial expansion and glomerular fibrosis. These results showed that QBF could improve renal dysfunction in DKD rats.

Recently, integrative Chinese and Western medicine is considered an effective approach for DKD treatment.[19,43] In addition, Chinese medicines has the properties of effect-enhancing and toxicity-reducing.[44] Thus, we then investigated the protective effect of combination of QBF with Los on renal dysfunction in DKD rats. Compared with the QBF or Los alone treatment group, combination treatment further reduced kidney-to-body weight in DKD rats, attenuating renal hypertrophy. Moreover, combination treatment further improved renal dysfunction and inhibited renal fibrosis in DKD rats, compared with the QBF or Los alone treatment group. These results showed that combined treatment of QBF and Los could exert enhanced therapeutic effect compared with single treatment.

PI3K/AKT signaling pathway was shown to regulate a variety of cellular processes such as cell growth, survival, and metabolism.[45,46] Additionally, overactivation of PI3K/AKT and ERK signaling pathways are related to the progression of DKD.[47,48] Bozic *et al.* found that activation of PI3K/AKT pathway could exacerbate tubulointerstitial fibrosis in kidney tissues of mice with renal fibrosis.[49] Huang *et al.* found that Qufengtongluo decoction could reduce 24 h urinal protein level and prevent renal fibrosis in DKD rats *via* inhibiting PI3K/AKT signaling.[50] Furthermore, Xu *et al.* found that Liuwei Dihuang pill could prevent renal fibrosis and inflammation in DKD rats *via* suppressing ERK signaling.[51] These findings showed that multi-herbal formula could exert the renoprotective role in kidney diseases *via* modulating PI3K/AKT and ERK signaling pathways. In this study, we found that QBF or Los treatment significantly reduced the AKT and ERK1/2 protein expressions in kidney tissues of DKD rats. As expected, combination treatment further decreased PI3K, AKT and ERK1/2 protein expressions in DKD rats. These results showed that combined treatment of QBF and Los could inhibit the progression of DKD *via* inactivating PI3K/AKT and ERK signaling pathways.

PPARγ, a member of the nuclear receptor superfamily, is believed to be involved in the progression of DKD as well.[52,53] Activation of PPARγ could attenuate renal inflammation and renal interstitial fibrosis.[54,55] Wang *et al.* showed that pioglitazone, a PPAR-γ agonists, was able to attenuate renal fibrosis and dysfunc-

