EXPERIMENTAL ARTICLES

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EFFECTIVENESS OF NITROGEN-CONTAINING BISPHOSPHONATES IN REGULATION OF MINERAL METABOLISM IN ALIMENTARY OSTEOPOROSIS IN RATS

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The aim of the study was to investigate the effectiveness of nitrogen-containing bisphosphonates synthesized as promising substances for correction of mineral metabolism in osteoporosis. The study was carried out on a model of alimentary osteoporosis that was characterized by hypocalcaemia, hypophosphatemia, decreased 25-Hydroxyvitamin D_3 content in blood serum and severe bone tissue demineralization (reduced ash content and mineral components). It was found that synthesized novel nitrogen bisphosphonates (pyrazole-containing analogues), like reference drugs — metylene bisphosphonate (disodium salt of metylene bisphosphonic acid) and alendronate (4-amino-1-hidroxy-butyliden bisphosphonate), inhibit with the different efficiency demineralization of the bone tissue and increase the mineral metabolism in rats with alimentary (nutritional) osteoporosis that was assessed by the marker parameters of bone formation. In particular, drug administration (bisphosphonates I-12, I-40, I-42) resulted in elevation of calcium and phosphate levels and decreased the total activity of alkaline phosphatase and its isoenzymes in blood serum. The ash content and the levels of calcium and phosphorus in the ash of tibia and femur bones were shown to be markedly elevated. Bisphosphonate I-12 has shown more profound antiresorbtive activity and ability to correct mineral metabolism in alimentary osteoporosis, including such of reference drugs. It was found a significant decrease of 25-Hydroxyvitamin D_3 content in the serum that is considered as a profound vitamin D_3 deficiency associated with nutritional osteoporosis. As it was not compensated by bisphosphonates, we suggest that further investigations should be directed to the combined use of both: bisphosphonates as inhibitors of osteoclast activity that diminish bone resorption and vitamin D_3 as a key regulator of bone remodeling process and osteosynthesis activator.

Key words: nitrogen-containing bisphosphonates (pirazole-containing analogues), alimentary osteoporosis, mineral metabolism, vitamin D_3.

The loss of bone tissue is the main cause of many skeletal diseases that are mainly accompanied by the fractures of proximal part of tibia and spine. The main drugs that can slow down the loss of bone tissue due to inhibition of bone resorption are the following: calcium, estrogens, calcitonin, vitamin D_3, and bisphosphonates [1]. These drugs are called “bone antiresorbents”. Bisphosphonates are synthetic analogues of inorganic pyrophosphate. They are widely used for therapy of numerous metabolic disorders such as postmenopausal and glucocorticoid-induced osteoporosis, Paget’s disease, bone metastasis in cancer patients, hypercalcemia, hereditary skeletal disorders in children, etc. [2–4]. To regenerate bone tissue and renovate its structure after damage, it is used orthopedic implants based on the composites of bisphosphonates with nanocrystalline hydroxyapatite, nanoparticles of bioactive glass, natural and synthetic

polymers that possess biodegradability [5, 6]. Bisphosphonates have their therapeutical effect due to their ability to inhibit osteoclast activity and decrease hydroxyapatite degradation. Getting into osteoclasts, bisphosphonates inhibit the synthesis of some key metabolic enzymes, promote structural changes in cytoskeleton (actin and vinculin), and decrease the concentration of protein-markers for resorption of bone tissue and calcium in blood serum [7]. Indisputable advantage of bisphosphonates can be seen under disruption of bone tissue remodeling — the balance between osteosynthesis (mediated by osteoblasts) and bone resorption (bone demineralization) that mediated by osteoclasts. Molecular mechanisms of bisphosphonate action on bone tissue cells are dependent on their chemical structure. Bisphosphonates of first generation (etidronate, clodronate and tiludronate), due to their structural similarity to inorganic pyrophosphate (PPi), can interact with adenosine monophosphate and as a result, they form ATP-analogues, which are unable to hydrolysis and inhibit numerous ATP-dependent processes in cells that leads to apoptosis of osteoclasts. Bisphosphonates of second generation (nitrogen-containing bisphosphonates), in particular, alendronate, pamidronate and olpadronate due to their structural similarity to the substrate dimethyl allyl pyrophosphate in carbo cationic form can inhibit prenyl transferase (geranyl pyrophosphate synthase) — the enzyme that converts dimethyl allyl pyrophosphate into geranyl pyrophosphate. It leads to the decrease of geranyl pyrophosphate concentration. Besides, isopentyl pyrophosphate, reacting with ATP, forms isopentyl-ATP, the compound that initiates releasing of caspases and, therefore, apoptosis of osteoclasts and other macrophages. Bisphosphonates of third generation (nitrogen-containing bisphosphonates) such as risedronate, ibandronate, zoledronate are structurally similar to geranyl pyrophosphate and inhibit the next stage — conversion of geranyl pyrophosphate into farnesyl pyrophosphate or geranyl-geranyl pyrophosphate (by inhibiting farnesyl pyrophosphate synthase) [8, 9].

