1α,25-dihydroxyvitamin D₃ promotes osseointegration of titanium implant via downregulating AGEs/RAGE pathway in T2DM

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Abstract

Diabetes-induced advanced glycation end products (AGEs) overproduction would result in compromised osseointegration of titanium implant and high rate of implantation failure. 1α,25-dihydroxyvitamin D₃ (1,25VD₃) plays a vital role in osteogenesis, whereas its effects on the osseointegration and the underlying mechanism are unclear. The purpose of this study was to investigate that 1,25VD₃ might promote the defensive ability of osseointegration through suppressing AGEs/RAGE in type 2 diabetes mellitus. In animal study, streptozotocin-induced diabetic rats accepted implant surgery, with or without 1,25VD₃ intervention for 12 weeks. After killing, the serum AGEs level, bone microarchitecture and biomechanical index of rats were measured systematically. In vitro study, osteoblasts differentiation capacity was analyzed by alizarin red staining, alkaline phosphatase assay and Western blotting, after treatment with BSA, AGEs, AGEs with RAGE inhibitor and AGEs with 1,25VD₃. And the expression of RAGE protein was detected to explore the mechanism. Results showed that 1,25VD₃ could reverse the impaired osseointegration and mechanical strength, which possibly resulted from the increased AGEs. Moreover, 1,25VD₃ could ameliorate AGEs-induced damage of cell osteogenic differentiation, as well as downregulating the RAGE expression. These data may provide a theoretical basis that 1,25VD₃ could work as an adjuvant treatment against poor osseointegration in patients with type 2 diabetes mellitus.

Introduction

As the continuous improvement of implant design and surgical technique, implant restoration has become the first manner for patients with tooth deficiency. However, there existed local or systemic factors can affect osseointegration, which is critical for implanting, and increase the failure rate, type 2 diabetes mellitus (T2DM) is one of them (1, 2). The pathological changes of T2DM, such as microangiopathy, immunity decline and collagen degradation, can reduce the resistance of the soft and hard tissue to local pathogenic factors (3) and jeopardize the healing process (4). And the high venture of implantation in diabetic patients has been confirmed by many researches (5, 6). Therefore, it is of great significance to improve the osseointegration of implants in T2DM patients.

Advanced glycation end products (AGEs), pernicious outgrowth of non-enzymatic glycosylation, can be inevitably produced and accumulate in tissues with aging (7, 8). In addition, the formation of AGEs is accelerated under hyperglycemia condition (9), which is the dominant factor for chronic complications of diabetes. When combing with their receptors (receptor for AGEs, RAGE), AGEs trigger oxidative stress and inflammatory reaction...
(10), then lead to function changes of osteoblasts and osteoclasts (11, 12) and impair bone formation eventually. Moreover, David et al. argued that the formation of AGEs in high glycemic conditions, may contribute to a slower rate of osseointegration that negatively affects implant stability (13). Clinical research has also indicated AGEs may be considered as a potential marker of inflammation levels in diabetic individuals with peri-implantitis (14).

Vitamin D is an essential steroid hormone to human body, and the intriguing finding has certified that the supplementation of vitamin D could reduce the deposition of AGEs in the medial layer of the aortic wall and systemic oxidative stress in diabetic rats (15). 1α,25-dihydroxyvitamin D3 (1,25VD3) acts as the main active form of vitamin D, which combines with vitamin D-binding protein in the plasma, reaching target tissues to play an endocrine role (16). The well-acknowledged function of 1,25VD3 includes the regulation of Ca and P metabolism (17, 18), accordingly to facilitate bone mineralization. Recently, the epidemiological cross-sectional studies on diabetes have demonstrated that the incidence of type 2 diabetes increased significantly in population with low level of serum 1,25VD3 and accumulating of AGEs in T2DM patients may lead to implantation failure. The central hypothesis of this research is that the topical or systemic application of 1,25VD3 might promote the defensive ability of osseointegration by suppressing AGEs/RAGE in T2DM. Further, the relevant mechanisms will be clarified in the current study.

Materials and methods

Animals

Animal experiments were approved by Institutional Animal Welfare and the Animal Ethics Committee of Shandong University (Jinan, China). Fifteen age-matched male Sprague–Dawley rats, weighing (200±20) g, were purchased and fed in the Experimental Animal Center of Shandong University (Jinan, China) under optimum rearing condition.

Inducement of T2DM model

After 1-week adaptive breeding, five rats were selected as normal control group randomly, which continued to be given ordinary feed. Rest ten ones were fed with high-fat and high-carbohydrate diet to induce T2DM model. Four weeks later, rats in model group were intraperitoneally injected with 30mg/kg streptozotocin (STZ, Sigma) solution after 12-h fasting, and another group rats were treated with citrate buffer. Fasting blood glucose (FBG) ≥11.1 mmol/L was validated as hyperglycemia for further research after 1-week injection.

