Vitamin D is involved in the effects of the intestinal flora and its related metabolite TMAO on perirenal fat and kidneys in mice with DKD

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Abstract

Vitamin D directly exerts a protective effect on the kidneys of individuals with diabetic kidney disease (DKD) in our previous study. However, whether it has an effect on perirenal adipose tissue (PRAT) or the intestinal flora and its metabolites (trimethylamine N-oxide, TMAO) is unclear. We found that 1,25-(OH)$_2$D$_3$ could improve the dysbiosis of the intestinal flora of mice with DKD, increase the abundance of beneficial bacteria such as lactic acid bacteria, decrease the abundance of harmful bacteria such as Escherichia, reduce the pathological changes in kidney histopathology, reduce fat infiltration, and downregulate the mRNA expression of TLR4 and NF-$\kappa$B in kidney tissue. We also found that the serum TMAO concentration in mice with DKD was significantly higher than that of the control group, and serum TMAO content was significantly positively correlated with urine ACR. In addition, vitamin D stimulated the expression of the surface markers PGC1$\alpha$, UCP-1 and VDR in the PRAT in mice with DKD, and TMAO downregulated the expression of PRAT and renal VDR. The above results show that the renal protective effect of 1,25-(OH)$_2$D$_3$ on mice with DKD may also be related to the improvement of the intestinal mucosal barrier, composition of the intestinal flora and its metabolites, inhibition of the TLR4/NF-$\kappa$B inflammatory pathway and reduction in PRAT metabolite effects on the kidney. This study provides a theoretical basis for the use of hypoglycemic drugs combined with vitamin D therapy to improve diabetic nephropathy.

Introduction

The intestinal microbial metabolites are absorbed by the host act on receptors in organs such as the liver, intestine, brown adipose tissue (BAT), white adipose tissue (WAT) [1]. Dietary choline is metabolized by the gut flora to trimethylamine (TMA) and further oxidized in the liver to trimethylamine N-oxide (TMAO) [2]. In animal models, TMAO accumulation impairs liver function and increases hepatic triglyceride accumulation and lipogenesis. High TMAO levels are associated with vitamin D deficiency and nonalcoholic fatty liver disease (NAFLD) [3]. The abundance and composition of the gut microbiota have been proved that it could influence lipid metabolism in mouse and human blood and tissues [4]. Recent reports have shown that TMAO is associated with a high risk of development of renal fibrosis and renal impairment, as well as reduced rates of long-term survival[5, 6]. However, the mechanistic link between TMAO and diabetic kidney disease (DKD) remains to be explored.

In recent years, following the study of the "intestinal-renal axis" and urinary toxins, TMAO has become the subject of a node of research activity in the field of nephropathy as a metabolite of intestinal bacteria and a urine toxin affecting the prognosis of CKD [7]). The roles of vitamin D/VDR receptor (VD/VDR) have been well documented in kidney protection. Studies have shown that the interaction between VD and gut microbes is closely related to inflammation [8, 9], VD favors probiotics rather than pathogenic microbial colonization when the body is in an inflammatory state [10, 11], and VDR activation exerts anti-inflammatory effects by inhibiting the activation of NF-$\kappa$B in tubular and mesangial cells [12]. The association between decreased serum 25(OH)D levels in animal models of DKD and intestinal flora characteristics has been confirmed in many studies and in our previous studies [13, 14]. However, it is
unclear whether VD is involved in the changes in the intestinal flora seen in mice with DKD or in the relationship between TMAO and the kidneys.

There are three types of fat around the kidney: pararenal fat, antral fat, and perirenal adipose tissue (PRAT). PRAT is located in the retroperitoneal space and is considered as a simple connective tissue that protects the kidneys and renal vessels from external physical stimuli [15]. Because PRAT is anatomically in direct contact with the kidneys, its presence can lead to various pathological abnormalities when its expansion due to obesity or diabetes [16]. Some scholars speculate that the expansion of PRAT means an increase in white adipocytes, which promote the progression of kidney damage [17]. With the development of device, it has been found that a large amount of BAT is around the kidney and has high metabolic activity [16]. Adipose tissue is considered as an endocrine organ that secretes various adipokines, rather than just storing energy. As an endocrine organ, PRAT contains highly activated beige adipose cells transformed by white adipocytes [18], which has the effect of increasing lipid catabolism and mitochondrial respiration, accelerating the energy expenditure of WAT, and reducing inflammation.

