

## EXPERIMENTAL WORKS

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### THE IMPACT OF VITAMIN D<sub>3</sub> ON BONE REMODELING IN DIFFERENT TYPES OF EXPERIMENTAL PATHOLOGY

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*Osteoporosis is a progressive systemic skeletal disease characterized by a decrease in bone density, impairment of its microarchitectonics, and an increased risk of fractures that occur under minimal or no mechanical stress. One of the main causes of osteoporosis is vitamin D deficiency, which leads to disruption of normal bone remodeling. The aim of our study was to analyze the features of the process of bone tissue remodeling by measuring the key biochemical markers of bone formation/resorption in primary and secondary osteoporosis, as well as to investigate the potential corrective effect of vitamin D<sub>3</sub> supplementation. The work was conducted on rats with different osteoporosis models: alimentary, dysfunctional and secondary osteoporosis associated with diabetes mellitus. We used ELISA to measure 25(OH)D content in blood serum. Blood serum and bone tissue calcium, and alkaline phosphatase activity were determined with bioassay kits. The content of inorganic phosphate in blood serum and ash was assayed by the Dyce method. It was shown that all the studied pathological conditions were accompanied by vitamin D deficiency, which led to impaired absorption of calcium in the intestine and reabsorption of inorganic phosphates by the kidneys, reducing, as a result, their concentration in the blood serum. Hypocalcemia and hypophosphatemia contributed to the disruption of normal bone remodeling, excessive activation of alkaline phosphatase, and a decrease in the content of calcium and phosphate in bone tissue. Thus, sufficient vitamin D bioavailability was confirmed to be critical for effective bone remodeling in primary and secondary osteoporosis.*

*Key words:* vitamin D, osteoporosis, type 1 diabetes mellitus, bone remodeling.

The World Health Organization (WHO) defines osteoporosis as a systemic disease characterized by metabolic changes in the structure of bone tissue, leading to a decrease in bone mass and strength, as well as a significant increase in the risk of fractures with minimal or even no traumatic impact. Based on the materials presented at the World Congress on Osteoporosis in 2020, which was devoted to the clinical and economic aspects of osteoporosis, it can be noted that this pathology is one of the most common in the world and, along with cardiovascular diseases, diabetes and cancer, is at the forefront in the structure of morbidity and mortality from various diseases [1].

In general, osteoporosis is divided into primary and secondary [2]. In turn, primary osteoporo-

sis (POP) includes postmenopausal osteoporosis (type I) and osteoporosis of the elderly (type II), while secondary osteoporosis is induced or exacerbated by other diseases or drug exposure [3]. The underlying mechanism for the development of POP is a deficiency of estrogens and androgens in adolescence and after menopause, which leads to changes in bone metabolism and the predominance of bone resorption processes over bone formation. At the same time, the mechanisms of secondary osteoporosis are more complex and less studied, and depend on the initial cause of its development, in particular on the primary disease (endocrine disorders, gastrointestinal and kidney dysfunction, celiac disease, inflammatory and autoimmune diseases, etc.) or the type of medications used by the patient (glucocor-

ticoids, thyroid hormones, proton pump inhibitors, H<sub>2</sub>-histamine receptor blockers, antiepileptic drugs, antidepressants, thiazolidinediones, antiretroviral drugs, anticoagulants, some chemotherapy drugs, loop diuretics, etc.). Diabetes-induced osteoporosis (DIOP) is one of the types of secondary osteoporosis that has been actively studied in recent years [4]. In our previous study, we demonstrated a potential molecular mechanism that may underlie the DIOP-dependent imbalance of bone remodeling – a malfunction of the receptor activator of nuclear factor  $\kappa$ B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG; decoy receptor for RANKL) axis in bone marrow cells [5]. However, this is only one of the potential molecular mechanisms involved in impaired bone remodeling and, therefore, further studies are needed to comprehensively establish the pathogenesis of osteoporosis.

Vitamin D<sub>3</sub> (cholecalciferol) is a bioactive substance formed from cholesterol in the human body, and it can also be obtained from food, especially cod liver and other types of oily fish [6]. In the body, cholecalciferol is converted into a hormonally active form – calcitriol (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>), and after the formation of a complex with a specific receptor (vitamin D receptor, VDR), it can regulate the expression of more than 1000 genes [7]. Although much attention has been paid in recent years to the study of the non-classical (non-calcemic) effects of vitamin D<sub>3</sub>, it is also important to keep its classical functions in mind. These functions include regulation of intestinal calcium and phosphate absorption, control of bone metabolism, and, along with parathyroid hormone, maintenance of physiological serum ionized calcium concentrations. Furthermore, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> is able to locally regulate bone remodeling by acting on bone cells such as osteoblasts and osteoclasts, as well as other cells (chondrocytes, endothelial cells, etc.), the correct functioning of which is critical for normal vascularization and bone remodeling [8].

The systemic review, provided by Amrein et al. in 2020 demonstrated the prevalence of vitamin D deficiency all over the world, especially in Europe, where more than 40% of adolescents have 25(OH)D (the main marker of vitamin D availability) level in blood serum less than <50 nmol/l (or 20 ng/ml) [9]. Unfortunately, Ukraine is not an exception to global trends regarding vitamin D insufficiency/deficiency. Thus, according to epidemiological studies, most of the population of Ukraine has vitamin D deficiency (81.8%), 13.6% exhibits insufficient and only 4.6% –

an adequate level of 25(OH)D in the blood serum [10]. Vitamin D insufficiency/deficiency is strongly associated with higher risk of age-related plethora pathologies development, in particular primary osteoporosis and diabetes mellitus. Secondary osteoporosis is known to be a common complication of both type 1 and type 2 diabetes mellitus [11].