Treatment

Rats were divided into control group, T2DM group and 1,25VD3-treated group with five rats per group. After weighing, the rats were anesthetized with 10% chloral hydrate (40mg/kg) via intraperitoneal injection. Special designed mini-Ti implants (1 mm in diameter and 10 mm in length) were implanted into intercondylar fossa under aseptic operation, which were parallel to the long axis of the femur. All animals received intramuscular antibiotic injection.

Three days after surgery, the treatment group received 1,25VD3 (Sigma) at a dose of 0.5μg/kg (22) during the whole experiment. All rats were killed at the 12th week and peripheral blood was collected. The specimens, bilateral femurs with the implant, were fixed in 4% polyformaldehyde and preserved at normal temperature.

Detection of FBG and body weight

After 12-h fasting, blood samples were collected from tail vein and blood glucose levels were measured with glucose oxidase method. The FBG was recorded before STZ inducement (as 0 day), then on 7th day and every 2 weeks after 1,25VD3 treatment. And the rats in different groups were weighted every 2 weeks throughout the experiment.

The serum value of AGES

The level of AGES was investigated by fluorescence spectrophotometry. The method is shown below: 0.2mL serum was diluted 10-folds with distilled water, and then the excitation and the fluorescence wavelength were adjusted to 370 nm and 440 nm respectively, and the slit was set as 5 nm. The serum value of AGES was expressed by U/mg prot, which indirectly reflected the AGES content in bone tissue.
Microscopic computerized tomography (micro-CT) analysis

Fixed specimens were placed in the micro-CT instrument (Rigaku, USA). After obtaining all the images, a radius of 200 μm around the implant was regarded as the region of interest for 3D reconstruction, so as to observe the osteogenesis around the implant. The indexes of bone volume per total volume (BV/TV), percentage of osseointegration (%OI), which was the ratio between bone and total voxels in direct contact with implant (21), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were analyzed by Mimics 19.0 and associated software of micro-CT.

Histological analysis

Tissue blocks were dehydrated in gradient ethanol for 48h, infiltrated and then embedded with light-cured resin. After embedding, the specimen was sectioned with hard tissue slicer (Leica, Germany), whose thickness was set at 100 μm. Methylene blue-acid fuchsin staining was observed under light microscope. The index of bone-implant contact (BIC) was measured by NIS-Elements image software, which was defined as the length percentage of direct bone-implant interface to total implant surface in the cancellous bone (23).

Pull-out test

The maximum pulling force of the implant under tensile force is measured by universal material testing instrument (Shimadzu, Japan), which represents the maximum retention force obtained by the implant in the bone. The specimen was fixed on the machine, setting stretch speed was 1 mm/min and the distal of cylindrical implant was exposed as a gripper handle. The maximum force was recorded at the moment of departure.

Cell culture

Osteoblasts were obtained from neonatal rat (<24 h old) calvaria, which were cut into 1.0 mm³ fragments and inoculated in culture flask. After two hours, the flask was turned over and added to 5 mL DMEM (HyClone) medium containing 10% foetal bovine serum (BI, USA), 100 U/mL penicillin G and 100 U/mL streptomycin (Beyotime), culturing at 37°C in 5% CO₂. Cells at passages 3–5 were used to undertake future experiment.

Osteoblasts were seeded in multiple-well plates and treated as follow groups: BSA, 200 μg/mL AGEs-BSA (24), 200 μg/mL AGEs-BSA with RAGEs inhibitor and 200 μg/mL AGEs-BSA supplemented with 10⁻⁸ mol/L 1,25VD₃ (25). AGEs-BSA were prepared as follows: BSA(Roche) and d-glucose were dissolved in phosphate buffer (PBS) and incubated under sterile conditions at 37°C for 10 weeks. As a control, BSA was incubated in parallel without d-glucose. Then the preparations were dialyzed in PBS to remove free glucose and no endotoxin was detectable in them.

Alizarin red staining

After 4-week osteogenic induction, the formation of mineralized nodule was detected by alizarin red staining. Subsequently, cells were fixed with 4% paraformaldehyde and rinsed with PBS, and then stained with 0.1% alizarin red (Sigma) diluting with Tris–HCl. After 20 min, mineralized nodule was observed by light microscope.

