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Research Article

Evaluation of osteogenesis processes against the background of experimental osteoporosis therapy

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ABSTRACT

BACKGROUND: Osteoporosis is a problem all over the world with important clinical and economic consequences. A significant contribution to solving the problem of the spread of osteoporosis can be the creation of drugs based on unique biologically active compounds.

AIM: The aim was to evaluate the processes of osteogenesis, according to the formation of an organic matrix of bone tissue, as well as to evaluate markers of bone remodeling in blood serum at the stages of anti-osteoporosis therapy.

MATERIALS AND METHODS: The study was performed on an experimental model of osteoporosis using biochemical methods for analyzing markers of osteoporosis in blood serum, as well as atomic absorption spectroscopy and X-ray densitometry.

RESULTS: According to the results of the study, the specific anti-osteoporotic activity of the new drug based on succinic acid salts was proved: a significant increase in the organic component — the total collagen in bone tissue and the mineral component as the main elements in bone tissue in both young and old senile animals. Evaluation of the dynamics of the content of markers of bone remodeling showed the high effectiveness of the new drug in monotherapy, and in combination with vitamin D_3 in the activation of osteogenesis processes in experimental osteoporosis.

CONCLUSIONS: The effectiveness of the proposed anti-osteoporotic agent is shown, which is more pronounced in senile rats and is due to a proportional increase in the organic and mineral components of bone tissue.

Keywords: osteoporosis; experimental model; bone remodeling; collagen; markers of osteogenesis; anti-osteoporosis agent.

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Научная статья

Оценка процессов остеогенеза на фоне терапии экспериментально индуцированного остеопороза

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АННОТАЦИЯ

Актуальность. Остеопороз представляет проблему во всем мире с важными клиническими и экономическими последствиями. Существенным вкладом в решение проблемы распространения остеопороза может стать создание препаратов на основе уникальных биологически активных соединений.

Цель — оценка процессов остеогенеза, по данным образования органической матрицы костной ткани, а также по оценке маркеров костного ремоделирования в сыворотке крови на этапах антиостеопорозной терапии.

Материалы и методы. Исследование выполнено на экспериментальной модели остеопороза с применением биохимических методов анализа маркеров остеопороза в сыворотке крови, а также атомно-абсорбционной спектроскопии и рентгеноденситометрии.

Результаты. По результатам исследования доказана специфическая антиостеопорозная активность нового препарата на основе солей янтарной кислоты: существенное увеличение органического компонента — суммарного коллагена в костной ткани и минерального компонента как основных элементов в костной ткани — как у молодых, так и у старых сенильных животных. Оценка динамики содержания маркеров костного ремоделирования показала высокую эффективность нового препарата при монотерапии, и в комбинации с витамином D_3 в активации процессов остеогенеза при экспериментальном остеопорозе.

Заключение. Показана эффективность предлагаемого антиостеопорозного средства, которая более выражена у сенильных крыс и обусловлена пропорциональным нарастанием органического и минерального компонентов костной ткани.

Ключевые слова: остеопороз; экспериментальная модель; костное ремоделирование; коллаген; маркеры остеогенеза; антиостеопорозное средство.

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BACKGROUND

According to the World Health Organization, osteoporosis (OP) is one of the most common diseases today, ranking first among myocardial infarction, stroke, cancer, and sudden death in the structure of morbidity and mortality of the population. OP and induced fractures have become major causes of illness, disability, and death, accounting for a considerable healthcare cost [29, 33, 34]. Postmenopausal women are the most susceptible and four times more likely than males to suffer from the disease [29, 33]. OP is a worldwide problem with important clinical and economic implications [25, 31]. Indeed, OP fractures cause a significant increase in morbidity, disability, and mortality, particularly in older adults, with significant implications for healthcare costs [25, 26, 31].

Osteoporosis is a systemic metabolic disease of the skeleton characterized by a decrease in bone mass and disruptions in bone tissue microarchitectonics, which increases bone fragility and the risk of bone fractures [19]. According to the International Osteoporosis Foundation [28], OP affects about 75 million people in Europe, the United States, and Japan [27, 28]. Hip bone fractures caused by OP are expected to increase by 240% in females and 310% in males by 2050 as life expectancy increases worldwide. In European countries, the incidence of disability due to OP exceeds that of cancer (except lung cancer) and is comparable with other chronic noncommunicable diseases (rheumatoid arthritis, asthma, and arterial hypertension) [32, 34, 39].