Inhibition of prenyl transferase (geranyl pyrophosphate synthase and farnesyl pyrophosphate synthase) essentially decreases the formation of farnesyl pyrophosphate and geranyl pyrophosphate — compounds that are necessary for prenylation (isopentylation) of small signal G-proteins (Rho, Ras, Rac, cdc 42). This posttranslational covalent modification of signal G-proteins is necessary for normal function and intercellular interactions of osteoclasts. In particular, attaching the lipid (isoprenoid) "tails" to signal molecules provide their involvement into regulation of specific osteoclast functions, such as final stages of differentiation, joining, endocytosis, maintenance of cell shape, and apoptosis [10].

As nitrogen presence and its position in the molecular structure of bisphosphonates may play a crucial role for their properties, the aim of the work was to investigate biological effectiveness of newly synthesized bisphosphonates, which contain nitrogen in pyrazole nucleus, on regulation of mineral metabolism in rats under experimental alimentary osteoporosis.

Materials and Methods

Approaches for design and synthesis of nitrogen-containing bisphosphonates (pyrazole-containing analogues) were carried out by colleagues from Institute of Organic chemistry NAS of Ukraine, who are co-author of this paper.

To investigate the biological effectiveness of synthesized nitrogen-containing bisphosphonates, female Wistar rats (one month, weight 90 ± 5 g) were used. Experimental alimentary osteoporosis was induced by keeping the rats on D-hypovitaminosis diet for 30 days according to The State Standard (GOST) 11222-65 with the balanced ratio of calcium and phosphorus (calcium content — 1.2%, phosphorus — 0.8%, the ratio Ca^{2+}/P — 1.5). The selection of animals and group formation were done by the method of "random number". After keeping on D-hypovitaminosis diet for 30 days, experimental animals recieved intragastrically (in 0.1 ml) the preparations by gavage once a day. Control animals got a full ration according to vivarium standards.

Animals received light ether anesthesia during all manipulations. All studied bisphosphonates: methylene bisphosphonate (dihydrate disodium methylene bisphosphonic acid, the preparation was obtained in Palladin Institute of Biochemistry of NAS of Ukraine), alendronate (commercial preparation "Alendronate-stoma"), and synthesized nitrogen-containing bisphosphonates (I-12, I-40, I-42) were administered *per os* (1.7 mg/kg of body weight) as an aqueous suspension. Vitamin D_3 bioavailability was estimated by the content of $25OHD_3$ in blood serum.

For that purpose we used the enzyme linked immunosorbent assay (ELISA) according to the protocol of 25-Hydroxy Vitamin D EIA kit (IDS, USA). Registration of the signal was carried out on an automatic microplate reader ER-500 (Sinnowa) at $\lambda = 450$ nm.

Calcium level in blood serum was determined using bioassay kits Lachema, Czech Republic. Standard solution of 25.0 mM $CaCO_3$ was prepared in 1.7% HCI. Inorganic phosphate content was measured after protein precipitation with 12% trichloroacetic acid (TCA) according to Dyce [11]. Total alkaline phosphatase activity (ALP) was determined using bioassay kits Lachema, Czech Republic. Activity of isoenzymes of alkaline phosphatase, in particular, the bone thermolabile isoform was determined after incubation of the probes at 55 °C; L-phenylalanine was used as an inhibitor for the intestinal isoform [12, 13].

Ash content of bone tissue was determined by the method of dry mineralization at 500–600 °C after degreasing of animal tissue with hexane for 7 days and the obtained results were calculated on the weight of bone tissue. Content of mineral components in ash was determined using the mentioned methods after ash dissolution in 0.5 ml of concentrated HCl. To the obtained salt solution we added 9.5 ml of bidistilled water with further dilution of the sample with bidistilled water at a ratio 1:40. For osteometric reserches tibia and femur bones were used. According to the standard procedure, we measured in frontal surface the total length, the width of the distal epimetaphysis of femur bones and proximal epimetaphysis of tibia bones, as well as, their thickness between the middle and lower thirds [14].

The data were statistically processed using Microsoft Excel. Statistical analysis of the results was was carried out using Student's t-test, the difference was considered as significant with $P < 0.05$.

