UDC 577.161.2+616.379-008.64 doi: https://doi.org/10.15407/ubj90.02.056

OSTEOPROTECTIVE EFFECTS OF VITAMIN D_3 IN DIABETIC MICE IS VDR-MEDIATED AND REGULATED VIA RANKL/RANK/OPG AXIS

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There is growing evidence that vitamin D_3 deficiency could be a contributing factor in the development of different chronic diseases and their complications. A better understanding of how diabetes influences bone tissue metabolism may become an underlying basis for effective prevention and treatment of skeletal disorders in diabetes. This study was performed to define diabetes-associated impairments in bone tissue remodeling in relation to vitamin D bioavailability and to estimate the effects of cholecalciferol treatment. We established that chronic hyperglycemia in diabetes was accompanied by a 2.15-fold decrease of 25OHD content in the serum. Vitamin D deficiency correlated with impairments of tibia biomechanical properties (decline of bone maximal load and stiffness values). μCT analysis of tibia showed respectively 3.0-, 2.1- and 1.3-fold decreases in trabecular bone volume per tissue volume, trabecular number and cortical thickness in diabetes indicating the development of secondary osteoporosis. Diabetes led to up-regulation of NF-κB/phosho-p65, RANKL, RANK (2.3-, 1.51-, 1.72-fold respectively) and down-regulation of OC, OPG and VDR (1.5-, 1.6- and 1.8-fold respectively) in tibial tissue of diabetic mice. Diabetes-associated abnormalities in the serum levels of RANKL, OPG and TRAP were also detected. Restoration of circulatory 25OHD content was achieved due to cholecalciferol treatment. Better vitamin D availability and increased VDR expression resulted in normalization of RANKL/RANK/OPG- and NF-κB-associated pathways that attenuated diabetes-induced structural and biomechanical abnormalities in bone tissue.

K e y w o r d s: experimental type 1 diabetes, secondary osteoporosis, vitamin D deficiency, cholecalciferol, bone remodeling.

Convincing evidence suggests that both type 1 and 2 diabetes mellitus is associated with harmful metabolic changes in bone tissue, formation and resorption [1]. This leads to a significant loss of bone mass and to changes in its microarchitectonics that in most cases is recognized as secondary osteoporosis [2]. Alterations of remodeling at the molecular level are due to increased non-enzymatic glycosylation, prooxidant processes in bone tissue and bone marrow cells, imbalance of the Wnt signaling pathway and deregulation of cytokine function [3]. An impaired calcium metabolism in secondary osteoporosis leads to changes in the functioning of the parathyroid hormone-calcitonin-vitamin D axis [4].

Numerous findings from experimental, clinical and epidemiological studies have previously shown that low bioavailability of vitamin D increases the risk of developing a number of common chronic human diseases, which can be associated with a disrupted implementation of both classical (calcicemic) and nonclassical (noncalcemic) effects of vitamin D_3 (cholecalciferol). Until recently, they were not considered to be fully associated with vitamin D deficiency and, therefore, have not been sufficiently investigated. It is now becoming increasingly clear that significant disturbances in the expression of a large number of key cellular genes in various tissues, including bone tissue, can be the result of vitamin D deficiency associated with diabetes [5].

1,25-Hydroxylated cholecalciferol is estimated to control via vitamin D receptors (VDR) the transcription of about 3% of the genes in human cells. Notably, vitamin D provides some non-genomic physiological effects by regulating the membrane and biochemical processes involved in the transduction of cellular signals in target tissues [6, 7]. More recently, it has also been shown that the signaling pathway of the receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and its decoy receptor osteoprotegerin (OPG) play an important role in the maintaining of structure and functional properties of bone tissue. In particular, the triad RANKL/RANK/OPG determines the consistency between osteoblastogenesis and osteoclastogenesis, being one of the key cytokine bone remodeling systems [8]. The expression of its components is regulated by bone-seeking hormones such as parathyroid hormone and 1,25-dihydroxyvitamin D_3 [9]. Therefore, by investigating the mechanisms of diabetes-induced osteoporosis in connection with the bioavailability of vitamin D_3 and the action of its hormonal forms through VDR – vitamin D receptors. we might gain more insight into the role of the endocrine-paracrine system of cholecalciferol in regulating crucial osteotropic systems in bone tissue.

The present study was designed to evaluate the relationship between vitamin D_3 status, VDR expression and the RANKL/RANK/OPG signaling pathway underlying disturbances of bone remodeling associated with experimental type 1 diabetes (T1D).

