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Active Vitamin D_3 Protects Against Diabetic Kidney Disease by Regulating the JNK Signaling Pathway in Rats

Xiaoju Fan[1, †], Linlin Liu[2, †], Wenzhuo Zhu[1], Yumei Zhao[1], Haowei Zhang[1], Yang Wu[3], Jia Fu[1], Hongmin Liang[4, *], Rui Han[1, *]

[1]Department of Diabetes, The First Affiliated Hospital of Kunming Medical University, Kunming, People's Republic of China

[2]School of Forensic Medicine, Kunming Medical University, Kunming, People's Republic of China

[3]Department of Endocrinology, The Second People's Hospital of Kunming, Kunming, People's Republic of China

[4]Department of Ultrasound, The First Affiliated Hospital of Kunming Medical University, Kunming, People's Republic of China

Email address:

1021589617@qq.com (Xiaoju Fan), liull0818@126.com (Linlin Liu), zhuwenzhuo8116@163.com (Wenzhuo Zhu), zhaoyumei423@sohu.com (Yumei Zhao), 46404375@qq.com (Haowei Zhang), kmwuyang@126.com (Yang Wu), 714650932@qq.com (Jia Fu), kmlianghongmin@163.com (Hongmin Liang), hanrui_201207@163.com (Rui Han)

[*]Corresponding author

† Xiaoju Fan and Linlin Liu are co-first authors.

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Abstract: Diabetic kidney disease (DKD) is an inflammatory disease caused by metabolic disorder. As an important signaling pathway in the inflammatory response, the JNK signaling pathway plays an crucial role in kidney injury in DKD. Vitamin D_3 can reduce the inflammatory reaction and delay or even reverse DKD progression. Unfortunately, the mechanism by which vitamin D_3 regulates DKD pathogenesis is unclear. This research established a DKD rat model and vitamin D_3 and irbesartan were used as interventions. Then, urine and blood biochemistry; and inflammatory cytokine (IL-1 and IL-6), phosphorylated JNK pathway protein (MEK-4 and JNK1/2/3) and downstream factor (AP-1 and ATF-2) expression were assessed. We found that the DKD group showed body weight and insulin secretion were significantly decreased; significantly increased FPG, HOMA-IR and blood lipids; and significantly increased 24-h urinary protein (UPro) compared with normal group. Additionally, the levels of IL-1 and IL-6 and phosphorylated JNK pathway proteins were significantly elevated. These changes were improved by vitamin D_3, especially at a low dosage. These results suggest that active vitamin D_3 protects against DKD in rats by reducing IL-6 and IL-1 release, downregulating the JNK inflammatory signaling pathway, and inhibiting downstream transcription factor AP-1- and ATF-2-mediated kidney damage. This research provides a new theoretical support for vitamin D_3 treatment of diabetic nephropathy.

Keywords: Diabetic Kidney Disease (DKD), Active Vitamin D_3, IL-1, IL-6, JNK

1. Introduction

Diabetic kidney disease (DKD) is one of the most common and serious complications of diabetes. According to statistical studies, the number of diabetes patients worldwide will increase to 366 million, and the number of DKD patients will exceed 100 million by 2030. [1]. Glomerular hypertrophy, glomerulosclerosis and interstitial fibrosis caused by extracellular matrix protein deposition and progressive renal insufficiency are important characteristics of DKD. If left untreated, DKD may eventually cause end-stage renal disease (ESRD) [2-5]. With the increasing incidence of diabetes, DKD has become the main cause of ESRD worldwide [6-8]. Because of its high morbidity and mortality and because it severely impacts the quality of life of patients, ESRD has become a serious public-health issues.

The pathogenesis of DKD is multifaceted and mainly includes environmental and genetic factors, glucose

metabolism disorders, microcirculatory disorders, cytokines and inflammatory factors [9]. Several studies have found that leukocytes, macrophages and monocytes are involved in the process of DKD [2, 10-12] and that pro-inflammatory cytokines and circulating inflammatory markers are closely related to the risk of complications of diabetic [2, 13-15]. The activation of inflammatory signaling pathways, the release of inflammatory factors such as monocyte chemoattractant protein-1 (MCP-1), C-reactive protein, and interleukin, promote macrophage infiltration, renal tubular fibrosis, and glomerulosclerosis and ultimately accelerate renal injury in DKD [16, 17]. In contrast, immunosuppressive strategies can reduce the accumulation of megaphagocytes in the kidney and attenuate the development of DKD [18, 19]. Therefore, DKD may be an inflammatory disease caused by metabolic disorders, and inflammatory reactions are a important factor in the development of DKD [2, 16].

