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VITAMIN D_3 AND METHYLENEBISPHOSPHONIC ACID IN THE CORRECTION OF MINERAL METABOLISM DISORDERS AND BONE REMODELING ASSOCIATED WITH GLUCOCORTICOID-INDUCED OSTEOPOROSIS

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The study was aimed at evaluating therapeutic efficacy of vitamin D_3 (VD_3, 1000 IU/kg of b.w., 30 days) and sodium salt of methylenebisphosphonic acid (MBPA, 17 mg/kg of b.w., 30 days) monotherapies as well as their effect in combination in preventing mineral metabolism and bone remodeling disturbances associated with glucocorticoid(GC)-induced osteoporosis. Osteoporosis in rats was induced by long-term (30 days) administration of the synthetic glucocorticoid prednisolone (5 mg/kg of b.w.). Calcium and inorganic phosphate levels, the activity of alkaline phosphatase (ALP) in serum, bone tissue and bone marrow were determined spectrophotometrically. The protein levels of VD_3 receptor (VDR), receptor activator of nuclear factor kappa-B (RANK), its ligand (RANKL), and osteoprotegerin (OPG) in bone tissue were determined by Western blotting. Serum 25-hydroxyvitamin D_3 ($25OHD_3$) content was assayed by ELISA. It was shown that prednisolone caused the development of hypocalcemia and hypophosphatemia, increased the alkaline phosphatase activity in the blood serum, while downregulating its activity in bone tissue and bone marrow. GC-induced osteoporosis was accompanied by a profound deficiency of VD_3 and a decrease in the content of VDR. Evaluation of the NF-κB-associated cytokine axis RANK/RANKL/OPG, which regulates the balance of osteoblasts/osteoclasts, showed a simultaneous decrease in the RANK content and OPG/RANKL ratio. Vitamin D_3 restored mineral metabolism and $25OHD_3$ level that led to the normalization of VDR-mediated signaling and RANK/RANKL/OPG functions in bone tissue. It has been shown that the administration of MBPA had a corrective effect on the content of mineral components in the blood serum and bone tissue, as well as on the activity of alkaline phosphatase only in combination with vitamin D_3, indicating a low efficiency of bisphosphonate monotherapy in GC-induced vitamin D_3 deficiency and osteoporosis.

K e y w o r d s: vitamin D_3, methylenebisphosphonic acid, glucocorticoid-induced osteoporosis, bone remodeling, RANK/RANKL/OPG axis.

Glucocorticoids (GCs) are considered indispensable medications due to their anti-inflammatory and immunosuppressive effects. They are widely used to treat various systemic inflammatory diseases, including allergies, rheumatoid arthritis, chronic obstructive pulmonary disease, systemic lupus erythematosus, inflammatory bowel disease, and other acute and chronic conditions [1]. Moreover, synthetic glucocorticoids, in particular dexamethasone and prednisolone, have shown significant efficacy in preventing mortality among critically ill patients with COVID-19 [2].

However, a number of adverse effects associated with systemic (oral and parenteral) glucocorticoid therapy have been repeatedly reported. In particular, depending on the nature of the side effects caused by GCs, they can be classified into musculoskeletal, endocrine, gastrointestinal, neuropsychiatric, cardiovascular, dermatological, ocular or immunological [3]. Among the most frequent, we can highlight a

destructive effect on bone tissue and, as a result, an increased risk of developing glucocorticoid-induced osteoporosis (GIOP) and osteonecrosis associated with high doses of GCs and a long course of treatment. Under the influence of GCs, bone resorption and fragility significantly increase that, in turn, disrupts bone microarchitectonics, leading to bone mass loss and to a high risk of fractures [4, 5].

A number of studies have made significant progress in explaining the molecular mechanisms of GIOP development. In particular, high doses of GCs induce apoptosis of osteoblasts and osteocytes by enhancing the production of reactive oxygen species (ROS), activating endoplasmic reticulum (ER) stress, and inhibiting the Wnt/β-catenin signaling pathway, which is critical for maintaining bone mass [6]. In addition, glucocorticoids cause a decrease in the synthesis of osteocalcin and affect the coupling of angiogenesis/osteogenesis that generally leads to deterioration in the main biomechanical parameters of the femur in osteoporosis [7]. Furthermore, GCs were found to inhibit bone formation by suppressing osteoblast differentiation, which is under the control of the canonical Wnt/β-catenin signaling pathway. The mechanism of GC action is based on the induction of sclerostin (SOST) and Dickkopf-related protein 1 (DKK1) expression, both of which serve as antagonists of the Wnt/β-catenin signaling pathway. While the synthesis of Wnt proteins is inhibited and bone morphogenetic protein (BMP) signaling is blocked, mesenchymal stem cell differentiation is mainly directed towards adipocytes rather than osteoblasts that generally leads to inhibition of bone formation [8]. The adipogenic differentiation of bone marrow mesenchymal stem cells is facilitated by an increased expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), thus leading to fat accumulation in the bone marrow and to bone loss in patients chronically supplemented with glucocorticoids. It is noteworthy that Wnt/β-catenin signaling pathway-activating compounds can prevent the development of GIOP [9], including vitamin D_3 (VD_3).

Bone remodeling is a precisely coordinated process of functional interaction between cells, in particular, osteoblasts, which provide bone formation, and osteoclasts, responsible for bone resorption and demineralization. One of the key players involved in osteoclast differentiation, activation, and maturation is the nuclear factor κB (NF-κB)-associated signaling through RANK (receptor activator of nuclear factor κB)/RANKL (RANK ligand)/OPG (osteoprotegerin) pathway. We also suggest that an additional mechanism is involved, which consists in GC-induced impairment of RANK/RANKL/OPG axis in bone marrow cells that may result in maturation and activation of osteoclasts [10].

To prevent bone loss elicited by glucocorticoids, various osteoanabolic and antiresorptive drugs are widely used in medical practice, the effect of which is shown to be mediated through increased osteogenesis and inhibition of osteoclastogenesis. Treatment protocols for osteoporosis include bisphosphonates, calcium, vitamin D_3, denosumab, teriparatide, raloxifene, and strontium ranelate etc [11, 12]. However, the question remains regarding the effectiveness of their combination for the treatment of secondary osteoporosis of various origins.

As antiresorptive medications, bisphosphonates (BPs) are the most commonly used for the treatment of bone diseases such as osteoporosis, Paget's disease, fibrous dysplasia, hypercalcemia, and bone loss associated with inflammation. BPs are synthetic chemically stable analogs of pyrophosphate, a physiological regulator of calcification and bone resorption. In BPs, the P–O–P bond of pyrophosphate is replaced by a P-C-P bond, which is resistant to chemical and enzymatic hydrolysis [13]. The mechanism of inhibition of bone resorption by bisphosphonates is based on their affinity to bone mineral hydroxyapatite. BPs are selectively adsorbed on mineral surfaces and bind to hydroxyapatite crystals in the bone, where they interfere with the functions of bone-resorbing cells – osteoclasts, and disrupt the bone's ruffled edges [13, 14].

