

# Therapeutic efficacy of liraglutide on diabetic nephropathy mice by inhibiting inflammatory factors

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## Abstract

This study was to investigate the role and mechanism of liraglutide in the treatment of diabetic nephropathy (DN) mice. A mouse model of streptozotocin-induced DN was established. The mice were intraperitoneally injected with liraglutide at a dose of 200 µg/kg for 6 weeks. The expression of interleukin-6 (IL-6), tumor necrosis factor (TNF), and nuclear factor kappa B (NF-κB) messenger RNA (mRNA) in renal tissue of mice was examined by real-time quantitative polymerase chain reaction (PCR). Meanwhile, the expression of IL-6 and TNF protein in renal tissue of mice was detected by western blot, while the expression of NF-κB protein in renal tissues of each group was detected by immunofluorescence. After 6 weeks of intervention, the blood glucose (GLU), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), and weight of the liraglutide group were significantly lower than those of the DN group ( $P < 0.01$  or  $P < 0.05$ ), whereas high-density lipoprotein (HDL) was significantly increased ( $P < 0.05$ ). At the same time, the microscale albuminuria (MAU) and *N*-acetyl-β-D-glucosaminidase (NAG) in the liraglutide group were significantly lower than those in the DN group ( $P < 0.05$ ). Moreover, the urea (UR), creatinine (CR), and uric acid (UA) in the liraglutide group were significantly lower than those in the DN group ( $P < 0.01$  or  $P < 0.05$ ). In addition, the mRNA and proteins of IL-6, TNF, and NF-κB in the liraglutide group were significantly lower than those in the DN group ( $P < 0.05$ ). In conclusion, the mechanism of liraglutide in the treatment of DN may be related to the inhibition of the expression of genes and proteins of inflammatory factors.

## Keywords

diabetic nephropathy, IL-6, inflammatory factor, liraglutide, NF-κB, TNF

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## Introduction

Diabetic nephropathy (DN) is a common diabetic microvascular complication and is currently considered to be the leading cause of end-stage renal failure.<sup>1</sup> In recent years, it has been shown in the studies that type 2 diabetes is a chronic low-grade inflammatory disease, and a variety of inflammatory factors are involved in the occurrence and development of type 2 diabetes and its complications.<sup>2</sup> Abnormal increase in interleukin-6 (IL-6), tumor necrosis factor (TNF), nuclear factor kappa B (NF-κB) messenger RNA (mRNA) and the elevation of their protein expression levels are related

to the occurrence of diabetes.<sup>3</sup> Therefore, the treatment of DN can be performed by targeting

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inflammatory factors. Glucagon-like peptide 1 (GLP-1) is an intestinal hormone composed of 30 amino acid residues.<sup>4</sup> Under physiological conditions, GLP-1 can be secreted by L cells of the ileum and colon mucosa in a large amount under the stimulation of food, thereby activating the GLP-1 receptor of islet  $\beta$  cells, promoting insulin secretion, and inhibiting glucagon secretion. Eventually, the effect of further regulating and maintaining the stability of blood glucose is achieved. Because of this, it was further verified in some studies that GLP-1 has a certain anti-inflammatory effect. Liraglutide is a long-acting analogue of human GLP-1, which has 97% or more homology with native GLP-1 and is not easily degraded. In addition, liraglutide has a certain anti-inflammatory effect.<sup>5</sup> At present, there are few reports on the effects of liraglutide on DN. Therefore, the therapeutic effect of liraglutide on DN mice was explored in this study to determine the mRNA and protein expression of IL-6, TNF, and NF- $\kappa$ B and to investigate the role of liraglutide in the treatment of DN mice by affecting inflammatory factors, thereby laying the experimental foundation for the development of new target drugs for treating DN mice.

