



Protective Effect of Adalimumab on Diabetic Nephropathy by Regulating TNF- α Signal Pathway

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Abstract

Background: To evaluate the inhibitory effect of adalimumab on diabetic nephropathy (DN) through animal models.

Methods: We carried out the study in Weifang People's Hospital, Weifang 261041, China in December 2020. Streptozotocin was used to induce DN in model animal Sprague-Dawley (SD) rats. The DN animal model was given treatment with adalimumab, and the inhibitory effect of adalimumab on the development process of DN was evaluated by detecting changes in blood glucose and urinary albumin levels. Meanwhile, the content of UN, Cr and CysC of the blood in different experimental groups was tested by weighing the ratio of kidney and performing ELISA to evaluate the protective effect of adalimumab on kidney of DN animal model. In addition, the changes in the transcription and translation levels of tumor necrosis factor α (TNF- α) and its downstream regulatory factors MCP-1 and NF- κ B in kidney of different experimental groups were detected by fluorescence quantitative PCR and Western blot tests to further reveal the molecular mechanism of adalimumab inhibiting the diabetic nephropathy.

Results: adalimumab could significantly downregulate blood glucose and urinary albumin levels ($P < 0.05$). The renal body weight ratio and the contents of UN, Cr and Cysc in blood in the adalimumab group were significantly lower than those in the placebo group ($P < 0.05$). Meanwhile, adalimumab could significantly downregulate the expression of these molecules ($P < 0.05$).

Conclusion: adalimumab could exert its therapeutic effect on diabetic nephropathy through its specific targeting TNF- α signaling pathways.

Keywords: Diabetic nephropathy; Adalimumab; Kidney; TNF- α signal pathway

Introduction

Diabetic nephropathy (DN) is the main cause of end-stage renal disease (ESRD). Compared with healthy people, the mortality of patients with diabetic nephropathy within 10 yr has increased by at least six times (1).

DN was characterized by multiple morphological changes in all parts of kidney, which will affect the functions of organs, including thickening of the glomerular basement membrane (GBM) and intraglomerular nodular sclerosis (2). The pathways that causes diabetic nephropathy involve



many aspects: hemodynamic changes, such as the arteriolar resistance imbalance, metabolic factors causing oxidative stress, cell signaling and transcription factors, proinflammatory molecules, etc (3). All these pathways can lead to apoptosis, podocyte loss, and reduced glomerular filtration rate.

TNF- α is a multi-effect inflammatory cytokine produced not only by hematopoietic cells such as monocytes, macrophages and T cells, but also by intrinsic renal cells such as mesangial cells, endothelial cells, dendritic cells and renal tubular cells. TNF- α acts on apoptosis and necrotic cell death through direct and autocrine mechanisms and altering endothelial cell permeability. It is also associated with reactive oxygen species in a variety of cells, including glomerular mesangial cells that can lead to impaired glomerular capillary wall barrier function, to increase albumin permeability (4). The level of TNF- α in blood and urine of patients with DN is significantly higher than that of non-diabetic patients; and TNF- α impaired blood flow between the glomeruli of kidney in related study on the pathogenesis of DN (5).

Adalimumab is the first human recombinant IgG1 monoclonal antibody specific to human TNF- α in the world. Experimental results in vitro showed that the application of adalimumab suppressed the major inflammatory effect of TNF- α in endothelial cell activation, endothelial monocyte adhesion, and endothelial cell leakage (6). The in vivo results of animal experiments showed that the treatment with adalimumab can attenuate serum IgE, TH2 and TH1 derived inflammatory cytokines (IL-4 and IFN- γ) in ovalbumin-induced bronchoalveolar lavage fluid, inhibit the recruitment of bronchoalveolar lavage fluid and inflammatory cells in lungs and neutrophil growth in bronchoalveolar lavage fluid. Meanwhile, adalimumab can significantly improve the epithelial metaplasia and bronchial fibrosis goblet cells (7). All the results mentioned above showed that as a specific monoclonal antibody against TNF- α , adalimumab has a good prospect in the treatment of diseases related to TNF- α signal pathway (8). Since TNF- α signal pathway plays an important role in DN, the research and application of ada-

limumab in DN has not been deeply involved. The SD rat model was used in this study to explore the good therapeutic effect of adalimumab on DN, and its role through the TNF- α signal pathway. The authors would like to expand the application of adalimumab in related fields, and provide a theoretical basis for the clinical treatment of patients with DN.

