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# Treatment of berberine alleviates diabetic nephropathy by reducing iron overload and inhibiting oxidative stress

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**Summary.** Diabetic nephropathy (DN) has become one of the major fatal factors in diabetic patients. The aim of this study was to elucidate the function and mechanism by which berberine exerts renoprotective effects in DN. In this work, we first demonstrated that urinary iron concentration, serum ferritin and hepcidin levels were increased and total antioxidant capacity was significantly decreased in DN rats, while these changes could be partially reversed by berberine treatment. Berberine treatment also alleviated DN-induced changes in the expression of proteins involved in iron transport or iron uptake. In addition, berberine treatment also partially blocked the expression of renal fibrosis markers induced by DN, including MMP2, MMP9, TIMP3, β-arrestin-1, and TGF-β1. In conclusion, the results of this study suggest that berberine may exert renoprotective effects by ameliorating iron overload and oxidative stress and reducing DN.

**Key words:** Diabetic nephropathy, Iron overload, Oxidative stress, Berberine

## Introduction

The incidence of diabetes has increased dramatically in most countries around the world lately. In China, the incidence of diabetes is increasing year by year, and has become a serious health threat (Cole and Florez, 2020; Dragan et al., 2020). As one of the major complications, diabetic nephropathy (DN) has become one of the main fatal factors in diabetic patients. The main cause of DN is renal hemodynamic changes caused by long-term hyperglycemia, which adversely affects renal function (Sagoo and Gnudi, 2020). The progression of DN not only places a serious economic burden on patients, but also poses a significant threat to their health and life. At present, the treatment strategies for DN include glycemic and blood pressure control, low-protein diet, lipid-lowering drugs and interference with the renin-angiotensin system. Although progression of DN can be slowed by treatment, the health threat posed by the disease remains for patients, and most patients with DN will eventually develop end-stage renal disease (ESRD) (Warren et al., 2019; Calle and Hotter, 2020; Sagoo and Gnudi, 2020). Therefore, it is of great clinical value to explore the molecular mechanism underlying DN progression and develop effective therapeutic drugs for treatment.

The pathogenesis of DN is quite complex, involving multiple factors such as abnormal glucose metabolism, metabolic hemodynamic disorders, cytokine over expression, and oxidative stress (Sagoo and Gnudi, 2018; Lin et al., 2018; A/L B Vasanth Rao et al., 2019). Increasing evidence suggests that ongoing oxidative stress and the resultant inflammation are consistent features of the progression of diabetic complications. Oxidative stress can lead to the production and excessive accumulation of reactive oxygen species (ROS), which leads to the imbalance of the antioxidant defense system. Oxidative stress can cause morphological damage and the functional decline of renal cells, and eventually induce the occurrence of DN (Sagoo and Gnudi, 2018; Østergaard et al., 2020). With the deepening of understanding of the mechanism in the induction of oxidative stress, iron overload has gradually been found to be a key mediator. Iron is an essential element in some basic metabolic processes (such as DNA synthesis, ATP synthesis, etc.). However, because iron can lead to ROS production, its homeostasis is essential for the body, while high levels of free iron are detrimental (Sung et al., 2019). Studies have shown that, on one hand, iron overload causes oxidative stress damage to islets which disturbs insulin secretion to induce hyperglycemia; on the other hand, iron overload produces large amounts of

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ROS which cause tissue damage and the occurrence of diabetes (Galaris et al., 2019). Considering that ROS production is one of the key etiological factors of DN, we inferred that iron overload may play an important role in the development of DN.

In recent years, traditional Chinese medicine (TCM) has been widely used in the treatment of diabetes and its complications. Many advantages have been found for TCM in the prevention of diabetic complications, such as multiple approaches and multiple targets (Wang et al., 2020). Key components in TCM can facilitate the repairment of lipid metabolism, reduction of blood viscosity, improvement of hemorheology and renal hemodynamics, inhibition of oxidative stress and inflammatory response, suppression of proteinuria excretion, and the protection of renal function (Lu et al., 2019). Therefore, the exploration for TCM that can treat DN through antioxidant effects has attracted the attention of a lot of researchers. Berberine is an isoquinoline alkaloid extracted from Coptis chinensis, which has good antibacterial effect and few side effects, and is clinically used for the treatment of some gastrointestinal diseases caused by bacteria infection. With the deepening of research, it has been shown that, in addition to antibacterial function, berberine also has the effects of anti-oxidation, anti-hypertension, anti-tumor and neuroprotection (Wang et al., 2017; Song et al., 2020). Although the therapeutic effects on DN as well as the renoprotective effects of berberine have been frequently investigated (Ni et al., 2015a,b), the mechanism of action remains unclear. Therefore, the aim of this work was to verify whether berberine plays a renoprotective role by regulating iron overload in DN.