## Materials and methods

### *Drugs, reagents, and main instruments*

Liraglutide injection (trade name: Victoza) was purchased from Novo Nordisk, Denmark; streptozotocin (STZ) was purchased from Sigma, USA; SYBR Green Master was purchased from Roche, USA; and RNA extraction kit was purchased from Omega, USA. A large number of antibodies, including  $\beta$ -actin murine primary antibody, IL-6 murine primary antibody, TNF murine primary antibody, NF- $\kappa$ B murine primary antibody, and goat anti-mouse IgG H&L (HRP) antibody, goat anti-mouse IgG H&L (FITC) were all obtained from Beijing Abmaking Biotechnology Co., Ltd. *Taq* enzyme and reverse transcription kit were purchased from Takara Co., Japan. Citric acid and sodium citrate as well as sodium carboxymethyl cellulose are purchased from Shanghai YuanMu Biological Technology Co., Ltd, China. The UV spectrophotometer was purchased from Shimadzu Corporation, Japan. The real-time quantitative polymerase chain reaction (PCR) amplification instrument was purchased from ABI Company,

USA. The glucometer was purchased from Sinocare Inc., China, and the micronucleic acid detector was purchased from Beijing Yuanping HAO Biological Technology Co., Ltd, China.

### *Laboratory animals and feed*

C57BL/6 mice, 30 males, weighing 18 to 23 g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd, China. Mouse feed is purchased from Shanghai genegen Biological Technology Co., Ltd.—standard feed: 18% protein, 4% fat, 49% carbohydrate, 5% fiber, 8% ash, 7% moisture, 7% compound amino acid, 1% compound vitamin, and 1% mixed salt. High-fat feed: 18% protein, 35% fat, 18% carbohydrate, 5% fiber, 8% ash, 7% moisture, 7% compound amino acid, 1% compound vitamin, and 1% mixed salt. All experimental procedures were approved by the Ethics Committee of Kunming Medical University. The approval number assigned by the committee was no. 83617382. This study conformed to institutional guidelines for the care and use of experimental animals.

### *Establishment and experimental grouping of DN mouse model*

In total, 30 C57BL/6 mice aged 6 weeks were fed adaptively for 1 week and 20 mice were injected with STZ solution in the left lower abdominal cavity at a dose of 50 mg kg<sup>-1</sup> to make DN model. The STZ was prepared with citric acid–sodium citrate buffer (pH 4.5) before use. The mice were injected every other day for three times and were fed with high-fat diet at the same time. In all, 10 mice in the control group were injected with the same amount of citric acid–sodium citrate buffer, and a standard feed diet was administered. Moreover, the mice were housed in the same animal room. After 4 weeks, random blood glucose was measured for three consecutive days. If the blood glucose levels of the mice in the model group were  $\geq 2$  g L<sup>-1</sup>, the urine protein and renal function indexes were significantly higher than those of the control group, indicating that the mouse model of DN was successfully established.

The experimental animals were divided into three groups: normal control group (control), DN group (DN), and liraglutide treatment group (Liraglutide), with 10 rats in each group. In the liraglutide group, mice with successful modeling were intraperitoneally injected with liraglutide at a

daily dose of 200  $\mu\text{g/kg}$ , and the same amount of saline was given in the mice of the DN group. A total of 10 normal C57 mice were taken as the control group and injected with the same amount of normal saline. The three groups of mice were continuously intervened for 6 weeks. Blood glucose is measured every 2 weeks during this period, followed by weighing of body mass once a week and administration of standard diet.

#### *Collection of animal specimens and determination of biochemical indexes*

The experimental mice were fasted for 12 h, and the urine samples were collected from the metabolic cages of mice; microscale albuminuria (MAU) in urine and urinary *N*-acetyl- $\beta$ -D-glucosaminidase (NAG) were detected by automatic biochemical analyzer. The body weight of the mice was weighed at 9 a.m., the mice were anesthetized, and blood was taken from the eyelids. Then, the blood was collected in a centrifuge tube and incubated at 37°C for 30 min. Then, the blood sample was placed in a 4°C refrigerator for 2 h, and centrifuged at 3000 $\times$ g conditions. After centrifugation for 10 min, the serum was aspirated and analyzed by an automatic biochemical analyzer. Biochemical indicators, including blood glucose (GLU), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) in serum, and renal function indicators, including urea (UR), creatinine (CR), and uric acid (UA), were detected.