According to studies by the Research Institute of Rheumatology of the Russian Academy of Medical Sciences, 33.8% of females and 26.9% of males over the age of 50 yr have OP in Russia, whereas 43.3% of females and 44.1% of males exhibit symptoms of osteopenia [1, 10]. Thus, 14 million people (10% of the population) in Russia have OP, 20 million people have IPC conditions corresponding to osteopenia, and 34 million people are at risk of osteoporotic fractures. The number of OP patients in Russia is expected to increase by one-third by 2050 due to population aging [10]. According to global trend analysis, fractures, such as femoral neck fractures, are expected to double between 2005 and 2050 due to the aging of the worldwide population alone [26].

The problem of OP has gained particular importance in recent decades as the population of elderly and senile people, particularly postmenopausal women, has increased. About one-third of the total life expectancy of females falls in the postmenopausal period, which increases the risk of developing postmenopausal and senile OP [6, 18]. Due to the increasing number of elderly people, this disease is becoming a medical and social problem.

The ineffectiveness of the proposed prevention and treatment programs for OP can be explained by more complex causes of its development than simple calcium deficiency. Therefore, such treatments and preparations should be used to treat and prevent OP, as they correlate to the biology of age development and the pathophysiology of OP development. The creation of drugs based on novel biologically active compounds can significantly contribute to solving the OP problem.

The use of a complex of acid salts of succinic acid in drug therapy, which potentially affects the uptake of macroelements and microelements by bone tissue and the biotransformation of vitamin D_3 and increases the bioavailability of its active forms in the body, is a new approach in the prevention and treatment of senile and postmenopausal OP and vitamin D_3 deficiency in particular [1, 12, 13, 15, 21]. Among natural substrate-metabolites, acid salts of succinate are the most potent modulators of orphan receptors and L-type calcium channels, and they activate Ca^{2+} accumulation inside the cell via the endoplasmic and sarcoplasmic reticulum and mitochondria, as well as the limiting step in cholesterol metabolism, namely, entry into mitochondria and subsequent biotransformation into active steroid forms [11, 12, 15, 37]. In an experimental study, a succinate-containing complex preparation increased bone mass index and estrogen and androgen synthesis under hormone deficiency conditions [13, 16, 21]. The expected outcome of the technology's implementation is an effective therapy in the prevention and treatment of vitamin D_3 deficiency OP and, consequently, a decrease in musculoskeletal, endocrine, and cardiovascular diseases and a reduction in the risk of disability and premature death [7, 8, 13, 12, 20].

The study aimed to investigate the pharmacological properties of the novel drug in an OP experimental model in female rats of different ages over 30 days.

MATERIALS AND METHODS

The experiment included 40 sexually mature female Wistar rats, for whom an experimental model was developed in accordance with the research objective.

The method of developing an experimental OP model is described in several studies [1, 9, 22]. The method consists of bilateral surgical removal of female rats' ovaries, followed by twice-daily administration of prednisolone. The experiment used intact female Wistar rats weighing 240–260 g at 4–6 months (young) and 360–420 g at 12–14 months (senile). Bilateral ovariectomy was performed in accordance with the recommendations of Bunoc's (1968) manual. The animals were anesthetized with ether and placed on the operating table in the abdominal position. The hair on the back was clipped from the pelvis to the rib arch, and the skin was treated with alcohol and a diluted alcoholic iodine solution. A scalpel was used to make a 1.5 to 2 cm-long longitudinal

incision along the midline of the back. A puncture was made in the posterior region of the abdominal cavity by alternately moving the incision to the left and right. Once the right or left horn of the uterus was found, it was transported outside via a puncture. The ovary was then found, and electrocautery was used to separate it from the horn of the uterus. The second ovary was also removed in the same way. The peritoneal punctures and dorsal incision were treated with streptocide. The dorsal incision was sutured, and the suture was treated with 5% iodine tincture. After surgery, the animals were placed in a clean cage, and daily wound treatment with disinfectants was conducted for the first 4–5 days. On days 7–9, the wound healed. Female rats were given a 25 mg/kg intraperitoneal injection of prednisolone solution 3 weeks after surgery. The second injection was given at a 15 day interval. The technique of lifelong validation of this pathology by identification of bone remodeling markers in blood was used to assess the severity of OP [2].