BPs are divided into nitrogen-containing and non-nitrogen-containing compounds. Nitrogen-containing BPs, including the well-known drugs alendronate, ibandronate, risedronate, and zoledronate, suppress the mevalonate pathway of cholesterol synthesis by inhibiting the enzyme farnesyl diphosphate synthase and blocking the prenylation of small GTPases, resulting in impaired osteoclast function. Non-nitrogen-containing BPs, such as etidronate, clodronate, and methylenebisphosphonic acid (MBPA), inhibit bone resorption through various mechanisms, namely by incorporation into intracellular non-hydrolysable ATP analogues that are not capable of releasing energy that leads to the death of osteoclasts [15]. MBPA is the simplest bisphosphonate analogue of pyrophosphoric acid (PPi) in which the bridging oxygen P-O-P is replaced by methylene carbon. MBPA has an affinity for and adheres to the

surface of hydroxyapatite crystals in the bone matrix, accumulating at the sites of osteoid mineralization [16].

Among osteoanabolic compounds, the most promising one is vitamin D_3, a steroid prohormone synthesized in the skin under the influence of ultraviolet radiation and/or supplied with food. VD_3 plays an important role in the regulation of calcium-phosphate balance by increasing calcium absorption in the intestine and reducing renal excretion that, all in all, ensures the maintenance of mineral homeostasis and an adequate rate of bone remodeling. Along with bone calcification, vitamin D_3 controls various osteoblast functions such as differentiation, proliferation, and expression of specific proteins and growth factors [17].

The biologically active metabolite of VD_3 is synthesized in the body after a two-stage hydroxylation. At the first stage, vitamin D_3 is converted into the main transport form – 25-hydroxyvitamin D_3 ($25OHD_3$) under the action of 25-hydroxylases (CYP2R1 and CYP27A1) in the liver. At the second stage, $25OHD_3$ is converted to the hormonally active form of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol) in the kidneys and other tissues in a reaction catalyzed by the enzyme CYP27B1 [18, 19]. Hormonally active $1,25(OH)_2D_3$ binds to specific vitamin D receptor (VDR) in target cells [20]. The VDR is considered to be a ligand-activated transcription factor that acts in combination with the retinoid X receptor (RXR) to positively and/or negatively regulate gene expression. In osteoblasts and osteocytes, the liganded VDR-RXR heterodimer induces, at least, the expression of RANKL, a cytokine that acts as an autocrine/paracrine regulator of bone resorption by activating osteoclasts [21].

Taking into account the established direct effects of VD_3 and bisphosphonates on bone remodeling, the aim of our study was to investigate the combined effect of methylenebisphosphonic acid, which suppresses osteoclast activity and the demineralization process, and vitamin D_3, one of the key regulators of osteogenesis, in experimental glucocorticoid-induced osteoporosis.

Materials and Methods

Animals and experimental design. Female Wistar rats (100 ± 5 g) were housed under standard animal conditions and allowed free access to standard rodent diet and water. After acclimatization for 1 week, animals were randomly divided into 5 groups, each including 10 animals: 1 – the control group; 2 – the group that received synthetic glucocorticoid prednisolone (water solution) at a dose 5 mg per kg of body weight (*per os*, daily for 30 days); 3 – the group that received prednisolone at a dose 5 mg per kg of body weight and 1000 IU of vitamin D_3 (oil solution) per kg of body weight (*per os*, daily for 30 days); 4 – the group of prednisolone-administered rats that simultaneously received sodium salt of methylenebisphosphonic acid (water solution) at a dose 17 mg per kg of body weight (*per os*, daily for 30 days); 5 – the group of prednisolone-administered rats that simultaneously received 1000 IU of vitamin D_3 per kg of body weight and 17 mg of sodium salt of MBPA per kg of body weight (*per os*, daily for 30 days). All experimental procedures with animals were performed in accordance with national and international guidelines and laws concerning animal welfare: the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986), Bioethical appraisal of preclinical and other scientific research conducted on animals (Kyiv, 2006).

Mineral metabolism assessment. Indicators of mineral metabolism (calcium, inorganic phosphate, alkaline phosphatase) were determined in blood serum, bone tissue and bone marrow extracts. Calcium levels were measured spectrophotometrically using a biotest kit (LAHEMA, Czech Republic). The content of inorganic phosphate was assessed after protein precipitation with a 12% trichloroacetic acid solution according to the Dice method [22]. The activity of total alkaline phosphatase (ALP) was determined spectrophotometrically using a biotest kit (LAHEMA, Czech Republic). The content of mineral components in the bone tissue was studied after protein extraction by dry mineralization at a temperature of +600-800°C. The mineral components of bone ash were determined spectrophotometrically after dissolution in 0.5 ml of hydrochloric acid and subsequent 20-fold dilution in distilled water.

Serum $25OHD_3$ measurement. Commercial ELISA kit (The IDS 25-Hydroxy Vitamin D EIA, Immunodiagnostic Systems, UK) was used for quantification of 25-hydroxyvitamin D_3 level in rat serum according to the manufacturer's protocol.

Western blot analysis. The levels of target proteins VDR, RANK, RANKL and OPG in bone tissue were determined by western blot analysis. Protein extracts from bone tissue samples were prepared using a standard protocol with RIPA buffer (20 mM

Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM EGTA; 0.1% SDS; 1% sodium deoxycholate; 10 mM sodium pyrophosphate). Bone tissue samples (100 mg) were lysed for 20 min in RIPA buffer with protease inhibitor cocktails (PIC, Sigma, USA), then sonicated and centrifuged for 20 min (14000 g) at +4°C. Protein concentration in supernatants was determined by Lowry method. Equal amounts of protein (50 μg per track) from each sample were loaded onto 15% PAAG for electrophoresis, and then transferred onto nitrocellulose membranes (3550 mA, 1h). Membranes were blocked with 5% non-fat milk in PBS with 0.05% Tween-20 (PBST) for 1h followed by incubation overnight at +4°C with primary antibodies against VDR (1:500; Invitrogen, USA), RANK (1:400; Santa Cruz Biotechnology, USA), RANKL (1:250; Santa Cruz Biotechnology, USA), and OPG (1:250; Santa Cruz Biotechnology, USA) in PBS supplemented with 0.1% Tween-20 and 5% non-fat milk. Primary-antibody-bound membranes were then incubated with HRP-conjugated secondary antibodies: anti-rabbit IgG (H+L)-HRP conjugate (1:4000; Bio-Rad Laboratories, Inc., USA) or anti-goat IgG (H+L) (1:2500; Invitrogen, USA) for 1 h at room temperature. Thereafter the membranes were developed with chemiluminescent agents: p-coumaric acid (Sigma, USA) and luminol (AppliChem GmbH, Germany). Target protein levels were normalized to β-actin (1:10000; Sigma, USA). Quantification of western blots was performed by measuring the optical densities of the respective bands on the films using Gel-Pro Analyzer 32, v3.1.

Statistical analysis. Statistical analysis was performed using Origin Pro 8.5 software (OriginLab Corporation, Northampton, MA, USA). The results of all experiments were expressed as mean ± SEM. Each experiment was repeated at least three times. The hypothesis of normality distribution of data was tested by the Shapiro-Wilk test. Statistical differences between the groups were compared using the ANOVA test followed by Tukey post-hoc test. The difference between groups was considered significant when $P \leq 0.05$.

Results and Discussion

Bone remodeling is the process that ensures tight coordination between the intensity of bone resorption and bone formation under normal physiological conditions, and is repsonsible for the maintenance and restoration of mineral homeostasis under pathological conditions. During bone remodeling, old bone tissue is removed by osteoclasts (terminally differentiated polynuclear myeloid cells) and replaced by the new bone tissue with the participation of osteoblasts – the bone-forming cells that develop from pluripotent mesenchymal stem cells, express osteoclastogenic factors, matrix proteins and ensure mineralization [5, 6].