To date, according to WHO recommendations, T-scores of bone mineral density (BMD) are considered the main diagnostic marker of osteoporosis. Despite this, there are several key serum biochemical markers of bone turnover that can be divided into markers of bone formation (bone isoenzyme of alkaline phosphatase, osteocalcin, carboxyterminal propeptide of type I collagen) and markers of bone resorption (cross-linked C-telopeptide of type I collagen, tartrate-resistant acid phosphatase etc.) [12, 13]. In addition, examination of total Ca<sup>2+</sup> and inorganic phosphate in blood serum, as well as determining the status of vitamin D in patients with osteoporosis is also very important for choosing the right therapeutic tactics.

The purpose of our work was to analyze the processes of bone tissue remodeling by measuring the key biochemical markers of bone formation/resorption in primary and diabetes-induced secondary osteoporosis, as well as to study the potential corrective effect of vitamin D<sub>3</sub>.

## Materials and Methods

*Animals and experimental modeling.* All experimental pathological conditions represented in this work were designed on Wistar rats. Primary osteoporosis (POP) developed when female rats (weighing 100 ± 5 g) were kept on a D-hypovitaminosis diet for 30 days [14]. Control animals were on a regular vivarium diet during all period of experiment. A model of dysfunctional osteoporosis (DOP) was induced in adult female rats (7 months old, weighing 300 ± 10 g) by keeping the animals in special cages of small size and volume for 90 days. Control animals were on a regular vivarium diet and in normal cages without limitation of motor activity during all experiment. Diabetes-induced secondary osteoporosis (DIOP) was modulated in young male rats (weighing 140 ± 5 g) by administration of streptozotocin (STZ, Sigma; USA) at a dose of 55 mg/kg b.w., dissolved in 50 mM citrate buffer, pH 4.5. Two weeks after STZ administration, the animals were tested for blood glucose levels using a One Touch Select glucometer (LifeScan Inc.; Switzerland) and

divided into experimental groups. Control animals were injected with 500 µl of 50 mM citrate buffer solution. During the week-long adaptation period and throughout the experiment, all animals were kept in the vivarium of the Palladin institute of biochemistry of the National Academy of Sciences of Ukraine at a temperature of 18-22°C, humidity 50-60%, natural day-night light regime. Control animals were fed a standard diet. All procedures with animals were carried out in accordance with international recommendations of the European Convention for the Protection of Vertebrate Animals used for Research and Scientific Purposes (Strasbourg, 1986) and the “Bioethical appraisal of preclinical and other scientific research conducted on animals” (Kyiv, 2006).

The regimen of vitamin D<sub>3</sub> administration was as follows: for POP and DOP – 400 IU/kg of body weight of cholecalciferol orally for 30 days after a month of osteoporosis development; for DIOP – 600 IU/kg body weight of cholecalciferol per os for 30 days, two weeks after STZ injection. Vitamin D<sub>3</sub> in sunflower oil solution (Cholecalciferol, Sigma; USA) was used in all experiments.

*Determination of the 25(OH)D level in blood serum of experimental animals.* The concentration of 25-hydroxyvitamin D in blood serum was performed by using in-house developed ELISA kit according to previously described procedure [15].

*Measurement of the ash content of the bone tissue, and mineral components in blood serum and bone tissue.* The content of Ca<sup>2+</sup> in blood serum and bone ash was determined with a bio-test kit (Lachema; Czech Republic) using 25 mmol/l CaCO<sub>3</sub> dissolved in 1.7% HCl as a standard solution according to the manufacturer’s instruction. The content of inorganic phosphate (P<sub>i</sub>) in the blood serum and bone ash was determined by Dyce method after precipitation of proteins with 12% solution of trichloroacetic acid [16].

The ash content of bone tissue was determined by the method of dry mineralization at a temperature of 500-600°C after its degreasing with hexane for 7 days and was calculated relative to the mass of bone tissue. The content of mineral components in the ash was determined by the methods described above after the ash was dissolved in 0.5 ml of concentrated HCl and further diluted in bidistilled water.

*Determination of total alkaline phosphatase (ALP) activity and its bone isoform in blood serum.* ALP activity was determined using a reagent kit provided by Lachema, Czech Republic, according to

the manufacturer’s protocol and calculated using the formula:

$$X = \varepsilon_{\text{sample}} / \varepsilon_{\text{standard}} \times K,$$

where X is the number of micromoles of 4-nitrophenol released under the action of the enzyme contained in 1 l of serum per 1 min at 37°C.

The activity of the bone isoform of ALP was calculated as the difference between the activity of total alkaline phosphatase and thermostable alkaline phosphatase, which was determined after incubation of samples in a water bath at a temperature of 56-57°C [17].

*Statistics.* All data are expressed as mean ± SEM for at least six rats per group. For determination of the type of data distribution we used Shapiro-Wilk test. One-way ANOVA with Tukey post-hoc test was used to indicate statistical differences between the experimental groups. Differences were considered significant when  $P \leq 0.05$ . All statistical analysis was performed using Origin Pro 8.5 (OriginLab Corporation, Northampton; USA).

## Results and Discussion

Vitamin D deficiency has long reached pandemic status worldwide [18]. Among the factors that can cause the development of vitamin D deficiency are pregnancy and postmenopausal period, dark skin color, insufficient sun exposure, obesity in childhood and adulthood, and plethora of diseases (autoimmune, endocrine, gastrointestinal) [18, 19]. On the other hand, low vitamin D status is closely associated with a higher risk of developing various pathologies, such as type 1 and type 2 diabetes mellitus, certain types of cancer, infectious diseases, in particular COVID-19 [20, 21]. Adequate circulating levels of 25(OH)D are essential for normal bone formation and remodeling, while subclinical vitamin D deficiency, more prevalent worldwide, is a key risk factor for osteoporosis and increased fracture rates [22].

Our results are in full agreement with the literature data as we have shown that the level of 25(OH)D in the blood serum of experimental animals was significantly lower in both primary and secondary diabetes-induced osteoporosis compared with controls (Fig. 1). It was found that in the POP group the concentration of 25(OH)D was 70% lower than in the control group. Studies on the DOP model showed a decrease in 25(OH)D by 62%, and in DIOP – by 34.4% compared with the control group (Fig. 1).

