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Effects of 1, 25-dihydroxyvitamin D3 on expression of TGF-β1, CD68 and MCP-1 in type 2 diabetic nephropathy rat.

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Abstract

Objective: This study aims to investigate the role and mechanism of 1, 25-dihydroxyvitamin D3 in type 2 diabetic nephropathy rats.

Methods: There were 30 male Sprague-Dawley (SD) rats that were randomly divided into 3 groups: the normal control group (NC group, n=10), the diabetic nephropathy model group (T2DN model group, n=10), and the diabetic nephropathy group treated with 1, 25-dihydroxyvitamin D3 (vD3-T2DN group, n=10). After 15 weeks, the changes of renal tissues morphology, renal function, and 24 h urinary protein quantification were measured. The expressions of Transforming Growth Factor beta 1 (TGF-β1), CD68 and Monocyte Chemoattractant Protein-1 (MCP-1) in renal cortex were also detected by Immunohistochemistry (IHC).

Results: The weight of rats were significantly decreased in T2DN model group and vD3-T2DN group than in NC group (P<0.05) at the end of 15th week, while the blood glucose, 24 h urinary protein and triglyceride were significantly increased (P<0.01). The expression of TGF-β1, CD68 and MCP-1 in T2DN model group and vD3-T2DN group were significantly higher than in NC group (P<0.01). Compared with NC group, the triglyceride (P<0.01) and serum creatinine (P<0.05) were significantly higher in T2DN model group. In vD3-T2DN group, the expression of TGF-β1, CD68 and MCP-1, the content of 24 h urinary protein (P<0.01), and triglyceride (P<0.05) were significantly than in T2DN model group.

Conclusion: Through repressing the expression of TGF-β1, CD68 and MCP-1, 1, 25-dihydroxyvitamin D3 can inhibit invasion of macrophages to protect kidney of T2DN rats.

Keywords: Type 2 diabetic nephropathy, 1, 25-dihydroxyvitamin D3, Transforming growth factor beta 1 (TGF-β1), CD68, Monocyte chemoattractant protein-1 (MCP-1).

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Introduction

Diabetic Nephropathy (DN) is one of common and serious chronic complication of Diabetes Mellitus (DM). DN is a severe microvascular complication, which is the common etiology of end-stage renal diseases [1,2]. In recently, a large number of studies [3-6] found that immune inflammatory cells, cytokines, acute phase reactive protein and other inflammatory mediators played important roles in the genesis and development of DN [7]. It is found that Monocyte Chemoattractant Protein-1 (MCP-1) is the main factor for monocyte-macrophage cell aggregation, which directly stimulated mesangial cell to express Transforming Growth Factor beta 1 (TGF-β1) to accelerate renal fibrosis [8]. TGF-β1 is a powerful fibrosis factor, which is involved in glomerular hypertrophy and Extracellular Matrix (ECM) progressive accumulation process through promoting ECM components accumulation, such as synthesis of collagen and fibronectin proteins and inhibition of degradation enzymes for ECM components, and ultimately leads to glomerulosclerosis [9-11].

Recent studies showed that 1, 25-dihydroxyvitamin D_3 (1, 25-$(OH)_2$ D_3, active vitamin D_3, calcitriol) is not only involved in the incidence of type 2 diabetes, but also protects kidney of DN

by reducing urinary protein, inhibiting aggregation of ECM, epithelial-mesenchymal, anti-inflammation and Renin Angiotensin Aldosterone System (RAS) [12-18]. In this study, we investigated the protective roles of vitamin D_3 in type 2 DN (T2DN) rats and explored the related molecular mechanisms.

Materials and Methods

Animal modeling and grouping

Totally 30 male SD rats (provided by Experimental Animal Center, Xinjiang Medical University) with weighing about 80 g, were divided into 3 groups. The Normal Control group (NC group) was fed with normal diet, while the diabetic nephropathy model group (T2DN model group) and the 1, 25-dihydroxyvitamin D_3 treatment diabetic nephropathy group (vD_3-T2DN group) were fed with high-fat and high-sugar diets (containing 10% refining lard, 20% sucrose, 2% cholesterol, 1% pig bile and 67% of normal diet). After 6 weeks, the T2DN model group and vD3-T2DN group were subjected to the tail intravenous injection with 30 mg/kg STZ (Sigma, St. Louis, MO, USA), which dissolved in pH4.5 citrate buffer after 12 h fasting. One week later, the Fasting Plasma Glucose (FPG) and 2 h plasma glucose (2hPG) were tested, and rats with FPG ≥ 7.0 mmol/L and/or 2hPG ≥ 11.1 mmol/L were considered as Type 2 diabetic (T2DN) models.