## Materials and methods

### *DN animal models*

60 SD male rats of the same age (body weight 180±20 g) were purchased from Changsha Tianqin Biotechnology Co, Ltd, China. All the rats were housed in 6 different metabolic cages, 10 rats were housed in each metabolic cage, and adaptively fed normal rat chow for one week (free access to food and drinking water, temperature 20±2°C, humidity 60±5%, in a 12h light and dark cycle). The rats in normal group (n=10) were intraperitoneally injected with sodium citrate-citric acid buffer (30 mg/kg), followed by feeding of normal diet and normal drinking water. The other five groups (n=10) of rats (fed with high sugar and high fat diet for 4 weeks) were given intraperitoneal injection of streptozotocin (STZ, 30 mg/kg). Blood glucose was measured 72h later. Blood glucose higher than 16.7 mmol/l was set as the standard of the successful construction of diabetic rats. Then, 24 hours urinary protein quantification and urinary protein/creatinine ratio were measured weekly until the increase in albuminuria was equivalent to the elevated levels in human (typically ~10-fold). After the urinary protein excretion rate was reached, it was determined that the DN rats were successfully constructed (Betz and Conway, 2016).

3 groups of DN rats (n=10) were treated with berberine (low dose: 50 mg/kg; moderate dose: 100 mg/kg; high dose: 200 mg/kg) for 20 weeks. For rats in negative control group, after the DN modeling, they were treated with sodium citrate-citric acid buffer (30 mg/kg, 1 time per day), and fed with normal diet and normal drinking water. The animal experiments in this study were approved by the Ethical committee of Haikou Hospital Affiliated to Xiangya Medical College of Central South University (No.2016-027)

### *H&E staining*

At the end of the animal experiments, all the rats were anesthetized and decapitated, renal tissue samples were taken from rats in each group and fixed in 10% buffered formalin. Renal tissues were embedded in paraffin, which was serially sectioned, spread, baked, dried, deparaffinized with xylene, washed with alcohol and water, and subjected to histopathological examination with hematoxylin & eosin (H&E) staining.

### *Determination of urinary protein, iron and creatinine*

After disinfecting and washing the metabolic cages, the rats were placed in the metabolic cages to collect fresh urine and 24h urine samples. Then, urinary total protein was measured by the Pyrotriphen red colorimetric method; urinary iron content was measured by the ferrozine method; urinary creatinine was measured by the sarcosine oxidase method.

### *Measurements of serum creatinine, ferritin, hepcidin, rat total antioxidant capacity and superoxide dismutase*

Orbital venous blood was collected from rats in each group. Serum and plasma blood samples were obtained separately. Serum samples were used for detecting serum creatinine by enzymatic colorimetric method.

Ferritin (#CSB-EL010124RA, Cusabio, Wuhan, China), hepcidin (#CSB-E08826r, Cusabio, Wuhan, China), Rat total antioxidant capacity (TAOC) (#JYM0902Ra, JYMBIO, Wuhan, China) and superoxide dismutase (#CSB-EL022398RA, Cusabio, Wuhan, China) were detected by corresponding enzyme linked immunosorbent assay (ELISA) kit according to the instructions provided by the manufacturer.

### *Immunohistochemical analysis*

Paraffin-embedded renal tissue sections were deparaffinized. Antigen retrieval was performed using citric acid, followed by incubation with 3% $H_2O_2$ for 8 min at room temperature and blocking with goat serum. After the excess PBS on the tissue sections was shaken off, the primary antibodies, including anti-β-Arrestin 1 (#30036, CST, 1:300) and anti-TGF-β1 (#ab25715,

abcam, 1:400), was added and incubated with the tissue sections in a refrigerator at 4°C for 24h. Subsequently, the sections were placed at room temperature for 30 min. Then NovoLink polymer detection system RE7159 (Leica, Germany) was added. The sections were further placed in a 37°C incubator for 30 min. DAB chromogenic solution was next added to stain for 3-5 minutes before the color development was terminated with water. Hematoxylin staining was used for counterstaining for 1 min. After that, the sections were differentiated with 0.5% hydrochloric acid alcohol for 3 s, rinsed with running water for 5-10 min, dehydrated, transparented, mounted, and microscopically examined.

