ORIGINAL ARTICLE

Exploration on the protective effect of liraglutide on renal injury in diabetic nephropathy rats based on ROS-NLRP3 inflammasomes

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ABSTRACT

Objective: To investigate the effect of liraglutide on diabetic nephropathy in rats and its regulatory mechanism.
Methods: The diabetic nephropathy rat model was constructed with high-glucose-high-fat diet in combination with STZ, and was randomly divided into normal saline group and liraglutide group. The rats in liraglutide group were given sc 200 μg/kg of liraglutide, and the rats in normal saline group were given sc 20 mg/kg of normal saline twice a day for 4 weeks. The normal control group was not treated with any treatment. The biochemical indexes such as rat body weight, 24-hour urine total protein quantification (UTP), fasting blood glucose (FPG), triglyceride (TG), cholesterol (TC), blood urea nitrogen (BUN) and serum creatinine (Scr) were measured. HE staining, Masson staining and PAS staining were used to observe the pathologically morphological changes in renal tissues. Western-blot was used to detect the expression of NLRP3 inflammasome-related protein in renal tissues. Elisa was used to measure the serum levels of interleukin-18 (IL-18) and IL-1β. SPSS 26.0 statistical software and Graph Prism 9.0 software were used for analysis and mapping. The t-test was used for the comparison of measurement data between two groups, one-way ANOVA was used for the comparison among multiple groups, and Tukey's test was used for the comparison in the same group.
Results: Compared with the normal saline group, FBG, UTP, BUN, Scr, TC and TG in the liraglutide group were significantly decreased ($p < .01$), the glomerular basement membrane was slightly thickened, with the tubular lumen slightly dilated and the lesion damage alleviated; the expression levels of NLRP3, ASC and Caspase-1 inflammasome-related proteins were decreased ($p < .01$), and the levels of IL-18 and IL-1β were decreased ($p < .01$).
Conclusions: Liraglutide can inhibit the activation of NLRP3 inflammasome mediated by oxidative stress in renal tissues through ROS-NLRP3 inflammasome pathway, thereby inhibiting the inflammation, and finally playing an anti-diabetic nephropathy renal injury role.

Key Words: Diabetic nephropathy, Liraglutide, ROS-NLRP3 inflammasome

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1. INTRODUCTION

At present, the specific pathogenesis of T2DM is still unclear, and long-term exposure to hyperglycemia will affect the macrovascular and microvascular systems, which will have a significant impact on patients' life quality and overall life expectancy. Diabetic nephropathy (DN) is one of the most common complications in patients with diabetes mellitus and the main cause of mediating chronic kidney disease (CKD) and end-stage renal disease (ESRD).[1] DKD is a metabolic disease mediated by a variety of inflammatory factors such as hyperglycemia, abnormal lipid metabolism, oxidative stress and advanced glycation end products, which run through the entire course of the disease.[2] Evidence from clinical and experimental studies suggests that the inflammatory response is the culprit in the pathogenesis of DKD. NLRP3 inflammasome links the perception of metabolic stress in renal tissues with the activation of the pro-inflammatory cascade by inducing the secretion and maturation of IL-18 and IL-1β; the kallikrein-kinin system promotes the inflammatory process by producing bradykinin and activating its receptors, which further activate protein kinase receptors leading to renal fibrosis.[3] NLRP3 inflammasome is composed of NLRP3 protein, apoptosis-associated speck-like protein containing CARD (ASC), and cysteinyl aspartate-specific protease-1 (Caspase-1).[4] Therefore, to intervene the NLRP3 inflammasome pathway will provide new therapeutic strategies and intervention targets for the prevention and treatment of diabetic nephropathy.