The body of diabetes mellitus (DM) is often in a state of inflammation or oxidative stress, accompanied by increased levels of inflammatory factors, acute phase reactants and other stress molecules, thus activating corresponding stress signal pathways, for instance, the c-Jun N-terminal kinase (JNK) signaling pathway [20]. The JNK pathway is an extremely important signaling pathway of inflammatory response. JNK signaling pathway can be significantly activated in DM or high glucose environment and then participate in the renal inflammatory response and oxidative stress injury [21]. Moreover, the JNK pathway can be specifically activated by IL-1 [22] and IL-6 [23], and then influence biological processes [24]. In the process of DM, phosphorylated JNK can promote glomerular mesangial matrix expansion and type IV collagen deposition in the kidney, leading to glomerulosclerosis, renal fibrosis and tubular injury [25-27]; furthermore, it can accelerate the injury and apoptosis of podocytes in kidney tissue, damage the integrity of the glomerular filtration barrier, and finally lead to proteinuria [28]. After treatment with drugs or JNK inhibitors, the expression of phosphorylated JNK protein decreased significantly, and the above situation was significantly improved [29, 30]. Therefore, JNK plays an crucial role in the process of DKD kidney injury and participates in its pathogenesis [31-33].

Increasing evidence shows that vitamin D deficiency is an important risk factor for the occurrence and development of DKD [34]. Vitamin D deficiency is common in DKD patients, and 47.01% of diabetic nephropathy patients have vitamin D_3 deficiency [35]. Studies have shown that vitamin D_3 is correlated with the occurrence and development of inflammatory diseases, and vitamin D_3 can inhibit the release of IL-1, IL-6 and IL-8 and reduce the inflammatory reaction [36]. Oral administration of calcitriol and other analogs significantly reduced the levels of cytokines such as TNF-α, IL-6 in serum and peripheral blood mononuclear cells [37] as well as urinary protein levels [38, 39], which significantly improved renal interstitial fibrosis and glomerulosclerosis in mice [40]. Vitamin D_3 can delay or even reverse the progression of DKD by reducing the expression of inflammatory factors to a certain extent and thus has a protective effect against DKD [41]. Unfortunately, the mechanism by which vitamin D_3 regulates DKD development is not clear. Studies have shown that vitamin D_3 can significantly improve liver lesions in rat models of type 2 diabetes by downregulating the JNK inflammatory pathway and reducing downstream inflammatory factor-mediated liver injury [42].

However, whether vitamin D_3 can improve the renal damage caused by DKD by downregulating the JNK inflammatory pathway has not yet been reported. Therefore, this research intended to observe the effects of vitamin D_3 on the JNK pathway in the kidney tissue of DKD rats, to explore the protective effect and mechanism of vitamin D_3 on renal tissues and to provide a new theoretical basis for the prevention and treatment of DKD.

2. Materials and Methods

2.1. DKD Modeling and Grouping

70 female Sprague-Dawley (SD) rats (6 weeks, 160-220 g) were obtained from the Experimental Animal Center of Kunming Medical University. After one week of acclimation, they were fed a regular diet for one week and then randomly divided into DKD model group (n=60) and normal control group (n=10). The normal control group was kept on a regular diet, while the DKD model group was fed a high-fat diet (66.5% conventional feed, 20% sucrose, 10% lard, 2.5% cholesterol, 1.0% cholate) [43]. After 10 weeks of consuming the high-fat diet, the model group received an intraperitoneal injection of freshly prepared 35 mg/kg body weight streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) in pH 4.5, 0.1 mol/L citrate buffer; the control group received an injection of the same dose of citrate buffer based on body weight. Fasting plasma glucose (FPG) was measured on 3 consecutive days beginning 72 hours after the injection of STZ. When FPG stabilized at 16.7 mmol /L, the diabetes model was successfully established. After 8 weeks of continuously feeding the diabetic rats, rats with 24-hour urinary protein (24-h UPro) >20 mg and urine volume >150% of the original urine volume were considered to successfully model type 2 diabetic nephropathy [44, 45]. The 50 rats that were successfully modeled were randomly divided into the peanut oil group, positive control irbesartan group, low-dosage vitamin D_3 group, medium-dosage vitamin D_3 group, and high-dosage vitamin D_3 group with 10 rats in each group (Figure 1). The low-, medium- and high-vitamin D_3 dosages were 0.03 μg/kg/d, 0.06 μg/kg/d and 0.12 μg/kg/d calcitriol (Shanghai Roche Pharmaceutical Co., Ltd., Shanghai, China) (dissolved in peanut oil) for 6 weeks; the peanut oil group was given the same amount of peanut oil according to body weight, and the irbesartan group (Sanofi) was administered 50 mg/kg/d irbesartan [46]. During this process, 2 rats in the peanut oil group died due to hyperglycemia, 2 died in the irbesartan group due to hyperglycemia and infection, 2 died in the low-dosage

