Early 1,25-Dihydroxyvitamin D$_3$ Supplementation Effectively Lowers the Incidence of Type 2 Diabetes Mellitus via Ameliorating Inflammation In KK-A$^+$ Mice

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Summary Few studies have been performed to investigate the effect of vitamin D supplementation and T2DM in type 2 diabetic animal models. The present study aimed to explore the relationship between early 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] and the incidence of T2DM and determine whether early 1,25(OH)$_2$D$_3$ supplementation was associated with inflammation in KK-A$^+$ mice. The KK-A$^+$ mice were divided into 4 vitamin D treatment groups, the low-dose vitamin D supplementation group (VDS-L, 1.5 $\mu$g/kg 1,25(OH)$_2$D$_3$), moderate-dose vitamin D supplementation group (VDS-M, 3.0 $\mu$g/kg 1,25(OH)$_2$D$_3$), high-dose vitamin D supplementation group (VDS-H, 6.0 $\mu$g/kg 1,25(OH)$_2$D$_3$) and the model control group (MC). C57BL/6J mice were used as the controls. The treatment period lasted for 9 wk. During this treatment period, fasting blood glucose (FBG) level of the mice was measured on a weekly basis. The levels of lipid profile, insulin and inflammation biomarkers were determined after 9 wk of 1,25(OH)$_2$D$_3$ intragastric gavage. After 9 wk of 1,25(OH)$_2$D$_3$ intragastric gavage, FBG level was significantly decreased in the vitamin D treatment groups compared with the MC group. The number of T2DM incidence in the VDS-L group ($n=7$), VDS-M group ($n=5$) and VDS-H group ($n=3$) was lower than those in the MC group ($n=10$) on week 9. Moreover, serum C-reactive protein (CRP) and interleukin-6 (IL-6) in the vitamin D treatment groups were significantly suppressed by 1,25(OH)$_2$D$_3$ administration compared with the MC group. Early 1,25(OH)$_2$D$_3$ supplementation could effectively lower the incidence of T2DM via ameliorating inflammation in KK-A$^+$ mice.

Key Words vitamin D, type 2 diabetes mellitus, inflammation, insulin resistance

Type 2 diabetes mellitus (T2DM) is a serious metabolic disorder that has become increasingly prevalent throughout the world. Insulin resistance (IR) is a major feature of T2DM (1). The formation mechanism of IR is complex, and its molecular mechanism remains unclear. In recent years, more and more evidences show that T2DM is an inflammatory disease (2–4), and reducing inflammation may have beneficial effects on IR (5).

1,25-Dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] is a biologically active metabolite of vitamin D that has been shown to have immunomodulatory or anti-inflammatory effects (6). 1,25(OH)$_2$D$_3$ can exert its anti-inflammatory activity by combining with the vitamin D receptor (VDR), which is involved in a large variety of immune cells, such as macrophages, dendritic cells, T helper cells and B cells (7). Epidemiologic evidences linking poor vitamin D status to T2DM suggested that insufficient vitamin D might be involved in the etiology of T2DM (8–12). In the last decade, more than ten well-designed, randomized trials evaluated the effect of vitamin D supplementation on glucose homeostasis in subjects at risk for T2DM and showed inconsistent results. Tang et al. (13) published a meta-analysis and did not find an effect of vitamin D supplementation on the incidence of T2DM. However, the authors suggested a possible dose-response effect of vitamin D supplementation to improve glucose and insulin metabolism among non-diabetic adults. They postulated a possible benefit of taking vitamin D supplements in higher doses for the primary prevention of T2DM.