#### *Detection of IL-6, TNF, and NF- $\kappa$ B mRNA expression in renal tissues by real-time PCR*

Kidney tissue of 50 mg (both cortex and medulla) was taken out from the -80°C refrigerator, placed in a pre-cooled mortar, ground to a fine powder on ice, and then total RNA was extracted according to the RNA extraction kit. RNA of 2  $\mu\text{g}$  was taken, and complementary DNA (cDNA) was synthesized by reverse transcription reaction using oligo (dT) as a universal primer—the upstream primer of the IL-6 gene: 5'-ACCAGAGGAAATTTTCAAT-AGGC-3' and the downstream primer: 5'-TGATGCACTTGCAAGAAAACA-3'; the upstream primer of TNF gene: 5'-TCAGCCTCTTCTCATTC-3' and the downstream primer:

5'-TTGGTGGTTTGCTACGAC-3'; the upstream primer of the NF- $\kappa$ B gene: 5'-ACCGCCGTG CAGGATGAGA-3' and the downstream primer: 5'-ACGGCCAAGTGCAGAGGTGTC-3'. The real-time PCR reaction mixture was composed of the following: 1  $\mu\text{L}$  of cDNA, 1  $\mu\text{L}$  of primers, 10  $\mu\text{L}$  of SYBR Green fluorescent dye, and 7  $\mu\text{L}$  of sterile distilled water. The reaction procedure is as follows: heating at 50°C for 2 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing at 60°C for 1 min, with the amplification for 40 cycles, and the relative expression levels of IL-6, TNF, and NF- $\kappa$ B mRNA were measured.

#### *Expression of IL-6, TNF, and NF- $\kappa$ B proteins in the renal tissues detected by western blot*

Take the renal tissues (both cortex and medulla) of three groups of experimental mice, add 200  $\mu\text{L}$  phosphate-buffered saline (PBS) + sucrose solution, grind on the ice with the grinding rod, then blow with 1 mL of injection needle, centrifuge at 700 $\times$ g for 30 min at 4°C. Then, take appropriate amount of protein solution, measure the protein concentration with a micro-nucleic acid detector, and place the remaining protein solution in a -80°C refrigerator for later use. The above protein solution (100  $\mu\text{g}$ ) was aspirated, subjected to electrophoresis loading, and then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the films were transferred so as to transfer the proteins onto polyvinylidene fluoride (PVDF). Then, the membranes were blocked with 5% bovine serum albumin (BSA) buffer (TBST) for 2 h, and they were incubated overnight with antibodies of IL-6, TNF, and NF- $\kappa$ B at 4°C. Consequently, the membranes were incubated with goat anti-mouse IgG H&L (HRP) antibody at room temperature for 2 h; finally, the horseradish peroxidase chromogenic reagent (DAB (3,3'-diaminobenzidine)) was added for color development, and the results were observed within 30 min. At the same time,  $\beta$ -actin served as an internal control; then, the density of each group of proteins was detected by an imaging system and photographed; the optical density was calculated by ImageJ software.

**Table 1.** Comparison of blood biochemical indexes in mice of each group ( $\bar{x} \pm s$ ).

Groups	n	GLU (g L <sup>-1</sup> )	TC (mg L <sup>-1</sup> )	TG (mg L <sup>-1</sup> )	HDL (mg L <sup>-1</sup> )	LDL (mg L <sup>-1</sup> )	Weight (g)
Control	10	1.16 ± 0.07	825.34 ± 6.41	647.32 ± 15.68	563.32 ± 27.28	117.54 ± 6.73	31.12 ± 1.71
DN	10	3.65 ± 0.48 <sup>ΔΔ</sup>	1558.25 ± 7.19 <sup>Δ</sup>	1031.67 ± 78.15 <sup>ΔΔ</sup>	445.17 ± 52.23 <sup>ΔΔ</sup>	159.12 ± 8.93 <sup>ΔΔ</sup>	35.11 ± 1.94 <sup>ΔΔ</sup>
Liraglutide	10	3.03 ± 0.25*	786.44 ± 9.23*	862.21 ± 80.16*	658.05 ± 64.74*	112.14 ± 5.07*	33.73 ± 1.80*