Investigation of the organic component of bone tissue

The state of collagen metabolism in bone tissue was assessed by the total collagen content in the femoral epiphysis homogenate calculated by the amount of hydroxyproline [5, 23].

Investigation of the mineral component of bone tissue

The elements in femur bone tissue were analyzed using atomic adsorption spectrometry (Varian spectrometer). Markers of bone remodeling, such as osteocalcin (OK), sclerostin (Skl), osteoprotegerin (OPG), fibroblast growth factor 23 (FGF-23), and nuclear factor kappa-β activator ligand (RANKL), in OP serum were determined using ELISA kits for enzyme-linked immunosorbent assay.

The object of the study was a drug based on succinic acid salts that was used to treat OP (hereinafter referred to as drug X3, patent for invention RU No. 2582973) [17]. This study, which is part of the complex preclinical studies required for product registration, aims to establish the property of the test object after multiple oral administrations at a fixed dose (62.5 mg/kg). The study was conducted in accordance with GLP laboratory research standards [14]. Using this method allowed us to reduce the number of animals in the experiment, conduct the study in accordance with humane principles of animal treatment, and comprehensively investigate the drug's effect on the main functional systems and organs of experimental animals.

RESULTS AND DISCUSSION

Collagen and calcium content in bone tissue of female rats with experimental osteoporosis

X-ray densitometry was used to study the processes of bone remodeling and determine the development and degree of OP and the possibility of its correction; atomic adsorption spectroscopy was used to measure the weight characteristics and the content of macronutrients, including Ca^{2+}; and the biochemical method was used to determine collagen calculated by the amount of hydroxyproline in the femoral bone homogenate.

The data presented in Figure 1 show changes in bone tissue composition after experimental OP indication. A significant decrease in the content of calcium and collagen, the leading indicators of organic and mineral components of bone tissue, was reported in senile animals (OP2). Moreover, the decrease in calcium in young rats (OP1) was inconsistent compared with the intact control, most likely due to the counteraction of strong compensatory mechanisms of calcium homeostasis in bone tissue.

The change in collagen content allows us to study the nature of changes in the protein matrix of bone tissue. The state of collagen metabolism in bone tissue was assessed by the total collagen content in the femoral epiphysis homogenate calculated by the amount of hydroxyproline (Figure 1). An increase in total collagen content in bone tissue compared with the control group suggested that collagen synthesis was accelerated. The content of oxyproline in the bone tissue of animals in various experimental groups changed differently after treatment with drug X3. Drug X3 significantly increased the content of oxyproline in young rats with experimental OP (by 17.5%, $p < 0.05$); however, it did not reach the values of the intact group. This was most likely due to the short duration of administration and the peculiarities of bone metabolism in young rats, which implies that drugs should be administered for a longer period of time to restore and complete bone remodeling. Collagen reduction after OP formation was more prominent in senile females (Control 2), and drug administration had a more significant therapeutic effect. The increase in oxyproline concentration, in particular, was 21.2% ($p < 0.05$).

The findings suggest that the proposed anti-osteoporosis drug X3 is effective in increasing the protein matrix of bone tissue and restoring the organic component of bone tissue to an acceptable level compared with the control group. Furthermore, the drug X3 gently increases the calcium content of bone tissue without causing apparent calcium excess, which may improve bone tissue fragility.

Thus, 30-day oral administration of X3 significantly increased total collagen content in bone tissue in both elderly and young rats compared with the control group. The intensity of collagen synthesis was more pronounced in senile rats.

Assessment of bone remodeling markers in peripheral blood

Bone remodeling markers representing osteogenesis and bone resorption (OK, Skl, OPG, FGF-23, and RANKL) were studied in the serum of female rats with experimentally induced OP.