Recent studies have shown that the production of TMAO is associated with inhibition of the activities of the beige and white adipose tissue [19]. In addition, the TMAO synthesis pathway is becoming an increasingly attractive therapeutic target for obesity-related conditions such as diabetes and DKD [20]. Typical markers of WAT browning are upregulation of uncoupling protein 1 (UCP1) expression and mitochondrial biosynthesis. However, it is uncertain whether TMAO can promote inflammatory changes in perirenal adipocytes in DKD mice and whether vitamin D plays a role in the effects of TMAO through VDR in the PRAT and the kidneys of DKD mice. Therefore, this study explores the effects of the administration of different concentrations of 1,25-(OH)\(_2\)D\(_3\) on the TLR4/NF-κB pathway in the intestinal flora and kidneys in DKD mice, as well as the relationship between serum TMAO and urine ACR. Further, it explores whether TMAO has an effect on the metabolism of PRAT to understand its possible relationship with active VD participation in the intestinal flora on the kidneys of DKD mice. Finally, it proposes new insights for the prevention and treatment of DKD.

**Materials and methods**

I. Animals

Sixty 8-week-old male spontaneous KKay (type 2 diabetes) mice and 10 C57BL/6 mice (Beijing Huafukang Biotechnology Co., Ltd. (license number: SCXK (Beijing) 2020-0004)), all weighing 18–22 g, were raised in an SPF animal room.

II. Methods

1. Experimental groups: Fifty-six KKay mice were divided into the DM group (n = 10, raised to 14 weeks of age) and DKD group (n = 46, raised to 20 weeks of age), and C57BL/6 mice were used as the control group (n = 10, raised to 20 weeks of age). The 56 mice in the DKD groups were divided into the placebo group (n = 10), low dosage (LVD, 0.3 µg/kg/d) (n = 10), medium dosage (MVD, 1.4 µg/kg/d) (n = 16), and
high dosage (HVD, 2.5 µg/kg/d) (n = 10) concentration 1,25-(OH)$_2$D$_3$ early intervention groups. The same volume of normal saline and the different concentrations of 1,25-(OH)$_2$D$_3$ were injected daily beginning when KKay mice reached 18 weeks of age for 14 days. Six of the DKD mice were administered 0.12% TMAO at the same time as medium-dose 1,25-(OH)$_2$D$_3$ injection and housed until 20 weeks of age. At the end of the experiment, mice were sacrificed for feces, urine, serum, perirenal adipose tissue and kidney tissue collection for subsequent experiments. This experimental setup was approved by the Affiliated Hospital of Zunyi Medical University and the Animal Ethics Committee.

2. Weight, blood sugar and urine protein measurements:

The weights of the mice were measured by a scale. Blood glucose was measured by tail clipping. Urine samples were collected in metabolic cages, and the urine protein excretion rate, urine microalbumin content and urine creatinine content were detected by electrochemiluminescence method. Urine ACR = urine microalbumin/urine creatinine ratio (unit: mg/g). The urine tests were completed on the automatic biochemical analyzer (Beckman Coulter AU5821).

3. Serum TNF-α and TMAO content determination:

Ocular venous blood was taken and ELISA was used to detect serum TNF-α levels (R&D Systems, USA). Serum TMAO was detected by LC–MS assay under chromatographic conditions: ACQUITY UPLC® BEH HILIC Column (2.1×100 mm, 1.7 µm, Waters, USA).

4. Urine tubular injury molecule (KIM-1) and TMAO determination:

ELISA was used to detect urinary KIM-1 (Abcam, Inc., ab213477). Urine TMAO detection was performed by LC–MS assay, chromatographic conditions: ACQUITY UPLC® BEH HILIC Column (2.1×100 mm, 1.7 µm, Waters, USA).