The test strip for urinary microproteins was used to measure continuously the morning urine of the T2DN rats after STZ injection at six weeks and seven weeks. If the results were positive, these rats were labeled as T2DN model, and they were continuously measured 24 h proteinuria. The T2DN model rats were given by gavage with 0.03 μg/kg vitamin D_3, dissolved in 0.05 ml peanut oil. Meanwhile, the T2DN model group were also treated by equal amount of peanut oil. Three groups of rats were sacrificed after 15 weeks. About 4 to 6 milliliters of venous blood and 24 h-urine prior to sacrifice were collected, and bilateral kidneys were removed and weighted. Renal cortex of 1 mm^3 was obtained from the kidney, and then fixed in 2.5% glutaraldehyde. Remaining kidney tissues were fixed in 4% paraformaldehyde.

Detection of biochemical parameters

Enzyme immunosorbent assay kit (Beyotime Institute of Biotechology, Haimen, Jiangsu, China) was adopted to measure 24 h-urinary albumin. Hitachi 7600 Automatic Biochemical Analyzer (Hitachi Science and Systems, Tokyo, Japan) was used to detect the content of blood glucose, creatinine, urea nitrogen, cholesterol and triglycerides.

Light microscopy examination

The light microscope samples were fixed in 4% paraformaldehyde and dehydrated by different concentration of alcohol (70%, 80%, 90%, 95%, and 100%). After hyalinized by xylene, these samples were embedded with paraffin, and then continuously cut into 2 μm sections. Hematoxin Eosin (HE) staining, Periodic Acid Schiff (PAS) reaction, Periodic-Acid-Silver Metheramine (PASM) staining and Masson's trichrom staining were performed before light microscope examination.

Transmission electron microscopy examination

The samples for transmission electron microscope oil were eluted with alcohol. After cutting into 1 mm^3 sections, the tissue was immediately fixed with glutaraldehyde liquid and osmic acid at 4°C. After dehydrated by acetone and alcohol, the samples were moved into acetone with epoxy resin, then cut into ultramicrocuts on ultramicrotome with 70-90 nm thickness after epoxy resin embedding, drying, fast repair and location. The sections were stained with uranyl acetate and lead Azusa citric acid, and then subjected to transmission electron microscopy on JEM100CX 2II electron microscope (Image Processing Center of Beihang University, Beijing, China).

Immunohistochemistry (IHC)

Paraffin sections were treated with normal procedures, then incubated with Rabbit anti-rat MCP-1 polyclonal antibody (l: 100 dilution; Boster Biological Technology, Wuhan, Hubei, China), mouse anti-rat CD68 (l: 200 dilution; Boster Biological Technology, Wuhan, Hubei, China) and TGF-β1 polyclonal antibody (l: 100 dilution; Santa Cruz, Texas, USA), at 37°C for 30 min respectively. The negative control was also dealt with PBS rather than antibody under same condition. Then secondary antibodies were added to incubate the sections. After stained with DAB chromogenic reagent (Beijing ZhongshanJinqiao Biotechnology Co., Ltd, Beijing, China) and counterstained with hematoxylin, these sections were visualized under microscopy with the CM-2000B biomedicine image analysis system (Beihang, Beijing, China). Five fields were randomly selected under high magnification (X400), and brown staining was considered as positive. The average number of positive cells in every 2 mm^2 were counted and calculated.

Statistical analysis

SPSS 17.0 software was used for statistical analysis, and data were expressed as mean ± Standard Deviation (SD). ANOVA was used for the group comparison and pairwise comparison. Chi-square test was used to analyse enumeration data. Correlation analysis was used to analyse the relationship of two variables, and $P<0.05$ was considered statistically significant.