To the best of knowledge, human epidemiologic studies supported a link between vitamin D status and T2DM, however, few studies have been performed to investigate the effect of early vitamin D supplementation on the incidence of T2DM in type 2 diabetic animal models. Nishizawa et al. have demonstrated that lower levels of vitamin D in a T2DM model, compared with controls (14). Norman et al. have reported that the decline in insulin secretion observed in T2DM might be due in part to vitamin D deficiency (15). Vitamin D supplementation could significantly reduce blood glucose levels by 40–60% in rats with streptozotocin induced diabetes (16). However, it is still unclear whether early vitamin D supplementation can reduce the incidence of T2DM and whether vitamin D supplementation con-
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Vitamin D contributes to the T2DM through influencing the inflammation response. Thus, we hypothesized that early vitamin D supplementation might modify glucose metabolism and prevent occurrence of T2DM, possibly through inflammation, in a type 2 diabetic animal model.

MATERIALS AND METHODS

Animals and experimental groups. Forty male KK Cg-Ay/J (KK Ay) mice (3 wk old) and ten male C57BL/6 (C57) wild-type mice (3 wk old) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). This strain was obtained by Japanese scholars who transferred the Ay gene into KK mice (17). The animals were housed in a SPF laboratory animal room under controlled photocycle (12 h light/12 h dark) and temperature (24 ± 2˚C) with ad libitum access to food and water. All mice were housed for 1 wk prior to vitamin D treatment. Ten male C57BL/6 (C57) mice were considered as normal control (NC) group, which were fed with normal diet (AIN-93G). Forty male KK A' mice were randomly divided into model control (MC) group, normal diet plus intragastric gavage with 1.25(OH)2D3 (VDS-L) group, normal diet plus intragastric gavage with 3.0 μg/kg 1.25(OH)2D3 (VDS-M) group, normal diet plus intragastric gavage with 6.0 μg/kg 1.25(OH)2D3 (VDS-H) group. The mice in the MC group, VDS-L group, VDS-M group and VDS-H group were fed with the normal diet (AIN-93G) daily and intragastric gavage with 1.5 mg/kg, 3.0 mg/kg and 6.0 mg/kg 1,25(OH)2D3 within 5 min of its preparation. 

Intragastric gavage with 1,25(OH)2D3. Mice in the VDS-L group, VDS-M group and VDS-H group were intragastric gavage with 1,25(OH)2D3 every other day during the experimental procedure. 1,25(OH)2D3 was dissolved in the refined peanut oil (Lu Hua Group Co., Ltd., Shandong, China) and intragastric gavage (1,25(OH)2D3 dose: 1.5 mg/kg BW, 3.0 mg/kg BW and 6.0 mg/kg BW) within 5 min of its preparation. 

Determination of serum 25(OH)D3. Serum levels of 25-hydroxyvitamin D3 [25(OH)D3] were determined using high-performance liquid chromatography (HPLC). Samples were treated with methanol protein deposition method and extracted by n-hexane. The mixture of methanol and deionized water (95 : 5 v/v) was used as mobile phase. For chromatography we used 600E-based HPLC (Waters). Separation was performed on a Venusil MP-C18 (250 mm x 4.6 mm, 5 μm) maintained at 30˚C. The flow rate was kept constant at 1.0 mL/min. Optimum response of 25(OH)D3 was observed when ultraviolet detection wavelength was set at 265 nm.

Determination of fasting blood glucose. The tail veins of the mice were pierced by a needle. Blood was collected from the tails following a 10-h fast and subsequently used to determine the FBG level using a glucometer (Roche, Basel, Germany). Measurements were obtained per week, and the FBG level was expressed in millimoles per liter (mmol/L) [18]. All animal procedures were performed in accordance with protocol approved by the Tianjin Medical University Animal Ethics Committee (permission code: TMUaMEC 2016002; permission date: 20160412). Furthermore, this manuscript reporting adheres to the ARRIVE guidelines for the reporting of animal experiments. We made every effort to minimize the number of animals used. The experimenters were blinded to the pharmacological treatment while processing data. Mice were euthanized by decapitation when the experimental procedure was finished on week (wk) 9.
units of mmol/L. Mice were considered diabetic if their FBG level was higher than 11.1 mmol/L (18).