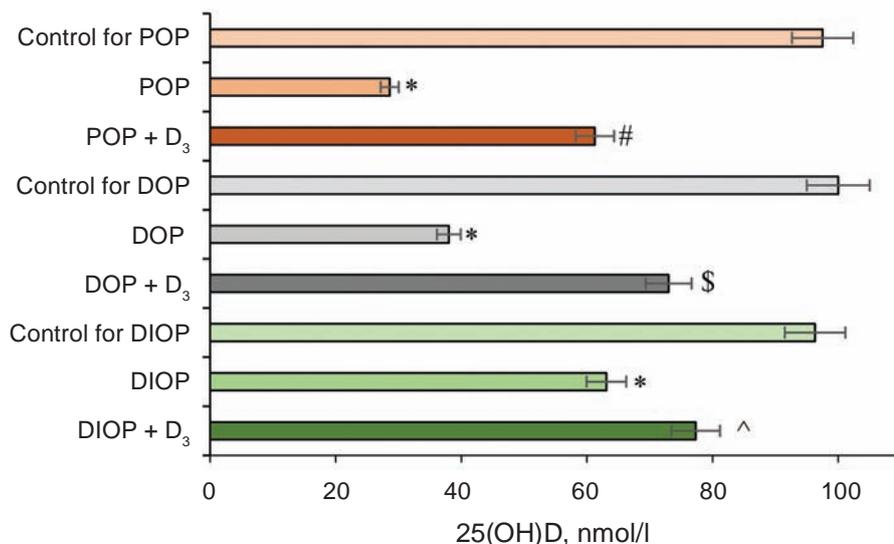


Fig. 1. The content of 25-hydroxyvitamin D in blood serum of rats with different types of osteoporosis. The data represented as mean  $\pm$  SEM,  $n = 6$ . \* $P \leq 0.05$  vs. appropriate control; # $P \leq 0.05$  vs. POP group; \$ $P \leq 0.05$  vs. DOP group; ^ $P \leq 0.05$  vs. DIOP group

This dramatic decline in vitamin D availability in various types of osteoporosis may have several explanations. The main mechanism that may contribute to the development of vitamin D deficiency in osteoporosis is an impairment of 25(OH)D formation in the liver. The first stage of vitamin D hydroxylation occurs in hepatocytes under the action of enzymes of the cytochrome P450 family – CYP2R1 (microsomal isoenzyme) and CYP27A1 (mitochondrial isoenzyme). In previous studies, we have demonstrated that in secondary osteoporosis associated with glucocorticoid therapy, the total activity of vitamin D 25-hydroxylases was strongly suppressed [23]. We observed the same trend in rats with experimental diabetes mellitus [24]. The prevalence of the mitochondrial isoform, which has a lower affinity for cholecalciferol and metabolizes the latter at concentrations much higher than physiological, with a simultaneous decrease in the activity of the main enzyme responsible for 25-hydroxylation of vitamin D, CYP2R1, could be the main reason for the development of vitamin D deficiency [25].

Vitamin D binding protein (VDBP), encoded by the *Dbp* gene, appears to be another important component of the auto/para/endocrine vitamin D-system, the disruption of which can contribute to vitamin D deficiency. Notably, *Dbp*-null mice showed a decrease in serum levels of 25(OH)D and  $1\alpha,25(\text{OH})_2\text{D}$  [25]. At the same time, studies on experimental type 1 diabetes revealed not only a drop

in the level of *Dbp* mRNA, but also a decrease in the synthesis of megalin and cubulin proteins, surface receptors of the 25(OH)D-VDBP complex responsible for the transmembrane transport of 25(OH)D [26].

While 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase, responsible for the formation of  $1\alpha,25(\text{OH})_2\text{D}$ , is regulated in many ways, including signals from parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and  $1\alpha,25(\text{OH})_2\text{D}$ , there are no reliable data on the regulation of CYP2R1 [27]. However, CYP2R1, as an inducible enzyme, may respond to vitamin D<sub>3</sub> loading during therapy and help restore normal vitamin D status in the treatment of osteoporosis. Our results are consistent with this assumption. We showed that after a 30-day course of cholecalciferol treatment, the level of 25(OH)D was significantly higher in all studied models of osteoporosis. Fig. 1 depicts a 53% increase in 25(OH)D in POP compared to the osteoporosis group. In the DOP group, this indicator was higher by 47%, and in the DIOP group – by 32% compared to the osteoporosis.

Prolonged D-hypovitaminosis can be considered as one of the leading factors, along with others, in the mechanism of osteoporosis development due to an abnormality in the hormonal regulation of calcium metabolism through changes in the functioning of the parathormone-calcitonin-vitamin D<sub>3</sub> system. Calcium and phosphates are known to be vital mine-

rals and structural elements of the bone mineral matrix. At the same time, calcium provides many other physiological functions, including muscle contraction, release of neurotransmitters, transmission of intracellular signals, blood clotting, etc [28]. Phosphate, in turn, is part of ATP, nucleic acids and phospholipids. The critical importance of calcium and phosphates in providing physiological and cellular processes has necessitated strict regulation of their concentration in a narrow range of fluctuations in the blood. Plasma levels of both minerals are regulated by the coordinated action of a group of bioactive substances called phosphocalcitic hormones, which include  $1\alpha,25(\text{OH})_2\text{D}$ , PTH, and FGF23. It is important to emphasize that the concentration of calcium and phosphate in plasma is regulated interdependently [29].