Results

Comparison of biochemical characters among NC group, T2DN model group and vD3-T2DN group

In order to investigate the effect of vitamin D_3 on biological characters in T2DN model rats, we firstly measured the body weight, kidney weight, blood glucose content, 24 h urinary protein, triglycerides, cholesterol, blood urea nitrogen and

creatinine content among NC group, T2DN model group and vD_3-T2DN group. As shown in Table 1, the body weight and kidney weight was significantly lower in T2DN model group and vD_3-T2DN group than in NC group ($P<0.05$), while the blood glucose and 24 h urinary protein was significant higher in T2DN model group and vD_3-T2DN group than in NC group ($P<0.05$). However, the 24 h urinary protein was significant lower in vD_3-T2DN group when compared with T2DN model group. We also found that the concentration of triglycerides, cholesterol and creatinine in T2DN model group were all significant higher than in NC group. It was shown that the content of triglycerides was significant decreased in vD_3-T2DN groups compared with T2DN model group (Table 2).

Table 1. *Comparison of body weight, renal weight, blood glucose and 24 h urinary protein excretion among different groups (mean ± SD).*

Group	Number of rats	Body weight (g)	Renal weight (g)	Blood glucose (mmol/L)	Proteinuria (g/24 h)
NC group	10	486.8 ± 33.29	4.00 ± 0.34	6.96 ± 9.76	0.06 ± 0.51
T2DN group	10	404.8 ± 63.52*	3.92 ± 0.62	27.40 ± 2.96**	1.15 ± 0.34**
vD_3-T2DN group	10	414.6 ± 52.83*	3.98 ± 0.47	25.95 ± 4.62**	0.33 ± 0.14**▲▲

Note: Compared with NC group, *P<0.05, **P<0.01; compared with T2DN group, P<0.05, P<0.01.

Table 2. *Comparison of the content of triglycerides, cholesterol, blood urea nitrogen and creatinine among different groups (mean ± SD).*

Group	Number of rats	Triglycerides (mmol/L)	Cholesterol (mmol/L)	Blood urea nitrogen (mmol/L)	Creatinine (mmol/L)
NC group	10	0.88 ± 0.92	0.78 ± 0.38	8.89 ± 0.80	44.2 ± 4.64
T2DN group	10	8.30 ± 5.15**	28.10 ± 19.47**	11.78 ± 4.62	306.40 ± 257.94*
vD3-T2DN group	10	4.28 ± 3.94▲	28.79 ± 24.26**	10.95 ± 3.33	180.11 ± 329.33

Note: Compared with NC group, *P<0.05, **P<0.01; compared with T2DN group, P<0.05, P<0.01.

Light microscopy examination of the renal tissues among NC group, T2DN model group and vD3-T2DN group

To elucidate the effects of vitamin D_3 on renal pathology in T2DN model rats, Masson staining on renal tissues was performed. Compared with NC group, mesangial cells were hyperplasia from mild to moderate in T2DN model group, and the area of mesangial area was increased in the glomerular capillary (Figure 1). The tubulars were expanded and infiltrated with a large number of inflammatory cells. Compared to T2DN model group, kidney tubulars were slightly expanded and less inflammatory cells were infiltrated in vD_3-T2DN group than in T2DN model group.

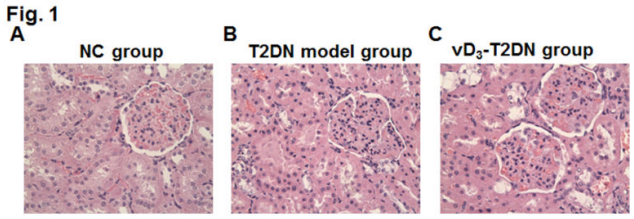

Figure 1. *Masson staining was performed to assess the pathological changes in renal tissues among NC group (A), T2DN model group (B) and vD3-T2DN group (C). Magnification: X400.*

TEM examination of the renal tissues among different groups

To further confirm the therapeutic effects of vitamin D_3 on renal tissues in T2DN, TEM detection was also performed. As shown in Figure 2, several characters were observed in T2DN model group rather than in NC group, such as mesangial endothelium swelling, lipid droplets hyperplasia, podocytes Golgi proliferation and actin enrichment, endotheliocytes vacuole degeneration, renal tubulars nuclear condensation, nuclear gap widened, thick clumps of heterochromatin and endoplasmic reticulum expansion. While in the vD_3-T2DN group, mild edema and denser massive structures were obvious in podocytes, valuolar degeneration changed slightly in endotheliocyte and mesangial cells, capsular space and vessel opened less, fat degenerated slighter in lipid droplets, and podocytes edema was slighter. These results validated the therapeutic effects of vitamin D_3 in T2DN.