Materials and Methods

Experimental Materials and Instruments

Non-specific pathogen-grade (SPF) SD rats (8 weeks old) were from Shanghai Xipur-Bikai Experimental Animals Co., Ltd. PBS buffer and 0.22 μ m sterile needle filters were from Biyuntian Biological Company, China, Art.No.: C0221A, FF362. Streptomycin was from American SIGMA Company, Art.No.: S0130. Streptomycin was dissolved in PBS, prepared into 100mg/ mL storage solution, filtered with a 0.22 μ m needle filter, and stored separately at -20 °C. adalimumab was from Selleckchem, Art.No.: A2010, adalimumab was dissolved in PBS buffer and prepared into 5mg/ mL storage solution for later use. RNA extraction reagents TRIzol was from Thermo Fisher, Art.No.: 15596018. RIPA protein lysate and BCA protein concentration assay kit were from Beibiyuntian Biological Company, Art.No.: P0013B. Albumin test kits were from SIGMA Biology, USA, Art.No.: MAK124. CysC specific ELISA kit was from Dr. De Biological, Wuhan, Art.No.: EK0679. Reverse transcriptome kit was from TAKARA Biology, Art.No.: R R037B. The fluorescent quantitative PCR reagent was from Roche, Art.No.: 4913850001. TNF- α , MCP-1, and NF- κ B antibodies were from CST, Art.No.: 3707S, 2027S, 6956T. HRP labeled sheep anti-rabbit and sheep anti-mouse second antibody were from Beijing Whole Type Gold Biological Company, Art.No.: HS101-01, HS201-01. The other inorganic reagents were from Xilong Science Co., Ltd.

The study was approved by the Ethics Committee of Weifang People's Hospital.

Automatic biochemical analyzer was from Japan Hitachi Company, Model: 917. The thermo scientific microplate reader was from American Bio-Rad Company, Model: 450. The small desktop cryogenic centrifuge was from the United States Thermo Fisher Company, Model: 75002456. Electrophoresis apparatus and transfer printing apparatus for Western Blot were from Bio-Rad Company, Model: 1645050.

Animal Model Establishment and Sample Collection

SPF-grade SD rats were randomly divided into A, B and C groups. Of which, A, B groups was induced DN by streptomycin (9). Eight-week-old SD rats were intraperitoneally injected with streptozotocin at 60mg/kg for 5 days. Twenty-one days later, SD rats in a group were treated with adalimumab intraperitoneal injection, named as adalimumab group. SD rats in B group were treated with the same amount of saline, named as Placebo group. C group was not given any treatment, named as Control group. The rats were weighed 26 weeks later, and blood samples (1.0 ml) and urine samples (0.5 ml) were collected from tail veins before feeding in the morning. After placing at room temperature for 20 min, the samples were centrifuged for 20 min at 2000 rpm/min, to collect serum and urine serum. Then the serum were separated, frozen and stored at -20 °C for the later use. Subsequently, the experimental animals in each group were euthanized, kidney tissue was removed after dissection, and their kidney weight was recorded.

Blood Glucose and Urinary Albumin Assay

The progress of DN was evaluated by detecting fasting blood glucose and urinary albumin in rats (10). Methods: The blood glucose content was detected by Hitachi 917 automatic biochemical analyzer, and the urinary albumin content was detected by specific kit.

Evaluation of Renal Function Injury

Blood urea nitrogen, creatinine (Cr) and serum cystatin (Cysc) were tested to evaluate the degree of kidney injury in SD rats treated with different treatments (11).

UN test: The serum samples were measured by Hitachi 917 automatic biochemical analyzer;

Cr test: The Cr in serum was determined by Astra 8 autoanalyzer;

CysC test: The expression level of CysC in serum was detected by ELISA kit with American Bio-Rad 450 microplate reader.