Results and Discussion

Antiresorptive properties of bisphosphonates are determined by P-C-P structure that provides greater stability and resistance to enzymatic hydrolysis in bone tissue as compared with P-O-P structure in PP_i molecule (Fig. 1). All bisphosphonates, which are used in clinical practice, contain a hydroxyl group at the central carbon; it is defined as R_1-position (the replacement of the hydrogen atom). Flanking (side) phosphate groups of bisphosphonates provide

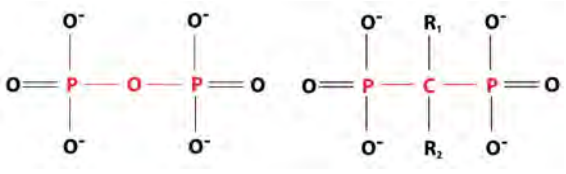
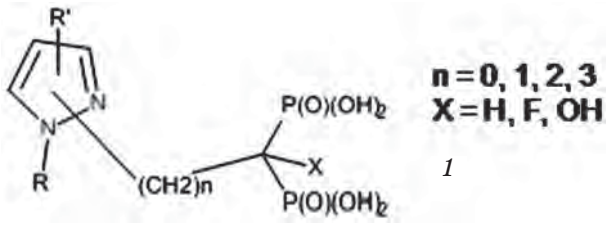


Fig. 1. **Chemical structure of pyrophosphate and bisphosphonates**

high affinity to hydroxyapatite crystals in bones (similarly as in the case of PP_i), while hydroxyl groups determine high affinity of bisphosphonates to bind calcium. Phosphate and hydroxyl groups jointly provide high affinity of bisphosphonates to the components of bone matrix, that determines the high specificity of bisphosphonate effect just on in bone tissue.

Other structural component of the molecule (R_2-position) is attached to the central carbon atom. It forms the site that is responsible for bisphosphonate (BP) ability to inhibit bone resorption. Chemical modification of side chains of carbon atom in R_2 –position in BP gives the possibility to synthesize many analogues that are characterized by both: higher antiresorptive potential and higher BP affinity to the bone tissue. Incorporation of nitrogen atom or amino group in BP structure was extremely efficient. It increases antiresorptive ability of bisphosphonates from 10 conventional units (CU) in case of etidronate to 10,000 CU as in case of zoledronate. The most important issue is not only the presence of nitrogen atom, but also its position in BP molecule [3, 8].

Analysis of MDDR data, which was carried out within the target complex program of fundamental research of NAS of Ukraine, proved the absence of pyrazole derivatives among the known BP [15]. Taking into consideration the patent clearance, researchers from Institute of Organic Chemistry of NAS of Ukraine developed new approaches and preparative methods for synthesis of 4-R-1,3-dialkyl pyrazoles and pyrazole-containing hydroxyl bisphosphonates. These approaches include serial conversion of correspondent pyrazole-containing carboxylic acids into their chloro anhydrides with further interaction with tris (trimethylsilil) phosphite and hydroxyl-containing aliphatic compounds [16]. It was synthesized for the first time the group of nitrogen-containing bisphosphonates (general structure *1*) with pyrazole nucleus:



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Among synthesized pyrazole-containing bisphosphonates, it was selected and synthesized three the most promising compounds with the different length of the linker that binds bisphosphonate fragment to pyrazole nucleus and has different way of its attachment with nitrogen atom or pyrazole (bisphosphonates I-12, I-40 and I-42). It was studied their biological activity as potential drugs, that normalize mineralization and inhibit the development of experimental alimentary osteoporosis.

To study effectiveness of pyrazole-containing bisphosphonates it was developed the model of alimentary osteoporosis in rats. The model is characterized by severe hypocalcemia, hypophosphatemia, and an increased activity of alkaline phosphatase in blood serum that can be considered as an enzyme biomarker of the formation and resorption of bone tissue [12]. Osteoporosis development in rats led to inhibition of cell growth, disorders in the structure of compact bone tissue and epiphyseal cartilage, decreased mass and ash content of tibia, reduced mineral components, and to weakening of cellular immunity (reduction in the number and inhibition of phagocytic activity of granulocytes and monocytes) [17].