Glucagon-like peptide 1 (GLP-1) is secreted by cells in the gastrointestinal tract, and circulating GLP-1 increases insulin secretion and inhibits glucagon secretion. Due to its extremely short half-life in vivo, its analogues have been developed, and GLP-1 analogues can also exert the same hypoglycemic effect when binding to their receptors. Liraglutide is a long-acting GLP-1 receptor agonist that has been widely used in clinical practice. Multiple studies have shown that liraglutide not only has a good hypoglycemic effect, but also has an effect of antioxidative stress and reducing inflammation in the body.[3] GLP-1 can not only control the inflammation in multiple parts (such as kidneys and blood vessels), but also activate the pathway of cyclic adenosine monophosphate protein kinase A (cAMP-PKA), protecting the kidneys from inflammatory damage.[5] Therefore, GLP-1 would be a promising therapeutic method to prevent DKD by regulating the inflammatory reaction. This study is designed to investigate the relationship between the regulation of NLRP3 inflammasome by liraglutide and renal protection, in order to provide new clues for the clinical treatment of DKD.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 *Research subjects*

4-week-old healthy male Wistar rats (SPF grade, body weight 180-200 g, production license for laboratory animals: SCXK [Beijing] 2019-0010) were purchased from Beijing Sipeifu Biotechnology Co., Ltd. They were kept in the animal room with a constant ambient temperature of $(22 \pm 2)^{\circ}$C and a humidity of 50%-60%, and had free access to food and water with the bedding regularly changed.

2.1.2 *Main reagents*

High-fat and high-sugar foods: Ratio per 100 grams: 60 grams of ordinary feed, 10 grams of milk powder, 13 grams of lard, 10 grams of egg yolk, 7 grams of sugar, and 10 drops of concentrated cod liver oil; Streptozotocin (STZ) was purchased from Beijing Solarbio Science & Technology Co., Ltd.; Liraglutide was purchased from Novo Nordisk; Serum creatinine (SCr), blood urea nitrogen (BUN), urine total protein (UTP), total cholesterol (TC) and serum triglyceride (TG) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute; IL-18 and IL-1β enzyme-linked immuno sorbent assay (ELISA) was purchased from Jiangsu Zeyu Biotechnology Co., Ltd.; NLRP3, ASC, Caspase-1 primary antibody and sheep anti-rabbit secondary antibody were all purchased from Beijing Bioss Technology Co., Ltd.

2.2 Experimental methods

2.2.1 *Animal model preparation and grouping*

All animal experiments were carried out according to the Animal Ethics Therapy Guidelines with the approval of Institutional Animal Care and Use Committee, and 22 healthy male Wistar rats were experimentally fed with normal diet for 1 week, and were randomly divided into two groups: normal control group (n = 6) and diabetic nephropathy model group (n = 16). The rats in the control group were fed with ordinary feed, and the rats in the diabetic nephropathy model group were fed with high-sugar and high-fat feed, after 4 weeks of feeding, the rats in the model group fasted and did not water for 12 hours, weighed themselves in the early morning of the next day, intraperitoneally injected with 30 mg/kg of the ready-made STZ solution, and after 72 hours, the tail vein blood was taken to measure the random blood glucose of the rats, and the random blood glucose $\geq$ 16.7 mmol/L for 3 times, that is, the T2DM rat model was successfully constructed, and the rats were continued to be fed with high-sugar and high-fat feed for 12 weeks. The rats were placed in the metabolic cage to collect the urine of each group of rats for 24 hours, and the urine protein > 20 mg was regarded as the successful establishment of the DKD rat model, and the rats that were successfully modeled were randomly divided

into liraglutide group (n = 8) and normal saline group (n = 8). The liraglutide group was given 200 μg/kg of liraglutide subcutaneously twice a day; the normal saline group was given subcutaneous injection of the same volume of normal saline. No treatment was given to the control group. After 4 weeks of intervention, the relevant laboratory indicators were tested.