**Glucose tolerance test (GTT).** After 6 h of fasting, blood glucose was measured. Glucose (2 g/kg BW) was injected intraperitoneally. Blood glucose concentration was measured at 30, 60 and 120 min after glucose administration using a caudal vein puncture. Blood glucose was determined with a glucometer (Roche). A glucose concentration-time plot was then prepared to compare the changes in glucose with time for each treatment and to calculate the integrated area under the curve (AUC) during the GTT, using the following equation:

\[
\text{AUC} = 0.5 \times [(BG_{0 \text{ min}} + BG_{30 \text{ min}}) \times 0.5 + (BG_{30 \text{ min}} + BG_{60 \text{ min}}) \times 0.5 + (BG_{60 \text{ min}} + BG_{120 \text{ min}}) \times 1]
\]

where \(BG_{0 \text{ min}}\), \(BG_{30 \text{ min}}\), \(BG_{60 \text{ min}}\) and \(BG_{120 \text{ min}}\) are the blood glucose (BG) concentrations measured at 0, 30, 60 and 120 min, respectively.

**Determination of insulin resistance index.** The fasting insulin (FINS) was determined in samples using ELISA kits (Merck Millipore, USA and Cusabio Biotechnology, Wuhan, China) according to the instructions of manufacturer. The homeostasis model assessment of IR (HOMA-IR) was calculated according to FBG and FINS levels. The formula was as follow:

\[
\text{HOMA-IR} = \frac{\text{FBG (mmol/L)} \times \text{FINS (mIU/L)}}{22.5}
\]

**Measurement of serum lipid profile.** Serum total cholesterol (TC) and triglyceride (TG) were measured by routine enzymatic methods. Low-density lipoprotein cholesterol (LDL-C) was measured using colorimetric method. All the lipid profiles were measured using the biochemical analysis kit (Mei Kang biological Polytron Technologies Inc., Ningbo, China) according to the instructions of manufacturer. The levels of lipid profile were measured enzymatically on the Dirui CS-7600 analyzer (Dirui Industrial Co., Ltd., Changchun, China).

**Measurement of inflammatory biomarkers.** The C-reactive protein (CRP), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-6 (IL-6) were determined using ELISA kits (Merck Millipore and Cusabio Biotechnology) according to the instructions of manufacturer.

**Statistical methods.** All statistical procedures were performed using SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation (SD), and analyzed by one-way analysis of variance (ANOVA) followed by Bonferonni post hoc comparison for between-group comparisons. \(p\) values <0.05 were considered statistically significant.

**RESULTS**

**Changes of body weight and the total food intake**

The BW significantly increased in the MC group compared to that in the NC group per week (Fig. 2A), and the total food intake also significantly increased in the MC group compared to that in the NC group per week (Fig. 2B). During 9 wk intervention, there were no significant differences in BW and total food intake between vitamin D treatment groups (VDS-L group, VDS-M group and VDS-H group) and MC group per week (Fig. 2).

**Serum 25(OH)D\(_3\)**

The serum 25(OH)D\(_3\) level was detected thrice to make sure the vitamin D supplementation activated to exert biological effects during 1,25(OH)\(_2\)D\(_3\) treatment. After 9 wk of 1,25(OH)\(_2\)D\(_3\) intragastric gavage, a significant increase in serum 25(OH)D\(_3\) level was observed in
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the VDS-L group, VDS-M group and VDS-H group, compared to the MC group (p<0.05, Fig. 3). Moreover, the serum 25(OH)D3 level in the VDS-H group was higher than those in the VDS-L group and VDS-M group on wk 9 (p<0.05).