GLU: blood glucose; TC: total cholesterol; TG: triglyceride; LDL: low-density lipoprotein; HDL: high-density lipoprotein; DN: diabetic nephropathy. Compared with the control group, <sup>ΔΔ</sup> $P < 0.01$ , <sup>Δ</sup> $P < 0.05$ ; compared liraglutide group with the DN group, <sup>\*\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$ .

**Table 2.** Comparison of microprotein content in urine of mice in each group ( $\bar{x} \pm s$ ).

Groups	n	MAU/CR (mg/μmol)	NAG/CR (U/μmol)
Control	10	0.85 ± 0.18	4.62 ± 1.54
DN	10	3.37 ± 1.01 <sup>ΔΔ</sup>	9.66 ± 3.01 <sup>ΔΔ</sup>
Liraglutide	10	1.66 ± 0.77*	8.01 ± 1.99*

MAU: microscale albuminuria; NAG: N-acetyl-β-D-glucosaminidase; DN: diabetic nephropathy. Compared with control group, <sup>ΔΔ</sup> $P < 0.01$ ; compared liraglutide group with DN group, <sup>\*</sup> $P < 0.05$ .

### Statistical analysis

The experimental data were represented by  $\bar{x} \pm s$  and analyzed by SPSS 23.0 statistical software. The comparison of multiple groups was performed by one-way analysis of variance (ANOVA).  $P < 0.05$  was considered to be statistically significant.

## Results

### Comparison of blood biochemical indexes in mice of each group

The GLU, TC, TG, LDL, and weight in the DN group were higher than those in the control group ( $P < 0.01$  or  $P < 0.05$ ), while the HDL was lower than that of the control group ( $P < 0.01$ ). After 6 weeks of intervention, the GLU, TC, TG, LDL, and weight of the liraglutide group were significantly lower than those of the DN group ( $P < 0.01$  or  $P < 0.05$ ), and HDL significantly increased when compared with DN group ( $P < 0.05$ ), as shown in Table 1.

### Comparison of microprotein content in urine of mice in each group

The MAU and NAG in the DN group were higher than those in the control group ( $P < 0.01$ ). After 6 weeks of intervention, the MAU and NAG in the liraglutide group were significantly lower than those in the DN group ( $P < 0.05$ ), as shown in Table 2.

### Comparison of UR, CR, and UA contents in blood of each group of mice

UR, CR, and UA in the DN group were higher than those in the control group ( $P < 0.01$ ). After 6 weeks of intervention, UR, CR, and UA in liraglutide group were significantly lower than those in DN group ( $P < 0.01$  or  $P < 0.05$ ), as displayed in Table 3.

### Comparison of relative mRNA expression IL-6, TNF, and NF-κB in renal tissues of each group

The relative mRNA levels of IL-6, TNF, and NF-κB in the DN group were significantly higher than those in the control group ( $P < 0.01$ ). After 6 weeks of intervention, the relative levels of IL-6, TNF, and NF-κB mRNA in the liraglutide group were significantly decreased when compared with those of the DN group ( $P < 0.05$ ), as shown in Figure 1.

### Comparison of IL-6, TNF, and NF-κB protein expression in renal tissues of mice

The expression of IL-6, TNF, and NF-κB in renal tissue of the DN group was significantly enhanced when compared with those of the control group ( $P < 0.01$ ). After 6 weeks of intervention, the expression of IL-6, TNF, and NF-κB in renal tissue of the DN group was significantly decreased when compared with those of the DN group ( $P < 0.05$ ), as shown in Figure 2.