2.2.2 *Collection and preservation of specimens*

The surgical instruments required for the experiment were sterilized in advance, and urine samples were collected for 24 hours at 4 weeks, 12 weeks, 16 weeks, and 20 weeks, and the urine output was recorded from 7 am to 7 am the next day, and 10 ml of samples were collected at room temperature at 3,000 r/min, centrifuged for 10 minutes, and stored in a -80°C freezer for the determination of 24 hUTP. After fasting for 12 h, rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium 50 mg/kg, and after laparotomy, 3-5 ml blood was collected from the abdominal aorta, which was left for 15 min at room temperature and then centrifuged (3,000 RPM/min, 15 min), and the supernatant was taken, labeled and packaged with -80 Frozen storage in refrigerator for later detection of renal function, lipids and related inflammatory factors. After the kidneys were fully exposed, the capsule was peeled off to take out the renal tissues, rinsed with normal saline, dried with filter paper, the wet weight of the kidneys was weighed, and the renal index (the sum of bilateral kidney mass/body weight $\times$ 100%) was calculated accordingly; The renal tissues were divided into two parts in the sagittal plane and fixed in 4% paraformaldehyde. The remaining renal tissues were labeled and stored in a -80°C freezer for subsequent detection of the protein of interest.

2.2.3 *Indicator measurement*

The supernatant was obtained by blood centrifugation, and the serum levels of SCr, BUN, TC, TG were determined by use of kits. ELISA was used to detect serum levels of IL-18 and IL-1β; Fasting tail vein blood was collected, and fasting blood glucose (FBG) was measured by a blood glucose meter.

2.2.4 *Detection of the expression of NLRP3 inflammasome by Western blot*

0.1 g of renal tissues were ground in liquid nitrogen until there were no visible particles, with 500 μl of protein lysate added, fully lysed on ice, centrifuged, and the supernatant was taken. Protein concentration determination was performed by bicinchoninic acid (BCA) method. Protein concentration determination was performed by bicinchoninic acid (BCA) method. Furthermore, sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) electrophoresis was performed, the protein was transferred to a polyvinylidene flooride (PVDF) membrane, sealed with 5% skimmed milk powder for 1 h, and washed with TBST 3 times for 10 min each time. NLRP3, ASC, and Caspase-1 primary antibodies were added, incubated overnight at 4°C, and washed with TBST 3 times for 10 min each time. Secondary antibody was added and incubated for 1 h, washed with TBST 3 times for 15 min each time, and colored by use of the enhanced chemiluminescence (ECL) kit. ImageJ was used to analyze the gray values of NLRP3, ASC, and Caspase-1 proteins.

2.3 Statistical treatment

SPSS26.0 statistical software and Graph Prism 9.0 software were used for analysis and plotting. The measurement data were represented by mean $\pm$ standard deviation ($\bar{x} \pm s$), t-test were used in the comparison between two groups, one-way ANOVA was applied to the comparison among multiple groups, and the Tukey test was used for pairwise comparison within the group. The difference ($p < .05$) was statistically significant.

3. RESULTS

3.1 General condition

The rats in the diabetic nephropathy model group were listless, with coarse hair, reddish-brown neck hair, greasy and dull hair all over the body, with the bedding damp and odorous. The blood glucose levels of 2 rats in the liraglutide group was not up to standard, and 2 rats in the normal saline group died during the experiment, which may be an acute complication caused by hyperglycemia.

3.2 Comparison of body weight, kidney weight and renal index of rats in each group

Compared with the control group, the body weight of rats in the normal saline group were decreased, and the kidney weight and renal index were increased on the contrary, with the difference statistically significant ($p < .01$), while there was no difference between the control group and the liraglutide group ($p > .05$). Compared with the normal saline group, the liraglutide group had an increase in body weight, and a decrease in kidney weight and renal index ($p < .01$, see Table 1).

3.3 Comparison of biochemical parameters in each group

Compared with the control group, the normal saline group had a significant increase in FBG, UTP, SCr, BUN, TC and TG ($p < .01$), and the liraglutide group had a significant increase in FBG, UTP, SCr and BUN ($p < .01$). Compared with the normal saline group, the liraglutide group had a significant decrease in FBG, UTP, SCr, BUN, TC and TG ($p < .01$; see Table 2).