Effect of vitamin D on blood glucose and incidence of T2DM in KK-Ay mice

Vitamin D treatment groups (VDS-L group, VDS-M group and VDS-H group) had a significantly reduced blood glucose compared to the MC group on wk 4 and wk 9 (p<0.05) (Fig. 4A), which indicated a direct effect of 25(OH)D3 on the control of the blood glucose levels. And the blood glucose in the VDS-H group was lower than that in the VDS-L group on wk 9. There was no significant difference in blood glucose reduction between VDS-H group and VDS-M after 9 wk intervention (p>0.05) (Fig. 4A). The number of T2DM incidence in MC group (n=10) was higher than those in the VDS-L group (n=7), VDS-M group (n=5) and VDS-H group (n=3) on wk 9 (Fig. 4B).

Effect of vitamin D on GTT in KK-Ay mice

Besides blood glucose level, GTT was also detected (Fig. 4C). GTT in the vitamin D treatment groups (VDS-L group, VDS-M group and VDS-H group) were significantly lower than those in the MC group on wk 9 (p<0.05), indicating that vitamin D also had effect on glucose tolerance in KK-Ay mice.

Effect of vitamin D on insulin resistance in KK-Ay mice

The HOMA-IR level in the VDS-L group, VDS-M group and VDS-H group were significantly lower than those in the MC group, and VDS-H group was significantly lower than those in the VDS-L group on wk 9 (p<0.05) (Fig. 4D).

Effect of vitamin D on serum lipid profile in KK-Ay mice

The serum levels of TC and LDL-C in the VDS-L group, VDS-M group and VDS-H group were significantly lower than those in the MC group on wk 9 (p<0.05) (Fig. 5A, C). There were no significant differences among VDS-L group, VDS-M group and VDS-H group after 9 wk of 1,25(OH)2D3 administration (Fig. 5A, C). Compared to the MC group, the serum TG level exhibited a decreasing trend in vitamin D treatment groups (VDS-L group, VDS-M group and VDS-H group), but statistically non-significant (p>0.05) (Fig. 5B).

Effect of vitamin D on inflammatory biomarkers in KK-Ay mice

The serum level of CRP in the VDS-L group, VDS-M group and VDS-H group were significantly lower than that in the MC group on wk 9 (p<0.05) (Fig. 6A). And the CRP level in VDS-H group was lower than those in the VDS-L group and VDS-M group on wk 9, no significant difference was observed in CRP reduction between
VDS-L group and VDS-M after 9 wk intervention ($p>0.05$) (Fig. 6A). Compared to the MC group, the serum level of TNF-$\alpha$ exhibited a decreasing trend in the vitamin D treatment groups (VDS-L group, VDS-M group and VDS-H group), but statistically non-significant ($p>0.05$) (Fig. 6B). The serum level of IL-6 in the vitamin D treatment groups (VDS-L group, VDS-M group and VDS-H group) were significantly lower than that in the MC group on wk 9 ($p<0.05$), no significant difference was observed in the serum level of IL-6 between any two vitamin D treatment groups ($p>0.05$) (Fig. 6C).

**DISCUSSION**

Epidemiological studies strongly suggested that vitamin D deficiency was associated with incidence of T2DM in high-risk Asian subjects (19), although the causal relationship remains poorly understood. It is demonstrated that low-grade inflammation and autoimmune activation play important roles in development and progression of T2DM. The present study was a novel exploration of the effects of early vitamin D supplementation on the incidence of T2DM, as well as inflammation status in KK-A$^\text{Y}$ mice. It was suggested that early vitamin D supplementation could modify the glucose and inflammation response in KK-A$^\text{Y}$ mice compared to the KK-A$^\text{Y}$ mice with normal vitamin D status in our study. Especially, the high dose vitamin D supplementation tended to have benefit for glucose and lipid metabolism, inflammation response and IR. Therefore, vitamin D could play a role by reducing inflammation to control the IR which is a major contributor to the pathogenesis of T2DM (20, 21).