GIOP is classified as a metabolic disease of bone tissue, accompanied by the predominance of osteoclast-dependent bone resorption over its osteoblast-mediated bone formation and mineralization, which leads to the loss of bone density, disturbances of bone microarchitecture and, ultimately, increased risk of fractures. Thus, the determination of the molecular and cellular mechanisms underlying GC-induced osteoporosis and methods for correcting aberrant bone homeostasis caused by chronic glucocorticoid therapy is of extremely important theoretical and clinical importance. Calcium, estrogens, calcitonin, bisphosphonates, and VD_3 are thought to be agents capable of inhibiting bone loss [11]. Therefore, as an adjunctive therapy for the correction of GIOP, we chose two main mediators of bone remodeling – vitamin D_3, which primarily affects bone formation, and MBPA, strongly affecting bone resorption.

First, to assess the therapeutic efficacy of the studied compounds in experimentally glucocorticoid-induced osteoporosis, rats were administered with vitamin D_3, disodium salt of methylenebisphosphonic acid, and their combination for 30 days against the background of prednisolone supplementation. To confirm the disturbances of bone homeostasis the key parameters of mineral metabolism were investigated – the levels of calcium and inorganic phosphates, as well as the activity of alkaline phosphatase in blood serum, bone tissue and bone marrow.

Calcium and inorganic phosphate are essential minerals and structural elements of the bone matrix. A stable level of calcium regulates various physiological functions, including muscle contraction, release of neurotransmitters, intracellular signal transduction and blood coagulation. Calcium ions are considered one of the main intracellular second messengers, and their concentration is tightly regulated by calcium channels, ion exchangers, and pumps [23, 24]. At the same time, ionized extracellular calcium acts as a primary messenger, serving as a ligand for G protein-coupled membrane calcium receptors. These receptors are called calcium-sensing recep-

tors (CaSR), which detect the level of free calcium in the blood and regulate the secretion of parathyroid hormone. The key function of CaSR is to maintain calcium homeostasis by coordinating calcium absorption from the gastrointestinal tract, its excretion by the kidneys, and accumulation by bone tissue.

In bone tissue, ionized calcium is a substantial regulator of the proper balance between osteogenesis and bone resorption. In particular, Ca^{2+} inhibits the formation and activity of osteoclasts and stimulates the activity of osteoblasts. The ability of osteoblast cell lines, osteoblast progenitors, and primary osteoblasts to express CaSR supports a Ca^{2+}-mediated mechanism of osteoblastogenesis. In turn, a high level of Ca^{2+} suppresses the differentiation and activity of osteoclasts, and also leads to their apoptosis. As a result, CaSR activation inhibits the resorptive activity of osteoclasts [25].

The results presented in Table reflect the state of mineral metabolism in terms of calcium and inorganic phosphate levels in blood serum and two target tissues – bone tissue and bone marrow. It was found that prednisolone administration led to a decrease in the level of total calcium as an integral indicator of mineral metabolism in the blood serum (by 13.2% compared with the control). It is known that calcium in the blood is represented in several forms. A small part of it binds to proteins (albumins and globulins), and most of it is in free form, including ionized (up to 85%) and chelated calcium (up to 15%) – with citrates, phosphates and bicarbonates. The revealed changes in the content of calcium in blood serum occured mainly due to its biologically active fraction of ionized calcium, the relative content of which in rats treated with prednisolone decreased by 15.1%. The level of another fraction, protein-bound calcium, is considered a constant parameter, so we observed no statistically significant differences between all 5 experimental groups (Table). The ratio between the forms of calcium is a sensitive parameter that varies depending on the different physiological and pathological conditions. In our opinion, the detected decrease in the ratio of free to protein-bound calcium forms from 8.3 in control to 7.7 in prednisolone-administered animals indicated a pronounced impairment of mineral metabolism.

As confirmation of calcium homeostasis disturbances in GIOP, we considered a contradictory effect on the total calcium content in other critical tissues, in particular, a decrease in calcium content in the bone marrow by 25.1% and its elevation in bone tissue by 28.9% compared with control rats (Table). The revealed changes in mineral components under the action of prednisolone in blood serum and bone tissue can be explained by different mechanisms of their regulation. In blood serum, changes in the level of mineral components might be associated with an impairment of their transport in the intestine via membrane proteins, in particular, vitamin D receptor, calcium transport protein 1 (CaT1), calbindin-D9K, and transient receptor potential vanilloid type 6 (TRPV6) [26]. At the same time, alterations observed in the bone tissue can be linked to an abnormal formation of hydroxyapatite. Under the action of prednisolone, the process of demineralization is likely to be enhanced that leads to the release of calcium from the bound state and, accordingly, an increase in its content in bone tissue. In addition, elevated bone calcium may represent an adaptive response to increased demineralization due to osteoclast activity, since it is known that high calcium levels can suppress osteoclast resorptive activity, balancing prednisolone-induced bone remodeling.

Another important parameter of mineral homeostasis is the level of inorganic phosphate (Pi), which was also affected by prednisolone (Table). In particular, its content declined in the serum (by 16.2%, from 2.20 ± 0.06 mmol/l in the control to 1.86 ± 0.07 mmol/l), bone tissue (by 14.5%, from 7.37 ± 0.16 to 6.30 ± 0.11 mmol/g tissue) and to a lesser extent in bone marrow (by 6.4%, from 14.1 ± 0.3 to 13.2 ± 0.7 mmol/g tissue) compared with the values in the control group.

The source of Pi for bone mineralization (formation of hydroxyapatites in the extracellular matrix) is phosphate esters of organic compounds and inorganic pyrophosphate (PPi), which are cleaved by alkaline phosphatase. ALP is one of the key enzymes that provide a multi-stage process of bone tissue remodeling [27]. The ability of ALP to hydrolyze PPi, which is formed during the splitting of ATP into ADP and PPi, is especially important. PPi is known to be a major physiological inhibitor of mineralization and calcification of the pericellular matrix. By hydrolyzing PPi, ALP increases the free phosphate pool and maintains an optimal PPi/Pi ratio for bone mineralization.

A wide range of metabolic disorders is associated with an impaired ALP activity [28]. The presence of ALP isoforms and the specificity of their distribution in tissues make it possible to use the measurement of ALP activity for diagnosing a number of

T a b l e . Characteristics of mineral metabolism in blood serum (n = 10), bone tissue (n = 6) and bone marrow (n = 5) of rats with glucocorticoids-induced osteoporosis and after VD_3 and MBPA administration

Parameter	Control	Prednisolone	Prednisolone + vitamin D_3	Prednisolone + MBPA	Prednisolone + vitamin D_3 + MBPA
Blood serum					
Total calcium, mmol/l	2.20 ± 0.02	1.91 ± 0.05*	2.20 ± 0.03$^{\#}$	1.94 ± 0.03*	2.24 ± 0.04$^{\#}$
Protein-bound calcium, mmol/l	0.24 ± 0.03	0.22 ± 0.04	0.21 ± 0.04	0.22 ± 0.04	0.20 ± 0.04
Free (ionized) calcium, mmol/l	1.99 ± 0.04	1.69 ± 0.05*	1.99 ± 0.05$^{\#}$	1.72 ± 0.04*	2.04 ± 0.05$^{\#}$
Inorganic phosphate, mmol/l	2.22 ± 0.06	1.86 ± 0.07*	2.26 ± 0.04$^{\#}$	1.92 ± 0.03*	2.30 ± 0.02$^{\#}$
Bone tissue					
Total calcium, μmol/g tissue	1.49 ± 0.09	1.92 ± 0.11*	1.82 ± 0.05*	1.91 ± 0.08*	2.14 ± 0.03*$^{,\#}$
Inorganic phosphate, μmol/g tissue	7.37 ± 0.16	6.30 ± 0.11*	7.21 ± 0.17$^{\#}$	6.59 ± 0.13*	7.45 ± 0.14$^{\#}$
Bone marrow					
Total calcium, μmol/g tissue	22.7 ± 1.2	17.0 ± 0.4*	19.6 ± 0.6*$^{,\#}$	15.5 ± 0.4*$^{,\#}$	21.8 ± 0.3$^{\#}$
Inorganic phosphate, μmol/g tissue	14.1 ± 0.3	13.2 ± 0.7	14.6 ± 0.2$^{\#}$	14.9 ± 0.1*$^{,\#}$	16.0 ± 0.8*$^{,\#}$