5. Perirenal adipose tissue, kidney tissue and fecal specimen collection:

Approximately 0.5-1.0 g of feces from between the colon and rectum segments was collected and stored in a sterile cryopreservation tube in a freezer at -80°C for subsequent experiments.

6. qPCR: qPCR was used to detect the mRNA expression of TLR4, NF-κB, PGC1α and UCP-1 in kidney tissue. Primer sequences are listed in Table 1.

7. Fecal microbiota DNA extraction and variable region PCR amplification:
Table 1
PCR primer sequences

<table>
<thead>
<tr>
<th>gene</th>
<th>forward primer(5′–3′)</th>
<th>reverse primer(3′–5′)</th>
</tr>
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<tbody>
<tr>
<td>PGC1α</td>
<td>ATGGCATGGCTTACACCACC</td>
<td>GAGGCCAATTTTGCTCCACA</td>
</tr>
<tr>
<td>UCP-1</td>
<td>ATGGCATGGCTTACACCACC</td>
<td>GAGGCCAATTTTGCTCCACA</td>
</tr>
<tr>
<td>NF-κB</td>
<td>AGGCTTCTGGGCCCTTATGTG</td>
<td>TGCTTCTCTCGCCAGGAATAC</td>
</tr>
<tr>
<td>MCP-1</td>
<td>TCTCTTCTCCACCACCTATGCA</td>
<td>GGCTGAGACAGCAGTGAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>GGCTGTATTCCCCCTCCATCG</td>
<td>CCAGTTGGTAACAAATGCCATGT</td>
</tr>
</tbody>
</table>

The target sequence was selected after DNA was extracted from the feces from the colon and rectum, and the 16S rRNA V3-V4 region was amplified. The primers 338F (5′ACTCCTACGGGAGGCAGCAGCA3′) and 806R (5′GGACTACHVGGGTWTCTAAT3′) were used for amplification. 16S rRNA was sequenced to determine the variety, quantity and function of the bacteria composing the intestinal flora.

8. HE, PAS, and Masson staining to observe the pathological changes in kidney tissue:

The slide was observed under a light microscope (200× and 400×).

9. Immunofluorescence for podocin expression in the kidney:

Each slide was photographed under a 100×/400× fluorescence microscope.

10. Immunohistochemistry to reveal the expression of VDR, PGC1α and UCP-1 in kidney and adipose tissue:

Each slide was observed and photographed under a 100×/200× microscope.

11. Electron microscopy to observe pathological changes in the kidneys and perirenal adipose tissue

12. Construction of a VDR knockout vector:

The VDR gene sequence (Gene ID: 22337) was determined by querying the NCBI database, and the CDS fragment of VDR was synthesized. The overexpression plasmid was cloned into a lentiviral vector. Finally, titer detection was performed.

III NovaSeq sequencing, bioinformatics analysis and statistical processing:

NovaSeq Platform Sequencing (Paysenno Biomicrobiology LLC) was used to perform alpha and beta diversity and taxonomic composition analyses of the data. The analysis of between-group differences at the species and phylum levels was carried out using Metastats software. SPSS 25.0 software was used for statistical analysis. P < 0.05 indicated that the differences were statistically significant.
Results

I. Construction of DKD experimental mouse model

Mice were administered high-fat and high-sugar feed until 20 weeks of age. Three blood glucose readings from the tail vein that were greater than 16.7 mmol/L times on the same day indicated that the type 2 diabetes mellitus (T2DM) model had been successfully established (8). 24-hour urine protein excretion (24huAlb) > 30 mg (9) and/or urine microalbumin/creatinine ratio (ACR) > 30 mg/g, which determined that the DKD model was successfully constructed.