Dyslipidemia is known as a potential risk factor for cardiovascular events (22), which is common in patients with T2DM. To our knowledge, the serum TC concentration is affected by cholesterol absorption from the gut and endogenous biosynthesis of cells. Accumulation of excess cholesterol due to the presence of increased circulating LDL-C promotes endothelium dysfunction and activation, which is associated with increased production of pro-inflammatory cytokines (23). In addition, among genetic traits associated with LDL-C levels, single nucleotide polymorphisms (SNPs) in the CELSR2/PSRC1/SORT1 locus and in the APOE/APOC1/TOMM40 locus have also been associated with inflammatory-related phenotypes (24), which further supports the strong relationship between cholesterol and inflammation. Observational studies have demonstrated an inverse correlation between higher level of serum 25(OH)D$_3$ and lower levels of serum TC, LDL-C and TG (25), however, the results of randomized controlled trial (RCT) to evaluate the effect of vitamin D on lipid profile were conflicting. The current meta-analyses have revealed that vitamin D improved serum levels of TC and LDL-C, no significant reduction in serum TG level among patients with T2DM (26). Similarity, our
results have observed that early vitamin D supplementation could improve the serum levels of TC and LDL-C in diabetic model. Kane et al. (27) have reported the reduction of serum cholesterol precursors (campesterol) in statin-treated subjects who received vitamin D, which suggested that vitamin D might reduce intestinal cholesterol absorption. Vitamin D could reduce the foam cell formation and decrease LDL-C deposition in macrophages of patients with T2DM (28). Our study suggested that vitamin D could reduce the inflammation response via decreasing the lipid profile level. Moreover, we used the KK-A' mice as the animal model, the KK-A' mice is transferred the A' gene into KK mice, which is a mildly obese T2DM mouse model. Thus, we only observed that the serum TG level exhibited a decreasing trend in vitamin D treatment groups, but statistically non-significant.

The results of basic and clinical research support beneficial action of vitamin D in the reduction of IR and related pathologies (29). Vitamin D deficiency impairs insulin secretion and induces glucose intolerance in animal models (15, 16, 30), whereas repletion of vitamin D status is associated with improvements in insulin secretion and glucose homeostasis (31, 32). This effect of vitamin D on insulin secretion may be mediated by changes in intracellular calcium concentration in pancreatic β cells (33, 34). Furthermore, there is some evidence that vitamin D improves insulin sensitivity by its anti-inflammatory activity, which is consistent with our study. Our recent meta-analysis concluded that the vitamin D supplementation was beneficial for the reduction of hs-CRP in T2DM subjects (35). Incubation of isolated monocytes with 1,25(OH)2D3 attenuates the expression of proinflammatory cytokines involved in insulin resistance such as IL-1, IL-6, and TNF-α in T2DM patients (36), which may be related to the down-regulation of NF-kB activity (37). NF-kB is an essential component of inflammatory pathways in adipose tissue. The activation of NF-κB and translocation of p65 subunit to the nucleus is related to IκBα degradation (38). It has been demonstrated that 1,25(OH)2D3 inhibits LPS-stimulated IL-6 secretion in two human adipocyte models via interference with NF-κB signaling (39). In human and mouse adipocytes, an inhibitory effect of vitamin D on inflammatory markers via NF-κB and p38 MAPK inflammatory pathway was also demonstrated (40–42). The bioactive form of vitamin D significantly suppresses inflammation via inhibition of IκBα phosphorylation and subsequent translocation of p38 MAPK or NF-κB into the nucleus (43), which may result in reducing IR and lower the incidence of T2DM.

CONCLUSIONS

This study demonstrated that early 1,25-dihydroxyvitamin D3 supplementation effectively lowers the incidence of T2DM via ameliorating inflammation in KK-A' mice.

Authorship

Research conception and design: LT, GH and MZ; experiments: LT, YY and MJ; statistical analysis of the data: LT, YY and MJ; writing of the manuscript: LT, YY, MJ, GH, and MZ.

LT and YY contributed equally to this work.

Disclosure of state of COI

The authors declare no conflict of interest.

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