When rats with developed osteoporosis obtained reference drugs or synthesized pyrazole-containing bisphosphonates, it was found an essential influence on the mineral metabolism. Calcium level, as an integral marker of mineral metabolism, was decreased in blood serum of rats with osteoporosis by 29.5%. It was observed an increase in the calcium level in case of rat treatment with reference drugs or studied bisphosphonates. Introduction of metylene bisphosphonate led to the increase in calcium levels by 14.5%, alendronate — 8.2%, bisphosphonate I-12 — 22.8% as compared with osteoporosis (Table 1). Bisphosphonates I-40 and I-42 had a much smaller effect on the calcium content. It has to be noted, that the observed changes in calcium content in blood serum were due to the fraction of ionized (ultrafiltered) calcium, its relative content in control was 91%. The level of ionized calcium increased in case of metylene bisphosphonate by 16.4%, alendronate — 9.3%, bisphosphonate I-12 — 27.1%, bisphosphonate I-40 — 10.1%, I-42 — 10.7% as compared with osteoporosis. Bisphosphonates had no essential effect on the content of protein-bound calcium. It is known that there are some functional forms of calcium in blood serum. In particular, small amount of calcium is bound to the proteins (albumins and globulins). However,

Table 1. **Content of mineral components in blood serum of rats with alimentary osteoporosis and after administration of bisphosphonates**

Experimental groups	Calcium, $mM \cdot L^{-1}$			Inorganic phosphate $mM \cdot L^{-1}$
	Total calcium	Protein-bound calcium	Ultrafiltered calcium	
Control	2.24±0.12	0.20±0.01	2.04±0.10	1.95±0.09
Alimentary osteoporosis	1.58±0.08*	0.18±0.02	1.40±0.08*	1.46±0.07*
Alimentary osteoporosis+ methylene bisphosphonate	1.81±0.07#	0.19±0.02	1.63±0.07#	1.73±0.06#
Alimentary osteoporosis + alendronate	1.71±0.09	0.17±0.03	1.53±0.07#	1.50±0.06
Alimentary osteoporosis + bisphosphonate I-12	1.94±0.14	0.17±0.09	1.78±0.11#	1.76±0.08#
Alimentary osteoporosis + bisphosphonate I-40	1.70 ±0.9#	0.19±0.03	1.54±0.6#	1.52±0.07
Alimentary osteoporosis + bisphosphonate I-42	1.71±0.6	0.16±0.03	1.55±0.06	1.49±0.6

Note: here and after * — $P < 0.05$ compared with the control; # — $P < 0.05$ compared with osteoporosis group; $M \pm m$, $n = 9$.

the great amount of calcium is in an ultrafiltered form that contains ionized calcium (up to 85%) and chelated one (up to 15%): citrate- phosphate- and bicarbonate-bound calcium. The ratio between calcium forms can be changed due to the different physiological state and can serve as a marker of some pathological states [1]. The decrease in the ratio of ultrafiltered: membrane-bound calcium form from 10.2 (control value) to 7.8 (osteoporosis state) can be the evidence of the severe disorder of mineral metabolism. Investigated bisphosphonates were able to increase the above-mentioned ratio (bisphosphonate I-12 — up to 10.4, bisphosphonate I-40 —to 8.1, bisphosphonate I-42 — to 9.7) and they normalized to some extent mineral metabolism. However, the content of calcium and its functional forms in blood serum did not reach the value that was observed in the control group of animals.

Calcium level in blood serum is one of the constant characteristics of the body. Daily fluctuations of calcium level do not excess 3–5%. It is the necessary condition for normal functioning of neural system, muscles, coagulation system, maintenance of the structure and permeability of membranes as well as secretion and action of hormones, etc.

The level of extracellular Ca^{2+} in the body is kept within the narrow physiological range. It is based on the coordinated functioning of intestines, kidney, parathyroid glands and bone tissue. Direct regulation is carried out by parathyroid hormone, D_3 vitamin and calcitonin [18].

Ca^{2+} is considered to be one of the major intracellular second messengers rapid changes in its concentration are achieved by highly effective regulation of calcium channels, pumps and exchangers [19].However, ionized extracellular calcium acts as a primary messenger and serves as a ligand for membrane calcium receptors conjugated with G-protein (GPCR — G-protein-coupled-receptor). These receptors are called “calcium receptors”. They contain Ca^{2+} binding center CaSR (calcium sensing receptor) [20]. The main function of CaSR is to maintain homeostasis of Ca^{2+} through coordinating the processes of calcium absorption from gastrointestinal tract, its excretion by kidneys, and calcium releasing and accumulating by bone tissue. At the molecular level, CaSR provides integration of calcium signaling, that coordinates changes in the concentration of extracellular Ca^{2+} with intracellular signaling systems and is essential for the physiological response.

Calcium of the bone tissue is an important regulator for maintenance of osteoblast/osteoclast balance, i.e. for the processes of osteosynthesis and bone resorption.