Total serum calcium can be divided into several subtypes. Some part of  $\text{Ca}^{2+}$  is associated with such blood plasma proteins as albumins and globulins and performs an inactive storage role. The predominant part of  $\text{Ca}^{2+}$  exists in the ionized form, is transported through membranes, and is a biologically active form of calcium [30]. It is well known that optimal calcium supplementation is critical to preventing the development of osteoporosis [31]. In rats with primary osteoporosis induced by ovariectomy in combination with a multi-deficiency diet, it was found that the concentration of ionized calcium decreases significantly in the late periods after surgery and is accompanied by hyperparathyroidism [32]. The authors of the work consider the diet, defi-

cient in calcium and vitamin D, as the main cause of the revealed changes in the content of bioactive  $\text{Ca}^{2+}$ .

In the present study, we demonstrated that the content of protein-bound calcium remained practically unchanged in the blood serum of rats with primary osteoporosis compared to control animals (Table 1). Changes in the levels of total calcium in D-hypovitaminosis occurred due to its free ionized fraction, which decreased by 1.4 times compared with control animals. In a model of dysfunctional osteoporosis, we also found a significant 1.3-fold reduction of total serum calcium, both protein-bound and free (Table 1).

In secondary diabetes-induced osteoporosis, calcium alterations were not as significant as in the POP and DOP models. It can be assumed that the development of type 1 diabetes mellitus within 6 weeks is not enough for a critical decrease in the concentration of total calcium in the blood serum of animals. In this particular case, we can most likely argue about the initial stages of impaired bone remodeling.

The main classical (skeletal) function of vitamin D is the control of absorption of  $\text{Ca}^{2+}$  and phosphates in the intestine, as well as the regulation of bone metabolism by endocrine (through the transport of calcium and phosphates to the bone with subsequent formation of hydroxyapatite) and auto/paracrine mechanisms (regulation of gene expression in bone cells by  $1\alpha,25(\text{OH})_2\text{D}$ -VDR complex) [6]. Thus, the vitamin D status of the body is critical for controlling the formation of calcitriol, the hormo-

*Table 1. The content of  $\text{Ca}^{2+}$  and inorganic phosphate in blood serum of rats with different types of osteoporosis and after cholecalciferol administration*

Group	$\text{Ca}^{2+}$ (total), mmol/l	$\text{Ca}^{2+}$ (free ionized), mmol/l	$\text{Ca}^{2+}$ (bound to protein), mmol/l	$\text{P}_i$ , mmol/l
Control for POP	$2.24 \pm 0.04$	$2.04 \pm 0.01$	$0.20 \pm 0.01$	$2.10 \pm 0.01$
POP	$1.58 \pm 0.01^*$	$1.40 \pm 0.01^*$	$0.18 \pm 0.02$	$1.46 \pm 0.02^*$
POP + $\text{D}_3$	$2.10 \pm 0.02^\#$	$1.91 \pm 0.03^\#$	$0.19 \pm 0.01^\#$	$1.78 \pm 0.04^\#$
Control for DOP	$2.13 \pm 0.04$	$1.92 \pm 0.03$	$0.21 \pm 0.08$	$2.01 \pm 0.06$
DOP	$1.85 \pm 0.05^*$	$1.63 \pm 0.02^*$	$0.22 \pm 0.05$	$1.63 \pm 0.05^*$
DOP + $\text{D}_3$	$2.11 \pm 0.06^\#$	$1.85 \pm 0.04^\#$	$0.26 \pm 0.01^\#$	$1.98 \pm 0.05^\#$
Control for DIOP	$2.16 \pm 0.10$	$2.00 \pm 0.01$	$0.16 \pm 0.02$	$2.13 \pm 0.20$
DIOP	$1.97 \pm 0.07^*$	$1.83 \pm 0.02^\#$	$0.14 \pm 0.05$	$1.89 \pm 0.09^*$
DIOP + $\text{D}_3$	$2.14 \pm 0.10^\#$	$1.96 \pm 0.01^\#$	$0.18 \pm 0.01^\#$	$2.10 \pm 0.08^\#$

Note. The data represented as mean  $\pm$  SEM,  $n = 6$ .  $^*P \leq 0.05$  vs. appropriate control;  $^\#P \leq 0.05$  vs. appropriate osteoporosis

nally active form, and for the realization of its auto/para/endocrine effects.

We established that cholecalciferol supplementation to animals with POP for 30 days and the restoration of the 25(OH)D concentration resulted in almost complete normalization of the calcium content in the blood serum (Table 1). The same effect was observed in an experimental model of dysfunctional osteoporosis, where the  $\text{Ca}^{2+}$  concentration was restored to the control level after a month of vitamin  $\text{D}_3$  therapy. Despite the fact that we did not observe a critical decrease in the calcium content in the blood serum of animals with DIOP, the administration of vitamin  $\text{D}_3$  contributed to a slow increase in total calcium compared with the diabetic group (Table 1).

It is well known that vitamin D deficiency leads not only to hypocalcemia, but also to the development of hypophosphatemia [33]. The hormonally active form of vitamin D,  $1\alpha,25(\text{OH})_2\text{D}$ , is responsible for adequate transport of calcium and phosphate to bone from the intestine. Our results indicate a statistically significant decrease in the concentration of inorganic phosphate in the blood serum of animals with experimental osteoporosis. Thus, we demonstrated a 1.4-, 1.5-, and 1.2-fold reduction in blood phosphate levels in the POP, DOP, and DIOP groups compared to the respective controls (Table 1). Cholecalciferol supplementation ensued to almost complete recovery of phosphate concentrations in all experimental models of osteoporosis. One of the mechanisms that may cause the development of hypophosphatemia in osteoporosis is an increase in PTH production due to insufficient synthesis of calcitriol in the kidneys [34]. Thus, vitamin  $\text{D}_3$  loading of animals with experimental osteoporosis promotes the formation of  $1\alpha,25(\text{OH})_2\text{D}$ , as a result of which the content of inorganic phosphate returns to the control level.