All the tests were operated strictly according to the instructions, and 450 nm wavelength values were detected by the microplate reader.

Exploration of Mechanism

The mRNA levels of TNF- α , MCP-1 and NF- κ B in kidney tissues of different treatment groups were detected. One hundred mg renal tissue collected from different treatment groups was shredded and 1ml TRIzol reagent was added, which were fully homogenized and silenced at room temperature for 5 min. 0.2ml chloroform was added with vortex oscillation for 15 s, silencing for 2 min, and being centrifuged at 4 °C for 12,000g \times 15min. The supernatant was collected, with 0.5ml isopropanol added. After mixing, it was silenced for 10min at room temperature, and centrifuged at 4 °C for 12,000g \times 10min. The supernatant was abandoned, then 1ml 75% ethanol precooled was added on ice to wash RNA sediment, and centrifuged at 4 °C for 7,500 g \times 10 min. The supernatant was abandoned again, then the rest part was silenced at room temperature for 10 min. 5ul non-RNAase H₂O was added to dissolve RNA. Then 500ng total RNA was reverse transcribed into cDNA. Subsequently, fluorescence quantitative PCR was performed according to the reagent instructions to detect the changes in the mRNA relative expression levels of TNF- α , MCP-1, NF- κ B and reference gene GAPDH. The primers used are shown in Table 1.

Table 1: QRT-PCR primers

<i>mRNA</i>	<i>Forward Primer</i>	<i>Reverse Primer</i>
TNF- α	GAGAAGTTCCTCCAAATGGCCT	GAGAACCTGGGAGTAGACAA
MCP-1	CCC ACT CAC CTG CTG CTA CT	TCT GGA CCC ATT CCT TCT TG
NF-kB	TACAAGCTGGCTGGTGGGGA	GTCGCGGGTCTCAGGACCTT
GAPDH	AGAACATCATCCCTGCATCC	AGTTGCTGTTGAAGTCGC

The protein level of TNF- α , MCP-1 and NF-kB of kidney tissue in different treatment groups was tested: 100mg renal tissue collected from different treatment groups were mixed with 1ml RIPA lysate, which was fully homogenized and lysed on ice for 30 min. During this period, it was mixed by eddy oscillation every 10min, and then centrifuged at 4 °C for 12,000g \times 10min. The supernatant was collected and the protein concentration was determined by BCA method. 5 \times upper sample buffer solution was added to the extracted total tissue proteins, which was bathed in boiling water for 5 min, followed by Western blot test. After membrane transfer, the protein levels of TNF- α , MCP-1, and NF-kB were detected by specific antibody and GAPDH was used as internal reference protein for quantitative analysis. All experiments were carried out in strict accordance with the reagent instructions.

Data Analysis

All statistics were represented by averages \pm standard errors (Mean \pm SE). The differences between experimental groups were analyzed by multiple comparative corrected ANOVA or *t* test for statistical significance (Primer 5, GraphPad Software). $P < 0.05$ indicates a statistically significant difference.

Results

adalimumab Significantly Inhibit the Development of Diabetic Nephropathy

In this study, the levels of blood glucose and urinary albumin in SD rats induced diabetic nephropathy were firstly evaluated. The results showed that after DN were induced to SD rats, the blood glucose and urinary albumin levels in the adalimumab group and the control group were significantly lower than those in the placebo group ($P < 0.001$) (Fig. 1). It indicated that adalimumab had a significant therapeutic effect on the progression of DN.