In particular, Ca^{2+} inhibits the formation and activity of osteoclasts and stimulates the activity of osteoblasts. The ability of many osteoblast cell lines and primary osteoblasts to express CaSR confirms Ca^{2+}–mediated mechanism of osteoblastogenesis through JNK-signaling mechanism and its role in pathology [21]. Calcium receptor mutations cause the development of pathologies of calcium metabolism, which are coupled with hypercalcemia or hypocalcemia. Family hypercalcemia coupled with hypercalciurea, primary hyperparathyroidism of newborns, and autosomal dominant hypocalcemia coupled with hypercalciurea are the examples of these pathologies [22]. CaSRs are also expressed by osteoclasts and monocytes, which are precursors of osteoclasts. High level of Ca^{2+} inhibits the differentiation and activity of osteoclasts and can increase their apoptosis [23]. As a result, activation of CaSR inhibits osteoclast resorptive activity regarding the bone tissue.

Osteoporosis hypocalcemia is accompanied by slight hypophosphatemia; the content of phosphates in blood serum was decreased by 25.2% (Table 1). Introduction of studied drugs caused a moderate increase in PP_i content. In particular, in case of metylene bisphosphonate it increased by 21.9% and by 21.9% for bisphosphonate bisphosphonate I-12. Other bisphosphonates had no significant effect on the content of phosphate. The observed unidirectional changes in the content of calcium and PP_i provided the stable ratio of Ca^{2+}/P_i within 1.5, which is a constant value for the animal organism.

The changes in the content of mineral components in blood serum of rats with alimentary osteoporosis and under the influence of the studied bisphosphonates were accompanied by the alteration of ALP activity — a biomarker enzyme that characterizes the intensity of the process of mineralization in bone tissue. Serum ALP activity at osteoporosis significantly increased — by 67.6%. The contribution of the bone isoform was 82.9% of the total serum alkaline phosphatase activity. Its activity at osteoporosis grew to 78.2% (Table 2). Normalization of mineral metabolism under the influence of studied bisphosphonates and reference drugs was accompanied by a decrease of general activity

of ALP and its bone isoform in serum to the control values. Thus, the total ALP activity was decreased under the influence of metylene bisphosphonate by 27.2%; bisphosphonate I-12 — by 27.4% and bisphosphonate I-40 — by 22.1%. Activity of bone isoform of the enzyme was decreased after MBFK administration by 32.7%, bisphosphonate I-12 — by 32.3%, bisphosphonate I-40 — by 28.9%, and bisphosphonate I-42 — by 26.5%.

ALP (phosphohydrolase of phosphoric acid monoesters E.C. 3.1.3.1) is a membrane-bound ectoenzyme that catalyzes the cleavage of monophosphoric esters and pyrophosphate, releasing inorganic phosphate (P_i) at alkaline pH. Isoenzymes of ALP comprise four types: tissue nonspecific alkaline phosphatase isoforms (TNALP) which are found in bones, liver and kidneys, and tissue specific isoforms — intestinal, placental and fetal. Organic monophosphates, phosphoethanolamine, phosphocholine, pyridoxal-5-phosphate and PP_i are substrates for alkaline phosphatase. They are sources for the formation of hydroxyapatite in cells [24, 25]. ALP possesses very important ability to hydrolyze PP_i, produced in significant amounts during conversion of ATP into ADP and PP_i in the reaction catalized by nucleotide pyrophosphatase/phosphodiesterase (EC 3.1.4.1). The extracellular PP_i, as the main physiological inhibitor of mineralization and calcification of the extracellular matrix, inhibits the proliferation of hydroxyapatite crystals along the collagen fibrils. Providing PP_i hydrolysis, ALP supports PP_i/P_i that is optimal for bone mineralization process [24].

The presence of ALP isoforms enables measuring the enzyme activity in the diagnosis of certain diseases. In particular, increased enzyme activity can be determined in serum of rachitic children, patients with bone diseases associated with increased activity of osteoblasts or bone fractures, bone carcinoma, bone metastases, megakaryoblastoma with bone lesions. So, increased activity of alkaline phosphatase is observed not only in the period of intensive growth of bone tissue, but also in bone diseases — osteoporosis, osteomalacia [1].

Mutations (knockout) of the gene of tissue-nonspecific alkaline phosphatase and inhibition of its activity cause hypophosphatasia and lower mineralization of skeleton and teeth. These occures in case of rickets in children and osteomalacia in adults as the result of unability of ALP to cleave PPi. Consequently it was observed a significant increase in its concentration that inhibits mineralization process [25, 26]. There is a wide range of metabolic disorders associated with reduced activity of alkaline phosphatase due to loss of enzyme ability to hydrolyze extracellular pyridoxal-5-phosphate (PP) to pyridoxal. The last one, in contrast to PP, is able to penetrate through the membrane into the cell. Pyridoxal-5-phosphate, as an active form of vitamin B6, serves as a coenzyme in the reactions of transamination, decarboxylation that facilitates the formation of some neurotransmitters in the nervous system such as dopamine, serotonin, histamine, γ-aminobutyric acid and taurine [28].