According to Tanaka criteria: 1-4 points were evaluated according to the ratio of the brownish-yellow area of glomeruli or tubules to the area of the entire glomerular area, 1 point: positive area ≤25%; 2 points: 25% <positive area ≤50%; 3 points: 50% <positive area ≤75%; 4 points: positive area >75%; staining intensity of positive areas: 0 point for basically no staining, 1 point for light staining (yellowish), 2 points for moderate staining (brownish-yellow), and 3 points for dark staining (brownish-brown). The scores of both are added as the final value for quantitative analysis.

### *Western blot*

Fresh renal tissues were homogenized using a high-speed mechanical homogenizer and total protein was subsequently extracted using RIPA lysis buffer. 20 μg of protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking in 5% non-fat dry milk for 2h, the membranes were incubated overnight at 4°C with specific primary antibodies including MMP-2 (#ab86607, abcam, 1:1000), MMP-9 (#ab76003, abcam, 1:2000), TIMP3 (#ab187297, abcam, 1:400), DMT1 (#ab140977, abcam, 1:500) and TFR (#ab214039, abcam, 1:1000). The next day, the blots were washed with TBST, added with horseradish peroxidase-labeled secondary antibodies (#BA1054, 1:10000, #BA1050, 1:5000, Bosterbio, USA), and incubated at room temperature for 1h. After washing with TBST, the blots were developed and exposed by adding enhanced ECL chemiluminescence solution, photographed with a gel imaging system, and analyzed by Image-Pro Plus.

### *Statistical analysis*

Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, USA), and all data were expressed as mean±standard deviation. Analysis of variance was used to perform F-test, and multiple comparisons were performed with Duncan's test at P value <0.05. P value <0.05 was considered statistically significant.

## Results

### *Treatment with berberine alleviates pathology of DN rats*

In this study, a high-fat diet together with streptozotocin (STZ, 30 mg/kg) treatment was used for constructing diabetic rat models. After confirming the appearance of diabetic symptoms, urinary protein and