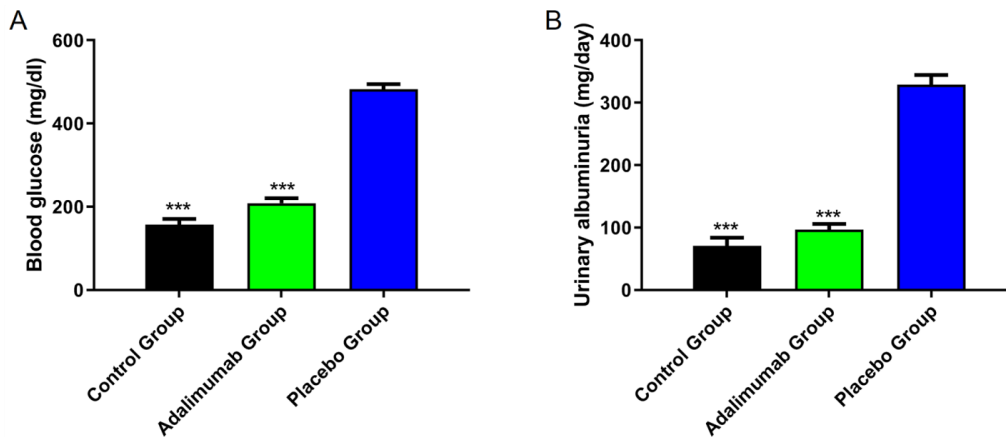


Fig. 1: adalimumab significantly decreased blood glucose and urinary albumin content. SD rats were given adalimumab treatment (adalimumab Group, n=8) or placebo treatment (Placebo Group, n=8) after 21 days of streptomycin injection. 26 weeks later, blood and urine samples were collected to detect blood glucose (A) and urinary albumin (B). *** $P < 0.001$, with placebo group as control group

adalimumab Significantly Improve the Degree of Renal Lesions Caused by Diabetic Nephropathy

Severe DN can lead to severe swelling of kidney and aggravate renal diseases. The effects of adalimumab on the renal lesions were investigated. After DN were induced to SD rats by urinary streptomycin, the kidney weight ratio was significantly increased ($P<0.01$), while the renal body weight ratio was significantly decreased after adalimumab treatment. It indicated that adalimumab can effectively improve the degree of nephropathy (Fig. 2).

Effects of adalimumab on Kidney

In the development of DN, renal macrophages gather around glomerular clusters and dilated tubes, and induce the release of related macrophage factors to damage the kidney to varying degrees. Through evaluating the kidney weight ratio of different treatment groups, we found that adalimumab can effectively improve the degree of renal lesions, and then we further evaluated the protective effect of adalimumab on the kidney. The content of UN, Cr, CysC factors in

blood of SD rats with DN was significantly increased, while the levels of these factors in adalimumab group were significantly lower than those in placebo group ($P<0.001$) (Fig. 3).

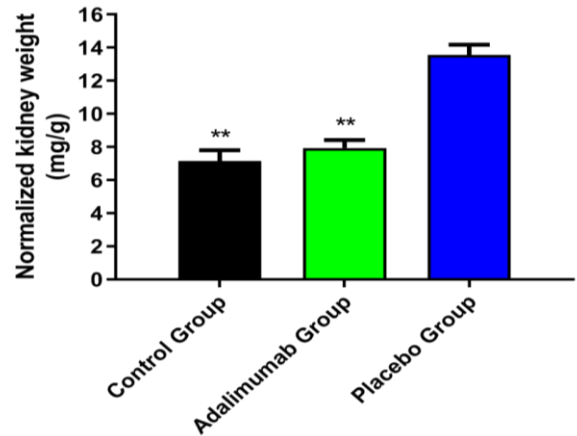


Fig. 2: adalimumab significantly improved the degree of renal lesions. After 21 days of injection of streptomycin SD rats, the kidney and body weight of different treatment groups were recorded and the ratio of kidney body weight was calculated. ** $P < 0.01$, placebo group