Note: Values are given as means ± SEM (n = 5-10); *$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. prednisolone-induced osteoporosis, $^{\&}P < 0.05$ vs. vitamin D_3 administration, $^{@}P < 0.05$ vs. MBPA administration

diseases. In particular, an increase in the activity of ALP in the blood serum is detected in children with rickets, in individuals with bone tissue carcinoma and tumor metastases in the bone tissue. Therefore, an increase in ALP activity can be considered a reliable marker of the development of pathological processes in bone tissue.

We established that glucocorticoid-induced hypocalcemia and hypophosphatemia were accompanied by an increase in the total activity of a valid marker of bone formation – ALP in blood serum, which was 52.9% higher than in the control group (227.0 ± 9.8 vs. 347.0 ± 14.9 μmol/min·l, Fig. 1, *A*). As shown earlier, an increase in ALP activity under the influence of prednisolone occurred mainly due to the elevated activity of its bone isoform [7]. Increased activity of alkaline phosphatase may reflect prednisolone-induced disturbances in the structural and functional state of bone tissue. Moreover, we suggest that increased ALP synthesis in bone tissue may indicate an increased number of chondrocytes due to the ability of GCs to inhibit and redirect osteoblast differentiation [29]. At the same time, ALP levels in target tissues declined after prednisolone administration: by 19.1% in bone tissue (Fig. 1, *B*) and by 20.2% in bone marrow (Fig. 1, *C*) compared with the intact animals. This may underlie the development of hypophosphatasia, which is characterized by inhibition of cartilage (rickets) and bone (osteomalacia) mineralization, spontaneous bone fractures and an increase in the concentration of extracellular inorganic pyrophosphate and, as a result, inhibition of bone tissue mineralization in GC-induced osteoporosis [30].

Thus, long-term administration of prednisolone revealed significant alterations in mineral metabolism, indicating the development of GIOP that is consistent with our earlier data on a decrease in the biomechanical parameters of the femurs in rats supplemented with GC [7].

The potential of various pharmacological agents, including calcium, estrogen, calcitonin, bisphosphonates, and VD_3, as well as their combinations, is being considered for inhibiting bone loss by

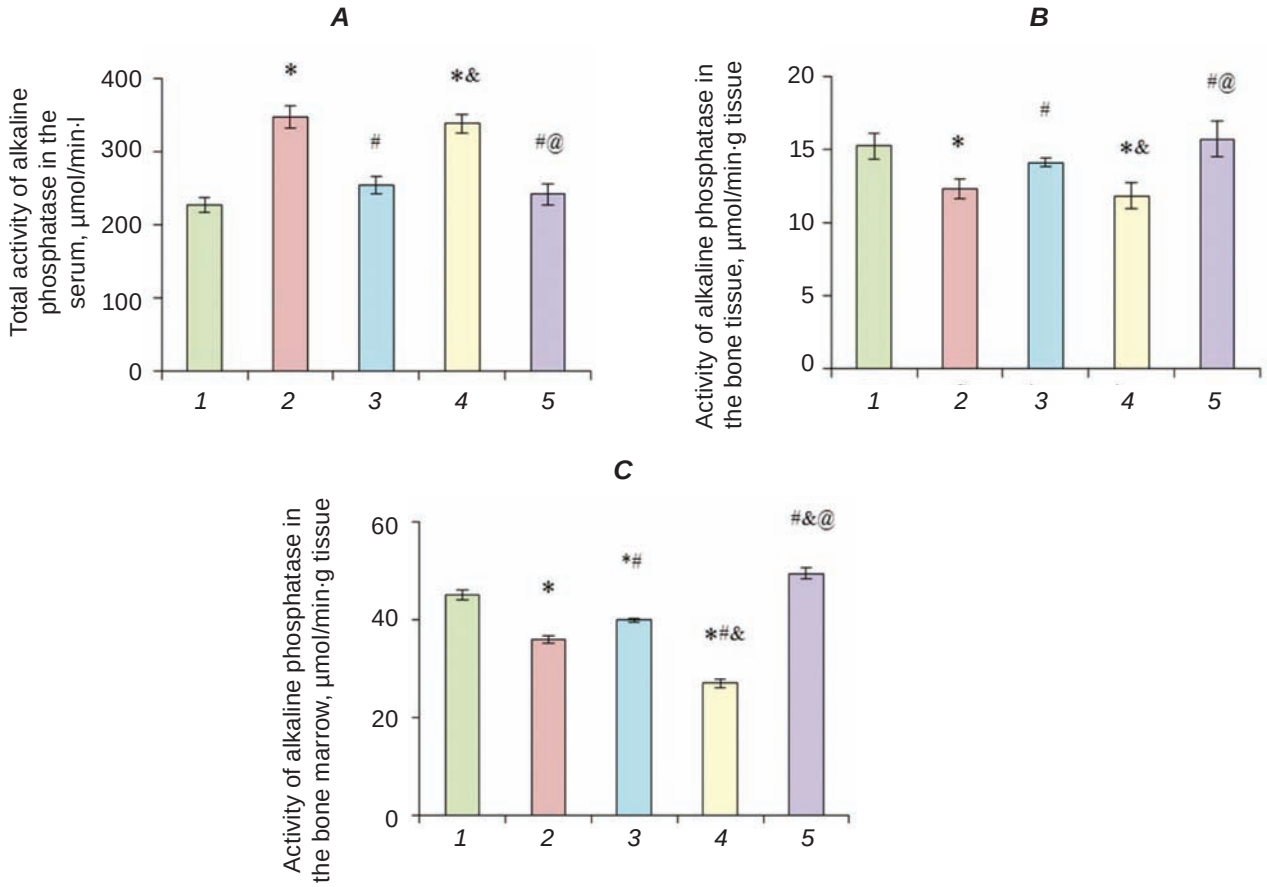

*Fig. 1. Effects of vitamin D_3 and MBPA administration on the activity of alkaline phosphatase in blood serum (n = 8), bone tissue and bone marrow (n = 5) in glucocorticoid-induced osteoporosis. ALP activity was measured spectrophotometrically in rat serum (**A**), bone tissue (**B**) and bone marrow (**C**) of 5 animal groups: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D_3 administration; 4 – prednisolone and MBPA administration; 5 – combined vitamin D_3 and MBPA administration against the background of prednisolone load. Results are shown as mean ± SEM of three independent experiments done in triplicate; *P < 0.05 vs. control, $^{\#}P < 0.05$ vs. prednisolone-induced osteoporosis, $^{\&}P < 0.05$ vs. vitamin D_3 administration, $^{@}P < 0.05$ vs. MBPA administration*

suppressing bone resorption [11]. Thus, to correct GIOP, we used two main mediators of bone remodeling – vitamin D_3, which primarily affects bone formation, and MBPA, which affects bone resorption, and also evaluated their possible additive effect.