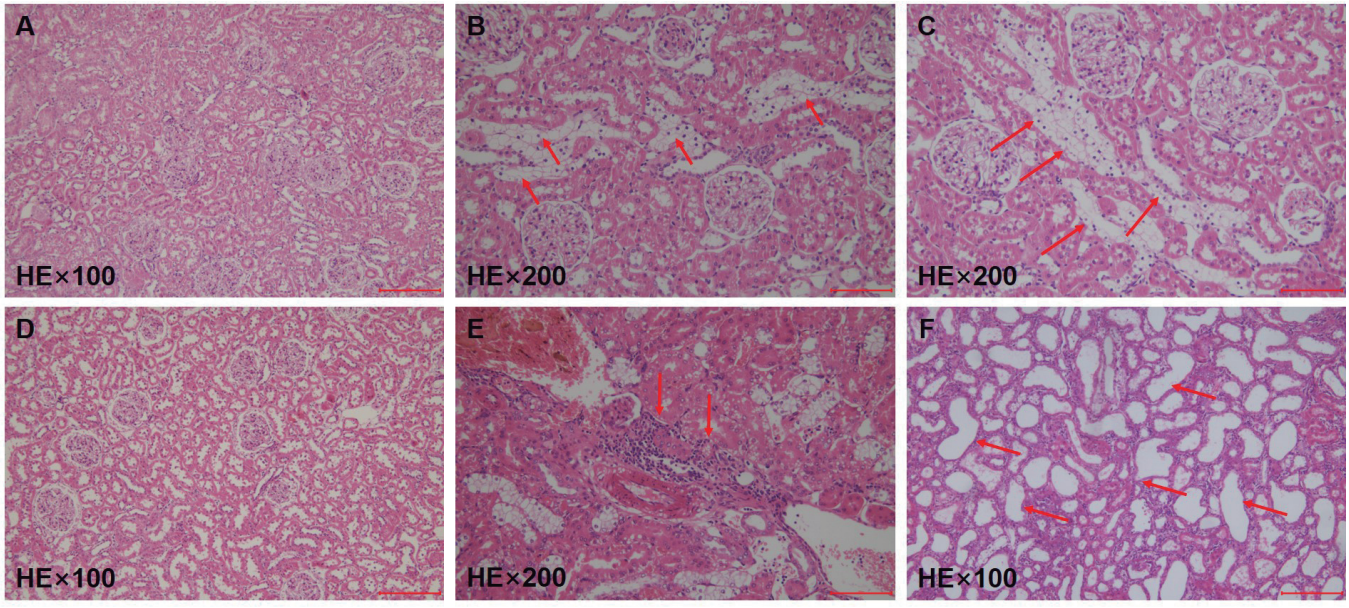


**Fig. 1.** H&E staining was performed to visualize pathological change of renal tissue. **A.** Renal tissue collected from normal rats. **B.** Renal tissue collected from DN rats treated with low dose berberine (50 mg/kg). **C.** Renal tissue collected from DN rats treated with moderate dose berberine (100 mg/kg). **D.** Renal tissue collected from DN rats treated with high dose berberine (200 mg/kg). **E.** Renal tissue collected from DN rats before berberine treatment. **F.** Renal tissue collected from negative control rats.

creatinine levels were subsequently detected once a week till the increase in albuminuria was equivalent to that in human (typically ~10-fold) as well as the successful construction of DN rats. Then, low dose (50 mg/kg), moderate dose (100 mg/kg) and high dose (200 mg/kg) of berberine were used for treating DN rats. As shown in Table 1, with the development of DN, the urinary protein and serum creatinine were both significantly elevated. After berberine treatment, especially at high dose, the ratio of urinary protein to creatinine was significantly lowered. Serum creatinine level also declined to a similar level with normal rats. Moreover, the pathological examination of DN rats (group E) and negative control rats (group F) based on H&E staining showed that, compared with rats in the normal rat (group A), significant vacuolar degeneration was observed in the proximal tubular epithelial cells; a small amount of inflammatory cell infiltration was observed in the interstitium; no hyperplasia of glomerular resident cells was observed; arteriolar endothelial cells were flat and no thrombosis could be observed; blood stasis was observed in some venules (Fig. 1). After berberine treatment, especially in the high-dose group, the pathological changes of renal tissues in DN rats were improved, especially the tubular and interstitial lesions (Fig. 1). Intact tubular epithelial cell structure could be observed (Fig. 1) with no significant vacuolar degeneration, no significant atrophy, and no significant inflammatory cell infiltration and fibrosis formation in the interstitium. On the other hand, it was also demonstrated that treatment of berberine significantly reduced serum glucose, suggesting the ability of berberine to alleviate diabetes (Table 1). These results indicated the renoprotection effects of berberine, which is in agreement with previous reports.

**Table 1.** Physiological characteristics of the rats in each group.

| | Urinary protein/ creatinine | Serum creatinine (μmol/l) | Serum glucose (mmol/l) |
|---|---|---|---|
| A (n=8) | 14.44±3.93d | 29.57±7.63a | 8.97±2.87e |
| B (n=7) | 83.86±10.00c | 35±5.76b | 19.86±2.87c |
| C (n=6) | 72.09±16.75c | 28.8±6.61b | 15.76±1.21cd |
| D (n=6) | 13.60±6.94d | 31.4±6.80b | 12.12±0.76de |
| E (n=9) | 137.36±35.00b | 34.29±4.42b | 27.29±1.52b |
| F (n=6) | 283.09±52.25a | 43.86±4.06b | 45.37±8.37a |
| P value | <0.0001 | =0.0008 | <0.0001 |

A, normal rats; B, DN rats treated with low dose berberine (50 mg/kg); C, DN rats treated with moderate berberine (100 mg/kg); D, DN rats treated with high dose berberine (200 mg/kg); E, DN rats before berberine treatment; F, negative control rats. The P value of multiple comparison (Duncan test) is 0.05, Statistical differences among groups were represented by a, b, c, d and e, respectively.

### *Treatment of berberine alleviates iron overload and oxidative stress in DN rats*

Next, we aimed to further explore the mechanism by which berberine executes its renoprotective functions. Considering the recently reported involvement of iron overload in the development of DN, urinary iron, serum

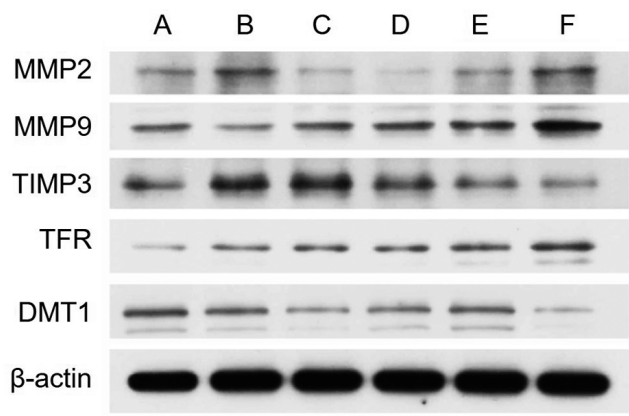


**Fig. 2.** The expression of indicated proteins was detected by western blot. **A.** Protein collected from normal rats. **B.** Protein collected from DN rats treated with low dose berberine (50 mg/kg). **C.** Protein collected from DN rats treated with moderate dose berberine (100 mg/kg). **D.** Protein collected from DN rats treated with high dose berberine (200 mg/kg). **E.** Protein collected from DN rats before berberine treatment. **F.** Protein collected from negative control rats.