II. Effects of vitamin D supplementation on renal function, intestinal flora characteristics, TMAO metabolites, and the TLR4/NF-κB pathway in mice with DKD

The urine ACR of mice in the DKD group was significantly higher than that of DM mice and of control mice. After 1,25-(OH)$_2$D$_3$ supplementation, the mental state and the diet of the mice improved. With an increase in the concentration of 1,25-(OH)$_2$D$_3$ administered, urine ACR began to decrease, and the HVD group had the most substantial decrease compared to the other groups. (see Fig. 1A). The pathological changes in renal tissues observed by HE and Masson staining showed mesangial stromal hyperplasia, and the glomerulus, tubular and interstitial fibrotic lesions and increased glomerular volume of mice with DKD. The size of the glomerular, tubular and interstitial fibrotic lesions were reduced with the administration of different concentrations of 1,25-(OH)$_2$D$_3$, and the HVD group improved significantly compared with the others (see Fig. 1B). Immunofluorescence observation of renal podocin expression showed that the glomerular volume of mice in the DM and DKD groups increased, and podocin expression decreased. Podocin expression in kidney tissues was upregulated with the administration of different concentrations of 1,25-(OH)$_2$D$_3$. The HVD group improved significantly compared with the others (see Fig. 1C).

The results of NovaSeq sequencing and bioinformatics analysis suggested that the abundance of Bacillota in the gut microbiota of the three groups was 56.46% in the control group, 43.25% in the DM group and 36.38% in the DKD group; the abundance of Bacteroidota was 41.62% in the control group, 34.31% in the DM group and 34.89% in the DKD group; the proportion of Pseudomonadota in the control group was very low, and the DM and DKD groups were increased by 18.27% and 25.22%, respectively (see Fig. 1D). At the genus level, Proteus, Lactobacillus, Staphylococcus and Shigella were the main genera. The control group had a higher relative abundance of Lactobacillus (P = 0.001), while the DKD group had a relatively dominant distribution of Shigella (P = 0.001). After 1,25-(OH)$_2$D$_3$ intervention, the richness index of the gut microbiota increased significantly, and the high abundance of G-bacteria that was expressed in the DKD group decreased after 1,25-(OH)$_2$D$_3$ intervention (see Fig. 1E). There was a significant difference in the gut microbiota makeup between DM and DKD mice. The richness and diversity of the gut microbiota of DKD mice were also reduced and the proportions of intestinal bacteria associated with TMAO production, such as Desulfovibrio, Clostridium, Enterococcus, Streptococcus and Acinetobacter, were significantly higher than those in the DM and control groups (see Fig. 1F).
Compared with the control group, the expression of TLR4 and NF-κB mRNA in kidney tissues in the DM and DKD groups was upregulated, and those of the DKD group were higher than that of the DM group. After 1,25-(OH)$_2$D$_3$ intervention, the mRNA expression of TLR4 and NF-κB in kidney tissues was downregulated, and the HVD group had significant improvement compared with the others (see Fig. 1G). Compared with the control group, TNF-α concentrations were increased in both the DM and DKD groups (P = 0.000). Compared with the DKD group, the serum TNF-α concentration of DKD mice decreased significantly after intervention with 1,25-(OH)$_2$D$_3$, and the serum TNF-α level showed a dose–response relationship with the increase in the concentration of 1,25-(OH)$_2$D$_3$. The difference was statistically significant (P = 0.001) (see Fig. 2H). The serum TMAO concentration of DKD mice was significantly higher than that in the control group, and serum TMAO was positively correlated with urine ACR (r = 0.4226, P = 0.0002) (see Fig. 2I). Serum and urine TMAO were downregulated after vitamin D treatment, and expression of the renal injury marker KIM-1 was also downregulated significantly (see Fig. 1J).

We observed that 3 mice died after high-concentration 1,25-(OH)$_2$D$_3$ intervention. Hypoglycemia occurred the day before death, so a medium concentration (1.4 µg/kg/d) of 1,25-(OH)$_2$D$_3$ was used for intervention for subsequent experiments.