The level of Ca^{2+} and phosphates in the blood is maintained within narrow physiological limits due to the joint work of the intestines, kidneys, parathyroid glands and bone tissue and is regulated by the closely coordinated action of a group of hormones, including parathyroid hormone (PTH), calcitriol (1,25-dihydroxyvitamin D_3), calcitonin, and fibroblast growth factor 23 (FGF23).

Considering the abovementioned, it is not surprising that after the supplementation of VD_3, we observed a corrective effect on the state of mineral metabolism, which manifested itself in the complete restoration of mineral components in the blood serum – calcium (total, free and the ratio of free and protein-bound form) and phosphates to control values (Table). In the bone marrow, a complete restoration of the content of phosphates (14.6 ± 0.2 µmol/g of tissue) to the control level was found, while the level of total calcium returned to normal only partially and was only 15.3% higher than in the prednisolone group. The character of bone tissue changes was as follows (Table). As in the bone marrow, after treatment with vitamin D_3, we found a complete recovery of the phosphate content (7.21 ± 0.17 µmol/g tissue) to the control level (7.37 ± 0.16 µmol/g tissue); at the same time, there were no significant changes in the level of total calcium compared with

the prednisolone group. This may indicate that the GC-induced changes in bone tissue were more profound, and normalization of bone mineral homeostasis required more time and, possibly, a higher dose of vitamin D_3.

Restoration of calcium-phosphate metabolism related to VD_3 action was accompanied by a decrease in total ALP activity in blood serum by 26.8% compared with prednisolone therapy (Fig. 1, *A*). A partial elevation of ALP activity in bone tissue (by 14.6%) and in bone marrow (by 10.8%) was esteblished compared with prednisolone-administered animals animals (Fig. 1, *B*, *C* respectively). However, these ALP values did not reach the control values, suggesting incomplete reversibility of bone changes caused by GC.

The study of the effect of methylenebisphosphonic acid on the content of mineral components (Table) and the ALP activity (Fig. 1) revealed no statistically significant effects on these parameters in the serum and bone tissue compared with the prednisolone action that strongly differs from the pronounced corrective effect of vitamin D_3. The obtained results are in agreement with the available data showing the low efficacy of bisphosphonates monotherapy against the background of VD_3 deficiency in relation to their ability to improve the structural and functional state of bone tissue in osteoporosis [31, 32]. Hovewer, it should be noted that in the bone marrow, we detected a pronounced lowering effect of MBPA treatment on the total calcium and ALP activity, not only compared with the control ones (by 31.7% and by 40.1% respectively), but also compared with the prednisolone group (by 8.8%, up to 15.5 ± 0.4 μmol/g tissue, and by 25%, up to 27.0 ± 1.3 μmol/g tissue respectively), as well as an increasing effect on the phosphate level (14.9 ± 0.1 μmol/min·g tissue) by 12.9% compared with the prednisolone action, and by 5.7% compared vs. the control group. Altogether, these data can be considered as evidence of abnormal mineral metabolism in the bone marrow following the administration of MBPA at a given dose in combination with prednisolone.

Finally, according to our results (Table, Fig. 1), MBPA only in combination with VD_3, which provides a sufficient level of $25OHD_3$ in blood serum, had a normalizing effect on mineral metabolism. In the group with the combined action of vitamin D_3 and MBPA a complete normalization of all studied parameters in the blood serum was observed, as in the group with only VD_3. Interestingly, the combination of vitamin D_3 and MBPA treatment increased calcium and phosphate levels in bone tissue and bone marrow to levels even higher than in the groups of animals with GC-induced osteoporosis treated with vitamin D_3 or MBPA alone (Table). A similar character of changes was observed with respect to the normalization of ALP activity in blood serum, bone tissue and bone marrow after the combination of vitamin D_3 and MBPA (Fig. 1, *A*, *B*, *C* respectively).

At the same time, some differences were found in the combined action of cholecalciferol and MBPA compared with the separate administration of vitamin D_3. In particular, when the phosphate content and ALP activity in the bone tissue were restored, the calcium level increased by 43.6% compared to all other groups, including control rats (2.14 versus 1.49 μmol/g of tissue). In the bone marrow, a similar effect was also detected in relation to phosphates – their level increased in comparison with all groups, including the control (by 13.5%). These data may indicate an intensification of mineral metabolism with the combined action of cholecalciferol and MBPA, however, further research is needed to determine whether this causes harmful or beneficial effects in the context of GIOP.

Sufficient bioavailability and optimal levels of VD_3 can reduce the manifestations of oxidative stress, enhance detoxification of xenobiotics, and increase antimicrobial and anti-inflammatory activity [33, 34]. In turn, vitamin D_3 deficiency is associated with the development of metabolic complications in various chronic diseases. It has recently been reported that the development of GC-induced osteoporosis may correlate with VD_3 deficiency due to impaired hepatic vitamin D_3 metabolism [35]. Thus, the question arose as to how the combined supplementation of vitamin D_3 and MBPA might affect the pool of circulating VD_3 in GIOP rats.

We have demonstrated that long-term administration of prednisolone at a dose of 5 mg per 1 kg of body weight causes a significant decrease in the level of $25OHD_3$ in blood serum by 2.9 times (Fig. 2, *A*) compared with the value of 97.5 ± 4.3 nmol/l in the serum of control animals. Thus, prednisolone-induced disorders of mineral metabolism were accompanied by a severe deficiency of vitamin D_3, reflecting a close relationship between the circulating pool of $25OHD_3$ and the maintenance of bone homeostasis. As expected, VD_3 administra-

tion raised $25OHD_3$ level by 63.8% compared to the prednisolone group (89.7 ± 5.2 vs. 34.0 ± 3.7 nmol/l), restoring it to the level of control animals. MBPA alone had no effect on $25OHD_3$ (39.7 ± 3.4 nmol/l) compared to prednisone-treated rats, while their combined action with vitamin D_3 restored $25OHD_3$ levels (84.5 ± 6.8 nmol/l) to control values. To our knowledge, this study is the first one to show that MBPA treatment does not affect vitamin D_3 metabolism that can be taken into account when developing treatment protocols for secondary osteoporosis.

It is known that there is a close relationship between VD_3 deficiency and reduced synthesis of its biologically active forms with an imbalance of osteoblastic/osteoclastic cells and impaired bone remodeling. Vitamin D_3 metabolites and proteins involved in vitamin D_3 transport, turnover, and signaling form the vitamin D_3-endo/auto/paracrine system. Its major component is VDR, which binds the hormonally active form, $1,25(OH)_2D_3$, heterodimerizes with PXR, and, after translocation into the nucleus, regulates the expression of many genes. In addition to organs involved in bone and mineral homeostasis (bones, intestines, kidneys), VDR is expressed in other body cells and mediates the pleiotropic regulatory action of $1,25(OH)_2D_3$ on a wide range of physiological and pathological processes. The importance of VDR in human bone physiology is evidenced by abnormalities in patients with hereditary hypocalcemic vitamin D-resistant rickets, which is an autosomal recessive disease with a mutation in the VDR gene. VDR knockout mice, in addition to impaired bone metabolism, have a significantly reduced lifespan [36]. In bone tissue, VDRs are detected on osteoblasts, osteocytes, chondrocytes and osteoclasts at the early stages of cell differentiation [37]. Since the expression of the main component of vitamin D_3-auto/paracrine system can reflect the process of osteogenesis (bone formation), it was important to evaluate the local status of the vitamin D_3-auto/paracrine system in bone tissue, based on the study of VDR protein level.