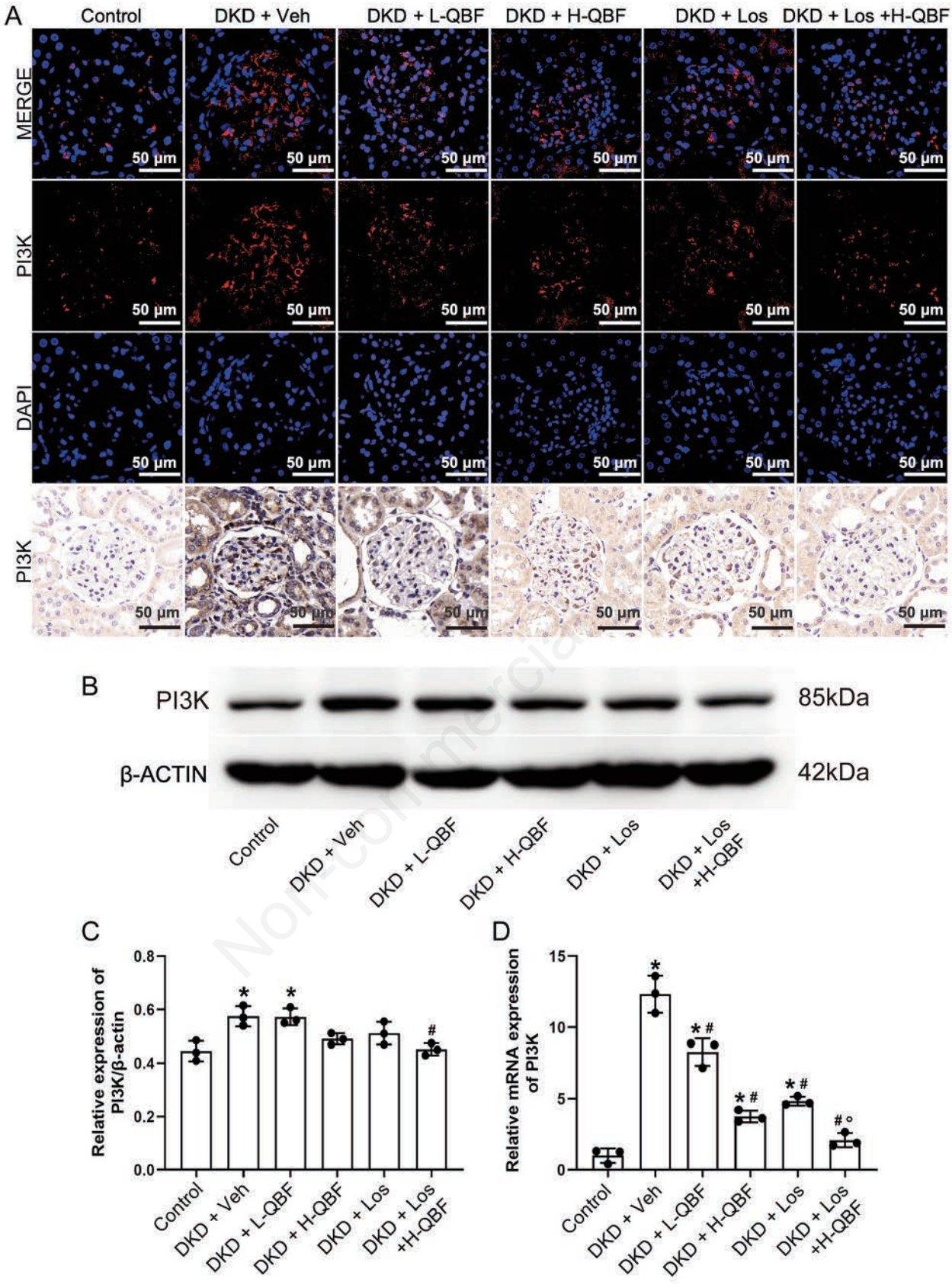

Figure 3. QBF treatment suppresses PI3K protein expression in DKD rats. A) IF and IHC analysis of PI3K expression in kidney tissues of DKD rats. B,C) Western blot and RT-qPCR (D) analysis of PI3K expression in kidney tissues of DKD rats. *p<0.05 *vs* control group; #p<0.05 *vs* DKD + Veh group; °p<0.05 *vs* Los group.