Materials and Methods

Animals. All studies were conducted on 4-month-old male C56Bl/J6 mice weighing 21 ± 3 g. T1D was induced by multiple (5 times) injections of streptozotocin (STZ, Sigma-Aldrich, USA) at a dose of 40 mg/kg body weight. Such a regimen of STZ administration is usually used to induce experimental autoimmune T1D in mice [10]. Six weeks after the induction of diabetes, animals with a blood glucose level of 21.4 ± 4.5 mmol/l were used for the study. Diabetic mice with stable hyperglycemia were given an aqueous suspension of vitamin D_3 (DSM, Netherlands) for 2.5 months (800 IU/kg body weight, *per os*), while the control group received saline solution. For the adaptation period (one week) and during the experiment animals were housed in the vivarium at a temperature of 18-22 °C, humidity 50-60%, natural light mode "day-night" in standard plastic cages with free access to their food and water. Mice were decapitated, using diethyl ether for anesthesia. Selection of animals and the formation of groups were performed by the method of "random numbers". The study was performed according to national (General ethical principles for experiments on animals, Ukraine, 2001) and international guidelines and laws (European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, Strasbourg, 1986) concerning animal welfare.

Serum analyses. The content of 25-dihydroxyvitamin D (25OHD), a main marker of vitamin D bioavailability, in serum was assayed using a commercial ELISA kit (IDS 25-Hydroxy Vitamin D EIA, USA) as described by the manufacturer. Serum content of the bone resorption marker tartrate resistant acid phosphatase (TRAP) was detected by the Mouse TRAPTM Assay (IDS, Finland). Serum levels of osteokines RANKL and OPG were assessed by Mouse/Rat ELISA kits (IDS, Germany).

Measurement of mechanical strength. After removal, tibias were stored at -70 °C. Before the mechanical testing, the bones were kept in PBS for 24 h. The biomechanics were analyzed with the three-point bending test (span length 55 mm, loading speed 0.155 mm/min) for the mid tibia using the Instron Universal Testing Machine (Instron 3366; Instron Corp.). The biomechanical parameters were calculated based on the recorded load deformation curves [11].

High-resolution microcomputed tomography (μCT) analysis. μCT analyses were performed on the proximal tibia by using the Skyscan 1072 scanner (Skyscan, Belgium), imaged with an X-ray tube voltage of 100 kV and current of 98 μA, with a 1-mm aluminum filter. The scanning angular rotation was 180° and the angular increment 0.90°. The voxel size was 6.51 μm isotropically. Datasets were reconstructed using a modified Feldkamp algorithm and segmented into binary images using adaptive local thresholding. In long bones, the trabecular bone proximal to the distal growth plate (femur) or distal to the proximal growth plate (tibia) was selected for analyses within a conforming volume of interest (cortical bone excluded) commencing at a distance of 338.5 μm from the growth plate, and extending a further longitudinal distance of 488 μm in the proximal direction. Trabecular thickness and separation were calculated by the sphere-fitting local thickness method [12].

Western blot analysis. The levels of nuclear factor κB (NF-κB) large subunit p65 phosphorylated at Ser311 (pNF-κB), VDR, RANK, RANKL, OPG and osteocalcin (OC) were determined in total lysates from tibias by western blot analysis. Murine tibia were lysed in RIPA buffer containing protease inhibitor cocktail (PIC, Sigma, USA). Protein concentrations in the lysates were determined as previously described (Lowry, 1951). Samples of 50 μg protein were loaded onto 10 or 12.5% SDS polyacrylamide gels. After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes [13]. Immunoblotting was conducted with primary antibodies against Ser311 phosphorylated NF-κB p65 (1:500; Santa Cruz Biotechnology, USA); VDR (1:200; Santa Cruz Biotechnology, USA); RANK (1:400; Santa Cruz Biotechnology, USA); RANKL (1:250; Santa Cruz Biotechnology, USA); OPG (1:250; Santa Cruz Biotechnology, USA); osteocalcin (1:500; Santa Cruz Biotechnology, USA) and β-actin (1:20000; Sigma, USA). Primary-antibody-bound membranes were incubated with peroxidase-conjugated secondary antibodies: anti-mouse IgG (Fab specific)-peroxidase (1:5000; Sigma, USA), anti-rabbit IgG (H+L)-HRP conjugate (1:4000; Bio-Rad Laboratories, Inc., USA) or anti-goat IgG (H+L) (1:2500; Invitrogen, USA). The bands were visualized by enhanced chemoluminescene agents p-coumaric acid (Sigma, USA) and luminol (AppliChem GmbH, Germany). Tissue levels of target proteins were normalized to β-actin. The immunoreactive bands were quantified with Gel-Pro Analyzer 32 (Media Cybernetics Inc, Italy).