It was found that after the action of prednisolone in the bone tissue, the content of the VDR protein significantly (by 36%) decreased, indicative of a possible reduction in the number of osteoblasts and osteocytes – cells that are very sensitive to the action of vitamin D_3 (Fig. 2, *B*). This effect is consistent with our previous study, which showed that the administration of prednisolone induces a decrease in the level of *Vdr* mRNA by 1.37 times that may contribute to anti-osteoblastic effects of GCs [7]. A reduced level of VDR in bone tissue may be attributable to a possible decrease in cellular reactivity to the action of VD_3 and, as a result, to an impairment of local auto/paracrine regulation of cell function by vitamin D_3. Cholecalciferol elevated the content of VDR protein by 26.5% compared with the GIOP group. The ability of VD_3 to enhance the expression of VDR in bone tissue can be explained by the presence of competitive interactions between vitamin D_3 and prednisolone for binding to the consensus sequence of promoter regions of target genes. MBPA supplementation had no significant effect on the content of VDR protein compared with the prednisolone group and was 45% lower than in control rats. The expression of VDR in bone tissue after the combined action of VD_3 and MBPA only slightly exceeded the effect of separate treatment with cholecalciferol.

Thus, the overall pattern of VDR expression turned out to be generally synchronized with the distribution of $25OHD_3$ between groups. There was a decrease in the circulatory level of $25OHD_3$ after GC treatment, and vitamin D_3 led to the normalization of $25OHD_3$ in blood serum and VDR in bone tissue to the values in control animals. In addition, no effect of MBPA on these parameters was found and, as a result, no combined effects were observed with GIOP treatment. Our results are in line with the data that bisphosphonates can effectively suppress bone demineralization and normalize mineral metabolism, but do not significantly affect the replacement and formation of bone tissue.

Proteins of the osteocytokine system RANK/RANKL/OPG are pivotal mediators of osteoclast activity in bone tissue remodeling. Bone resorption is stimulated by increased RANKL synthesis in osteoblastic cells. Binding of RANKL to its RANK receptor activates an NF-κB-dependent intracellular signaling pathway for osteoclastogenesis gene induction and enhances bone resorption. OPG acts as a decoy receptor that prevents RANKL from binding to RANK [38]. The study of the molecular mechanisms of osteoclastogenesis showed that signaling through the nuclear transcription factor kappa B in response to the action of the main "osteoclastogenic" cytokines, in particular, RANKL and M-CSF1, is extremely necessary in the process of osteoclast formation. RANKL and other cytokines activate NF-κB-related signaling pathways by inducing c-Fos and NFATc1 (nuclear factor of activated T cells) and also inhibit NFATc1 signaling repressors, thereby

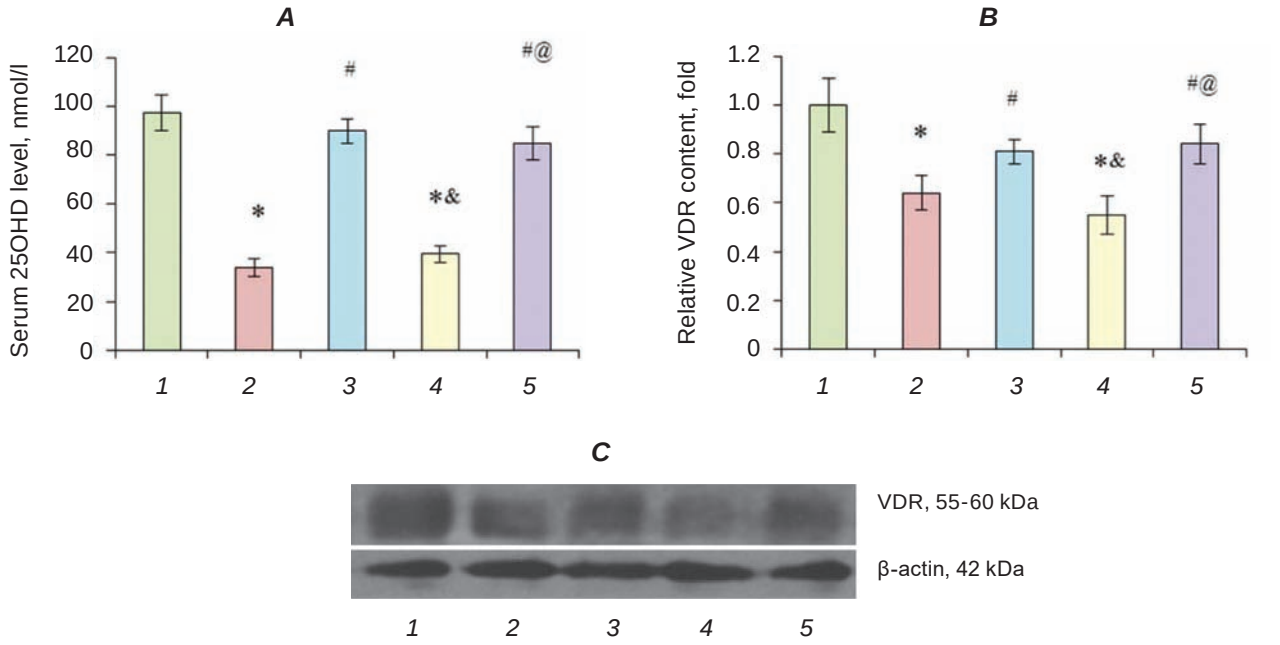

*Fig. 2. Effects of vitamin D_3 and MBPA administration on the 25-hydroxyvitamin D_3 level in blood serum (n = 10) and protein level of VDR in bone tissue (n = 5) in glucocorticoid-induced osteoporosis. 25OHD$_3$ (**A**) was evaluated by ELISA in rat serum of 5 animal groups: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D_3 administration; 4 – prednisolone and MBPA administration; 5 – combined vitamin D_3 and MBPA administration against the background of prednisolone load. Quantification of VDR level in bone tissue (**B**) and representative immunoblots (**C**) are presented. VDR protein level was normalized to β-actin. Results are shown as mean ± SEM of three independent experiments done in triplicate; *P < 0.05 vs. control, $^{\#}P < 0.05$ vs. prednisolone-induced osteoporosis, $^{\&}P < 0.05$ vs. vitamin D_3 administration, $^{@}P < 0.05$ vs. MBPA administration*

positively regulating osteoclast formation and function.

The exceptional importance of the RANK/RANKL/OPG system lies in the fact that the RANK signaling pathway can be a molecular mediator of the action of various bone remodeling regulators, including vitamin D_3 and its active forms. It is known that VDR in bone tissue controls the expression of genes encoding key effectors of bone and systemic homeostasis, including osteopontin, osteoprotegerin, RANKL, Runt-related transcription factor X2 (Runx2), and FGF23. Taking into account prednisolone-induced significant decrease in the level of VDR, a central component of the auto/paracrine system of vitamin D_3, we further investigated changes in the functioning of the NF-κB-associated cytokine system RANK/RANKL/OPG, the components of which control the balance of osteoblasts/osteoclasts. We also evaluated the corrective effect of VD_3 and MBPA during long-term supplementation of prednisolone. Moreover, these findings may be of interest in the context of a recent study showing that nitrogen-containing bisphosphonates, specifically zoledronic acid, inhibit osteoclastogenesis and osteoclast resorptive function by downregulating the RANKL-mediated NF-κB and c-Jun signaling pathways [39].