vitamin D_3 group due to hypoglycemia, 2 died in the medium-dosage vitamin D_3 group, and 2 died in the high-dosage vitamin D_3 group due to infection. The final number of rats in the model groups was 40, and each experimental group contained 8 rats. The study was approved by the animal ethics committee of Kunming Medical University.

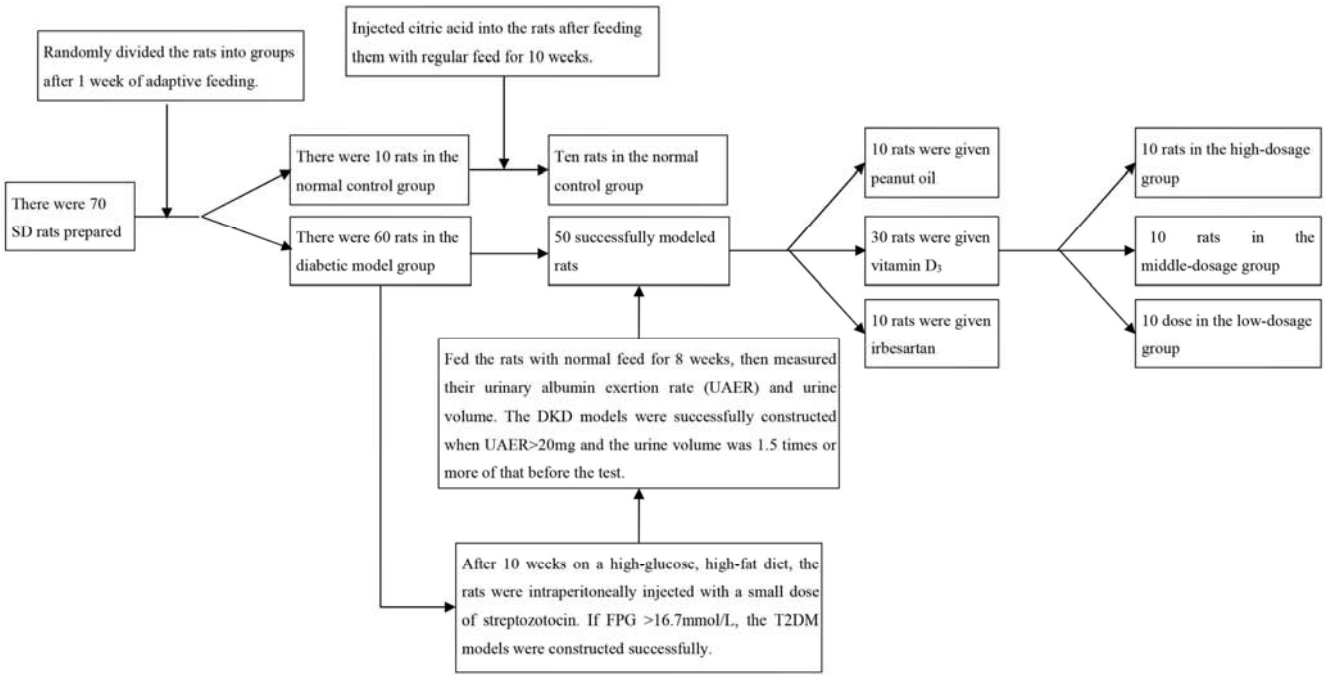

Figure 1. *Schematic diagram of the experimental rat groups and DKD model establishment.*

2.2. Tissue Sampling and Preservation

Upon completion of the 6-week treatment, the rats in each group were put into a clean metabolic cage one day before the end of the experiment. All rats were fasted and provided only water, and 24-hour urine was collected. At the end, blood samples were collected via cardiac puncture after anesthetization, centrifuged at 1,000 × g for 10 min, and the serum was quickly separated and frozen at -80°C for the analysis of other biochemical parameters. Immediately after blood sampling, the rats were dissected, and the kidneys were taken for subsequent analysis.