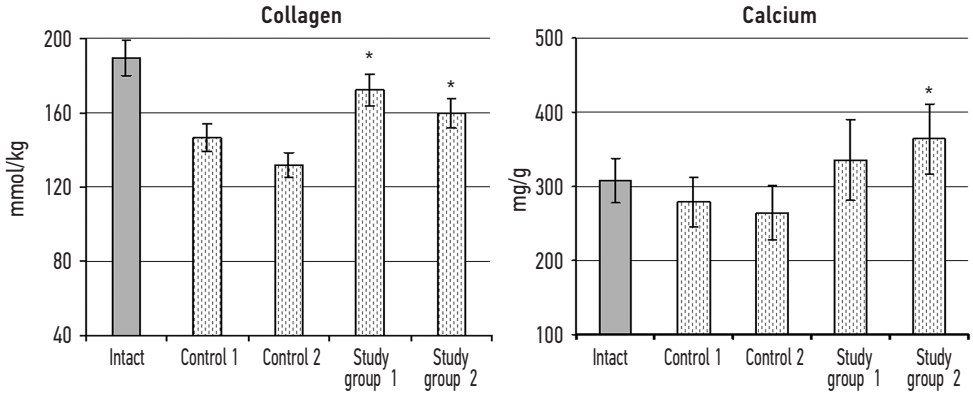

Fig. 1. The content of collagen and calcium in spongy bone tissue in female rats with experimental osteoporosis on the background of anti-osteoporosis drug therapy. ОП1 is an experimental group of 4–6-month-old young females receiving an anti-osteoporotic agent (62.5 mg/kg); ОП2 is an experimental group of 12–14-month-old females receiving an anti-osteoporotic agent (62.5 mg/kg). *$p < 0.05$, the difference is significant compared to the control and 12–14-month-old rats

Рис. 1. Содержание коллагена и кальция в губчатой костной ткани у самок крыс с экспериментальным остеопорозом на фоне терапии антиостеопорозным препаратом. ОП1 — опытная группа 4–6-месячных молодых самок, получавших антиостеопорозное средство (62,5 мг/кг); ОП2 — опытная группа 12–14-месячных самок, получавших антиостеопорозное средство (62,5 мг/кг). *$p < 0,05$, различие значимо по сравнению с контролем и 12–14-месячными крысами

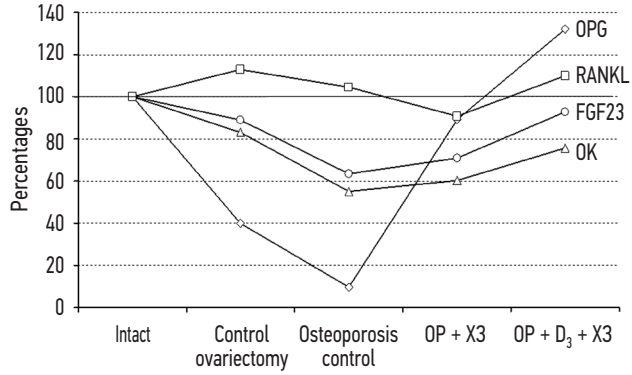

Fig. 2. Dynamics of the content of markers of osteoporosis OK, ORG, FGF 23 and RANKL in blood serum at the stages of formation of an experimental model of osteoporosis and its pharmacotherapy with X3 and vitamin D_3. The data of intact rats were taken as 100%. Here and further: o/э — ovariectomy; ОП — osteoporosis; ОП + X3 — the group receiving the drug X3; ОП + D_3 + X3 — the group receiving the drug X3 and vitamin D_3

Рис. 2. Динамика содержания маркеров остеопороза OK, OPG, FGF23 и RANKL в сыворотке крови на этапах формирования экспериментальной модели остеопороза и его фармакотерапии препаратом X3 и витамином D_3. Данные интактных крыс приняты за 100 %. Здесь и далее: о/э — овариоэктомия; ОП — остеопороз; ОП + X3 — группа, получавшая препарат X3; ОП + D_3 + X3 — группа, получавшая препарат X3 и витамин D_3

Bone remodeling is a continuous process in which bone is renewed to maintain its strength and mineral homeostasis, and bone formation and resorption are closely related. New bone tissue is formed to replace old bone during the remodeling process. The cycle of bone tissue remodeling lasts 5–6 months and is initiated by osteoblasts via the synthesis of RANKL and OPG. RANKL increases osteoclast differentiation and activity by binding to RANK receptors of osteoclasts and their precursors, whereas OPG inhibits RANK receptors, decreasing osteoclast function [38].

Figure 2 shows that the concentration of RANKL receptor ligands increases during OP formation, potentially as a compensatory response to bone tissue resorption activation, and, accordingly, it enters the circulating blood in greater amounts. The content of RANKL in the blood decreases in the subsequent stage of experimental OP correction, indicating a decrease in osteoblast synthesis due to the activation of osteoclastogenesis.