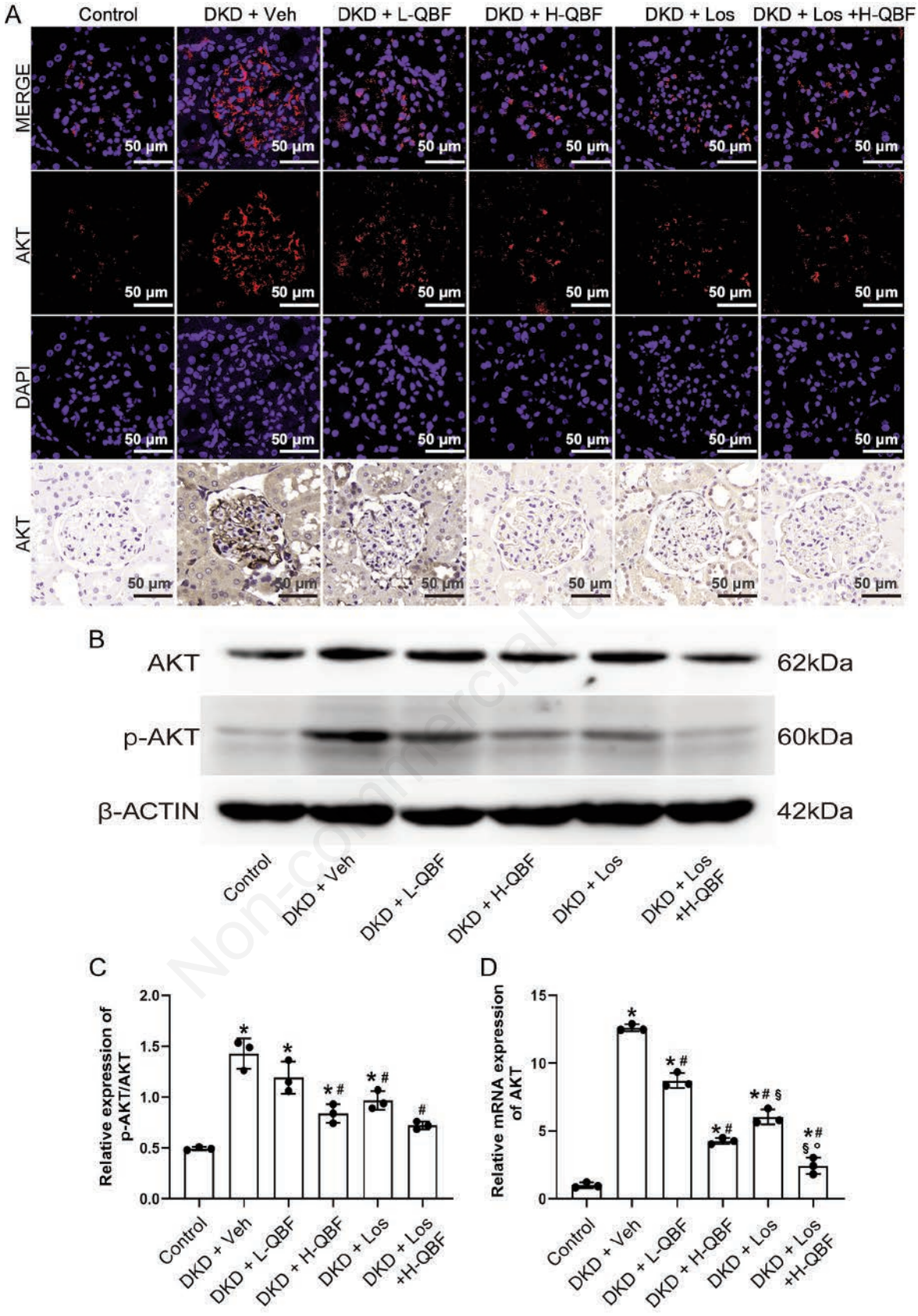

Figure 4. **QBF treatment suppresses AKT protein expression in DKD rats. (A) IF and IHC analysis of AKT expression in kidney tissues of DKD rats. (B, C) Western blot and (D) RT-qPCR analysis of p-AKT and AKT expressions sin kidney tissues of DKD rats. *p<0.05 *vs* control group; #p<0.05 *vs* DKD + Veh group; §p<0.05 *vs* H-QBF group; °p<0.05 *vs* Los group.**