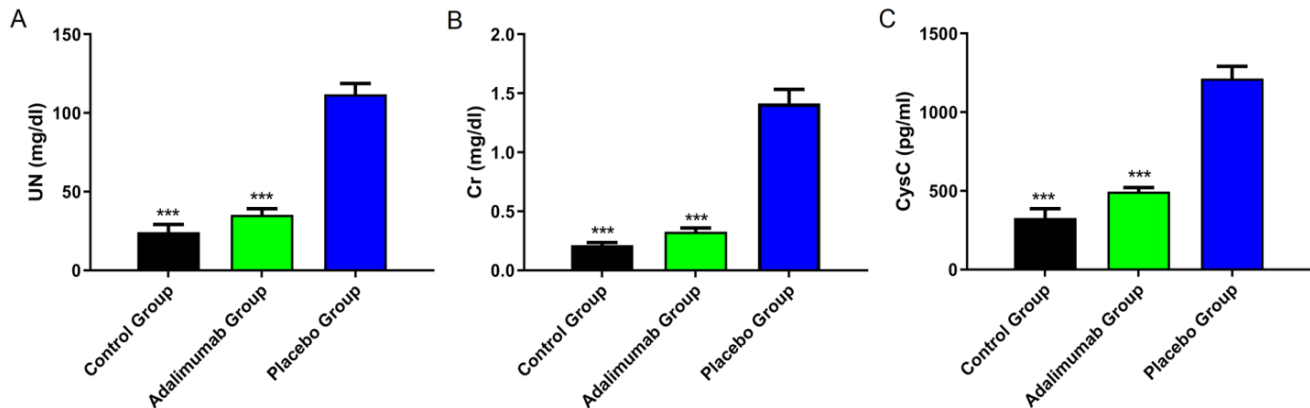


Fig. 3: adalimumab significantly improve renal injury caused by diabetic nephropathy. SD rats were injected with streptozotocin 21 days later, as shown in the figure. After 26 weeks, blood related factors were detected in different groups. A: serum urinary nitrogen content; B: serum creatinine content; C: serum cystatin content. *** $P < 0.001$, with placebo group as control group

adalimumab exerts its therapeutic effect on diabetic nephropathy by inhibiting TNF- α signal pathway

This study explored the effects of adalimumab on TNF- α signal pathway in DN. The results showed that the content MCP-1, NF- κ B TNF- α and its downstream important factors in renal

tissue of SD rats with DN was significantly increased ($P < 0.001$). Meanwhile, the expression of these factors in adalimumab group was effective-

ly reduced at transcription (Fig. 4) and translation level (Fig. 5) in rats.

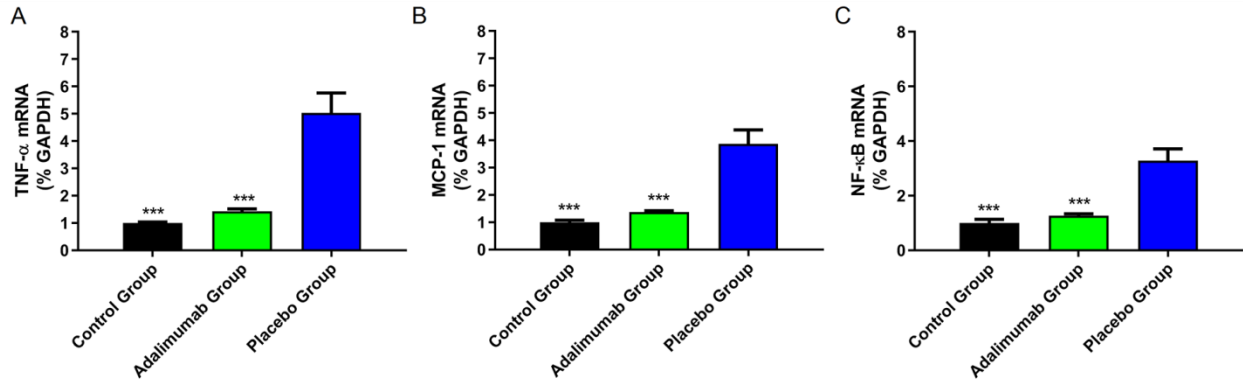


Fig. 4: Transcriptional expression level of adalimumab inhibits renal tissue TNF- α and its Downstream factors. SD rats were treated with streptozotocin 21 days after injection, as shown in the figure. After 26 weeks, relevant factors in renal tissues of different treatment groups were detected. A: TNF- α mRNA level in kidney tissue; B: MCP-1 mRNA level in kidney tissue; C: NF- κ B mRNA level in kidney tissues. *** $P < 0.001$, with placebo group as control group