2.3. Biochemical Analysis

Then, 24-h UPro, total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-c) were measured according to urine protein and blood lipid kits (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China) using a full-wavelength microplate reader (Molecular Devices). FPG was measured by a Roche blood glucose meter, and insulin (Wuhan Yi Lai Rui Te Biotechnology Co., Ltd., Wuhan, China) and C peptide (Cp; Bio-Swamp) were detected by ELISA. The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated by fasting blood glucose (mmol/L) multiplied by fasting plasma insulin (uU/ml) and divided by 22.5. This mathematical model is easy to test and highly reproducible and is widely used in the evaluation of islet β-cell function in clinical patients [47].

2.4. ELISA

After the kidney tissue was collected, the tissue was homogenized with a SCIENTZ-II D ultrasonic cell pulverizer, and the supernatant was extracted. Then, the concentrations of IL-1 and IL-6 were detected according to the ELISA instructions (Shanghai Baili Biological Co., Ltd., Shanghai, China).

2.5. Western Blotting

After ultrasonic homogenization, tissue samples were lysed with RIPA buffer, the supernatant was extracted after centrifugation, protein concentration was measured by BCA method, and Western blotting was performed according to the standard process. The main antibodies included in the analysis were phospho-ATF-2, phospho-JNK1/2/3, phospho-AP-1, and phospho-MEK-4 polyclonal antibodies (Wuhan Yi Lai Rui Te Biotechnology Co., Ltd., Wuhan, China); β-actin (Sigma-Aldrich) as an internal reference. A chemiluminescence substrate was used to detect the antibodies. Each band was digitally imaged and the intensity of band was measured bying Quantity One software (Bio-Rad Laboratories, Inc.).

2.6. Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. Differences were assessed using a t-test, and statistical significance was considered as $p < 0.05$. GraphPad Prism version 8 was used for graphical analyses.

3. Results

3.1. General Health of the Animals

In general, rats in the normal control group had a moderate body shape, smooth and shiny fur, and good appetite and were responsive. Compared with the control group, the body weight of DKD model group was decreased (Figure 2), the fur was dark yellow and dull. The response of rats in the DKD group was lower than that of the normal control group; moreover, their movement was slow; their appetite decreased slightly; and their drinking water intake, urine volume and feces volume increased significantly.