Hypocalcemia caused by impaired absorption of calcium in the intestine is one of the causes of increased bone resorption due to the development of secondary hyperparathyroidism. In addition, tumor necrosis factor (TNF)  $\alpha$  and interleukin (IL) 1 are involved in the mechanism of impaired bone remodeling, the concentration of which in blood serum increases in postmenopausal osteoporosis [35]. Morgan et al. demonstrated a dramatic decrease in total ash as well as bone calcium and phosphate levels in the femur of rats with postmenopausal ovariectomy-induced primary osteoporosis [35]. The

results of our research also showed a significant decrease in ash content (by 27 and 16% in the POP and DOP groups, respectively, vs. control), accompanied by a decrease in  $\text{Ca}^{2+}$  (by 54 and 10% in the POP and DOP groups, respectively, vs. control), as well as  $\text{P}_i$  (by 41 and 23% in the POP and DOP groups, respectively, vs. control) in the bone tissue of animals with primary osteoporosis (Fig. 2).

In the bone tissue of animals with experimental diabetes-induced secondary osteoporosis, there was no significant difference in total ash content compared to the control. At the same time, the amount of  $\text{Ca}^{2+}$  was 7%, and  $\text{P}_i$  8.8% lower than in the control group (Fig. 2). Bone loss in type 1 diabetes may depend on both sex and age [36]. Statistically significant loss of bone density occurs predominantly in postmenopausal women. In contrast, there is no difference in males in both adolescence and adulthood. However, experimental type 1 diabetes has also been shown to induce progressive loss of trabecular bone density and premature cessation of cortical bone growth [37]. Based on the literature data, we can speculate that the development of experimental type 1 diabetes within 6 weeks is not enough for the loss of bone mass and mineral components to the extent that they could be fixed by routine biochemical methods to determine the total ash content, as well as the percentage of  $\text{Ca}^{2+}$  and  $\text{P}_i$  content in bone tissue.

Cholecalciferol treatment led to complete normalization of the ash content in the bones of animals with experimental osteoporosis, with a further increase in the content of  $\text{Ca}^{2+}$  and  $\text{P}_i$  in the ash (Fig. 2). It should be noted that the biochemical response to the normalization of calcium intake is practically independent of the calcium concentration baseline, while the response to treatment with cholecalciferol is highly dependent on its initial level [38]. Unlike animals with POP and DOP, in which we observed the development of severe vitamin D deficiency, animals with DIOP were characterized by a less pronounced decrease in serum 25(OH)D levels, which naturally correlated only with a slight effect of such a decrease on ash content and the percentage of  $\text{Ca}^{2+}$  and  $\text{P}_i$  in bone tissue.

Biochemical markers of bone metabolism are conventionally divided into markers of bone formation and bone resorption [39]. Despite limitations in displaying overall bone turnover, these markers are critical for evaluating response to therapy in patients with osteoporosis. Alkaline phosphatase (ALP), the