## Discussion

More than 90% of diabetic patients are type 2 diabetes mellitus (T2DM). Insulin levels in patients with T2DM are not low, and some are even higher than normal levels. However, the presence of insulin resistance or resistance in patients, which results in insulin not being effectively exerted in the patients, leads to a relative lack of insulin in patients.<sup>7</sup> DN is the most common complication

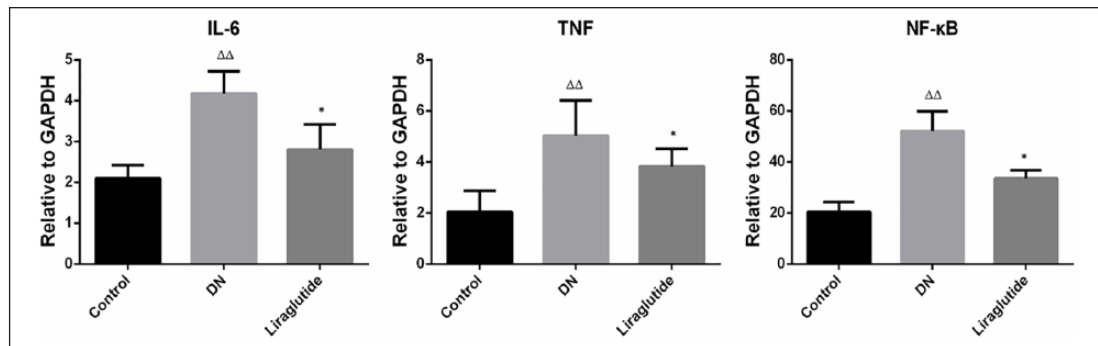


**Table 3.** Comparison of UR, CR, and UA contents in blood of each group of mice ( $\bar{x} \pm s$ ).

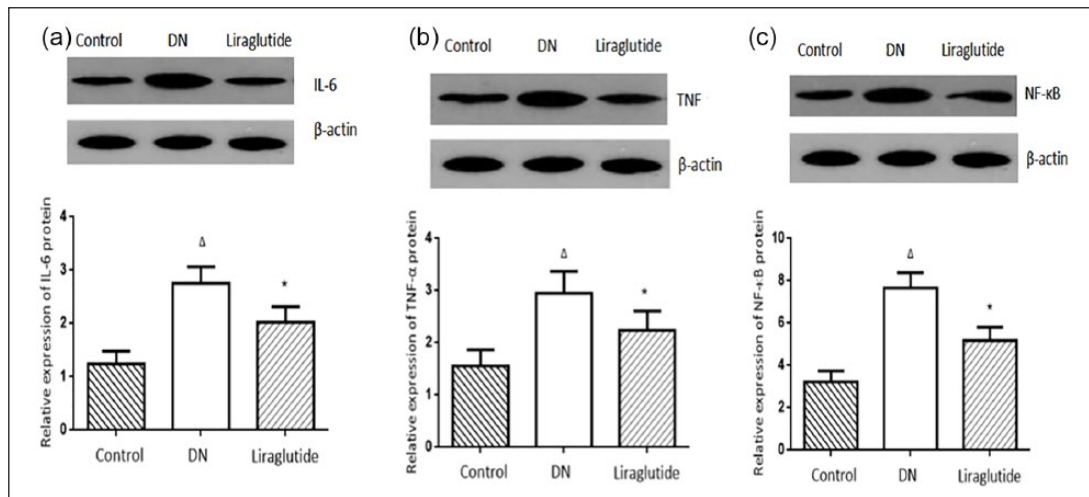
Groups	n	UR (mmol L <sup>-1</sup> )	CR ( $\mu$ mol L <sup>-1</sup> )	UA ( $\mu$ mol L <sup>-1</sup> )
Control	10	9.23 $\pm$ 0.71	31.67 $\pm$ 1.21	281.16 $\pm$ 32.67
DN	10	17.45 $\pm$ 1.78 <sup><math>\Delta\Delta</math></sup>	39.85 $\pm$ 0.93 <sup><math>\Delta\Delta</math></sup>	376.35 $\pm$ 8.71 <sup><math>\Delta\Delta</math></sup>
Liraglutide	10	11.92 $\pm$ 1.13*	34.46 $\pm$ 1.62*	324.15 $\pm$ 19.06*

UR: urea; CR: creatinine; UA: uric acid; DN: diabetic nephropathy.