III. VD played a role in the effect of TMAO on perirenal fat and kidney in mice with DKD

We collected kidney and perirenal adipose tissue samples from DKD mice (Figs. 2A, B) and found that the kidneys of DKD mice were surrounded by a layer of beige adipose tissue, and the fat mass of DKD mice was significantly higher than the control group (Fig. 2C). These samples were stained with HE staining (Fig. 2D) and perirenal fat appeared to shift towards a beige adipose tissue phenotype after vitamin D intervention, revealing multilocular adipocytes of various sizes. Electron microscopy (Fig. 2E) showed abundant mitochondria, mitochondrial swelling, crest fracture loss, and plate layer reduction in perirenal adipocytes in the DKD group; the expression of perirenal fat and renal VDR increased and the mitochondrial structure of adipocytes was significantly improved after vitamin D treatment. Compared with the control group, DKD mice fed a high-fat diet had more adipose cells and higher levels of hypertrophy. In DKD mice, intravenous injection with 1.4 µg/kg/d 1,25-(OH)$_2$D$_3$ and dietary supplementation with 0.12% TMAO caused a shift towards beige adipose tissue in the 1,25-(OH)$_2$D$_3$ intervention group. Adipose cells were more hypertrophied after TMAO administration. VDR expression decreased in the DKD group, and the expression of PGC1α (Fig. 2F) and UCP-1 (Fig. 3G) were increased after 1,25-(OH)$_2$D$_3$ intervention, in both the kidney and PRAT beige adipocytes. Vitamin D stimulation upregulated expression of VDR, while PRAT and renal VDR expression were downregulated after TMAO supplementation (Figs. 2H and 2I). Oil red O staining showed a significant increase in PRAT lipid infiltration after TMAO intervention and an effective reduction in PRAT lipid infiltration after 1,25-(OH)$_2$D$_3$ intervention (Fig. 2J), as well as lowered concentration of the inflammatory cytokine MCP-1 (Fig. 2K).

IV. Inhibition of VDR expression affected the function of DKD glomerular podocytes
Figure 3A–3C showed that the Lenti-shVDR transfection was effective. VDR-KO mouse renal VDR expression was significantly lower than that of the control group as determined by WB (see Fig. 3D). VDR expression was reduced in liver, intestine, kidney, and adipose tissue (see Fig. 3E). The intestinal mucosa was disrupted, and permeability was increased (see Fig. 3F). In PRAT, single cells were enlarged in the VDR-KO group, and the fat content increased at the same volume (see Fig. 3G–3H). The amount of PRAT did not change, and levels of indicators of energy metabolism and brown fat decreased (see Fig. 3I). Inhibition of VDR expression affected the function of DKD glomerular podocytes, which were in an inflammatory state. The expression of TNF-α was also increased (see Fig. 3L), the expression of podocin protein decreased (see Fig. 3K), and the expression of the fibrosis indicator collagen IV increased (see Fig. 3J).

Discussion

Following the development of sequencing technology, the intestinal flora has attracted much attention in recent years. The intestinal flora is strongly associated with many chronic diseases [21, 22], and Ackermannia species are abundant in patients with type 2 diabetes [23]. Studies have shown that SCFA-producing bacteria and Megasphaera in patients with T2DM and DKD may be involved in the development of DKD, while harmful bacteria such as Escherichia and Enterococcus are more abundant in DKD patients than in healthy people. In this study, 16S RNA sequencing showed that the abundance of intestinal flora in DKD mice changed: the abundance of beneficial bacteria such as lactic acid bacteria decreased, and the abundance of G-bacteria increased significantly. However, the effect of VD on the intestinal flora in patients with DKD has rarely been reported.

VD deficiency has been shown to be an important risk factor for the onset of CKD, and aggressive supplementation with VD can improve survival in patients with CKD [24, 25]. Studies have shown that the interaction between VD and gut microbes may be closely related to the regulation of inflammation [8, 9], but the specific mechanism is not clear. W. H. Wilson Tang et al. suggested that the gut microbiota relies on the TMAO synthesis pathway to promote the development of renal insufficiency and to increase the risk of death in CKD[6]. TMAO inhibits albumin uptake in human proximal tubular cells through PI3K and ERK signaling [26]. We speculate that these harmful and beneficial bacteria activate cytokines and produce systemic inflammatory responses by regulating the secretion of the intestinal metabolite TMAO; perhaps these intestinal metabolites directly participate in or aggravate the pathogenesis of DKD. This study also found that the serum TMAO concentration of mice with DKD was significantly higher than that in the control group, and serum TMAO content was positively correlated with urine ACR.