Statistics. Results were expressed as mean ± SEM for at least six mice per group. Statistical analysis of the data was performed using Origin Pro 8.5 (OriginLab Corporation, Northampton, MA, USA). Kolmogorov-Smirnov test was used for testing on normal distribution. Statistical differences between the various groups were compared by using ANOVA-test and considered significant when $P \leq 0.05$.

Results and Discussion

The spectrum of pathogenic effects of hypoinsulinemia and chronic hyperglycemia on bone tissue can be extremely wide in diabetes mellitus, since bone metabolism is known to be in close interaction with various regulators of the remodeling process. Considering the important role of growth factors and cytokines produced by bone cells for metabolism, bone tissue has emerged as an endocrine organ that affects normal physiology as well as various pathologies, including diabetes mellitus [14]. First, we established the level of glucose in the blood, which in diabetic mice reached a value 4.11-fold higher than in control mice (Table). Cholecalciferol had statistically insignificant glucose-lowering effects in diabetes.

It is important to note the existence of a close relationship between metabolic and remodeling disorders in bone tissue and deleterious changes in the metabolism of vitamin D3. A study of 25OHD content, the main bioavailability marker of vitamin D_3 and the precursor of its hormonally active form, showed a more than 2-fold decrease in the level of this derivative of cholecalciferol in the blood serum of diabetic mice compared to control animals (Table).This may be due to the diabetes-induced inhibition of vitamin D 25-hydroxylase, which is responsible for the synthesis of the 25OHD-active metabolite [15]. The range of serum 25OHD concentrations in health mice is 60-125 nmol/l (25-50 ng/ml), which is considered a vitamin D sufficiency. A decrease in the circulating serum 25OHD level below 25 nmol/l (>10 ng/ml) indicates the development of vitamin D insufficiency [16]. The long-term administration of vitamin D_3 to diabetic mice almost completely normalized serum 25OHD (Table).

Content of 25OHD and glucose in serum of mice with experimental diabetes and with vitamin D_3 administration, $M \pm m$, $n = 6$

Experimental groups	Content of 25OHD		Glucose concentration, mmol/l
	nmol/l	ng/ml	
Control	81.70 ± 4.12	32.68 ± 1.65	5.2 ± 1.1
Diabetes	37.90 ± 2.12*	15.16 ± 0.85*	21.4± 4.5*
Diabetes + D_3	77.30 ± 5.48$^{\#}$	30.92 ± 2.19$^{\#}$	15.2 ± 3.3*

*The difference compared with the control group is reliable ($P < 0.05$). $^{\#}$The difference compared with the "Diabetes" group is reliable ($P < 0.05$).

Against the backdrop of hyperglycemia-induced disturbances in vitamin D_3 metabolism and severe D-hypovitaminosis, significant changes in the biomechanical properties of bones and the development of secondary osteoporosis in diabetic mice were detected. The biomechanical parameters of mouse tibias in diabetes were investigated using the 3-point bending test. It was found that maximal load at failure (F_{max}) and stiffness of tibia bone in T1D were significantly reduced by 2.13- and 2.3-fold respectively (Fig. 1, $P < 0.05$). The μCT examination of the microscopic structure of tibia was also performed. The profound changes in the structure of the trabecular tissue of the tibia bones have been shown. Using the method of computed microtomography, we found a significant decrease in both volume of the trabecular tissue per total bone volume by 69.8% and the number of trabeculas by 53.2% in the trabecular bone tissue of the proximal metaphysis in diabetic mice (Fig. 2, $P < 0.05$). The results of our studies also showed a statistically significant decrease in the thickness of the tibia compact bone tissue in diabetic mice by 14.1% compared with control, indicating diabetes-induced significant changes in the structure of the cortical bone (Fig. 2, B2). It has been reaffirmed that experimental T1D conditions and diabetes-induced vitamin D_3 deficiency are accompanied with a disruption of bone tissue structure, function and growth. Such changes in biometric parameters are often accompanied by negative changes in the formation of the osteon structure and surrounding bone plates, misbalance in vascularisation and ossification of bone tissue and impairment of cartilage cell proliferation [17, 18].

It should be emphasized that the results obtained correlate with our earlier studies of bone mineral metabolism in experimental T1D. Previously, we found a decrease in the weight, length and diameter (diaphysis, proximal metaphysis) of the tibia of diabetic mice, as well as diminished mineral deposits in the bone tissue, which was accompanied by hypocalcemia and hypophosphatemia, increased serum alkaline phosphatase activity and its bone isoenzyme [15]. Here we additionally determined a significant elevation (by 1.92-fold) in the serum concentration of an important bone resorption marker TRAP in mice with experimental diabetes compared with the values of control animals (Fig. 3, $P < 0.05$).