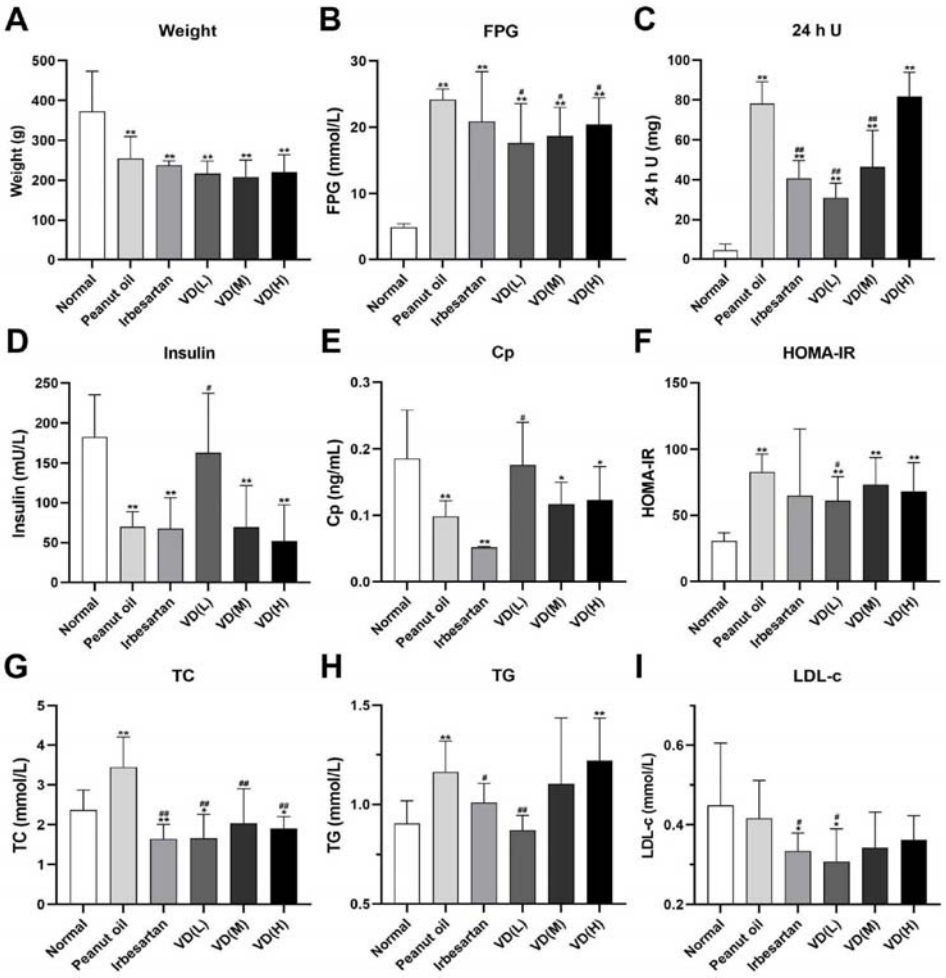

Figure 2. *Comparison of the body weight, blood glucose and related biochemical parameters of rats. (A) Weight; (B) FPG; (C) 24-h UPro; (D) insulin; (E) Cp; (F) HOMA-IR; (G) TC; (H) TG; (I) LDL-c. VD (L): low-dosage vitamin D_3 group, VD (M): medium-dosage vitamin D_3 group, VD (H): high-dosage vitamin D_3 group, *p < 0.05, **p < 0.01 vs the normal control group; #p < 0.05, ##p < 0.01 vs the peanut oil group.*

3.2. Effects of Active Vitamin D_3 on Body Weight and Biochemical Parameters

In order to evaluate the effects of vitamin D_3 on body weight, blood glucose, insulin secretion, renal function (24-h UPro), and blood lipids (TC, TG, and LDL-c) in DKD rats. The blood and urine biochemical parameters of rats with the different treatment were measured. The results of the body weight and biochemical parameter assessments are summarized in Figure 2. The DKD model group showed significantly increased FPG, HOMA-IR, TC, TG and 24-h UPro (p<0.01), and LDL-c showed a downward trend, compared with the normal group. Compared with peanut oil, the control drug irbesartan significantly reduced 24-h UPro, TC, TG and LDL-c levels (p<0.05). Compared with the peanut oil, middle-dosage vitamin D_3 showed significantly reduced FPG, 24-h UPro and TC levels (p<0.05), and high-dosage showed significantly reduced FPG and TC levels (p<0.05). However, low-dose vitamin D_3 significantly increased the synthesis of insulin and CP, decreased HOMA-IR (p<0.05), and significantly decreased FPG, 24-h UPro, and dyslipidemia (TC, TG, and LDL-c) (p<0.05).

3.3. Active Vitamin D_3 Partially Reverses DKD-associated Increases in IL-1 and IL-6

Compared with normal group, the expression of IL-1 and IL-6 in the DKD model groups were significantly increased ($p<0.05$). Compared with peanut oil group, the expression of IL-1 in the irbesartan group and all 3 vitamin D_3 groups were significantly decreased ($p<0.01$). The expression of IL-6 was similar to that of IL-1, but the difference was not significant in the medium-dosage vitamin D_3 group (Figure 3).

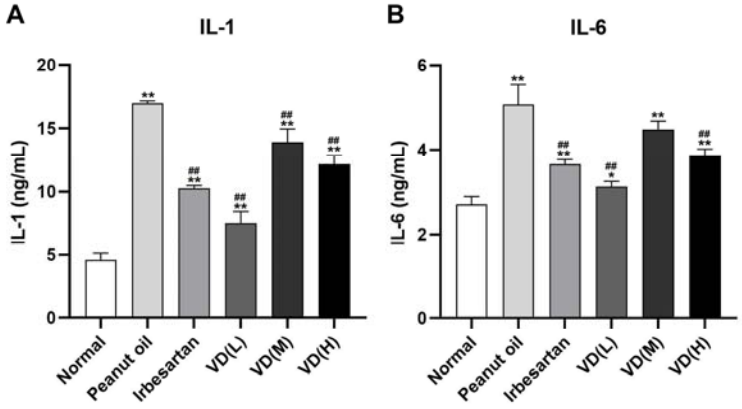

Figure 3. *Vitamin D partially reverses DKD-induced IL-1 and IL-6 levels in kidney tissues. (A) The expression of IL-1-type cytokines. (B) The expression of IL-6-type cytokines. VD (L): low-dosage vitamin D_3 group, VD (M): medium-dosage vitamin D_3 group, VD (H): high-dosage vitamin D_3 group, *p < 0.05, **p < 0.01 vs the normal control group; #p < 0.05, ##p < 0.01 vs the peanut oil group.*