In this study, it was also found that the intestinal microbiota richness index increased significantly after 1,25-(OH)₂D₃ intervention, indicating that the diversity of intestinal flora increased after 1,25-(OH)₂D₃ supplementation, and the abundance of Firmicutes gradually increased with increasing 1,25-(OH)₂D₃ concentration. This is similar to the results of recent studies [10, 11, 27, 28]. The effect of 1,25-(OH)₂D₃ on the gut microbiota is inconclusive, and our study is consistent with the findings of Bashir et al., which
found a decreased relative abundance of Proteus and increased relative abundance of Bacteroides after VD supplementation.

Adipose tissue is an important storage site for VD. VD deficiency is associated with abnormal fat production. This study found that VD supplementation improved renal fat infiltration in mice with DKD. High-fat foods are metabolized by gut microbes, and plasma TMAO concentrations subsequently become elevated [2].

Studies have also shown that increasing dietary vitamin D intake affects intestinal microbial regulation which plays a key role in maintaining immune homeostasis in healthy people [10, 29]. This study also found that as the expression of the TLR4/NF-κB inflammatory signaling pathway in kidney tissues of DM and DKD mice increased, the concentration of inflammatory factor TNF-α and urine protein levels increased, as well as the levels of the pathological changes of the glomerulus. Conversely, the expression of podocin was decreased. Compared with the control group, the expression of urine protein and markers of the TLR4/NF-κB signaling pathway were downregulated, podocin expression was increased, and the inflammatory state and pathological changes were improved after 1,25-(OH)₂D₃ intervention. In the VDR gene knockout experiments, it was further verified that inhibition of VDR expression affected the function of glomerular podocytes in diabetic nephropathy, which were in an inflammatory state, and the expression of podocin was reduced. This is similar to the results of previous studies.

Obeid et al. reported that vitamin D supplementation for 12 months is effective in reducing plasma fasting TMAO content and increasing plasma choline, a precursor to TMAO and an essential component for synthesizing phosphatidylcholine, which in turn is necessary to promote lipid output from the liver [30]. SHERRIFF J L speculates that the deficiency of choline is associated with the accumulation of lipids in the liver [31]. Vitamin D supplementation reduces choline-induced levels of TMA and TMAO by regulating the composition of the gut microbiota. Plasma levels of TMAO are inversely correlated with the abundance of Bacteroides and Ackermanella [32]. In previous studies, eight species and six genera of two different phyla (phloderma and Proteus) have shown significant choline depletion and TMA accumulation [33].

This study found that serum and urine TMAO levels were downregulated after vitamin D treatment, and that levels of the kidney injury marker KIM-1 was also downregulated. This is consistent with the findings of Obeid et al. [30]. This suggests that TMAO biosynthesis is dependent on vitamin D. Mateusz Ozorowski et al. had similar findings that cholecalciferol supplementation reduced leptin and TMAO levels in patients with obesity and vitamin D-deficiency [34].

It has been suggested that low vitamin D levels are associated with high TMAO levels and changes in the gut microbiota in obese individuals [3, 7]. A cross-sectional study found that vitamin D levels decreased significantly with increasing plasma TMAO concentrations; vitamin D levels were lowest and TMAO levels were highest in individuals with class III obesity [3]. All of the above studies suggest that vitamin D levels show a significant negative correlation with circulating TMAO levels. Our study partially explains
that the mechanism of this phenomenon may be related to TMAO promoting fat deposition and vitamin D reducing fat deposition.