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Table 1. Effects of liraglutide on body weight, kidney weight and renal index of rats in each group

	Control Group (n = 6)	Normal Saline Group (n = 6)	Liraglutide Group (n = 6)	F	*p*
Body Weight (g)	588.02±21.05	522.50±15.68$^{\#}$	563.82±12.21*	23.57	< .01
Kidney Weight (g)	2.75±0.75	3.90±0.22$^{\#}$	3.45±0.36$^{\#*}$	29.93	< .01
Renal Index (%)	0.47±0.04	0.69±0.05$^{\#}$	0.66±0.09$^{\#}$	22.80	< .01

Note. Compared with normal control group, $^{\#}p < .01$; Compared with normal saline group, $^{*}p < .01$.

Table 2. Comparison of biochemical parameters in each group

	Control Group (n = 6)	Normal Saline Group (n = 6)	Liraglutide Group (n = 6)	F	*p*
FBG (mmol/L)	5.83±1.69	28.25±1.70$^{\#}$	20.27±2.07$^{\#*}$	84.07	< .01
UTP (mg)	5.24±0.40	25.94±1.67$^{\#}$	21.87±0.74$^{\#*}$	148.6	< .01
Scr (μmol/L)	20.61±1.28	40.93±2.92$^{\#}$	30.27±1.12$^{\#*}$	162.51	< .01
BUN (mmol/L)	5.32±0.46	13.49±1.00$^{\#}$	7.79±0.55$^{\#*}$	207.09	< .01
TC (mmol/L)	0.92±0.22	2.05±0.34$^{\#}$	1.16±0.22*	26.30	< .01
TG (mmol/L)	0.97±0.20	2.01±0.33$^{\#}$	1.20±0.42*	20.48	< .01

Note. Compared with normal control group, $^{\#}p < .01$; Compared with normal saline group, $^{*}p < .01$.

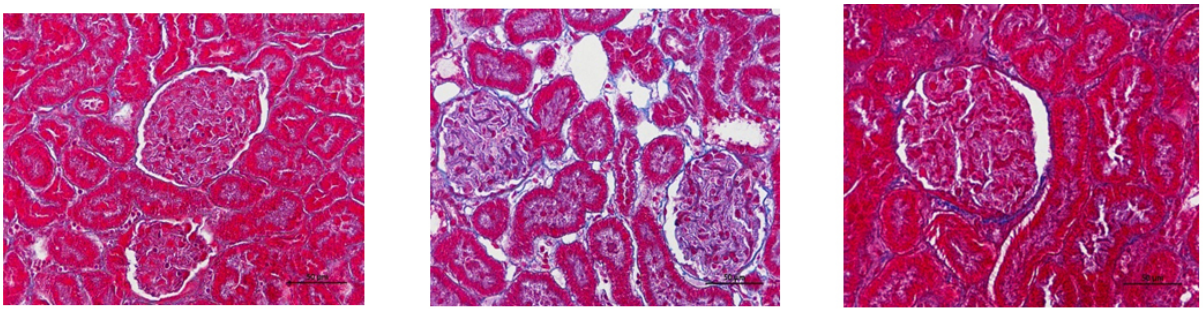
3.4 Effect of liraglutide on histomorphology in rats with DKD

3.4.1 *HE staining results of renal tissues*

HE staining showed that the glomerular structure of the control group was intact, the renal tubules were neatly arranged, and there was no matrix hyperplasia; In the normal saline group, the glomerular contour was not clear, the renal tubular vacuoles were increased, the renal tubules were disordered, inflammatory cells were infiltrated, and some cells were shed; the renal pathological condition in the liraglutide group was relieved in comparison with that in the normal saline group.