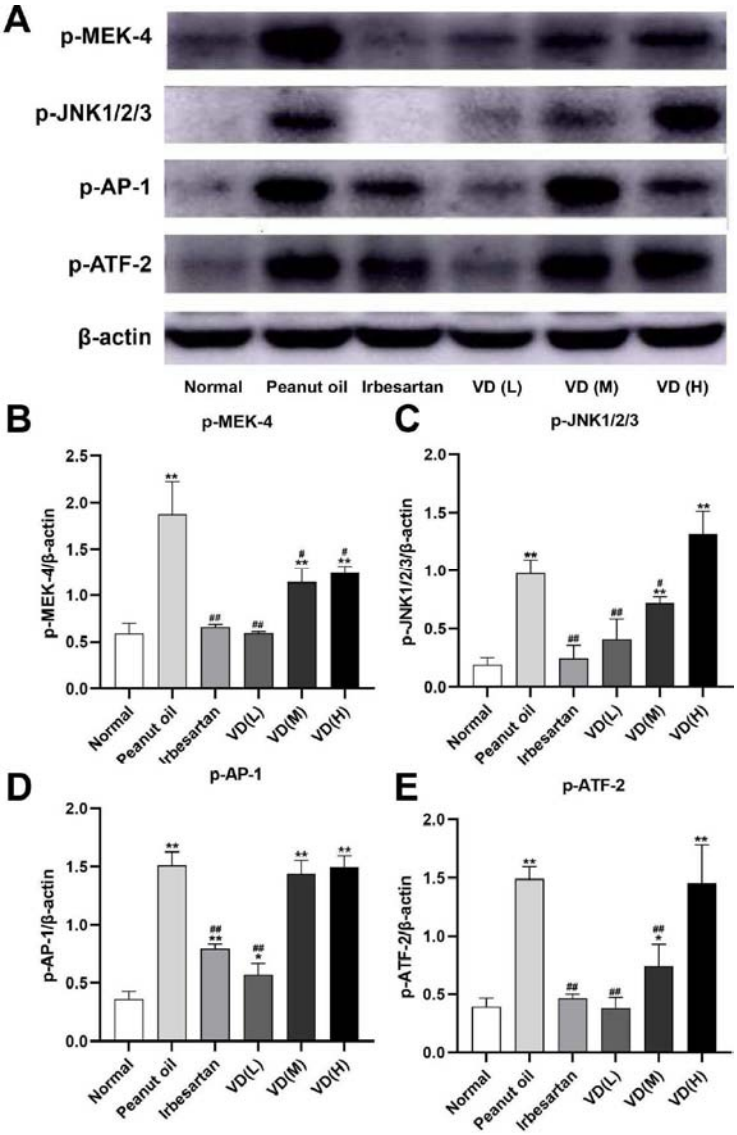

Figure 4. *The expression of p-MEK-4, p-JNK1/2/3, p-AP-1 and p-ATF-2 proteins in kidney tissues. (A) p-MEK-4, p-JNK1/2/3, p-AP-1 and p-ATF-2 detection bands were normalized to β-actin. The bar graph shows the quantification of p-MEK-4 (B), p-JNK1/2/3 (C), p-AP-1 (D), and p-ATF-2 (E). Data shown are mean±SD. VD (L): low-dosage vitamin D_3 group, VD (M): medium-dosage vitamin D_3 group, VD (H): high-dosage vitamin D_3 group, *p < 0.05, **p < 0.01 vs the normal control group; #p < 0.05, ##p < 0.01 vs the peanut oil group.*

3.4. Active Vitamin D_3 Partially Silences the DKD-related Activated JNK Signaling Pathway

The expression levels of p-MEK-4, p-JNK1/2/3, p-AP-1, and p-ATF-2 were detected by Western blot analysis. Compared with normal group, the expression level in the peanut oil group were significantly increased ($p<0.01$) (Figure 4). Compared with peanut oil group, the expression level in the irbesartan and low-dosage vitamin D_3 group were significantly decreased ($p<0.01$), the levels of p-MEK-4, p-JNK1/2/3, and p-ATF-2 were significantly decreased in medium-dosage vitamin D_3 group, and p-MEK-4 was significantly decreased in high-dosage vitamin D_3 group (Figure 4).