The presence of PRAT has recently been identified as an independent risk factor for CKD progression and is associated with cardiac and renal dysfunction [35]. In this study, we found that the lipid infiltration of PRAT increased significantly after TMAO intervention, PGC1α and UCP-1 expression increased after vitamin D intervention in both the kidney and in PRAT, and the concentration of the inflammatory cytokine MCP-1 was decreased. Comparing DKD mice PRAT with control mice PRAT, we found that the volume of PRAT in DKD mice was significantly increased, and we hypothesized that TMAO can act on PRAT and have an impact on kidney function, while vitamin D can antagonize the effect of TMAO.

However, this study did not thoroughly study the changes in intestinal and renal pathology or the changes in intestinal flora after supplementation with probiotics or fecal bacteria transplantation. This study also did not thoroughly explore the antagonistic mechanism associated with vitamin D and TMAO, which must be explored in depth in the future.

In summary, intestinal dysbacteriosis and its metabolite TMAO may be involved in the pathogenesis of DKD, and the protective effect of 1,25-(OH)₂D₃ on kidney tissue in DKD mice may be related to the inhibition of the activation of the renal TLR4/NF-κB inflammatory signaling pathway after improving intestinal dysbacteriosis. TMAO can affect PRAT and kidney function, but the effect of the microflora and its metabolites on kidney tissue and PRAT and the specific mechanism of action between kidney and PRAT in DKD needs to be further studied.

Declarations

Conflict of interest: The authors declare that they have no conflict of interest.

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References


Figures
**Figure 1**

A. Urine ACR results (n=6 mice per group) DKD: Diabetic Kidney Disease LVD: low dose vitamin D group MVD: medium dose vitamin D group HVD: high dose vitamin D group *p<0.05,#p<0.01

B. HE Masson staining. (18 images per 6 biological replicates for each group)

C. Immunofluorescence staining (with an anti-Podocin antibody). (18 images per 6 biological replicates for each group) sections of kidney. 200×

D. Histogram of the relative abundance of the top 10 bacteria at the phylum level (n=6 mice per group)
Figure 2
(A) kidney tissue of different groups after 1,25-(OH)2D3 intervention (B) Gross anatomy of perirenal adipose tissue (C) Comparison of fat mass (n=6 mice per group) (D) HE staining for Perirenal adipose tissue (18 images per 6 biological replicates for each group). Scale bar, 200x (E) Electron microscopy for Perirenal adipose tissue in DKD and 1,25-(OH)2D3 intervention group (VD) (n=6 mice per group). Red arrow: mitochondria, blue arrow: lipid droplets, N: nucleus. Scale bar, 100μm. (F)-(I) IHC-stained (with an anti-PGC1α and UCP1 and VDR antibody) sections of PPAT and kidney. Scale bar, 50μm. (J) Oil stained (18 images per 6 biological replicates for each group). (K) Kidney and PRAT MCP-1 and PGC1α and UCP-1 mRNA expression (1 technical replicate of 6 biological replicates per group).
Figure 3

(A) Fluorescence imaging of three different concentrations of viral fluid in 293T cells. Fluorescence imaging after 24 h transfer of 293T cells (upper layer, 100 ×); Fluorescence imaging after 48 h transfer of 293T cells (lower layer, 200×). NC: Blank control, no viral fluid injection; a: Viral fluid with an average titer of 3.02E+08; b: Viral fluid with an average titer of 2.60E+08; c: RT-PCR verification of 3 viral fluids with an average titer of 2.81E+08 (B) VDR silencing efficiency (C) renal tissue recombinant lentiviral
immunofluorescence validation (×200) (D) VDR-KO mouse kidney tissue recombinant lentiviral immunofluorescence verification (×200) (E) representative images of liver, intestine, kidney, adipose tissue, VDR protein expression (F), intestinal HE staining (×100) and ZO-1 fluorescence (×200). The intestinal mucosa is destroyed and permeability increases. (G) VDR expression decreased in PPAT histochemical VDR-KO group. Representative image of (×400)(H) Oil stained. (I) Representative image of UCP-1 immunofluorescence of kidney UCP-1 with reduced expression of oil red staining in the VDR-KO group. Representative image of (×400)(J) renal Collagen IV histochemistry. Representative image of (400x)(K) Podocin immunofluorescence. (200x)(L) TNF-α expression, p<0.05