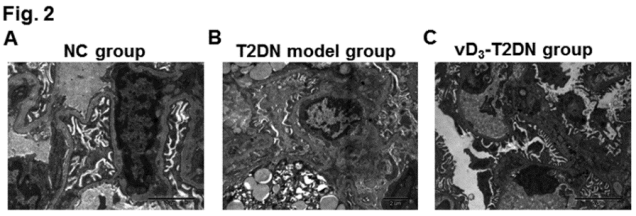

Figure 2. *TEM detection of the renal tissues among NC group (A), T2DN model group (B) and vD3-T2DN group (C). Scale bar: 1 μm.*

Expression of CD68, MCP-1 and TGF-β1 among NC group, T2DN model group and vD$_3$-T2DN group

To investigate the molecular mechanism of vitamin D_3 in the therapy on the T2DN, IHC was performed to detect the expressions of CD68, MCP-1 and TGF-β1 in renal tissues. As shown in Figure 3 and Table 3, CD68, MCP-1 and TGF-β1 were mainly expressed in the renal tubules and interstitial cytoplasm. The expression of CD68, MCP-1 and TGF-β1 were hardly detected in NC group, while they were highly expressed in T2DN model group and vD$_3$-T2DN group ($P<0.01$). In the vD$_3$-T2DN group, the expression of CD68, MCP-1 and TGF-β1 were higher than in NC group, and they were lower than in T2DN model group. These results indicated that vitamin D_3 treatment could regulate the expression of CD68, MCP-1 and TGF-β1, which contribute to its protection effect in T2DN.

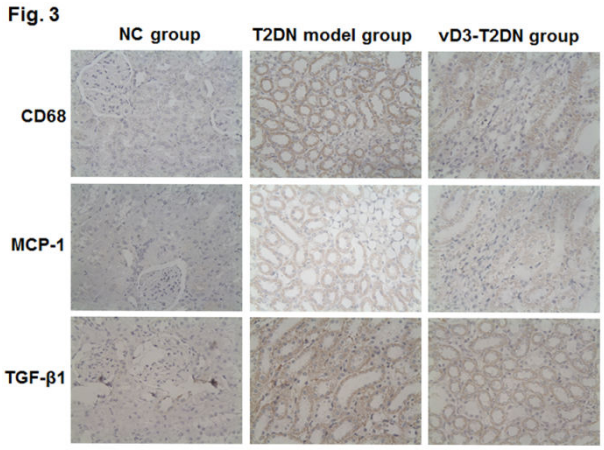

Figure 3. *Immunohistological staining was performed to assess the expression of CD68, MCP-1 and TGF-β1 protein among NC group, T2DN model group and vD$_3$-T2DN group. Magnification: X400.*

Table 3. *Comparison of TGF-β1, MCP-1 and CD68 expression among different groups (mean ± SD).*

Group	TGF-β1	MCP-1	CD68
NC group	2.58 ± 0.68	2.20 ± 0.79	2.56 ± 1.06
T2DN group	16.2 ± 2.12$^{**▲▲}$	15.3 ± 2.70$^{**▲▲}$	7.72 ± 2.04$^{**▲▲}$
vD3-T2DN group	5.02 ± 1.75$^{**▲▲}$	7.66 ± 3.09$^{**▲▲}$	7.72 ± 2.04$^{**▲▲}$

Note: Compared with NC group, $^{*}P<0.05$, $^{**}P<0.01$; compared with T2DN group, $P<0.05$, $P<0.01$.

Discussion

In recent years, several studies revealed that the key factors for the development of DN were inflammation and macrophages infiltration [19]. Moreover, the infiltration of mononuclear macrophages in renal tissues are correlated with accumulation of glomerular Extracellular Matrix (ECM) in glomerular mesangial and renal tubular, which is the characteristic pathological changes of DN [20]. MCP-1 is a cytokine secreted by renal tubular epithelial and mesangial cells, and its main function is to activate mononuclear macrophage, and its expression in renal tissue can serve as a biomarker of inflammation [8,21-23]. Cheng et al. found that MCP-1 could combine with glomerular mesangial cell membrane protein CCR2, which led to activate NK-κB pathway to induce TGF-β1 expression and increase the expression of fibronectin (Fn) in mRNA level and protein level. It was verified that the active TGF-β1 in DN was related to deposition of glomerular ECM [9], expression of type I collagen in mesangial cells [24], increased expression of Fn [25] protein and worsen renal function of DN.