4. Discussion

With the incidence rate of diabetes increasing yearly, DKD has become a serious public health problem [6-8]. DKD may be an inflammatory disease caused by metabolic disorder, and inflammatory reactions are the critical factor for the development of DKD was previously reported [2, 16]. The JNK pathway is an important signaling pathway in the inflammatory response. It may play an important role in the process of DKD kidney injury and participate in the pathogenesis of DKD [31-33]. Vitamin D_3 can inhibit the release of IL-1 and IL-6, reduce the inflammatory reaction, and delay or even reverse the progress of DKD [41]. Furthermore, vitamin D_3 may protect the liver from diabetic complications through the JNK pathway [42]. Unfortunately, whether vitamin D_3 can improve the DKD process by regulating the JNK inflammatory pathway and its mechanism remain unclear. This research established a DKD rat model, and different dosages of vitamin D_3 and irbesartan were used as the interventions. Then, the biochemical parameters of urine and blood; and the expression of inflammatory cytokines, phosphorylated JNK pathway proteins (p-MEK-4 and p-JNK1/2/3) and downstream factors (p-AP-1 and p-ATF-2) were further assessed. Our study found that the DKD group showed significantly decreased body weight and insulin secretion; significantly increased FPG, HOMA-IR and blood lipid indexes (TC and TG); and significantly increased kidney function indexes (24-h UPro). Additionally, the levels of IL-1 and IL-6 inflammatory cytokines and phosphorylated JNK pathway proteins were significantly elevated, Notably, these changes were improved by vitamin D_3, especially low-dosage vitamin D_3.

We used a high-sugar and high-fat diet combined with low-dose streptozotocin (STZ) [43-45] to successfully construct a type 2 DKD rat model with 24-h UPro >20 mg and urine volume >150%. At the end of the experiment, compared with the normal group, the body weight, insulin and Cp were significantly decreased and FPG, HOMA-IR, TC, TG and 24-h UPro were increased, which also proves that we successfully established the DKD model. Irbesartan is an angiotensin receptor antagonist that can reduce the urinary protein excretion, improve blood lipid levels and renal function in DKD, and reduce the production of cytokines and inflammatory mediators. It can be used to treat DKD [48, 49]. Therefore, this study use irbesartan as a positive control drug. Irbesartan reversed many of the negative changes in DKD rats to varying degrees, and the positive effect of irbesartan on diabetic nephropathy was confirmed in this study. In addition, in DKD rats, vitamin D_3 intervention, especially low-dose vitamin D_3, significantly inhibited 24-h UPro. The contents of TC, TG and LDL-C were decreased, and dyslipidemia was improved. The expression of IL-1 and IL-6 was decreased.

Dyslipidemia is closely associated with kidney disease [50], which can lead to the deposition of lipoprotein in the glomerulus, resulting in damage to the glomerulus, renal interstitium and renal tubules and increasing the excretion of urinary protein. Dyslipidemia promotes the occurrence and development of DKD, while DKD aggravates dyslipidemia [51, 52]. High levels of TC, TG and LDL are considered to be risk factors for renal degeneration in type 2 diabetes patients [53], and our DKD rat model also supports this point of view. Lipid-lowering therapy can delay the progress of renal disease and slow down the decline in renal function in DKD patients [51]. Our results show that irbesartan and low-dose vitamin D_3 can reduce the TC, TG and LDL-c contents and improve dyslipidemia. It was also found that vitamin D_3 can reduce 24-h UPro, consistent with previous studies. Major et al suggested that vitamin D_3 supplementation can reduce the levels of TC and LDL-c and improve dyslipidemia [54]. Active vitamin D_3 can regulate renal vascular endothelial function, inhibit renin expression, regulate the oxidative stress response, and reduce urinary protein excretion, thus improving diabetic nephropathy [55]. Timely and appropriate use of lipid-lowering drugs and improvement of dyslipidemia are important for delaying the progression of DKD. However, the detailed mechanism by which active vitamin D_3 regulates blood lipid levels has not been clarified. However, these findings support the possible use of vitamin D_3 to regulate dyslipidemia and protect against DKD.