**Table 2.** The level of several parameters of the rats in each group.

| | Urinary iron (mg/mmol) | Ferritin (ng/ml) | Hepcidin (pg/ml) | TAOC (U/ml) | Serum SOD (pg/ml) |
|---|---|---|---|---|---|
| A (n=8) | 0.4±0.57c | 18.2±7.66c | 123.17±23.22c | 2.23±0.06a | 12.65±3.18e |
| B (n=7) | 1.93±1.59b | 61.84±8.20b | 119.23±10.42c | 1.52±0.06c | 32.56±11.38c |
| C (n=6) | 1.88±1.03b | 56.01±9.28bc | 111.64±12.81c | 1.52±0.12c | 22.87±2.38d |
| D (n=6) | 2.29±1.32b | 39.09±18.8bc | 110.88±11.52c | 2.03±0.11b | 18.55±3.21de |
| E (n=9) | 1.12±0.57bc | 33.90±14.95a | 310.98±32.27b | 1.53±0.06c | 44.61±4.59b |
| F (n=6) | 4.78±1.56c | 63.15±3.35a | 342.05±25.23a | 2.29±0.06c | 54.06±2.94a |
| P value | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

A, normal rats; B, DN rats treated with low dose berberine (50 mg/kg); C, DN rats treated with moderate berberine (100 mg/kg); D, DN rats treated with high dose berberine (200 mg/kg); E, DN rats before berberine treatment; F, negative control rats. The P value of multiple comparison (Duncan test) is 0.05, Statistical differences among groups were represented by a, b, c, d and e, respectively.

ferritin and hepcidin were detected in all groups of rats. It was demonstrated that urinary iron concentration, serum ferritin and hepcidin level were increased significantly with the progression of DN, which was attenuated by the treatment of berberine, especially in high doses (Table 2). Consistently, the expression of DMT1, an iron transport protein, was downregulated and TFR, a key factor in iron uptake, was upregulated, along with the progression of DN, both of which were moderated by berberine treatment (Fig. 2). Since iron overload is an important factor in oxidative stress during DN development, we further revealed that the decreased levels of total antioxidant capacity (TOCA) and superoxide dismutase (SOD) induced by DN could be recovered by the intervention of berberine to some extent (Table 2). Collectively, we concluded that berberine may attenuate DN and play a renoprotective role by ameliorating iron overload and oxidative stress.

### *Treatment of berberine improves DN-induced renal fibrosis*

To further confirm the renoprotection effects of berberine, the expression level of some key markers in renal fibrosis, including MMP2, MMP9 and TIMP3 were examined by western blot. As shown in Figure 2, expression of MMP2 and MMP9 was distinctly upregulated in DN rats, representing more serious extracellular matrix accumulation and renal fibrosis (groups E&F vs. group A). TIMP3 is a negative regulator of iron overload induced inflammatory response and tissue fibrosis (Zhabyeyev et al., 2018). We found that TIMP3 was apparently downregulated along with the DN progression (groups E&F vs. group A) (Fig. 2). Moreover, according to the results of immunohistochemical staining, β-arrestin-1 expression was decreased (Fig. 3) while TGF-β1 expression was enhanced (Fig. 4)