Diabetic mice that received vitamin D_3 demonstrated normalization of the mineral metabolism, osteometric and biomechanical parameters (maximal load and stiffness, Fig. 1) of the tibias compared with diabetic animals. These changes also correlated with elevated trabecular bone volume in the proximal metaphysis of the tibia, trabecular number and an increase in the thickness of the cortical layer of tibial diaphysis (Fig. 2) in diabetic mice treated with vitamin D_3 vs. T1D mice.

Imbalance in the formation and resorption of bone tissue in osteoporosis may reflect impairments of the main mechanisms of systemic (hormonal) and local (cytokine) regulation of osteoblast and osteoclast activity. Therefore, we further investigated the intensity and direction of diabetes-elicited changes

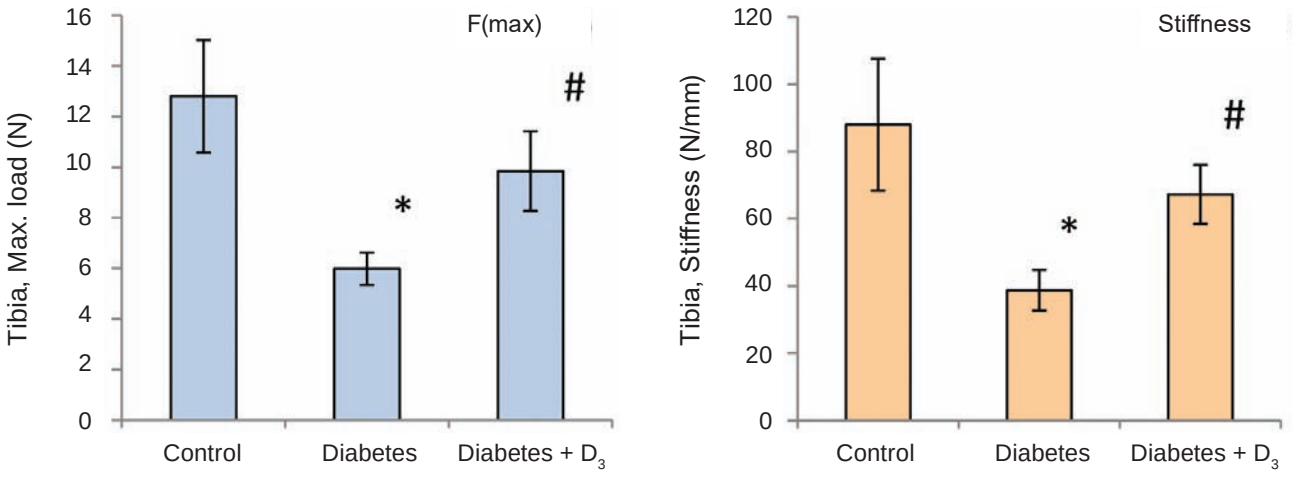

*Fig. 1. Three-point bending of tibia demonstrated a decreased maximal load at failure (F_{max}) and stiffness at failure in diabetic mice. These parameters increased with vitamin D_3 treatment. * $P < 0.05$ compared with control, # $P < 0.05$ compared with "Diabetes" group ($M \pm m$, $n = 6$)*