Effective control of blood glucose can hinder the progression of DKD. We found that vitamin D_3 also increased insulin content, decreased insulin resistance and improved blood glucose control in DKD rats, while irbesartan had no positive effect in this respect. Previous studies have confirmed that vitamin D_3 can improve insulin resistance by promoting insulin synthesis and secretion [56], reduce the level of insulin-degrading enzymes, and increase the phosphorylation of insulin receptors [57]. In conclusion, vitamin D3 may have more extensive positive effects in the treatment of DKD than irbesartan, suggesting the potential value of vitamin D_3 in the treatment of DKD.

A high-glucose environment can stimulate the expression of IL-6 and other inflammatory mediators and then destroy renal tubular epithelial cells, further aggravating renal disease [58, 59]. Additionally, IL-1 [22] and IL-6 [23] can activate the JNK signaling pathway. In a high-glucose or diabetes environment, the JNK signaling pathway can be significantly activated, regulate the expression of related genes and protein synthesis,

and then participate in the renal inflammatory response and in oxidative stress injury [21]. This leads to kidney injury and proteinuria [25-28]. In this study, we found that IL-1 and IL-6 were significantly upregulated, the JNK signaling pathway was highly activated, and the downstream factors ATF-2 and AP-1 were also significantly activated in the kidneys of DKD rats. Interestingly, increased expression of IL-1 and IL-6 and the hyperphosphorylation of JNK pathway proteins were reversed by irbesartan and vitamin D_3 intervention. Previous studies have shown that active vitamin D_3 can inhibit the release of the IL-1, IL-6, and IL-8 and reduce the inflammatory response [36]. Based on these theories and results, we hypothesized that activated vitamin D_3 could inhibit the release of the IL-1 and IL-6, reduce the activation of JNK signaling pathway, and reduce the activation and expression of downstream transcription factors such as AP-1 and ATF-2, thereby affecting the transcription and expression of related genes and proteins and ultimately protecting renal function.

Although vitamin D_3 plays an important role in the protection of DKD, this effect is closely related to the concentration of vitamin D_3. In our study, low-dosage vitamin D_3 more effectively reversed the changes in biochemical parameters, cytokines and the JNK signaling pathway in DKD rats than high-dosage vitamin D_3, which may be because supplementation with high dosages of vitamin D_3 can cause extensive deposition in rat kidney tissues, especially glomeruli and renal tubules, directly causing renal filtration and reabsorption dysfunction [60]. Thus, it is necessary to find the optimal concentration of vitamin D_3 in future research and clinical research is necessary. In addition, we previously confirmed that active vitamin D_3 can reduce the expression of the chemokine MCP-1 and improve renal injury by downregulating the NF-kB signaling pathway [61]. Therefore, vitamin D_3 participates in DKD regulation through multiple signaling pathways. To clarify the regulatory mechanism, it is necessary to conduct additional research, such as evaluating the effects of inhibitors of the corresponding signaling pathways, for further verification. We assessed renal function by measuring only 24-h UPro. For a more reliable renal function assessment, creatinine, urea nitrogen and glomerular filtration rate should be included in future work.

5. Conclusion

We constructed the DKD rat model and intervened with different doses of vitamin D_3 and irbesartan. The results showed that active vitamin D_3, especially low-dosage vitamin D_3 could reduce the release of IL-1 and IL-6, down-regulate the phosphorylation level of JNK inflammatory signal pathway proteins (p-MEK-4 and p-JNK1/2/3), inhibit the expression of downstream transcription factors AP-1 and ATF-2, affect the expression of related genes and proteins, thereby increase the synthesis of insulin and CP, and decreased fasting blood glucose, 24-h UPro, and dyslipidemia (TC, TG and LDL-c), improve dyslipidemia and insulin resistance, thereby protecting rats from DKD. In short, vitamin D_3 plays a positive protective role in DKD, this research provides a new theoretical support for the treatment of diabetic nephropathy with vitamin D_3.

Declarations

Ethics Approval and Consent to Participate

The study was approved by the animal ethics committee of Kunming Medical University.

Competing Interests

The authors declare that they have no conflicts of interest.

Statement

The study was carried out in compliance with ARRIVE guidelines 2.0.

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Authors' Contributions

RH and HL conceived and designed the study. XF, WZ, YZ, HZ, YW, and JF completed the animal experiment and prepared materials. LL, XF and RH carried out data acquisition and analysis. LL and XF drafted the manuscript. RH reviewed the manuscript. All authors read and approved the final manuscript.

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