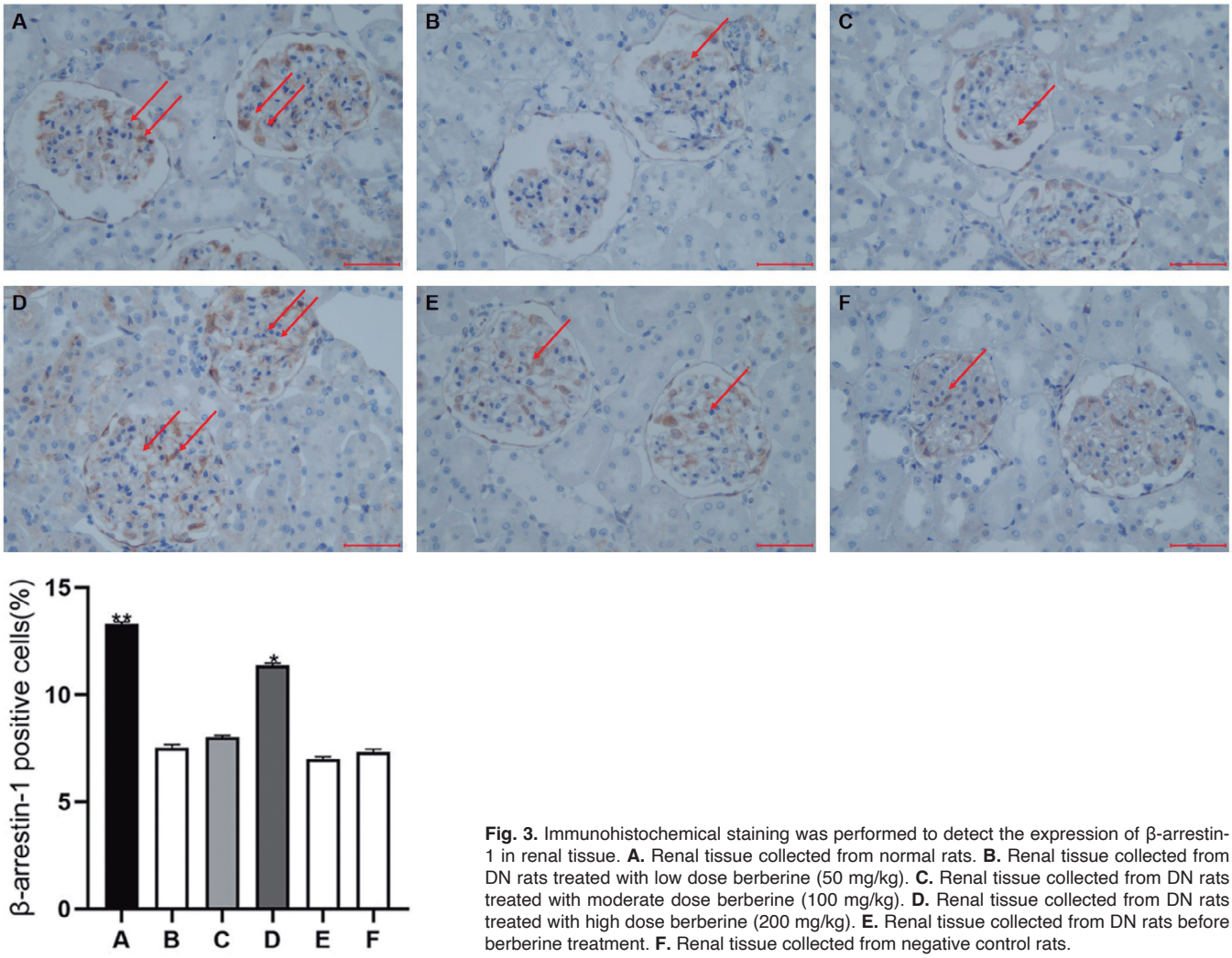


**Fig. 3.** Immunohistochemical staining was performed to detect the expression of β-arrestin-1 in renal tissue. **A.** Renal tissue collected from normal rats. **B.** Renal tissue collected from DN rats treated with low dose berberine (50 mg/kg). **C.** Renal tissue collected from DN rats treated with moderate dose berberine (100 mg/kg). **D.** Renal tissue collected from DN rats treated with high dose berberine (200 mg/kg). **E.** Renal tissue collected from DN rats before berberine treatment. **F.** Renal tissue collected from negative control rats.

in DN (groups E&F vs. group A), also indicating the renal fibrosis induced by DN. Notably, all the expression changes of MMP2, MMP9, TIMP3, β-arrestin-1 and TGF-β1 induced by DN could be partially blocked by berberine treatment (Figs. 2-4) (groups B&C&D vs. E&F).

## Discussion

In recent years, a large number of animal model studies as well as clinical studies have shown that berberine has a good anti-diabetic effect and displays high efficacy in the treatment of T2DM patients with dyslipidemia (Yin et al., 2008a; Zhang et al., 2008). Actually, berberine has been widely used to reduce blood glucose for T2DM patients in China (Yin et al., 2008a,b). Furthermore, some reports indicated that berberine can restore insulin sensitivity and reduce blood glucose for improving diabetes by various mechanisms, such as the activation of AMP-dependent protein kinase, regulation of islet function as well as intestinal microenvironment, and the upregulation of insulin receptor expression (Zhang et al., 2010a,b; Yin et al., 2012). Herein, we also indicate the ability of berberine to reduce blood glucose, as well as the antidiabetic effects.