3.4.2 *Masson staining results of renal tissues*

After Masson staining, the glomerular basement membrane and mesangial matrix of the normal control group were normal, and no interstitial fibrosis was observed; Compared with the normal control group, the renal tubules in the normal saline group atrophied, and a large amount of blue collagen deposition appeared in the glomeruli, renal tubules and renal interstitium; glomerular and renal interstitial collagen deposition was reduced in rats in the liraglutide group, as shown in Figure 1.

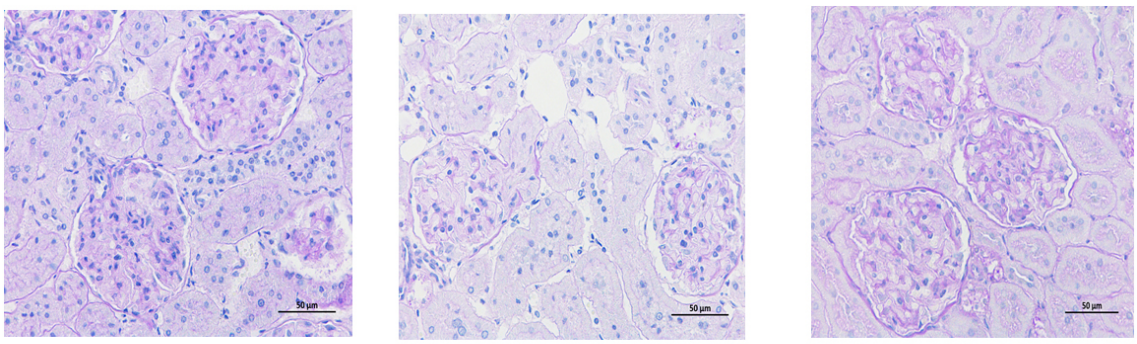


A. Control Group B. Normal Saline Group C. Liraglutide Group

Figure 1. Masson staining of renal tissues in each group (400×)

3.4.3 *PAS staining results of renal tissues*

After PAS staining, the glomeruli in the normal control group were uniform and smooth, and the tubular basement membrane and mesangial matrix were normal; Compared with the control group, the glomerular basement membrane in the normal saline group was significantly thickened, the mesangial cells and stroma were increased, and some renal tubules had vacuolar-like degeneration, and renal interstitial edema was observed; in the liraglutide group, the glomerular basement membrane was slightly thickened, the tubular lumen was slightly dilated, and the lesion damage was reduced. See Figure 2 for details.



A. Control Group B. Normal Saline Group C. Liraglutide Group

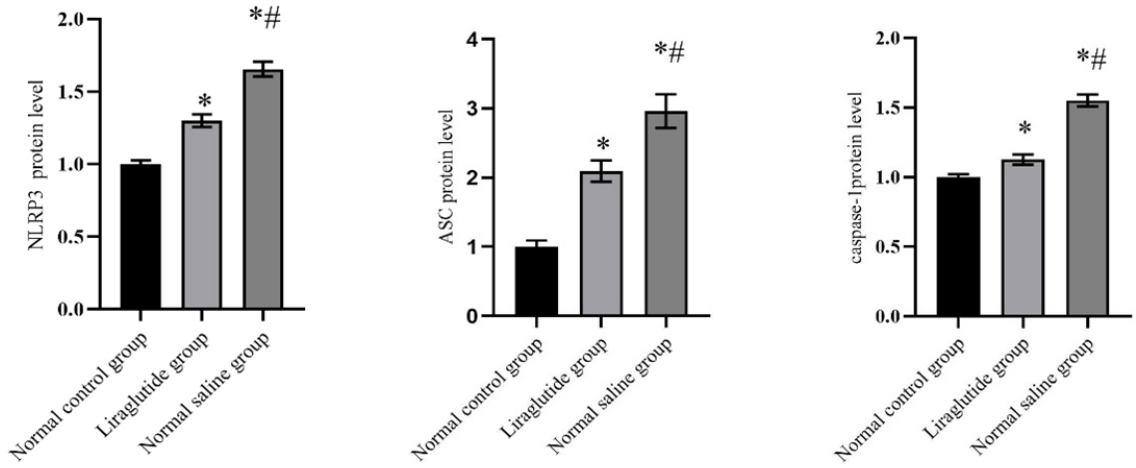
Figure 2. PAS staining of renal tissues in each groupc (400×)