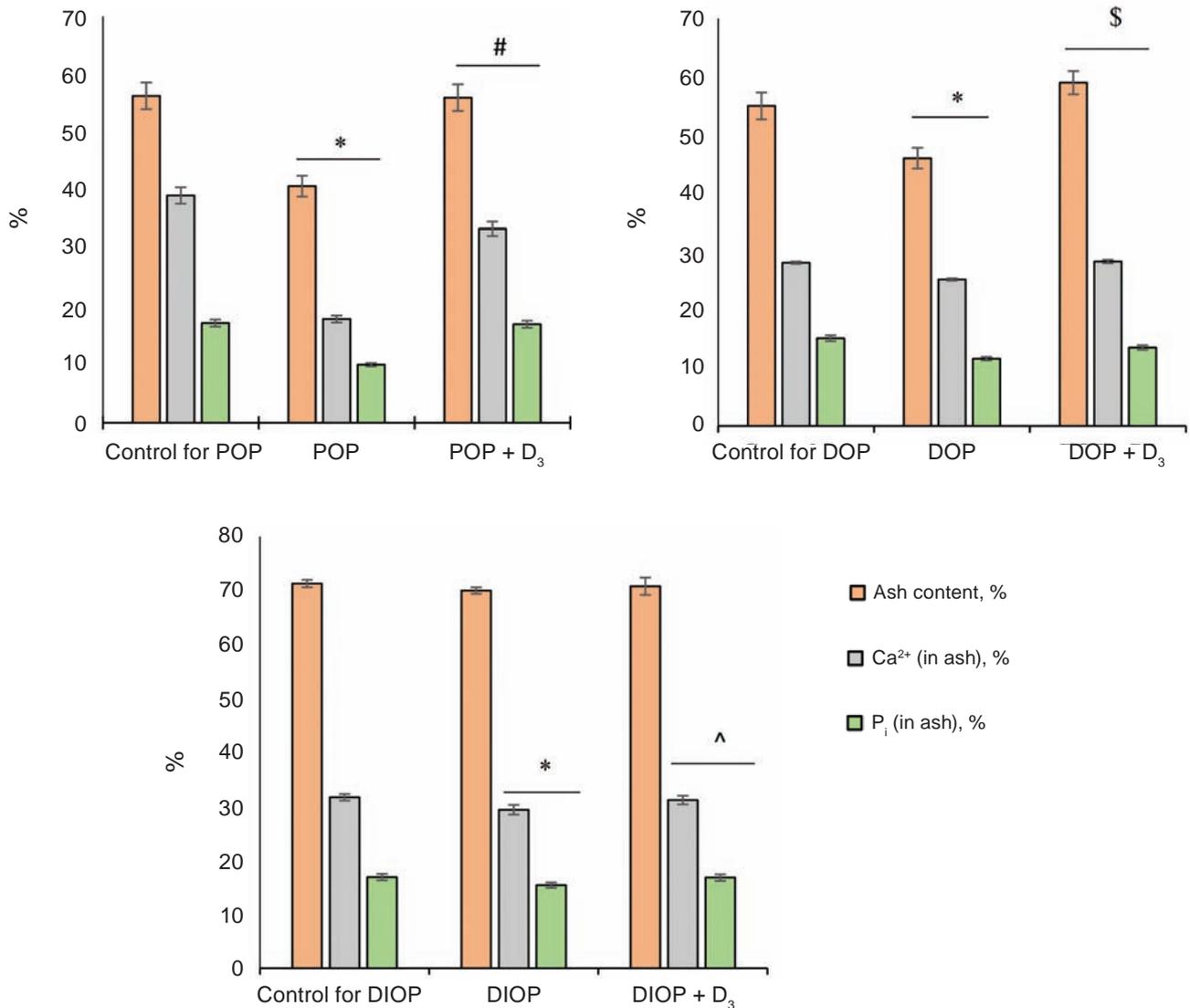


Fig. 2. Percentage of ash, Ca<sup>2+</sup> and inorganic phosphate in bone tissue of rats in different types of osteoporosis and after vitamin D<sub>3</sub> administration. The data represented as mean ± SEM, n = 6. \*P ≤ 0.05 vs. appropriate control; #P ≤ 0.05 vs. POP group; \$P ≤ 0.05 vs. DOP group; ^P ≤ 0.05 vs. DIOP group

total activity of which, like the activity of the bone isoenzyme, is determined in blood serum, can be considered one of the key markers of bone metabolism. It is a membrane-bound enzyme responsible for the release of inorganic phosphates from monophosphoric acid esters (phosphoethanolamine, phosphocholine, pyridoxal-5-phosphate) and pyrophosphate, followed by the formation of hydroxyapatite. At the same time, elevation of ALP activity was reported in blood serum of children with rickets, as well as in patients with bone diseases, accompanied by an increase in osteoclast activity [40].

We have shown that cholecalciferol deficiency is associated with a 1.6-fold increase in the activi-

ty of total alkaline phosphatase in the blood serum of animals with primary osteoporosis (Table 2). The elevation of ALP activity occurred mainly due to changes in the activity of its bone isoform (by almost 1.7-fold vs. control), suggesting the development of pathological processes in the bone tissue. In dysfunctional osteoporosis, an increase in the total activity of alkaline phosphatase and its bone isoenzyme, respectively, by 1.5 and 1.3 times compared with the control was revealed. A significant increase in ALP activity was also shown in the blood serum of rats with secondary diabetes-induced osteoporosis (by 1.5 times for both total ALP activity and its bone isoform) (Table 2).

Table 2. The impact of vitamin D<sub>3</sub> on alkaline phosphatase (total and bone isoenzyme) activity in blood serum of rats with experimental osteoporosis

Group	ALP (total), μmol/min·l	ALP (bone isoenzyme), μmol/min·l
Control for POP	224.76 ± 8.00	201.7 ± 6.0
POP	400.5 ± 1.0*	356.0 ± 1.0*
POP + D <sub>3</sub>	257.0 ± 1.0 <sup>#</sup>	222.8 ± 8.0 <sup>#</sup>
Control for DOP	300.15 ± 4.00	260.7 ± 4.0
DOP	460.1 ± 1.0*	330.0 ± 1.0*
DOP + D <sub>3</sub>	350.0 ± 8.0 <sup>#</sup>	261.8 ± 6.0 <sup>#</sup>
Control for DIOP	251.2 ± 1.0	232.2 ± 9.0
DIOP	383.0 ± 5.0*	349.0 ± 4.0*
DIOP + D <sub>3</sub>	299.0 ± 5.0 <sup>#</sup>	282.0 ± 4.0 <sup>#</sup>

Note. The data represented as mean ± SEM,  $n = 6$ . \* $P \leq 0.05$  vs. appropriate control; <sup>#</sup> $P \leq 0.05$  vs. appropriate osteoporosis

Summarizing, it can be noted that in dysfunctional osteoporosis, changes in the biochemical parameters of mineral and bone tissue metabolism were significantly more pronounced than in alimentary osteoporosis, while 25(OH)D decreased to approximately the same level (Fig. 1). This may indicate that, along with the development of vitamin D deficiency, an important role in the pathogenesis of DOP is played by the deficit of biomechanical loads on the animal skeleton, as well as possible disturbances in the hormonal background associated with aging and chronic immobilization stress.

It is noteworthy that, against the background of a significant increase in serum alkaline phosphatase activity in animals with DIOP, we did not find considerable changes in bone turnover, which may depend on the basal level of 25(OH)D, as we explained in the corresponding section.

Administration of cholecalciferol for 30 days caused almost complete normalization of ALP activity in all experimental models (Table 2). Based on our results, we can hypothesize that it is the increased activity of the bone isoenzyme of ALP that may be one of the reasons for the development of vitamin D deficiency/insufficiency in various models of osteoporosis. Literature data confirm that this isoform of ALP is capable of inhibiting the activity of

cytochrome CYP2R1, which is responsible for the synthesis of 25(OH)D [41]. In addition, calcitriol, a hormonally active form of vitamin D, can directly regulate ALP gene expression in osteoblasts and promote efficient bone mineralization [42].

Thus, vitamin D deficiency is associated with impaired absorption of calcium in the intestines and reabsorption of inorganic phosphate by the kidneys that leads to a decrease in their concentrations in the blood serum. Hypocalcemia and hypophosphatemia can cause disruption of normal bone remodeling, excessive activation of alkaline phosphatase, and reduce the content of calcium and phosphate in bone tissue.

*Conclusions.* Our results clearly demonstrate that dietary vitamin D deficiency, prolonged immobilization, and the development of type 1 diabetes mellitus equally inevitably lead to a decrease in the level of serum 25(OH)D as a reliable marker of vitamin D sufficiency/insufficiency/deficiency in experimental animals. In turn, a low concentration of 25(OH)D causes abnormal absorption of calcium and phosphate and, as a consequence, the development of hypocalcemia and hypophosphatemia, resulting in osteoporosis. Therapy with cholecalciferol restores normal mineralization of bone tissue by reducing the activity of alkaline phosphatase (both total and bone isoenzyme) and normalizes the absorption of the main mineral components of the bone, thereby contributing to the restoration of bone remodeling.