The identified changes in the content of mineral components and ALP activity in serum reflects the process of demineralization of bone tissue under condition of alimentary osteoporosis. The state of bone tissue of experimental rats, that were given the preparations, was characterized by ash content, calcium and phosphorus content and by osteometric indicators of femur and tibia bones.

Table 2. **Activity of alkaline phosphatase and its isoenzymes in blood serum of rats with alimentary osteoporosis and after introduction of bisphosphonates**

Experimental groups	Activity of alkaline phosphatase, IU/L of blood serum		
	Total activity	Intestinal isoenzyme	Bone isoenzyme
Control	230.2±11.7	48.9±2.2	190.9±12.3
Alimentary osteoporosis	386.0±14.0*	73.0±3.7*	340.2±20.9*
Alimentary osteoporosis+ methylene bisphosphonate	281.2±10.8	61.9±2.6#	229.0±14.5#
Alimentary osteoporosis + alendronate	301.6±16.4#	59.9±4.2#	280.7±13.6
Alimentary osteoporosis + bisphosphonate I-12	280.3±12.6#	68.7±3.2	230.3±11.5
Alimentary osteoporosis + bisphosphonate I-40	300.7±17.3#	66.4±3.9	241.9±15.7
Alimentary osteoporosis + bisphosphonate I-42	330.4±13.4	70.1±5.2	252.1±16.8

The data in Table 3 showed that in case of osteoporosis, tibia ash content decreased by 25.6%, the calcium content — by 34.3%, the content of P_i — by 33.8% compared to control. Administration of metylene bisphosphonate increased the ash content by 13.5%, calcium content — by 15.5%, and P_i — by 15.7%. The introduction of the bisphosphonate I-12 resulted in increased tibia ash content by 18.4%, calcium — by 31.9%, P_i — by 36.1%. Bisphosphonates I-40 and I-42 increased tibia ash content by 6.8% and 5.2%; calcium content — by 8.2% and 12.0% respectively. P_i contents under the effect of these bisphosphonates increased by 18. 5% and 10.2% respectively.

Changes in mineral metabolism are in accordance with osteometric data of femur and tibia bone under osteoporosis and after administration of bisphosphonates. In animals that were kept on the D-hypovitaminose diet for two months, we observed reduced length of the femur (by 25.0%) and tibia (by 21.2%) and reduction of thickness in distal (by 19.5%) and proximal (by 20.0%) distal epimetaphysis (Table 4) was also found. Administration of bisphosphonates to diseased rats revealed increase in length and thickness of the femur and tibia. In particular, the growth of bone was significantly greater in case of metylene bisphosphonate (length increased by 23.4% and 20.3% respectively) and bisphosphonate I-12 by 21.3% and 16.2%. At the same time, it was observed the increased thickness of the distal epimetaphysis under the influence of studied bisphosphonates. However, the changes did not reach values of control animals: neither osteometric indices nor the content of mineral components.

Table 3. **Content of mineral components in tibia of rats with alimentary osteoporosis and after administration of bisphosphonates**

Experimental groups	Ash content,% Dry mass	Calcium content, % in ash	Phosphate content, % in ash
Control	56.8±2.5	35.3±2.3	16.3±0.6
Alimentary osteoporosis	42.3±1.8*	23.2±1.6*	10.8±0.4*
Alimentary osteoporosis+ methylene bisphosphonate	48.0±1.7#	26.8±1.2#	12.5±0.3#
Alimentary osteoporosis + alendronate	45.0±1.7#	27.5±1.2#	11.8±0.3#
Alimentary osteoporosis + bisphosphonate I-12	50.1±3.2#	30.6±1.7#	14.7±0.8#
Alimentary osteoporosis + bisphosphonate I-40	45.2±2.9	25.1±1.4	12.8±0.7#
Alimentary osteoporosis + bisphosphonate I-42	44.5±2.8	26.0±1.4#	11.9±0.4#

Table 4. **Osteometric indicators of femur and tibia of rats with alimentary osteoporosis and after administration of bisphosphonates**