Studies have shown that vitamin D_3 plays its biological roles in cells through combining to vitamin specific receptor (VDR) [26-28]. Li et al. found that the diabetic mice with VDR deletion showed strongly active Renin-Angiotensin System (RAS) during their growth and development stage, accompanied with severe renal impairment [29]. However, the kidney injury was significantly alleviated through inhibition of RAS and therapy with vitamin D analogues. Zhang et al. confirmed that vitamin D_3 could inhibit the expression of renin and TGF-β1, reduce proteinuria and delay progression of renal failure through giving 1, 25-$(OH)_2$ D_3 to VDR knockout mouse model [30].

In this study, we firstly established T2DN rat model, and found that proteinuria and TGF -β1, MCP-1 and CD68 expression was significant higher in T2DN model group than in the NC group, while it was lower than in the vD$_3$-T2DN group. These results indicated that active vitamin D_3 could repress the expression of TGF-β1, MCP-1 and CD68, and protect the integrity of glomerular filtration membrane and diminish proteinuria. We also found that the body weight decreased significantly in T2DN model group and vD$_3$-T2DN group than in NC group. At the end of week 15, the content of glucose, 24 h urine protein and cholesterol was significantly increased in T2DN model group and vD$_3$-T2DN group than in NC group, as well as the expression of CD68, MCP-1, TGF-β1. Moreover, the content of triglycerides and creatinine was also significantly up-regulated in T2DN model group than in NC group. For the expression of CD68, MCP-1 and TGF-β1, it was similar to Tian et al. [31] when compared vD$_3$-T2DN group with T2DN model group. Meanwhile, the 24 h urine protein and triglycerides were significantly decreased in vD$_3$-T2DN group.

Furthermore, our results showed that the 24 h urine protein was obviously decreased in vD$_3$-T2DN group, whose kidney injuries were also alleviated, compared with T2DN model group. This result indicated that 1, 25-$(OH)_2$ D_3 could protect kidney in T2DN rats. In this study, it was indicated that Vitamin D may reduce proteinuria in diabetic nephropathy, which was consistent with reported clinical trials [32].

We also investigated the protection mechanism of 1, 25-$(OH)_2$ D_3 effect on T2DN rats and found that the infiltration of macrophages were significantly decreased in kidney tissues of vD$_3$-T2DN rats, and 1, 25 $(OH)_2$ D_3 could reduce the expression of CD68, MCP-1 and TGF-β1 [31]. TGF-β can be induced by angiotensin II, which is a powerful factor for mediating fibrosis [33]. Through stimulating the synthesis of

extracellular matrix proteins and reducing the degradation of matrix proteins, TGF-β can promote apoptosis of foot cells, which plays a key role in diabetic glomerulosclerosis [34]. And MCP-1 is monocyte chemoattractant protein-1, which can lead to kidney tissue injury through activating inflammatory cells and prompting the release of soluble medium from inflammatory cells [35]. CD68 is one kind of macrophage marker, and macrophage infiltration plays as an important mediator for renal interstitial fibrosis [36]. In this study, it was indicated that the expressions of TGF-β1, MCP-1 and CD68 were decreased in kidney tissues of DN rat, which further validated that vD_3 can alleviate diabetic nephropathy through inhibiting macrophage infiltration in kidney and relieving inflammatory pathways.

Vitamin D could not affect the blood glucose content, because the content of blood glucose showed no difference between in T2DN model group and in vD_3-T2DN group. This result indicated that the renal protective effect of vitamin D may be not reached by lowering glucose and reducing glycosylated productions. Zhao et al. developed a clinical trial that also indicated that vitamin D can protect diabetic nephropathy independent of the control of blood glucose and blood pressure [37].

Our study suggests that 1, 25-$(OH)_2$ D_3 could play protective roles in kidney through reducing the expression of MCP-1 in renal tissues, which inhibits the infiltration of macrophages and down-regulates TGF-β1 expression.

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Disclosures

All authors declare no financial competing interests. All authors declare no non-financial competing interests.

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