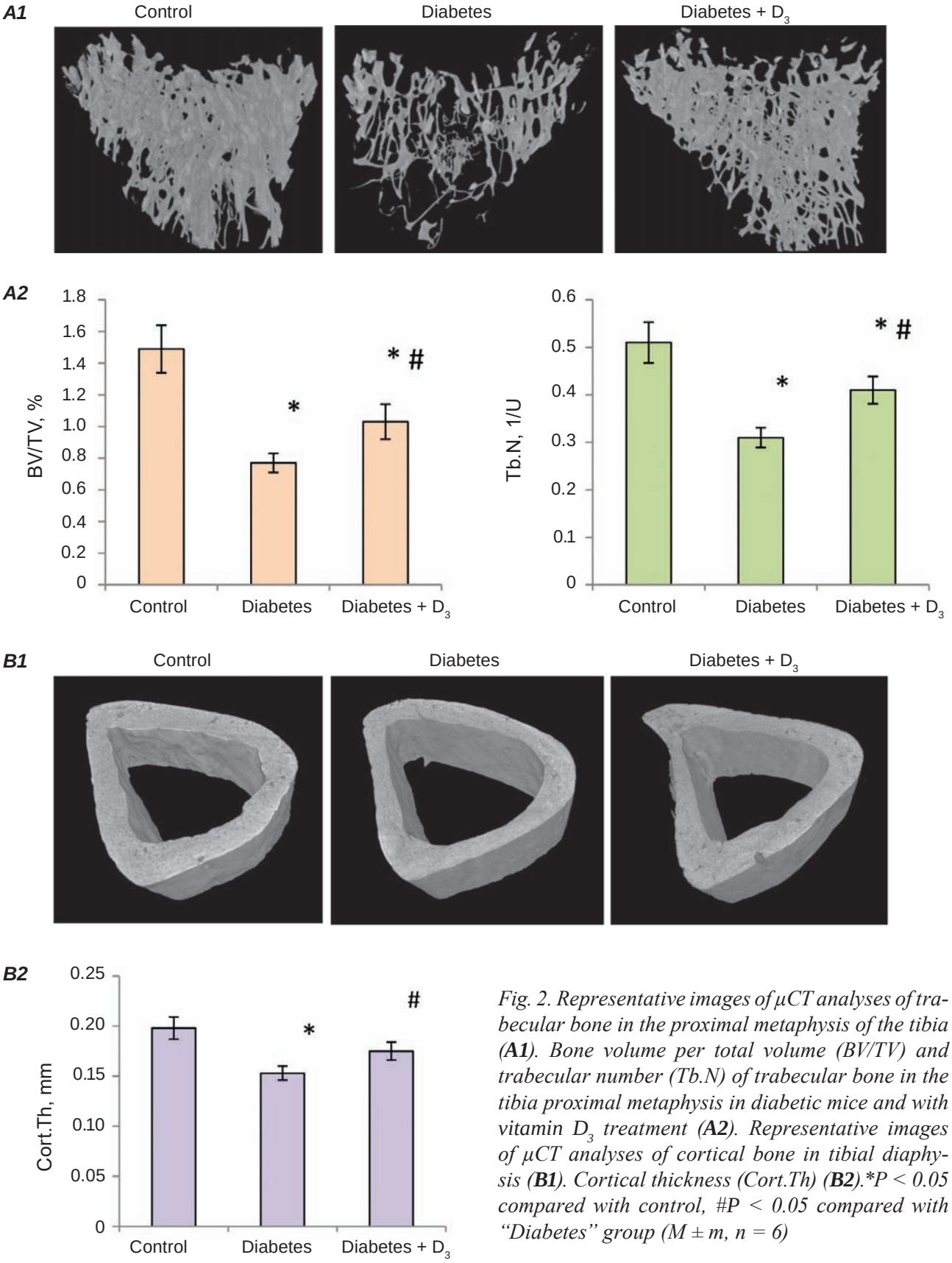

*Fig. 2. Representative images of μCT analyses of trabecular bone in the proximal metaphysis of the tibia (**A1**). Bone volume per total volume (BV/TV) and trabecular number (Tb.N) of trabecular bone in the tibia proximal metaphysis in diabetic mice and with vitamin D_3 treatment (**A2**). Representative images of μCT analyses of cortical bone in tibial diaphysis (**B1**). Cortical thickness (Cort.Th) (**B2**).*P < 0.05 compared with control, #P < 0.05 compared with "Diabetes" group (M ± m, n = 6)*