3.5 Determination of the expression of NLRP3 inflammasome protein in the liver of rats in each group by means of Western-blot

Western-blot showed that the expression of NLRP3 protein, ASC and Caspase-1 protein in the normal saline group were significantly increased, but decreased after liraglutide intervention in comparison with the control group. The results showed that liraglutide intervention significantly improved the expression of NLRP3 inflammasome in the kidney tissues of rats with diabetic nephropathy, preventing the progression of the disease course in rats with DKD (see Figure 3).

3.6 Determination of the expression of IL-1β and IL-18 in the serum by ELISA

Compared with the control group, the levels of IL-1β and IL-18 in the serum in the normal saline group were significantly increased, but decreased significantly after liraglutide intervention ($p < .01$; see Table 3, Figure 4). The results indicated that the intervention of liraglutide could significantly improve the levels of IL-1β and IL-18 inflammatory factors in the serum in DKD rats.



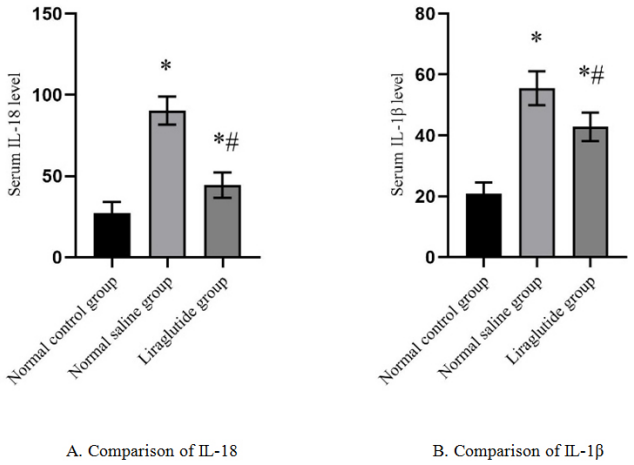
A. Comparison of NLRP3 B. Comparison of ASC C. Comparison of Caspase-1

Figure 3. Comparison of NLRP3, ASC, and Caspase-1 protein expression in rats of each group
*Compared with the control group, * $p < .01$; compared with the normal saline group, # $p < .01$.*

Table 3. Effect of Liraglutide on the expression of IL-18 and IL-1β in the serum in each group

	Control Group (n = 6)	Normal Saline Group (n = 6)	Liraglutide Group (n = 6)	F	*p*
IL-18 (pg/ml)	27.15±3.97	90.26±2.82$^{\#}$	44.38±2.43$^{\#*}$	104.5	.01
IL-1β (pg/ml)	20.87±1.01	55.53±0.89$^{\#}$	42.85±0.98$^{\#*}$	83.16	.01

Note. Compared with the control group, $^{\#}p < .01$; Compared with the normal saline group, $^{*}p < .01$.



A. Comparison of IL-18 B. Comparison of IL-1β

Figure 4. Comparison of the expression of IL-18 and IL-1β in the serum in rats of each group

*Compared with the control group, * $p < .01$; compared with the normal saline group, # $p < .01$.*