Experimenta groups	Femur		Tibia	
	Length, mm	Thickness of distal epimetaphysis, mm	Length, mm	Thickness of proximal epimetaphysis, mm
Control	32.8±2.3	6.7±0.4	36.3 ±2.3	7.0±0.3
Alimentary osteoporosis	24.6±1.2*	5.4±0.1*	28.6±8.1*	5.6±0.4*
Alimentary osteoporosis+ methylene bisphosphonate	29.6±2.7#	6.4±0.3#	35.3±3.3#	6.8±0.5#
Alimentary osteoporosis + alendronate	26.9±2.2#	5.8±0.5	30.9±2.2#	5.9±0.4#
Alimentary osteoporosis + bisphosphonate I-12	28.6±2.5#	6.2±0.4#	34.7±2.1#	6.2±0.6#
Alimentary osteoporosis + bisphosphonate I-40	27.3±3.1#	5.7±0.5	31.8±3.5#	6.0±0.7#
Alimentary osteoporosis + bisphosphonate I-42	26.4±2.2#	6.0±0.6#	32.9±3.1#	6.3±0.5#

Since nutritional osteoporosis was modeled by maintenance of rats for 3 months on D-hypovitaminosis diet, it was important to describe the state of vitamin D-sufficiency as a marker of structural and functional organization of bone and mineral metabolism in the body.

The most appropriate indicator of vitamin D_3-sufficiency is the content of its hydroxylated form, 25 hydroxycholecalciferol (25OHD3) in serum (plasma) blood, which normally reaches values of 100–150 nm · L^{-1} [3, 11].

Development of alimentary osteoporosis in our experiments was accompanied by a significant, almost 3-fold, reduction of 25OHD3 level in serum (34.0±3.7 nm · L^{-1} in osteoporosis and 97.5±4.3 nm · L^{-1} as the control level). According to the classification of vitamin D_3-sufficiency, such low level of 25OHD3 characterizes the state of deep D_3-deficiency [15, 16]. Investigated bisphosphonates, largely normalized the content of mineral components and alkaline phosphatase activity, but had virtually no effect on the level of 25OHD3 in serum. Only daily co-administration of 40 IU of vitamin D_3 for 30 days provided sufficient normalization of 25OHD3 (89.7 ± 5.2 nm · L^{-1}) in osteoporosis (Fig. 2).

Thus, the results of the studies indicate that the development of nutritional osteoporosis caused by keeping rats on D-avitaminosis diet, was accompanied by severe hypocalcemia, hypophosphatemia, increased activity of alkaline phosphatase and its bone isoenzyme and significant disorders of both osteometric indicators and the content of mineral components in rats. Administration of investigated pyrazole-containing bisphosphonates and reference drugs — metylene bisphosphonate (disodium salt of metylene bisphosphonic acid) and alendronate (4-amino-1-hidroxy-butyliden bisphosphonate) to osteoporosis rats inhibited demineralization process (resorption) of bone tissue and enhanced mineral metabolism in

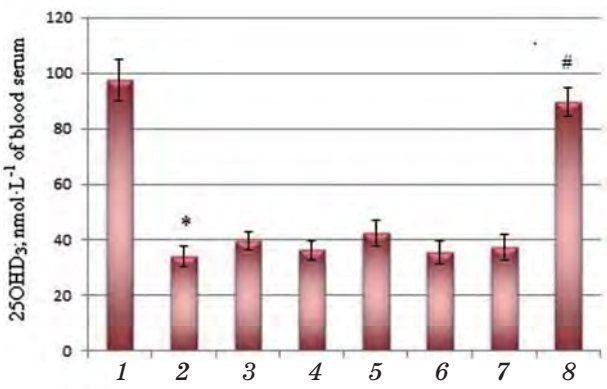

Fig. 2. **25OHD3 content in blood serum of rats with alimentary osteoporosis and after administration of bisphosphonates:**
1 — control;
2 — alimentary osteoporosis;
3 — alimentary osteoporosis + methylene bisphosphonate;
4 –alimentary osteoporosis + alendronate;
5 — alimentary osteoporosis + bisphosphonate I-12;
6 — alimentary osteoporosis + bisphosphonate I-40;
7 — alimentary osteoporosis + bisphosphonate I-42;
8 — alimentary osteoporosis + 40 IU D_3 vitamine

rats. Bisphosphonate I-12 has the highest antiresorptive efficiency and the ability to regulate nutritional mineral under condition of alimentary osteoporosis. Since deficiency of vitamin D_3 in this model of osteoporosis is not compensated by administration of studied pyrazole-containing bisphosphonates, further research should be aimed at studying of the combined effects of two powerful remodeling regulators of bone tissue: bisphosphonates, which inhibit osteoclast activity and decrease resorption, and D_3 vitamine as the main activator of osteosynthesis.