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*Conflict of interest.* Authors have completed the Unified Conflicts of Interest form at [http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi\\_disclosure.pdf](http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf) and declare no conflict of interest.

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## ВПЛИВ ВІТАМІНУ D<sub>3</sub> НА РЕМОДЕЛЮВАННЯ КІСТКОВОЇ ТКАНИНИ ЗА РІЗНИХ ВИДІВ ЕКСПЕРИМЕНТАЛЬНОЇ ПАТОЛОГІЇ

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Остеопороз — це прогресуюче системне захворювання скелету, що характеризується зниженням щільності кісткової тканини, порушенням її мікроархітекtonіки та підвищеним ризиком переломів, які виникають під час мінімального механічного навантаження або без нього. Однією з основних причин розвитку остеопорозу є дефіцит вітаміну D, який призводить до порушення нормального ремоделювання кісткової тканини. Метою дослідження було проаналізувати особливості процесу ремоделювання кісткової тканини шляхом визначення ключових біохімічних маркерів кісткоутворення/резорбції за первинного та вторинного остеопорозу, а також дослідити потенційний корегувальний ефект вітаміну D<sub>3</sub>. Експерименти проводили на щурах з різними моделями остеопорозу: аліментарний, дисфункційний та вторинний остеопороз, індукований цукровим діабетом. Для вимірювання вмісту 25(OH)D у сироватці крові щурів використовували імуноензимний аналіз. Вміст кальцію та активність лужної фосфатази у сироватці крові і кістковій тканині визначали за допомогою комерційних наборів. Вміст неорганічного фосфату в сироватці крові та золі кісток визначали за методом Дайса. Показано, що всі досліджувані патологічні стани супроводжувалися дефіцитом вітаміну D, що призводило до порушення всмоктування кальцію в кишечнику та реабсорбції неорганічних фосфатів нирками, та зниження їх концентрації в сироватці крові. Гіпокальціємія та гіпофосфатемія спричинювала порушення нормального ремоделювання кісткової тканини, надмірну активацію лужної фосфатази та зниження вмісту кальцію і фосфатів у кістковій тканині. Таким чином, підтверджено, що нор-

мальна біодоступність вітаміну D є критичною для ефективного ремоделювання кісткової тканини як за первинного, так і за вторинного остеопорозу.

**Ключові слова:** вітамін D, остеопороз, цукровий діабет 1 типу, ремоделювання кісткової тканини.

### References

1. WCO-IOF-ESCEO World congress on osteoporosis, osteoarthritis and musculoskeletal diseases. *Osteoporos Int.* 2020; 31(Suppl 1): 1-32.
2. Dobbs MB, Buckwalter J, Saltzman C. Osteoporosis: the increasing role of the orthopaedist. *Iowa Orthop J.* 1999; 19: 43-52.
3. Stein E, Shane E. Secondary osteoporosis. *Endocrinol Metab Clin North Am.* 2003; 32(1): 115-134.
4. Ala M, Jafari RM, Dehpour AR. Diabetes mellitus and osteoporosis correlation: challenges and hopes. *Curr Diabetes Rev.* 2020; 16(9): 984-1001.
5. Labudzynskyi DO, Shymanskyi IO, Lisakovska OO, Veliky MM. Osteoprotective effects of vitamin D<sub>3</sub> in diabetic mice is VDR-mediated and regulated via RANKL/RANK/OPG axis. *Ukr Biochem J.* 2018; 90(2): 56-65.
6. Malik AA, Baig M, Butt NS, Imran M, Alzahrani SH, Gazzaz ZJ. Bibliometric analysis of global research productivity on vitamin D and bone metabolism (2001-2020): learn from the past to plan future. *Nutrients.* 2022; 14(3): 542.
7. Carlberg C. Vitamin D: a micronutrient regulating genes. *Curr Pharm Des.* 2019; 25(15): 1740-1746.
8. Goltzman D. Functions of vitamin D in bone. *Histochem Cell Biol.* 2018; 149(4): 305-312.
9. Amrein K, Scherkl M, Hoffmann M, Neuwersch-Sommeregger S, Köstenberger M, Tmava Berisha A, Martucci G, Pilz S, Malle O. Vitamin D deficiency 2.0: an update on the current status worldwide. *Eur J Clin Nutr.* 2020; 74(11): 1498-1513.
10. Povorozniuk VV, Balatska NI. Vitamin D deficiency in the population of Ukraine and risk factors of its development. *Pain Joints Spine.* 2012; 4(8): 5-11. (In Ukrainian).
11. Berridge MJ. Vitamin D deficiency accelerates ageing and age-related diseases: a novel hypothesis. *J Physiol.* 2017; 595(22): 6825-6836.