Changes in the RANK/RANKL/OPG system were revealed against the background of prednisolone-induced disturbance of vitamin D_3 metabolism and the development of vitamin D_3 hypovitaminosis. We first determined the level of a key component of the RANK/RANKL/OPG osteokine system, RANK. RANK is expressed on the membrane surface of osteoclasts and their progenitors and provides signal transduction from RANKL to activate the process of osteoclast differentiation and maturation. It was shown that prolonged administration of prednisolone to animals elicited a simultaneous decrease in the level of RANK, a marker of mature osteoclasts (by 49%, Fig. 3, *A*, *D*) and OPG (by 31%, Fig. 3, *C*, *D*) compared with control rats, while the RANKL level remained unchanged in all

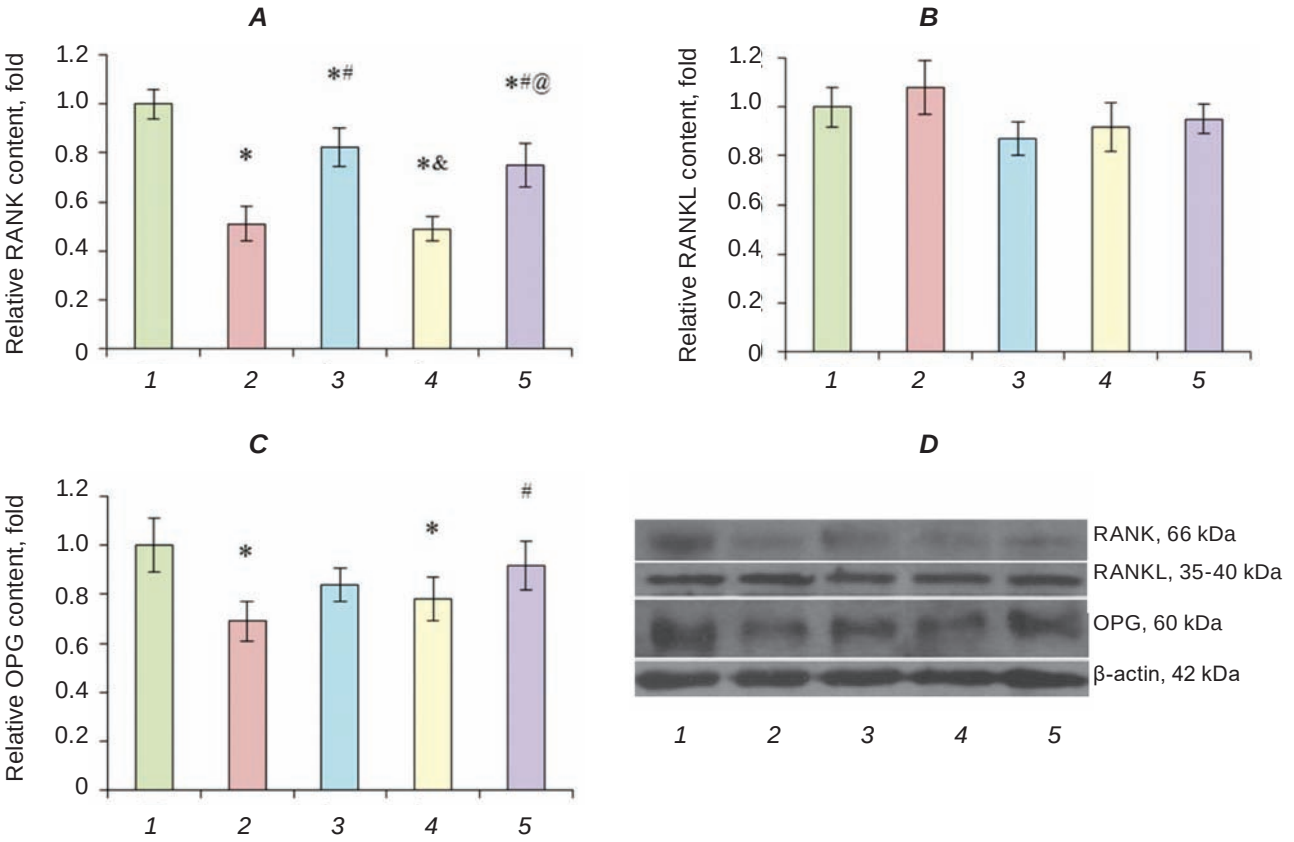

*Fig. 3. The influence of vitamin D_3 and MBPA administration on the protein levels of RANK, RANKL and OPG in bone tissue (n = 5) in glucocorticoid-induced osteoporosis. RANK, RANKL and OPG protein contents were evaluated by western blotting in bone tissue lysates of 5 animal groups: 1 – control; 2 – prednisolone administration; 3 – prednisolone and vitamin D_3 administration; 4 – prednisolone and MBPA administration; 5 – combined vitamin D_3 and MBPA administration against the background of prednisolone load. Quantification of RANK (**A**), RANKL (**B**), OPG (**C**) levels in bone tissue and representative immunoblots (**D**) are presented. Target protein levels were normalized to β-actin. Results are shown as mean ± SEM of three independent experiments done in triplicate; *$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. prednisolone-induced osteoporosis, $^{\&}P < 0.05$ vs. vitamin D_3 administration, $^{@}P < 0.05$ vs. MBPA administration*

experimental groups (Fig. 3, *B*, *D*). It is known that cells expressing RANK in bone tissue are mature multinucleated osteoclasts; therefore, a decrease in the relative content of RANK in bone tissue during GC loading may reflect a disruption in the normal functioning of osteoclasts. In turn, OPG in bone tissue is synthesized predominantly by osteoblasts and osteocytes and acts as an endogenous soluble decoy receptor for RANKL. Therefore, in our opinion, a decrease in its content may enhance RANKL-mediated bone resorption.

The OPG/RANKL ratio is an important factor in regulating the bone tissue remodeling. A decrease in the ratio of OPG/RANKL by 1.3 times compared with the control (0.64 vs. 1.0 a.u.) was established after the long-term action of prednisolone. A reduced OPG/RANKL ratio may contribute to the impaired formation and activation of osteoclasts, which are responsible for the process of bone resorption. Osteoprotegerin, by binding RANKL, prevents the activation of RANK on the cell surface of osteoclasts that can reduce both osteoclastogenesis and resorptive activity of osteoclasts. Despite the absence of significant changes in the expression of RANKL, the insufficient synthesis of OPG may indicate the inhibition of the functional activity of osteoblasts and the predominance of bone tissue resorption over the osteoblast-dependent osteosynthesis. Thus, the obtained results, in combination with the detected significant decrease in the OPG/RANKL ratio, indicate

deregulation of the RANK/RANKL/OPG system in bone tissue of glucocorticoid-treated animals.