Over the past decades, accumulating evidence has indicated that berberine can alleviate insulin resistance and glucose metabolism disorders through a variety of mechanisms, thereby protecting pancreatic β-cells and promoting insulin secretion to exert hypoglycemic effects (Yin et al., 2008b; Chang et al., 2014; Pang et al., 2015). Meanwhile, its therapeutic effects on DN have also been comprehensively studied (Sun et al., 2015; Zan et al., 2017; Zhang et al., 2020). It has been proved that berberine can attenuate renal injury, inflammatory response, and high glucose-induced podocyte apoptosis of STZ-induced DN rats by inhibiting the TLR4/NF-κB signaling pathway. Besides, berberine treatment reduced the kidney/body weight ratio, 24-hour urinary protein,

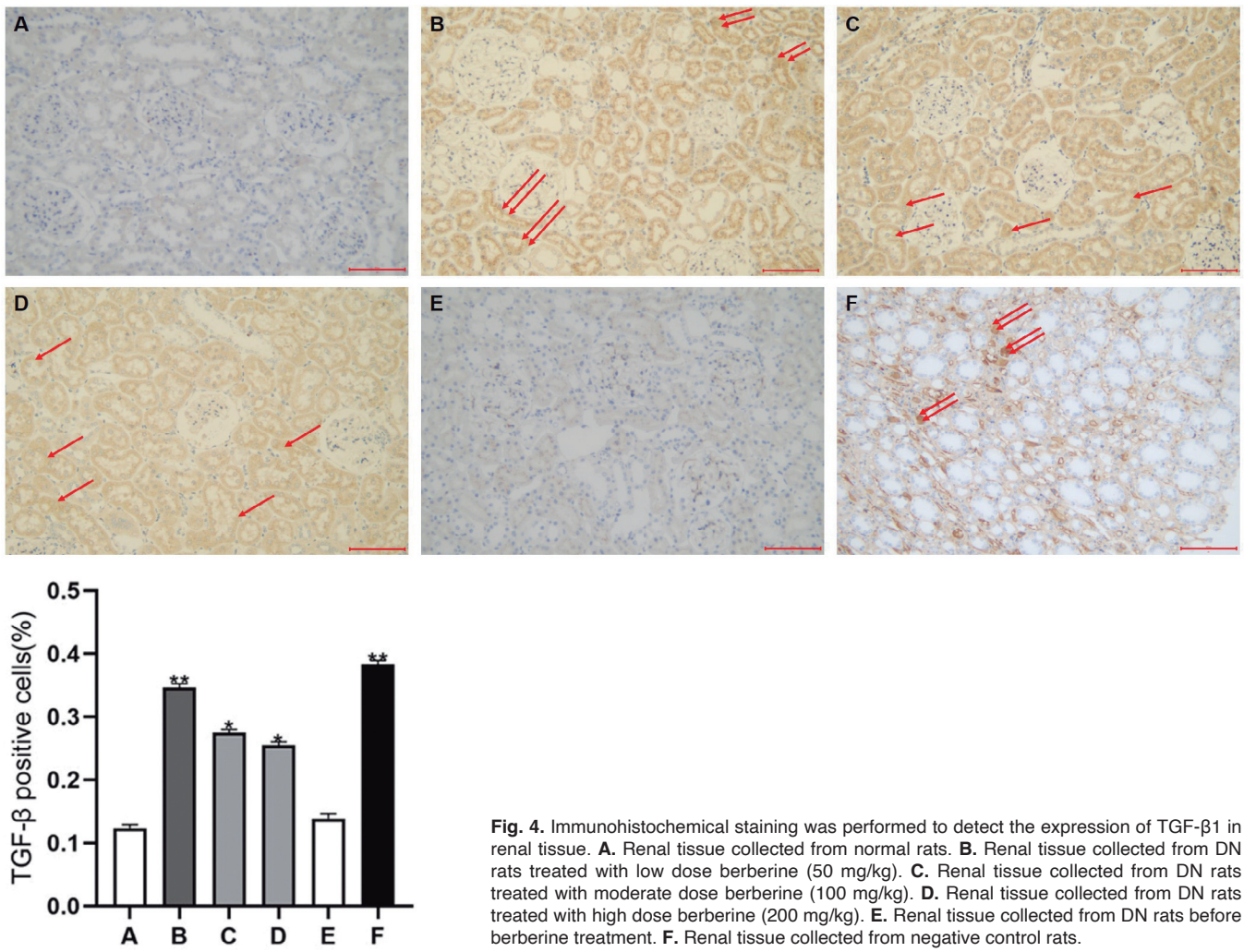


**Fig. 4.** Immunohistochemical staining was performed to detect the expression of TGF-β1 in renal tissue. **A.** Renal tissue collected from normal rats. **B.** Renal tissue collected from DN rats treated with low dose berberine (50 mg/kg). **C.** Renal tissue collected from DN rats treated with moderate dose berberine (100 mg/kg). **D.** Renal tissue collected from DN rats treated with high dose berberine (200 mg/kg). **E.** Renal tissue collected from DN rats before berberine treatment. **F.** Renal tissue collected from negative control rats.

serum creatinine, and hematuria nitrogen, thereby improving DN (Zhu et al., 2018). Similarly, the results in this work also showed that berberine treatment could significantly reduce the urinary albumin/creatinine ratio and the level of creatinine in DN rats and play a renoprotective function. On the other hand, a number of studies have suggested that the renoprotective function of Berberine is also reflected in the relief of renal fibrosis (Li and Zhang, 2017). For example, a previous study demonstrated that berberine can relieve symptoms of renal fibrosis by activating the Nrf2 pathway and inhibiting TGF-β/SMAD/EMT signaling (Zhang et al., 2016). Here, the inhibitory effects of berberine treatment on pathological changes of renal tissues induced by DN as well as TGF-β1 expression also clarified the involvement of suppressing renal fibrosis in berberine-related renoprotective effects. Otherwise, the therapeutic effects of berberine treatment on renal fibrosis were also represented by its influence on the expression of the MMPs/TIMPs system (Ni et al., 2015b). In addition, it has been reported that β-arrestin-1 as well as β-arrestin-2 is abnormally expressed in DN (Tang et al., 2016), and our study showed that this abnormal expression can be restored by the intervention of berberine.