12. Lane NE. Epidemiology, etiology, and diagnosis of osteoporosis. *Am J Obstet Gynecol.* 2006; 194(2 Suppl): S3-S11.
13. Zaitseva OV, Shandrenko SG, Veliky MM. Biochemical markers of bone collagen type I metabolism. *Ukr Biochem J.* 2015; 87(1): 21-32.
14. Riasniy VM, Apukhovska LI, Veliky NN, Shymanskyi IO, Labudzynskyi DO, Komisarenko SV. Immunomodulatory effects of vitamin D<sub>3</sub> and bisphosphonates in nutritional osteoporosis in rats. *Ukr Biokhim Zhurn.* 2012; 84(2): 73-80. (In Ukrainian).
15. Mazanova AO, Shymanskyi IO, Veliky MM. Development and validation of immunoenzyme test-system for determination of 25-hydroxyvitamin D in blood serum. *Biotechnol Acta.* 2016; 9(2): 28-36.
16. Dyce BJ, Bessman SP. A rapid nonenzymatic assay for 2,3-DPG in multiple specimens of blood. *Arch Environ Health.* 1973; 27(2): 112-115.
17. Komisarenko SV, Apukhovska LI, Riasniy VM, Kalashnikov AV, Veliky NN. "Mebivid" biopharmaceutical preparation efficacy against vitamin D<sub>3</sub> and calcium metabolism disorders in alimentary osteoporosis. *Biotechnol Acta.* 2011; 4(1): 74-81.
18. Holick MF. The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. *Rev Endocr Metab Disord.* 2017; 18(2): 153-165.
19. Pfothenhauer KM, Shubrook JH. Vitamin D deficiency, its role in health and disease, and current supplementation recommendations. *J Am Osteopath Assoc.* 2017; 117(5): 301-305.
20. Zmijewski MA. Vitamin D and Human Health. *Int J Mol Sci.* 2019; 20(1): 145.
21. Katz J, Yue S, Xue W. Increased risk for COVID-19 in patients with vitamin D deficiency. *Nutrition.* 2021; 84: 111106.
22. Chang SW, Lee HC. Vitamin D and health - The missing vitamin in humans. *Pediatr Neonatol.* 2019; 60(3): 237-244.
23. Khomenko AV. Cholecalciferol hydroxylation in rat hepatocytes under the influence of prednisolone. *Ukr Biokhim Zhurn.* 2013; 85(3): 90-95. (In Ukrainian).
24. Shymanskyi IO, Mazanova AO, Lisakovska OO, Labudzynskyi DO, Makarova OO, Komisarenko YuI, Veliky MM. The role of vitamin D-auto-/paracrine system in the development of metabolic inflammation of liver tissue in experimental type 2 diabetes. *Endocrinology.* 2021; 26(3): 271-280. (In Ukrainian).
25. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiol Rev.* 2016; 96(1): 365-408.
26. Mazanova A, Shymanskyi I, Lisakovska O, Labudzynskyi D, Khomenko A, Veliky M. The link between vitamin D status and NF-κB-associated renal dysfunction in experimental diabetes mellitus. *Biochim Biophys Acta Gen Subj.* 2022; 1866(7): 130136.
27. Bouillon R, Bikle D. Vitamin D metabolism revised: fall of dogmas. *J Bone Miner Res.* 2019; 34(11): 1985-1992.
28. Veklich TO, Nikonishyna YuV, Kosterin SO. Pathways and mechanisms of transmembrane calcium ions exchange in the cell nucleus. *Ukr Biochem J.* 2018; 90(4): 5-24.
29. Young K, Beggs MR, Grimby C, Alexander RT. Regulation of 1 and 24 hydroxylation of vitamin D metabolites in the proximal tubule. *Exp Biol Med (Maywood).* 2022; 247(13): 1103-1111.
30. Goldstein DA. Serum Calcium. In: Walker HK, Hall WD, Hurst JW, editors. *Clinical Methods: The History, Physical, and Laboratory Examinations.* 3<sup>rd</sup> edition. Boston: Butterworths; 1990. Chapter 143. Available at <https://www.ncbi.nlm.nih.gov/books/NBK250/>
31. Wawrzyniak N, Suliburska J, Kulczyński B, Kołodziejcki P, Kurzawa P, Gramza-Michałowska A. Calcium-enriched pumpkin affects serum leptin levels and fat content in a rat model of postmenopausal osteoporosis. *Nutrients.* 2021; 13(7): 2334.
32. Bauer NB, Khassawna TE, Goldmann F, Stirn M, Ledieu D, Schlewitz G, Govindarajan P, Zahner D, Weisweiler D, Schlieffke N, Böcker W, Schnettler R, Heiss C, Moritz A. Characterization of bone turnover and energy metabolism in a rat model of primary and secondary osteoporosis. *Exp Toxicol Pathol.* 2015; 67(4): 287-296.
33. Bikle DD. Vitamin D and bone. *Curr Osteoporos Rep.* 2012; 10(2): 151-159.
34. Song L. Calcium and Bone Metabolism Indices. *Adv Clin Chem.* 2017; 82: 1-46.
35. Morgan EN, Alsharidah AS, Mousa AM, Edrees HM. Irisin has a protective role against osteoporosis in ovariectomized rats. *Biomed Res Int.* 2021; 2021: 5570229.

36. Halper-Stromberg E, Gallo T, Champakanath A, Taki I, Rewers M, Snell-Bergeon J, Frohnert BI, Shah VN. Bone mineral density across the lifespan in patients with type 1 diabetes. *J Clin Endocrinol Metab.* 2020; 105(3): 746-753.
37. Silva MJ, Brodt MD, Lynch MA, McKenzie JA, Tanouye KM, Nyman JS, Wang X. Type 1 diabetes in young rats leads to progressive trabecular bone loss, cessation of cortical bone growth, and diminished whole bone strength and fatigue life. *J Bone Miner Res.* 2009; 24(9): 1618-1627.
38. Reid IR, Bolland MJ. Calcium and/or vitamin D supplementation for the prevention of fragility fractures: who needs it? *Nutrients.* 2020; 12(4): 1011.
39. Williams C, Sapra A. Osteoporosis Markers. In: StatPearls. Treasure Island (FL): StatPearls Publishing. Available at <https://www.ncbi.nlm.nih.gov/books/NBK559306/> (accessed, May, 2022).
40. Komisarenko SV, Volochnyuk DM, Shymanskyi IO, Ivonin SP, Veliky MM. Effectiveness of nitrogen-containing bisphosphonates in regulation of mineral metabolism in alimentary osteoporosis in rats. *Biotechnol Acta.* 2015; 8(4): 53-62.
41. Bellastella G, Scappaticcio L, Longo M, Carotenuto R, Carbone C, Caruso P, Maio A, Paglionico VA, Vietri MT, Maiorino MI, Esposito K. New insights into vitamin D regulation: is there a role for alkaline phosphatase? *J Endocrinol Invest.* 2021; 44(9): 1891-1896.
42. Van de Peppel J, van Leeuwen JP. Vitamin D and gene networks in human osteoblasts. *Front Physiol.* 2014; 5: 137.