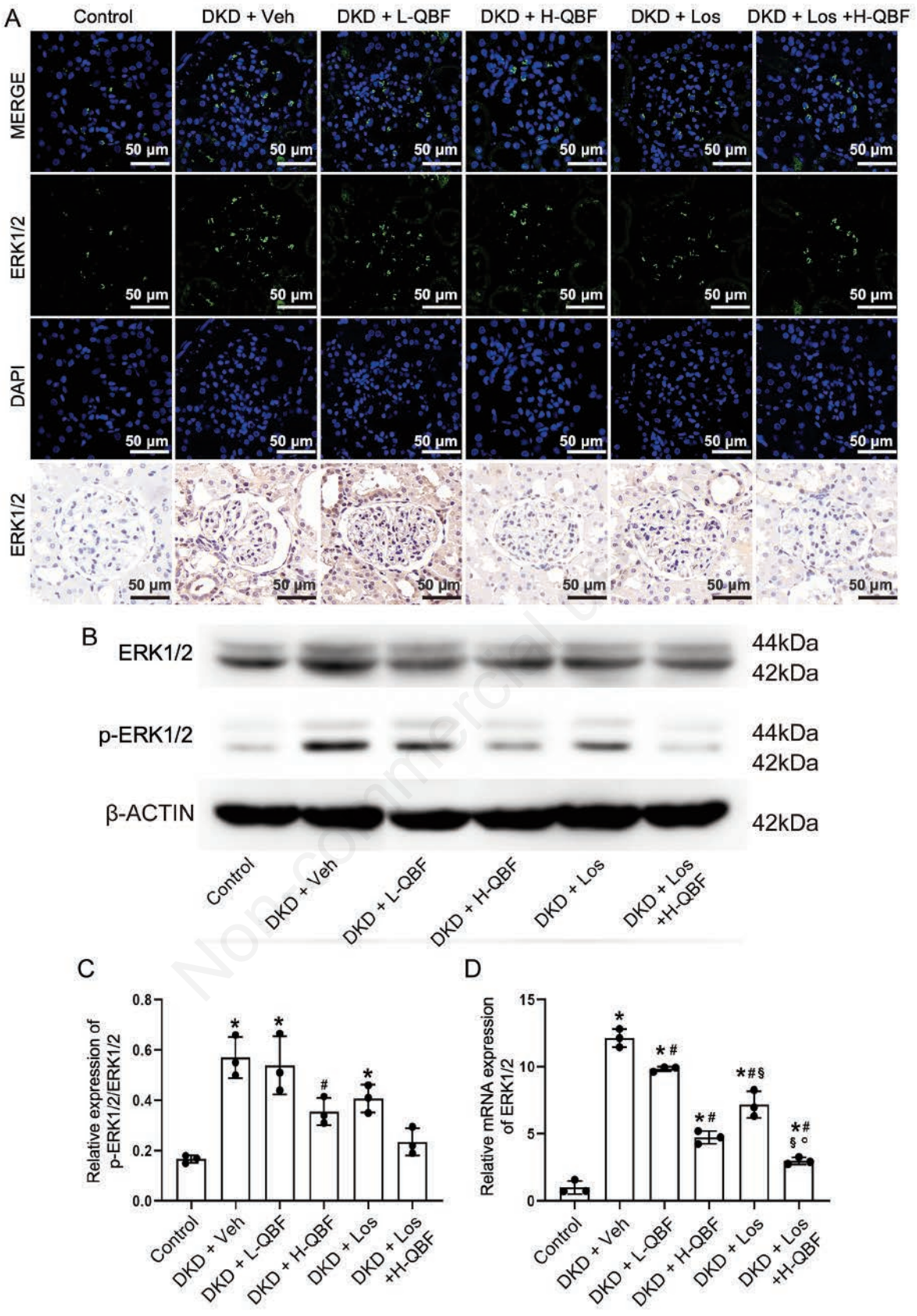

Figure 5. QBF treatment suppresses ERK1/2 protein expression in DKD rats.(A) IF and IHC analysis of ERK1/2 expression in kidney tissues of DKD rats. B,C) Western blot and RT-qPCR (D) analysis of p-ERK1/2 and ERK1/2 expressions in kidney tissues of DKD rats. *p<0.05 *vs* control group; #p<0.05 *vs* DKD + Veh group; §p<0.05 *vs* H-QBF group; °p<0.05 *vs* Los group.