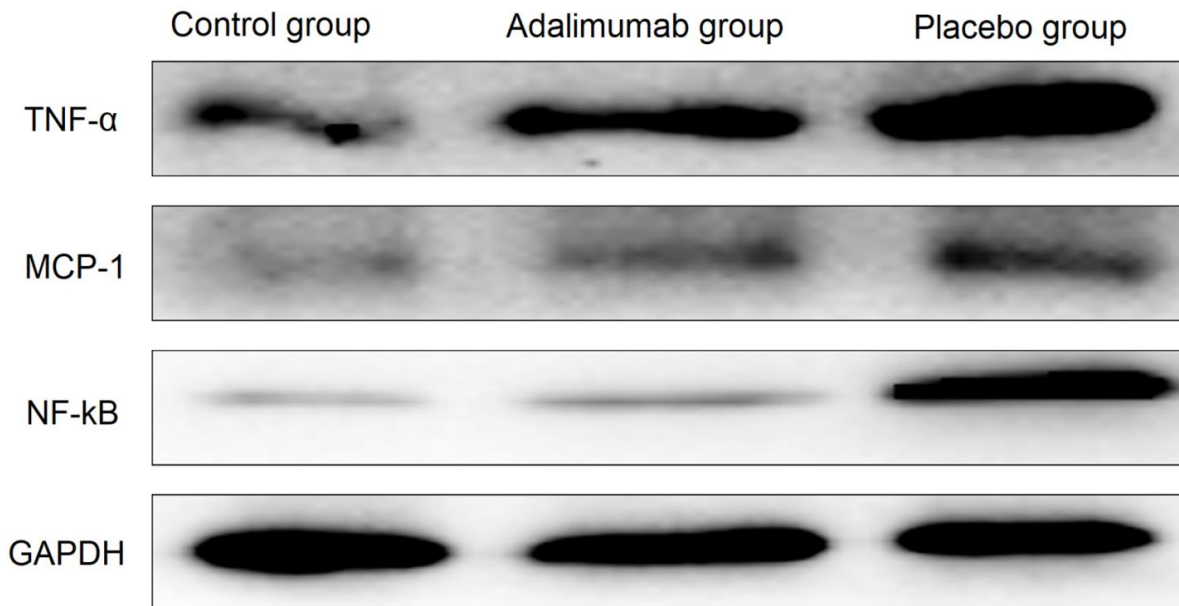


Fig. 5: Translational expression levels of monoclonal antibodies adalimumab inhibit renal tissue TNF- α and its Downstream factors. SD rats were treated with streptozotocin 21 days after injection, as shown in the figure. After 26 weeks, relevant factors in renal tissues of different treatment groups were detected

Discussion

DN is a kind of metabolic disease with high morbidity and mortality (12). At present, most

treatment options are limited to well-equipped large hospitals, and expensive blocking drugs are needed. Therefore, for the effective treatment in vast majority of patients, it is urgent to develop a

specific drug with significant effect, simple operation and affordable price.

In recent years, with the further study of cell and molecular levels, immune and inflammatory factors play important roles in the development of diabetic nephropathy and its diseases (13). In the occurrence and development of diabetic nephropathy, a variety of cells are involved, such as macrophages, white blood cells, monocytes, as well as a variety of small molecules such as cytokines, adhesion molecules, growth factors, enzymes and nuclear transcription factors (14).

The signature changes in DN are kidney hypertrophy and excess filtration. The two changes are closely related to the inflammatory process, particularly proinflammatory cytokines TNF- α signal pathway (15). TNF- α enhances sodium reabsorption by activating the distal tubular epithelial sodium channel. It triggers TGF- β release, and causes the development of kidney hypertrophy (16). A study in animal models of diabetes has also confirmed kidney hypertrophy and kidney weight increases in diabetic patients (17). We also found that in the rat model, DN causes a significant increase in kidney weight ratio. Kidney weight increase is one of the early markers of kidney hypertrophy and kidney damage in diabetic patients (18). Meanwhile, the increase of kidney weight is closely related to the gene expression level of IL-6 proinflammatory factors and the concentration in urine (19). These structural changes will affect the normal functioning of the kidneys, and lead to albumin leakage (20). Therefore, the earliest renal changes in diabetic patients are not changes in serum creatinine, but the hemodynamic changes caused by low glomerular perfusion and increased glomerular filtration rate, also known as hyperfiltration. This causes albumin leakage in the glomerular capillaries, resulting in proteinuria (21). In this study, a significant increase in urinary albumin was detected in a SD rat model of DN, showing that the data obtained by this animal model has considerable credibility. Given the global prevalence of DN and its negative impact on human health, many potential drugs are being actively developed and entered into clinical trials. However, the failure rate of