4. Discussion

Diabetic nephropathy is one of the main causes of morbidity and mortality in diabetic patients, with an incidence of up to 30% and 50% in patients with type 1 and type 2 diabetes, which seriously endangers patients' health.[6] There are no specific drugs for the treatment of diabetic nephropathy. A meta-analysis showed that GLP-1 receptor agonists could significantly improve the renal outcomes in patients with type 2 diabetes.[7] Studies have shown that liraglutide, as a long-acting GLP-1 receptor agonist, can relieve renal injury caused by diabetic nephropathy by lowering the level of blood glucose, inhibiting oxidative stress and inflammatory responses.[8,9] However, the mechanism of liraglutide on renal injury in type 2 diabetic nephropathy is unclear so far. In this study, the rat model of diabetic nephropathy was constructed by combining high-sugar and high-fat diet combined with low-dose STZ to observe the protective effect of liraglutide on renal injury in rats with diabetic nephropathy was observed. The results showed that after 4 weeks of modeling, the levels of 24hMAER, Scr and BUN were significantly increased, and the morphological structure of kidney tissues was obviously damaged, indicating that the rat model of diabetic nephropathy was successfully constructed. Liraglutide was able to improve renal function indicators and blood glucose level, and relieve renal damage in comparison to the control group. As an important pathological mechanism for the progression of diabetic nephropathy, inflammatory responses play a key role in the renal impairment caused by diabetic nephropathy. A large number of studies have shown that the inflammatory factors, signaling pathways and their downstream products in the process of the inflammatory response can significantly relieve the renal injury of diabetic nephropathy.[10,11] Studies have confirmed that moderate inflammatory responses can produce protective immune effects on the body, and excessive inflammatory responses such as inflammatory storm and cascade reaction will lead to the destruction of the body's immune function and the aggravation of renal injury caused by diabetic nephropathy.[12] The activation of NLRP3 inflammasome was observed in the renal biopsy from DN patients and in renal tissues from DN rats.[13] NLRP inflammasomes are a type of protein complexes made up of NLRP3, ASC and pro-Caspase 1. The results of this study showed that the increase in kidney weight in DN rats and glomerular hypertrophy led to an increase in kidney size and weight, which was a morphological marker of early DN. The HE staining results of renal tissues showed that the glomerular morphology of the liraglutide group was more regular, and the renal tubular edema was reduced. On the contrary, rats in the normal saline group showed obvious pathological changes, including irregular glomerular morphology and tubular edema with partial necrosis, indicating that liraglutide could exert a protective effect on DN. The study from Qiu et al.[14] showed that in patients with type 2 diabetes, there was an independent correlation between the rate of albumin excretion in urine and the level of IL-18 in serum and urine, and that the level of IL-18 in serum and urine was positively correlated with the degree of proteinuria, suggesting that these inflammatory markers may be independent risk factors for the development of DN. The elevated levels of IL-1β can lead to the proliferation of renal proximal tubular epithelial cells and the increase in endothelial cell permeability, induce the expression of intercellular adhesion factor-1 (ICAM-1), transforming growth factor-β1 (TGF-β1) and cadherin, and affect glomerular hemodynamic changes, further aggravating the renal damage. Sakai et al.[15] showed that inhibiting the activation and expression of NLRP3 inflammasome in DN patients can improve renal function and delay renal pathological changes. NLRP3 inflammasome links diabetic renal metabolic response to the activation of the pro-inflammatory cascade by inducing the generation of IL-1β and IL-18. The results of this study are consistent with this, after liraglutide intervention, the expression of NLRP3 inflammasome in rat renal tissues were decreased, and its downstream inflammatory cytokines IL-18 and IL-1β were also decreased, indicating that liraglutide may inhibit the activation of NLRP3 inflammasome, thereby exerting a protective effect on the kidney of DN rats. In conclusion, there exists the activation of NLRP3 inflammasome in DN, and liraglutide may delay the progression of DN by inhibiting the expression of NLRP3 inflammasome. NLRP3 inflammasome may become a new target for the prevention and treatment of DN, and it is needed to study its specific mechanism of action in the future.

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AUTHORS CONTRIBUTIONS
Jun Xue and Xiuqi Ma are responsible for the overall idea and review of the article, Xuehui Wu, Jiali He, Weiling Song and Hongju Li are responsible for data collection and writing, all authors read and approved the final manuscript.

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