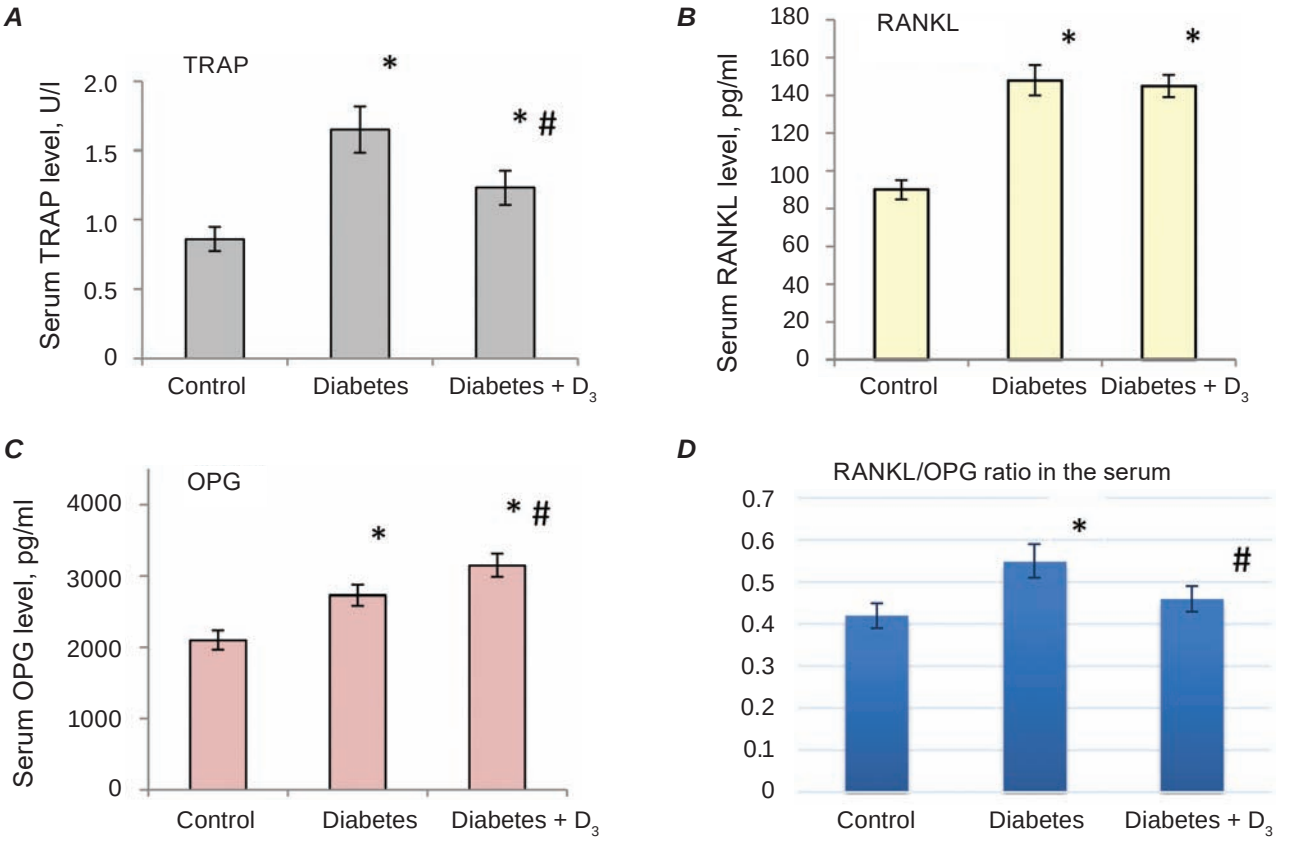

*Fig. 3. TRAP (**A**), RANKL (**B**), OPG (**C**) levels and RANKL/OPG ratio (**D**) in the serum of diabetic mice with or without vitamin D_3 treatment. *P < 0.05 compared with control, #P < 0.05 compared with "Diabetes" group (M ± m, n = 6)*

in the bone formation-resorption cycle in terms of the expression level of key markers of bone remodeling, and their relevance to vitamin D status of the animals.

Firstly, we assessed whether the changes of the RANKL/RANK/OPG axis are involved in the pathogenesis of diabetes-induced bone loss. According to the results depicted in Fig. 4, we observed marked increase in the bone tissue content of both RANK and its ligand RANKL by 1.51- and 1.72-fold, respectively, while the content of OPG, a decoy receptor for RANKL, was significantly decreased (by 1.59-fold) compared with the control ($P < 0.05$). These tendencies indicate diabetes-induced abnormalities in the RANKL/RANK/OPG signaling pathway that may contribute to disturbances of bone remodeling. The changes of RANKL and OPG levels in bone tissue were not fully consistent with the content of these osteotropic cytokines in the blood serum of diabetic animals, which we analyzed by ELISA. An increase in both RANKL and OPG levels in the serum of diabetic mice by 1.65 and 1.3 times, respectively, were seen in diabetes compared with the control animals (Fig. 3, $P < 0.05$). The RANKL/OPG ratio is considered to be an integral indicator of the osteoblast-dependent bone formation and the intensity of osteoclast-mediated bone resorption. Thus, the RANKL/OPG ratio is an important determinant of bone mass and skeletal integrity [19]. Our data showed a 1.4-fold elevation of this ratio in the serum of diabetic animals compared with the control (Fig. 3, $P < 0.05$), that reflects diabetes-induced prevalence of resorptive processes in bones.

RANK is a key component of the cytokine system, localized on the cellular surface of preosteoclasts, which facilitates their differentiation into osteoclasts and activation of mature osteoclasts responsible for enhancing the bone resorption process [20]. Therefore, the RANK protein content can correlate with both the number of osteoclastic cells and their activity in bone tissue [21]. Increased level of RANK protein, which we established in the present study, reflects most likely the intensification of osteoclastogenesis in diabetes-induced osteoporo-