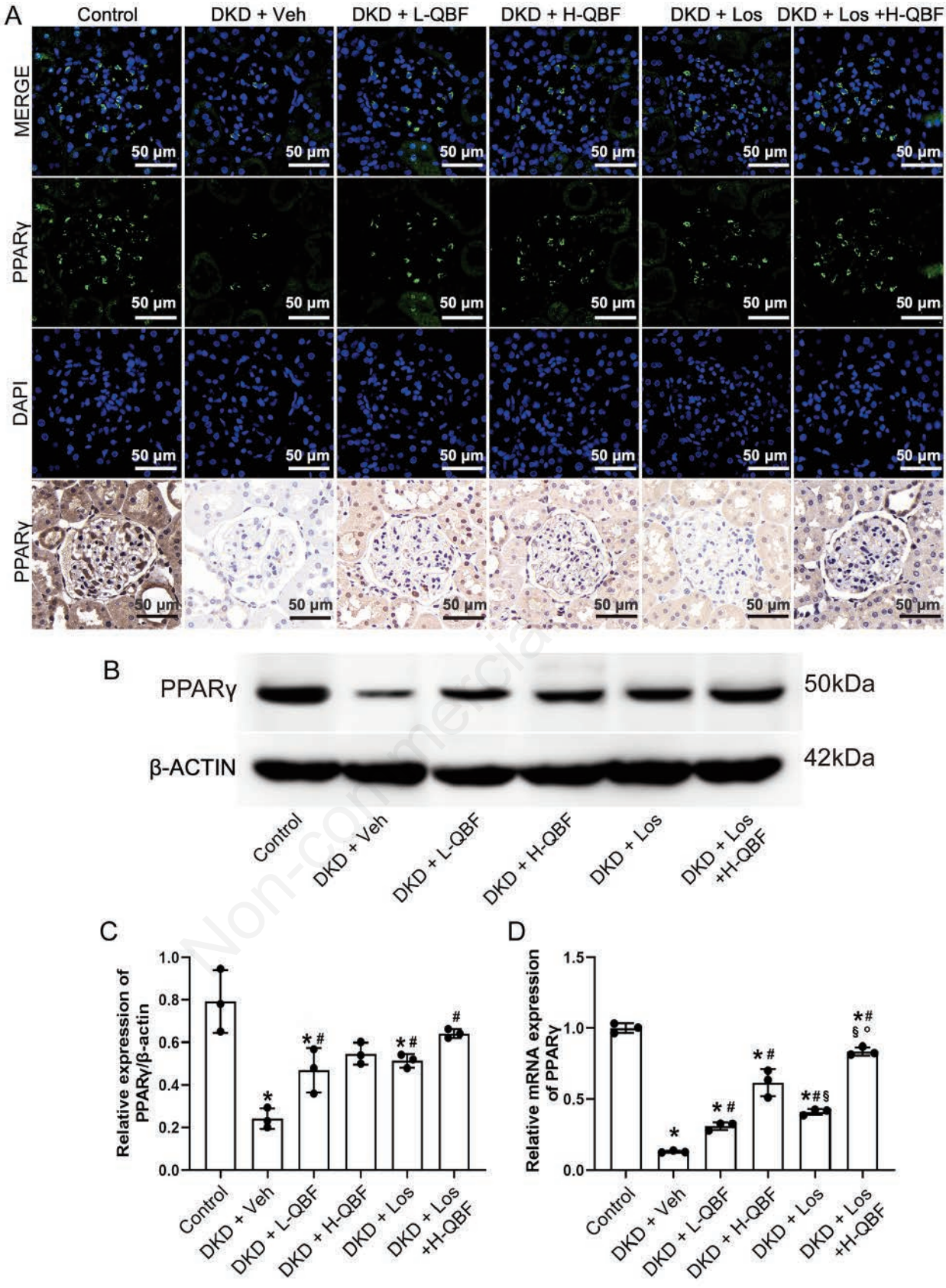

Figure 6. QBF treatment increases PPARγ protein expression in DKD rats. A) IF and IHC analysis of PPARγ expression in kidney tissues of DKD rats. B,C) Western blot and RT-qPCR (D) analysis of PPARγ expression in kidney tissues of DKD rats. *p<0.05 *vs* control group; #p<0.05 *vs* DKD + Veh group; §p<0.05 *vs* H-QBF group; °p<0.05 *vs* Los group.

tion in rats with diabetic fatty *via* downregulating Twist-1.[56] In this study, we found that QBF, Los or combination treatment notably increased the PPARγ protein expression in kidney tissues of DKD rats, suggesting that QBF and Los could prevent the progression of DKD *via* upregulating PPARγ.

Taken together, this study is the first to explore the effect of QBF and Los on HF with renal dysfunction. Our results showed that combination of QBF and Los could ameliorate renal injury and fibrosis in DKD rats *via* regulating PI3K/AKT, ERK and PPARγ signaling pathways. These findings highlight the therapeutic potential of QBF to prevent DKD progression. Additionally, combination of QBF with Los have a greater protective effect on kidney injury in DKD rats than QBF or Los alone. Thus, QBF may have the potential to improve the efficacy of Los for treating DKD. However, the therapeutic effects of combination of QBF and Los on DKD should be explored in clinical studies in the future.

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