Compared with control group,  <sup>$\Delta\Delta$</sup>  $P < 0.01$ ; compared liraglutide group with DN group, <sup>\*</sup> $P < 0.01$ , <sup>\*</sup> $P < 0.05$ .

**Figure 1.** Comparison of relative mRNA expression IL-6, TNF, and NF- $\kappa$ B in renal tissues of each group.

Compared with control group,  <sup>$\Delta\Delta$</sup>  $P < 0.01$ ; compared liraglutide group with DN group, <sup>\*</sup> $P < 0.05$ .

**Figure 2.** (a) Comparison of IL-6 protein expression in kidney tissues of each group, (b) comparison of TNF protein expression in kidney tissues of each group, (c) comparison of NF- $\kappa$ B protein expression in renal tissues of each group.

Compared with control group,  <sup>$\Delta\Delta$</sup>  $P < 0.05$ ; compared liraglutide group with DN group, <sup>\*</sup> $P < 0.05$ .

(the cumulative rate is as high as 30%–35%) and death factor of diabetes.<sup>8</sup> Therefore, research and development of new drugs for the prevention and treatment of DN will have important value and significance.

Liraglutide is an analogue of long-acting human GLP-1, which has been widely used as a new anti-diabetic drug.<sup>9</sup> It was demonstrated in the studies that liraglutide has the effect of controlling blood glucose, reducing body weight, improving insulin

resistance, and reducing the risk of cardiovascular disease.<sup>10</sup> Moreover, it was also showed in the studies that liraglutide significantly decreases the lipid content in the liver of diabetic rats, reduces liver lesions in diabetic rats, and reduces  $\beta$ -cell apoptosis induced by cytokines and free fatty acids.<sup>11</sup> In this study, liraglutide can improve biochemical indicators, such as GLU, TC, TG, LDL, and weight in mice with DN, and thus reduce the burden on the kidneys of diabetic mice. In

addition, it was further confirmed in the research that the trend of elevated MAU and NAG in the urine of mice with DN was inhibited by the treatment with liraglutide. Meanwhile, the results of renal function suggested that the UR, CR, and UA indexes in the blood of DN mice were recovered, and the function of affected kidneys was improved.

There is increasing evidence that inflammation is involved in the development of DN and can accelerate the onset of diabetes syndrome.<sup>2</sup> Inflammatory factors such as IL-6 and TNF were considered to be risk factors for T2DM.<sup>12</sup> IL-6 is a cytokine produced by activated T cells, which can stimulate the activation of B cells and secrete a large number of IgG, causing excessive activation of killer T cells, thereby leading to the death of islet  $\beta$  cells. TNF is an important inflammatory factor released by activated macrophages, which can increase vascular permeability and promote apoptosis of endothelial cells. NF- $\kappa$ B is a very important transcription factor in gene expression and signal transduction pathways, which plays an important role in inflammation, immunity, and cancer. It was discovered in this study that liraglutide can inhibit the expression of IL-6, TNF, and NF- $\kappa$ B mRNA in renal tissue of DN; reduce the expression of IL-6, TNF, and NF- $\kappa$ B proteins; and improve renal function. It is suggested that liraglutide can treat DN mice by inhibiting inflammatory factors.

In summary, the mechanism of liraglutide in the treatment of DN may be related to the inhibition of the expression of inflammatory factor genes and proteins.

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W.F. and Z.L. contributed equally to this article, and Z.L. is the co-first author.

### Declaration of conflicting interests

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