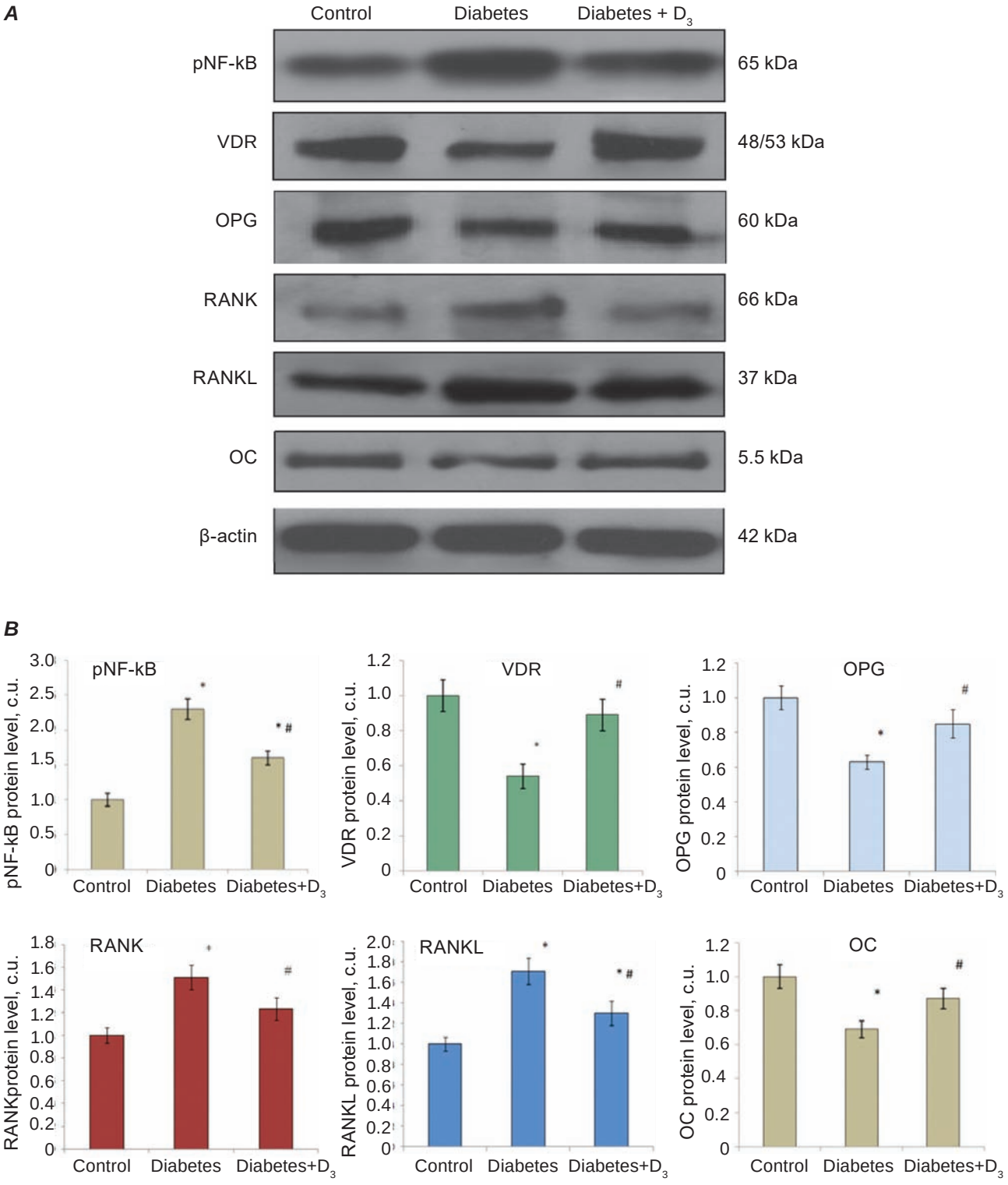

*Fig. 4. NF-κB, VDR, OPG, RANK, RANKL and OC protein content in the tibia bone lysates of diabetic mice (**A**). Graphical representations are expressed as means ± SD (**B**). Values are mean ± SEM; *P < 0.05 compared to control, #P < 0.05 compared to untreated diabetic group (M ± m, n = 6)*

sis. Moreover, the resorptive activity of osteoclasts and the nature of bone remodeling are mainly determined by the ratio of OPG and RANKL production. In bone tissue, OPG is synthesized mainly by osteoblasts and osteocytes and acts as an endogenous soluble decoy receptor for RANKL [20, 21]. Osteoprotegerin, by binding RANKL, prevents the activation of RANK on the cellular surface of osteoclasts, which reduces both osteoclastogenesis and resorptive activity of osteoclasts. Thus, the observed decrease in the level of OPG with a simultaneous increase in RANKL are most likely to enhance RANKL-mediated bone resorption. The likely activation of bone resorption is also confirmed by a more than 2-fold ($P < 0.05$) diabetes-elicited increase in the bone tissue level of the transcriptionally active form of NF-κB, with the large p65 subunit being phosphorylated at Ser311 (Fig. 4). It is known that binding of RANKL to RANK triggers the activation of NF-κB as its upstream target [22]. Transcriptional activation of NF-κB leads to preosteoclast differentiation into mature and active osteoclasts [23]. Thereby, out results demonstrate a significant role of the overexpression of the functional duo RANK - RANKL in the induction/activation of NF-κB-mediated bone resorption with the background of vitamin D3 deficiency.