Alkaline phosphatase (ALP) assay

ALP activity was evaluated by Alkaline Phosphatase Assay Kit (Beyotime). Seven days after osteogenic induction, the supernatant, which came from cells’ lysis and centrifugation, are added into contrast holes, gauge holes, sample holes in order. After 25-min incubation,

| Group | 0 day | 7th day | 2 weeks | 4 weeks | 6 weeks | 8 weeks | 10 weeks | 12 weeks |
|-------|-------|---------|---------|---------|---------|---------|----------|---------|
| Control | 4.6±0.3 | 4.7±0.2* # | 4.6±0.2* # | 4.5±0.4* | 4.6±0.2* # | 4.5±0.3* # | 4.7±0.2* # | 4.8±0.3* # |
| T2DM | 4.5±0.2 | 19.8±1.8 | 24.4±1.5 | 23.7±1.6 | 22.7±1.4 | 25.9±2.4 | 24.8±1.7 | 25.3±1.5 |
| 1,25VD₃-treated T2DM | 4.6±0.4 | 17.9±1.2 | 15.7±1.1* | 13.7±0.8* | 11.1±0.9* | 9.8±0.7* | 10.3±0.7* | 10.0±0.8* |

0 day represented before STZ inducement, 7th day and 2–12 weeks represented after 1,25VD₃ treatment, data are presented as mean±s.d.

*P<0.05, for T2DM vs other two group; #P<0.05, for 1,25VD₃-treated T2DM vs control.
the staining solution was added into each well for 15 min in the dark. The OD absorbance at 520 nm was measured by microplate spectrophotometer. The calculation of ALP activity was based on the concentration of phenol in the gauge well and the protein concentration.

**Western blotting**

After adding RIPA buffer (Solarbio, China), cells were lysed and followed by centrifugation at 4°C, 14,167 g for 15 min. The supernatant was collected and the protein concentration was determined after centrifugation. Proteins were separated by SDS-PAGE, then transferred onto polyvinylidene difluoride membrane, subsequently incubated with primary antibodies RUNX2 (1:1000, CST, USA), ALP (1:1000, CST, USA), COL1 (1:1000, CST, USA) and OCN (1:1000, CST, USA), anti-RAGE (1:1000, CST, USA) and GAPDH (1:10,000, Proteintech) overnight. After three times washing with TBST, the membrane was incubated with horseradish peroxidase-labeled secondary antibody. The protein bands were visualized by the ECL chemiluminescence detection system and the gray values were analyzed by ImageJ 1.8.0.

**Statistical analysis**

All data were performed using SPSS19.0 and expressed as the mean ± standard deviation (s.d.) of three to five independent experiments. Statistical differences among various treatment groups were determined by one-way ANOVA or the Student’s two-tailed t-test. Differences were considered statistically significant when P value less than 0.05.

**Results**

**1,25VD₃ improved FBG and maintained body weight**

After STZ injection, the blood glucose level of the T2DM rats increased significantly (P<0.05). With administration of 1,25VD₃ treatment, the beneficial effects on lowering blood glucose gradually emerged on the second week in T2DM rats (P<0.05), whereas still maintained high level compared with normal control group (P<0.05) (Table 1).

**Table 2**  Effect of 1,25VD₃ on body weight in experimental rats (n=5/group).

| Group                  | 0 day  | 7th day | 2 weeks | 4 weeks  | 6 weeks  | 8 weeks  | 10 weeks | 12 weeks |
|------------------------|--------|---------|---------|----------|----------|----------|----------|----------|
| Control                | 268 ± 10 | 280 ± 9  | 301 ± 11 | 336 ± 15* | 374 ± 14* | 393 ± 16* | 422 ± 11* | 443 ± 14* |
| T2DM                   | 281 ± 15 | 284 ± 8   | 289 ± 9  | 272 ± 10  | 263 ± 12  | 256 ± 8   | 242 ± 12  | 233 ± 11  |
| 1,25VD₃-treated T2DM   | 279 ± 9   | 286 ± 10  | 294 ± 11 | 311 ± 13* | 318 ± 14* | 322 ± 9*  | 345 ± 11* | 362 ± 12* |

*P<0.05, for T2DM vs other two groups.

**1,25VD₃ lessened serum value of AGEs**

Data in Fig. 1 displayed that serum AGE value of T2DM rats significantly clearly went up compared to controls (P<0.05). Moreover, it was restored to nearly normal level by 1,25VD₃ treatment. The results suggested that 1,25VD₃ might improve the osseointegration by decreasing the AGEs level.