A large number of studies in recent years have shown that iron has a close association with the pathophysiology of diabetes. When iron overload emerges, excessive iron induces the production of large amounts of ROS through the pyrogallol reaction, activates a series of oxidative stress reactions, activates various cytokines, transcription factors and inflammatory mediators, causes glomerular extracellular matrix deposition, and ultimately leads to renal fibrosis; it also reduces the body's antioxidant capacity, further aggravating renal injury, and accelerating the development of DN (Wood, 2015). Moreover, several studies have indicated the increased renal and urinary iron levels in patients or animal models with acute kidney injury or chronic kidney disease (Gutiérrez et al., 2012; Hashemieh et al., 2012; Moreno et al., 2012). Furthermore, animal studies for DN revealed that iron and ferritin levels in the renal proximal tubules were increased and positively correlated with urinary protein (Dominguez et al., 2015). In addition, it was demonstrated that iron chelators are able to effectively reduce macrophage infiltration, alleviate the inflammatory response, and inhibit the expression of fibrosis-related markers in DN renal tissues (Zou et al., 2017). Oral iron chelators can also induce a reduction in proteinuria, which is a major predictor of renal disease progression in patients with DN or non-diabetic glomerular disease (Rajapurkar et al., 2013). Herein, our results also confirmed that berberine fixed iron metabolism index while alleviating the oxidative stress induced by DN. DMT1 is a molecule which plays an important role in iron transport (Ward et al., 2005; Yanatori and Kishi, 2019). In our study, it was illuminated that berberine treatment can recover the DN-induced expression decline of DMT1, which may be an important reason for iron overload. In contrast, TFR, which has an important role in iron uptake (Kosman, 2020), was upregulated in DN and moderated by berberine treatment. These data showed that the regulation of iron overload by berberine may involve iron uptake and transport by TFR and DMT1, respectively.

In conclusion, the results in this study demonstrate that berberine attenuates DN and plays a renoprotective role through ameliorating iron overload and oxidative stress.

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*Author contributions.* Yujing Wang designed this program. Shuling Yue operated the cell and animal experiments. Wen Zhu conducted the data collection and analysis. Feng Cai produced the manuscript which was checked by Chunyun Li. All the authors have confirmed the submission of this manuscript.

*Conflict of interest.* The authors declare no conflict of interest.

*Data availability statement.* The data that support the findings of this study are available from the corresponding author upon reasonable request.

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