This deregulation of the RANKL/RANK/OPG cytokine system, the determinant for the osteoclastogenesis process, in diabetes mellitus was accompanied by a significant decline in the expression of the key bone formation marker – OC, by 1.54-fold ($P < 0.05$), compared to the control (Fig. 4). A significant decrease in the content of OC, a vitamin K-dependent non-collagen protein of bone tissue which is synthesized by mature osteoblasts and is a sensitive indicator of the intensity of bone tissue metabolism, suggests a pronounced inhibition of osteogenesis associated with type 1 diabetes.

Despite the osteoprotective effect of hormonally active vitamin D metabolites and its efficacy as an additional therapy for treatment of diabetes and its complications, little is known about the link between vitamin D status and diabetes-induced changes in the expression of key components of the RANKL/RANK/OPG axis. Administration of vitamin D_3 to diabetic animals resulted in a significant 24% reduction in RANKL and a more pronounced elevation (by 35%) of OPG in bone tissue ($P < 0.05$, Fig. 4). In turn, this did not lead to the normalization of the level of RANKL in blood serum, but the serum OPG content achieved a value that was 15% higher than in diabetes *(*$P < 0.05$, Fig. 3). The RANKL/OPG ratio was shown to be partially decreased in blood serum after vitamin D supplementation. These data are consistent with the ability of vitamin D to suppress RANKL production by osteoblastic cells [24]. Another possible mechanism can be related to osteoblast differentiation, since hormonally active forms of vitamin D may influence the differentiation of mesenchymal progenitors into osteoblastic cells, resulting in the suppression of RANKL synthesis [24]. It should be further emphasized that in bone tissue there was a diabetes-associated decrease in the level of OPG expression, in contrast to its serum level. This difference can be explained by the fact that a large pool of the serum OPG is expressed by the cells of the cardiovascular, immune and digestive systems, in addition to osteoblasts/stromal cells [25, 26].

Figure 4 illustrates that the RANK protein level in bone tissue decreased by 19% with the administration of cholecalciferol compared with diabetes ($P < 0.05$). This may contribute to the more than 2.5-fold reduction of phosphorylated NF-kB/p65 content compared with diabetic mice ($P < 0.05$), suggesting antiosteoclastic and proosteoblastic effects of vitamin D_3 in bone. Normalization of osteogenesis and improvement of metabolic processes in bone tissue was evidenced by an increase in the expression of OC, the relative content of which reached the level of control animals after vitamin D_3 treatment.

As diabetes-evoked failures in bone remodeling can be VDR-mediated and the biological effects of the hormonal form of vitamin D_3 in various cell types are realized through these receptors, their levels in bone tissue were further investigated. We found that in T1D, the VDR protein content significantly (by 1.82-fold) decreased in this tissue ($P < 0.05$). It should be noted that a decrease in the level of OC and VDR in bone tissue caused by diabetes testifies to the suppression of the functional activity of the cells responsible for bone formation (osteoblasts and osteocytes), since these proteins are considered as reliable markers for these types of cells. Our results have indicated that the restoration of vitamin D bioavailability led to normalization of the level of VDR expression in bone tissue as compared with control. Better vitamin D_3 availability and increased VDR expression might result in normalization of RANK-RANKL and NF-κB-associated pathways that attenuate diabetes-induced

structural and biomechanical abnormalities in bone tissue.

The identified structural changes in bone tissue and abnormal expression of bone regulatory cytokines, underlying the diabetes-induced impairment of bone remodeling, may partially be caused by the chronic inflammatory process, asssociated with the development of diabetes mellitus. It was prevously shown that a close relationship exists between the development of diabetes-induced secondary osteoporosis and the changes of the cytokine profile towards the proinflammatory status [27, 28]. The RANKL/RANK/OPG axis may be recognized as a key signaling pathway in the integration of osteoimmune interaction, especially under diabetic conditions. Vitamin D might have a large regulatory potential in osteoimmunity modulation [29, 30].

Our findings suggest that diabetes-induced alterations of bone tissue remodeling are most likely caused by a decline in vitamin D availability and can be linked to disturbances in the RANKL/OPG signaling pathway. It was demonstrated that diabetes-related impairments may efficiently be prevented or corrected by vitamin D_3 treatment.

Data on financial support and acknowledgements. The biomechanical part of the research was done at the laboratory of professor Juha Tuukkanen in the Institute of Cancer Research and Translational Medicine, Department of Anatomy and Cell Biology, Faculty of Medicine, University of Oulu (Oulu, Finland) with the financial support of The Centre for International Mobility CIMO (Decision 29.1.2015/TM-15-9520).

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Received 26.03.2018