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ЕФЕКТИВНІСТЬ НІТРОГЕНВМІСНИХ БІСФОСФОНАТІВ У РЕГУЛЮВАННІ МІНЕРАЛЬНОГО ОБМІНУ ЗА АЛІМЕНТАРНОГО ОСТЕОПОРОЗУ У ЩУРІВ

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Метою роботи було дослідити ефективність синтезованих нітрогенвмісних бісфосфонатів як перспективних субстанцій лікарських засобів у корекції порушень мінерального обміну за остеопорозу. Дослідження проводили на моделі аліментарного остеопорозу у щурів, розвиток якого характеризується гіпокальціємією, гіпофосфатемією, зниженням вмісту 25-гідроксивітаміну D_3 у сироватці крові та вираженою демінералізацією кісткової тканини (знижуються зольність і вміст мінеральних компонентів). Встановлено, що досліджувані синтезовані нітрогенвмісні бісфосфонати (піразолвмісні аналоги), подібно до препаратів порівняння — метиленбісфосфонату (динатрієвої солі метиленбісфосфонової кислоти) та алендронату (4-аміно-1-гідроксибутиліден-бісфосфонату), з різною ефективністю гальмують процес демінералізації кісткової тканини та посилюють мінеральний обмін у щурів з остеопорозом. Зокрема, за дії досліджуваних препаратів (бісфосфонати І-12, І-40 та І-42) зростав вміст кальцію, фосфатів і знижувалась активність лужної фосфатази та її ізоформ у сироватці крові. Підвищувалися зольність та вміст кальцію і фосфору в золі великогомілкової та стегнової кісток. Найвищу антирезорбтивну ефективність і здатність корегувати мінеральний обмін за аліментарного остеопорозу, в тому числі й вищі за аналогічні показники препаратів порівняння, продемонстровано для бісфосфонату І-12. Виявлене суттєве зниження вмісту 25-гідроксивітаміну D_3 у сироватці крові характеризує значний дефіцит вітаміну D_3 за аліментарного остеопорозу, що не компенсується бісфосфонатами, а тому подальші дослідження мають бути спрямовані на поєднане застосування бісфосфонатів, які пригнічують активність остеокластів і зменшують резорбцію, та вітаміну D_3 — основного регулятора процесу ремоделювання кісткової тканини та активатора остеосинтезу.

Ключові слова: нітрогенвмісні бісфосфонати (піразолвмісні аналоги), аліментарний остеопороз, мінеральний обмін, вітамін D_3.

ЭФФЕКТИВНОСТЬ НИТРОГЕНСОДЕРЖАЩИХ БИСФОСФОНАТОВ В РЕГУЛИРОВАНИИ МИНЕРАЛЬНОГО ОБМЕНА ПРИ АЛИМЕНТАРНОМ ОСТЕОПОРОЗЕ У КРЫС

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Целью работы было исследовать эффективность синтезированных нитрогенсодержащих бисфосфонатов как перспективных субстанций лекарственных средств в коррекции нарушений минерального обмена при остеопорозе. Исследования проводили на модели алиментарного остеопороза у крыс, развитие которого характеризуется гипокальциемией, гипофосфатемией, снижением содержания 25-гидроксивитамина D_3 в сыворотке крови и выраженной деминерализацией костной ткани (снижаются зольность и содержание минеральных компонентов). Установлено, что исследуемые синтезированные нитрогенсодержащие бисфосфонаты (пиразолсодержащие аналоги), подобно препаратам сравнения — метиленбисфосфонату (динатриевой соли метиленбисфосфоновой кислоты) и алендронату (4-амино-1-гидроксибутилиден-бисфосфонату), с разной эффективностью тормозят процесс деминерализации костной ткани и усиливают минеральный обмен у крыс с остеопорозом. В частности, при действии исследуемых препаратов (бисфосфонаты I-12, I-40, I-42) возрастало содержание кальция, фосфатов и снижалась активность щелочной фосфатазы и ее изоформ в сыворотке крови. Повышались зольность и содержание кальция и фосфора в золе большеберцовой и бедренной костей. Наиболее высокие антирезорбтивная эффективность и способность корректировать минеральный обмен при алиментарном остеопорозе, в том числе и выше аналогичных показателей препаратов сравнения, продемонстрированы для бисфосфоната I-12. Обнаруженное существенное снижение содержания 25-гидроксивитамина D_3 в сыворотке крови отражает значительный дефицит витамина D_3 при алиментарном остеопорозе, который не компенсируется бисфосфонатами, поэтому дальнейшие исследования должны быть направлены на сочетанное применение бисфосфонатов, подавляющих активность остеокластов и уменьшающих резорбцию, и витамина D_3 — основного регулятора процесса ремоделирования костной ткани и активатора остеосинтеза.

Ключевые слова: нитрогенсодержащие бисфосфонаты (пиразолсодержащие аналоги), алиментарный остеопороз, минеральный обмен, витамин D_3.