new drugs in clinical trials was more than 90%. Only a few of the drugs have entered phase three clinical trials (22). However, the main reason was that the pathogenesis of DN has not been fully revealed, and the safety of new drugs in human body was a great challenge (23).

In proinflammatory cytokines associated with the progression of DN, TNF- α signal pathways play an important role. Especially, during early kidney damage, the process has a direct connection with TNF- α (24). A lot of experimental data and clinical samples show that TNF- α signal pathways are closely linked to a variety of chronic immune-related diseases, such as rheumatoid arthritis (25), Crohn's disease (26), systemic lupus erythematosus (27), inflammation-related intestinal diseases (28) and neurodegenerative diseases (29). TNF- α signal pathways play an important role in many diseases that threaten human health. Therefore, at present, many specific small molecule inhibitors for TNF- α have entered clinical trials, in order to bring great benefits to some patients with inflammatory diseases.

The design of small molecular inhibitors that interfere with TNF- α signal pathway can effectively target TNF- α signal pathway related diseases. These clinical experimental data greatly increase the researchers' unremitting exploration of TNF- α inhibitors (30). The adalimumab used in this experiment was a monoclonal antibody specific to human TNF- α , which can be inactivated or degraded by specific binding TNF- α molecules (31). Compared with the other two biological treatments, antagonistic proteins and chimeric monoclonal antibodies, fully humanized adalimumab has many advantages. First, although all three programs have a good inhibitory effect on a variety of inflammatory-related diseases, when completely humanized adalimumab is applied to humans, it is safer compared with antagonistic protein and chimeric antibody (32). Considering that some inflammation-related diseases have a longer medication cycle, which may last for years, the cost of using adalimumab will be significantly lower than that of the other two treatments (33). adalimumab was approved for the treatment of rheumatoid arthritis in the United States in 2002

and has since been extended to include ankylost spondylitis, juvenile idiopathic (rheumatoid) arthritis, severe psoriasis and psoriatic arthritis, Crohn's disease and ulcerative colitis (34). adalimumab is considered a drug that can improve rheumatic diseases, and has been shown to improve inflammatory arthritis symptoms as well as joint and cartilage injury (35). Humira Pharmaceutical Company sells adalimumab as a pre-filled syringe of 40 mg/0.8 ml. The typical maintenance dose of adult adalimumab is 40 mg subcutaneous every other week (36). Meanwhile, because of the remarkable therapeutic effect of adalimumab on many diseases, there are adalimumab generic on the market (37), which further reduces the cost of adalimumab, bringing convenience to more patients. Meanwhile, because of the important role TNF- α play in the pathogenesis of DN, we evaluated the efficacy of adalimumab in the treatment of diabetic nephropathy in SD rats. adalimumab had significant inhibitory effect on DN, and could effectively improve DN caused by kidney injury phenomenon. These results revealed the important role of TNF- α signal pathway in the development of DN, and indicated the potential of adalimumab in the treatment of DN.

The SD rat model of DN involved in this study was induced by streptozotocin, and the reliability of this model has been confirmed by a large number of researchers (38). We also observed that the blood glucose concentration and urinary albumin increased significantly in the rats with DN, which was consistent with the statistical data of clinical patients. At the same time, the renal function of the rat model of DN was seriously affected, similar to that observed in clinic, so adalimumab had therapeutic effect on rats with DN. This will provide a certain theoretical basis and practical experience for the further experiment of adalimumab in human body, which not only further expands the application of adalimumab in human diseases, but also provides a new treatment for patients with DN.

Conclusion

adalimumab can exert its therapeutic effect on diabetic nephropathy through its specific targeting TNF- α signaling pathway.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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