Taking into account the presence of a marked deficiency of VD_3 during the long-term supplementation of prednisolone, the detected decrease in the content of the RANK indicates the direct involvement of vitamin D_3 as a hormonal regulator of NF-κB-mediated bone remodeling. This suggestion is confirmed by the observation that administration of vitamin D_3 resulted in the normalization of $25OHD_3$ content in blood serum to control values that led to an increase in RANK level by 61.4% (Fig. 3, *A*, *D*), and to a less extent – OPG by 21.7% (Fig. 3, *C*, *D*) compared with the prednisolone-treated rats. Accordingly, the ratio of OPG/RANKL was significantly elevated under the influence of VD_3 compared with the prednisolone group (0.96 vs. 0.64 a.u., respectively) that was probably mediated by an increase in the osteoblast-dependent process of bone tissue formation and a parallel decrease in osteoblast-mediated stimulation of the osteoclastogenesis.

The study of the action of MBPA did not reveal a significant effect on the content of the RANK protein in bone tissue compared with the prednisolone group. The RANK level was still 51% lower than in the control group (Fig. 3, *A*, *D*). However, a trend towards a slight increase in OPG levels after MBPA supplementation should be noted, as was observed in rats treated with vitamin D_3 (Fig. 3, *C*, *D*). The OPG/RANKL ratio in the MBPA group was 0.85, which is 11.5% lower than with vitamin D_3 alone. In general, the results obtained are consistent with the available data on the ineffectiveness of bisphosphonate therapy under conditions of VD_3 deficiency regarding the possibility of their positive regulatory effect on the functional state of bone tissue in secondary osteoporosis.

Finally, there were no additive effects on RANK, RANKL and OPG levels in the combined vitamin D_3 and methylenebisphosphonic acid group compared to vitamin D_3 treatment alone. The OPG/RANKL ratio was the same as in the vitamin D_3 group, indicating that MBPA was not effective in improving this parameter. Thus, based on the assessment of the state of RANK/RANKL/OPG, it can be concluded that bisphosphonate therapy should be accompanied by a correction of the vitamin D_3 status and ensuring the optimal content of $25OHD_3$ in the blood serum.

Notably, the gold standard of bisphosphonate therapy, zoledronic acid, has previously been shown to significantly attenuate osteoclastogenesis and bone resorptive capacity. In addition, zoledronic acid also suppressed NF κB activation and c-Jun N-terminal kinase phosphorylation, and also inhibited downstream factors c-Jun, c-Fos, and activated T cell nuclear factor c1, thereby reducing the expression of specific osteoclast markers [39]. Hereby, while zoledronic acid may have therapeutic potential in osteoporosis, MBPA has not shown an effect on the RANK/RANKL/OPG axis in GIOP and, therefore, can only be considered as a therapeutic agent in combination with vitamin D_3 therapy.

Our findings demonstrated that alterations of the RANK/RANKL/OPG system, which occur against the background of an insufficient level of VD_3, may be the cause of bone loss during long-term supplementation of prednisolone. At the same time, cholecalciferol and its combination with MBPA are able to partially normalize the identified bone remodeling disorders. Therefore, it can be argued that the determination of RANKL and OPG is not only an indicator of the process of bone formation and resorption, but can also serve as an indicator for assessing how effective the therapeutic potential of drugs is in correcting bone tissue remodeling.

Conclusions. Our data showed that prednisolone caused the development of hypocalcemia and hypophosphatemia, increased the activity of alkaline phosphatase in blood serum, but reduced the latter in bone tissue and bone marrow. GC-induced osteoporosis was accompanied by a decrease in serum levels of 25-dihydroxyvitamin D_3, a marker of vitamin D_3 status, against the background of abnormal synthesis of a key component of the vitamin D_3 auto/paracrine system, VDR, in bone tissue. GC-induced depletion of serum $25OHD_3$ elicited a simultaneous decrease in the synthesis of the osteoclastic marker RANK and the OPG/RANKL ratio. It has been found that vitamin D_3 supplementation normalizes mineral metabolism and $25OHD_3$ levels, which leads to the restoration of VDR-mediated signaling and the functioning of the RANK/RANKL/OPG system in bone tissue. It has been shown for the first time that the administration of MBPA has a corrective effect on the content of mineral components in the blood serum and bone tissue, as well as on the activity of alkaline phosphatase only in combination with vitamin D_3, indicating a low efficiency of bisphosphonate monotherapy in GC-induced vitamin D_3 deficiency.

In summary, the present study provided a comprehensive evaluation of the potential combination of vitamin D_3 and MBPA, as well as their monotherapies, in the treatment of osteoporosis associated with

chronic administration of glucocorticoids, based on the assessment of parameters of mineral metabolism and key components of RANK/RANKL/OPG and vitamin D_3 auto/paracrine systems that together form the molecular basis of bone remodeling.

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 and declare no conflict of interest.

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ВІТАМІН D_3 ТА МЕТИЛЕНБІСФОСФОНОВА КИСЛОТА У КОРЕКЦІЇ ПОРУШЕНЬ МІНЕРАЛЬНОГО ОБМІНУ ТА РЕМОДЕЛЮВАННЯ КІСТКОВОЇ ТКАНИНИ, ПОВ'ЯЗАНИХ ІЗ ГЛЮКОКОРТИКОЇД-ІНДУКОВАНИМ ОСТЕОПОРОЗОМ

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Метою дослідження було оцінити терапевтичну ефективність вітаміну D_3 (1000 МО/кг маси тіла, 30 днів) та натрієвої солі метиленбісфосфонової кислоти (МБФК, 17 мг/кг маси тіла, 30 днів) у запобіганні порушень мінерального обміну та ремоделювання кісткової тканини, пов'язаних із розвитком остеопорозу, індукованого введення глюкокортикоїдів (ГК). Остеопороз у щурів спричиняли тривалим (30 днів) введенням синтетичного глюкокортикоїдного препарату преднізолону (5 мг/кг маси тіла). З допомогою спектрофотометричних методів визначали вміст кальцію та неорганічного фосфату, активність лужної фосфатази (ЛФ) у сироватці крові, кістковій тканині та кістковому мозку. Методом вестерн-блот аналізу в кістковій тканині визначали рівень наступних протеїнів: рецептора вітаміну D_3 (VDR), рецептора активатора ядерного фактора каппа-В (RANK), його ліганду (RANKL) і остеопротегерину (OPG). Вміст 25-гідроксивітаміну D_3 ($25OHD_3$) у сироватці аналізували за допомогою імуноензимного аналізу. Показано, що преднізолон спричинював гіпокальціємію та гіпофосфатемію, підвищував активність ЛФ у сироватці крові, одночасно знижуючи її активність в кістковій тканині та кістковому мозку. ГК-індукований остеопороз супроводжувався глибоким дефіцитом вітаміну D_3 і зниженням вмісту VDR. Оцінка стану NF-κB-асоційованого цитокінового шляху RANK/RANKL/OPG, який регулює баланс між остеобластами та остеокластами, показала одночасне зниження вмісту RANK і співвідношення OPG/RANKL за дії преднізолону. Вітамін D_3 відновлював вміст основних показників мінерального обміну і рівень $25OHD_3$, що призводило до нормалізації VDR-опосередкованих сигнальних шляхів та цитокінової системи RANK/RANKL/OPG у кістковій тканині. Показано, що застосування МБФК мало коригувальний вплив на вміст мінеральних компонентів у сироватці крові та кістковій тканині, а також на активність лужної фосфатази лише у поєднанні з вітаміном D_3, що свідчить про низьку ефективність монотерапії бісфосфонатами у разі дефіциту вітаміну D_3, пов'язаного із розвитком ГК-індукованого остеопорозу.

Ключові слова: вітамін D_3, метиленбісфосфонова кислота, глюкокортикоїд-індукований остеопороз, ремоделювання кісткової тканини, сигнальний шлях RANK/RANKL/OPG.

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