**1,25VD₃ improved bone quality of T2DM rats**

After different treatments, the peri-implant cancellous bone among groups were compared through 3D micro-CT images. T2DM group exhibited the more bone loss, the
less and thinner bone trabecula, in contrast to control group. And 1,25VD$_3$ treatment ameliorated these injuries of trabecular in diabetic model significantly (Fig. 2A). The quantitative diagram (Fig. 2B, C, D, E and F) demonstrated visually the differences between with or without the 1,25VD$_3$ application in diabetic group. The decline of BV/TV, %OI, Tb.Th and Tb.N in the T2DM group were more dramatic than the control and 1,25VD$_3$-treated group. Conversely, the values of Tb.Sp in untreated diabetic rats rose by 38.7% compared to with 1,25VD$_3$-treated rats.

**1,25VD$_3$ promoted bone microarchitecture of T2DM rats**

Staining slices showed that when compared with control group, the local bone resorption around implant resulted in direct exposure to the medullary cavity, and the connection between the trabecular bones was largely disappeared and ill organized in T2DM group (Fig. 3A and B). And 1,25VD$_3$ treatment conspicuously increased peri-implant bone mass in diabetic rats, with the uniform and integrity of trabecular bone structure (Fig. 3C).
BIC of diabetic rats was presented as 23.2%, significantly less than control and 1,25VD$_3$, which respectively had 59.8 BIC and 41.8% BIC.

1,25VD$_3$ increased biomechanical index of T2DM rats

The pull-out test was used to record the maximum pulling force of implants, and evaluate the degree of osseointegration. Significant increases were observed in the values of the biomechanical index in 1,25VD$_3$ treatment group (Fig. 4): 1.6-fold increase in the maximal pulling force, when compared to T2DM model rats ($P<0.05$). No significant differences between 1,25VD$_3$ treated and control group in the index were found ($P>0.05$).

1,25VD$_3$ attenuated AGEs-mediated damage in osteogenesis

Osteogenesis function was tested by alizarin red staining, the activity of ALP and the expression of associated proteins in cells. Osteogenesis later stage, which manifested as the mineralization level, was delegated by alizarin red staining. There were significant differences in the number of mineralized nodules and the average area of single nodule under microscope (Fig. 5A). The nodule was not detected in the only AGEs-treated osteoblasts, but alizarin deposition increased when adding RAGE inhibitors and 1,25VD$_3$. The marker of early stage osteogenesis, ALP was detected at 7 days after induction. The results demonstrated that AGEs significantly reduced ALP activity, while the presence of 1,25VD$_3$ could increase the activity visibly ($P<0.05$), which had no difference with other two groups ($P>0.05$) (Fig. 5B). On the protein levels of osteoblast differentiation markers including RUNX2, ALP, COL1 and OCN, the data pointed toward the same trend (Fig. 5C, D, E, F and G).

Discussion

The successful establishment of animal models is the basis for in-depth study of the development and complications of diabetes. In our study, T2DM was induced by high-glucose and high-fat diet plus

1,25VD$_3$ promote osteogenesis by blocking AGES/RAGEs signaling pathway

Above results displayed that the osteogenic abilities of osteoblast in AGES group were enhanced by 1,25VD$_3$, but the exact mechanism was unclarified yet. Therefore, we detected the expression of RAGE protein by Western blot. After treatment with 1,25VD$_3$, the intensities analysis results showed that RAGE levels decline dramatically, in comparison with those in AGES group, while AGES group were significantly higher than those in control group (Fig. 6).

Figure 3
Bone microarchitectures were observed by histological staining (original magnification ×40). (A) Control group; (B) T2DM group; (C) 1,25VD$_3$-treated T2DM, (D) histograms of the BIC in quantitative analysis. $n=5$ specimens/group, *$P<0.05$, data were presented as mean ± s.d.

Figure 4
Biomechanical parameter was detected by pull-out test, $n=5$ specimens/group, *$P<0.05$, data were presented as mean ± s.d.
low-dose STZ injection. And the high glycemic index and low weight in diabetic group arose from the destruction of rat islet B cells by STZ (26). Osseous abnormality is a common undesirable phenomenon in diabetic patients, manifesting as osteopenia, and osteoporosis is considered as one of diabetic complications (27). Results in in vivo study demonstrated that there was evident deterioration of osseointegration, bone microarchitecture and implant fixation in T2DM rats.

Figure 5
1,25VD₃ ameliorated the impaired differentiation of osteoblasts induced by AGEs. (A) Alizarin red staining was performed to represent the mineralization levels of osteogenesis later stages after 4 weeks induction (original magnification ×40). (B) Osteoblasts differentiation was detected by ALP activity at 7 days. (C, D, E, F and G) The protein levels of RUNX2, ALP, COL1 and OCN assessed by Western blot and the quantification analysis, n=3 group, *P<0.05, for AGEs group vs other three groups, data were presented as mean ± s.d.

Vitamin D insufficiency is a risk factor for T2DM, whose active metabolite 1,25VD₃ may participate in the regulation of glucose tolerance by influencing islet B cells function and insulin sensitivity (28, 29). In our experiment, the data of micro-CT and histological staining showed that 1,25VD₃ therapy could obviously promote the formation and reconstruction of bone tissue around implant in diabetic rats and also improve the degree of osseointegration. The result can be explained by...
1,25VD₃ maintains glucose homeostasis, which reduces blood glucose, inhibits inflammation, increases insulin synthesis and improves insulin resistance (30), ulteriorly reduces the incidence and development of osteopenia in diabetes. Besides, 1,25VD₃ could exert bone-protective effects independent of its calcium-related effects (31). Whereas there was still a certain difference between the 1,25VD₃ treatment and normal rats, the possible cause of this difference is that 1,25VD₃ promoting glucose metabolism is limited, in accordance with the floating of blood glucose level and body weight. Results from pull-out test also indicated that the implant fixation enhanced, appearing as 1.6-fold increase in the maximal pull-out force after 12-week 1,25VD₃ treatment. The above results indicated that 1,25VD₃ not only can prevent osteopenia caused by T2DM, but also participate in ameliorating glucose homeostasis up to a point.

Our conclusion in study in vivo is consistent with the results published by Wu et al. (21) who were the first to indicate that 1,25VD₃ could reverse the impaired osseointegration of implants in diabetic rats, but the related mechanism was not attached. Therefore, we struggled to clarify the factors contributing to this therapeutic effect. It is generally known that the formation and accumulation of AGEs are one of the primary factors for inhibition of osteoblast viability (32) and destruction of bone quality caused by hyperglycemia. Furthermore, we found that the serum value of AGEs declined markedly in 1,25VD₃ treatment rats. Based on it, we assumed that these positive effects of vitamin D on bone metabolism may be achieved through this key factor. Therefore, we attempted to estimate the potential relationship between 1,25VD₃ and AGEs in osseointegration.

Osteoblast differentiation is the precondition and basis for leading bone formation, and undergo four stages, including proliferation, extracellular matrix maturation, mineralization and apoptosis (33, 34). The high expression of ALP activity serves as an early marker of osteoblast differentiation, which can mediate calcium phosphate into insoluble phosphate salts, in order to enhance calcification (35, 36). Data demonstrated that AGEs markedly decreased the ALP activity of osteoblasts, while 1,25VD₃ ameliorated with the same effects on RAGE inhibitor. In the process of osteogenesis, mineralized nodules are the markers of maturation, and also are the main morphological expression of osteoblasts to perform function (37). Alizarin red staining is one of the commonly used methods to observe it. With the addition of AGEs, malnourished mineralized nodes of osteoblasts were observed by staining while 1,25VD₃ could attenuate these adverse effects. Besides, the translation level of RUNX2, ALP, COL1 and OCN of osteoblast were detected by Western blot, which obtained similar results. All above evidence indicates that the impaired cell differentiation is improved by 1,25VD₃ treatment.

The interaction of AGEs and RAGE can initiate the changes in intracellular signal transduction and activate the nuclear transcription factor, which is the critical path leading to a variety complications of diabetes (38, 39). Research has certified that the supplementation of vitamin D could reduce the deposition of AGEs in the medial layer of the aortic wall (15). Consistently, the Western blot results displayed that 1,25VD₃ could inhibit RAGE expression, meaning that its pro-osteogenesis effect generate from impeding AGEs/RAGE. And the

![Figure 6](https://ic.bioscientifica.com)
thorough mechanisms how 1,25VD₃ acts on downstream innovation factor is not clarified. Xiong et al. treated diabetic mice lacking FoxO1 in osteoblasts by 1,25VD₃ and found FoxO1 might be involved in the regulation of 1,25VD₃ on implant osseointegration (40). It is reasonable to suggest that there may exists crosstalk between AGEs and FoxO1, which needs paying more attention to in the future research.

In conclusion, the present study reveals that the therapeutic effect of 1,25VD₃ on promoting implant osseointegration in T2DM through a new perspective. Results demonstrate 1,25VD₃ can inhibit the expression of RAGE and interrupt osteoblasts’ damages of AGEs. It provides a theoretical basis for prevention and treatment, that 1,25VD₃ could work as an adjunct treatment to suppress the AGEs/RAGE and own potential protective effects against poor osseointegration in diabetic patients.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by the Fundamental Research Funds of Shandong University (21330078614061); the General Financial Grant from The China Postdoctoral Science Foundation (grant number 2017M612294).

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