Osteocalcin plays a vital role in bone matrix synthesis. OK is a protein produced by osteoblasts capable of binding calcium and stabilizing the quaternary structure of collagen, hence controlling the formation of bone matrix known as osteon. OK levels decrease when OP is formed, and the decrease in blood concentration reflects the decrease in collagen production by osteoblasts (Figure 2). The anti-osteoporosis drug X3, administered alone and in combination with vitamin D_3, leads to a significant increase in the concentration of OK in the blood, indicating an increase in osteogenesis and osteoblastogenesis.

Along with X3 drug therapy, the concentration of RANKL in the blood decreases, implying osteoblast activation and a decrease in the need for osteoblast potentiation due to RANKL synthesis.

The dynamics of OPG are important. In bone remodeling, OPG, in turn, blocks RANK receptors, inhibiting osteoclast function [38]. OPG reduces osteoclast mobilization, proliferation, and activation by inhibiting the binding of RANK to RANK-ligand; therefore, an increase in RANKL synthesis leads to bone resorption and, consequently, bone mass loss. Figure 2 shows that the decrease in OPG is most pronounced during OP formation, when bone resorption is most pronounced due to osteoclast activation. OPG levels dropped significantly and multifold. Notably, a greater decrease in OPG occurs immediately after ovariectomy, indicating that this process is hormone-dependent. Administration of the drug X3 restores the initial level of OPG in blood, and the combination of X3 with vitamin D_3 causes a significant reliable increase in OPG concentration compared with the intact group.

An important indicator characterizing osteogenesis is FGF-23, which is mainly expressed in bone tissue and synthesized by osteocytes. Its primary role is to reduce serum phosphate levels [4]. FGF-23 regulates serum phosphate levels and vitamin D activity. Because vitamin D increases tubule reabsorption of phosphate, reducing its concentration in the blood suppresses reabsorption and lowers blood phosphate levels. Figure 2 shows that a decrease in FGF-23 levels correlates with a decrease in blood Ca and P levels [1].

Active forms of vitamin D_3 increase FGF-23 gene expression, resulting in negative feedback in the regulation of tubule phosphate reabsorption. Blood FGF-23 concentrations increased significantly in the OP group receiving combination X3 and D_3 therapy than in the X3 monotherapy (Figure 2). In turn, an increase in FGF-23 levels causes a decrease in the active form of vitamin D in the blood [24].

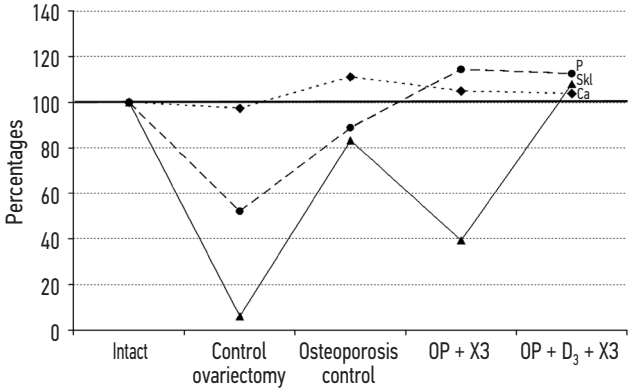

Fig. 3. Dynamics of sclerostin (Skl) and Ca and P ions in blood serum at the stages of formation of an experimental model of osteoporosis and its pharmacotherapy with X3 and vitamin D_3. The data of intact rats were taken as 100%

Рис. 3. Динамика склеростина (Skl) и ионов Ca и P в сыворотке крови на этапах формирования экспериментальной модели остеопороза и его фармакотерапии препаратом X3 и витамином D_3. Данные интактных крыс приняты за 100 %

Sclerostin is a glycoprotein family member produced in osteocytes and some chondrocytes and inhibits osteoblast bone formation. Skl binds to coreceptors on the osteocyte surface and contributes to the disruption of Wnt signaling, slowing down the process of osteoblastogenesis and bone tissue formation [30].

The Skl levels increase during OP formation and decrease after administration of the X3 drug. The Skl levels are normalized by combining X3 with vitamin D_3. Thus, Skl, which plays a vital role in bone tissue metabolism, regulates osteoblast activity via a negative feedback system [3]. Skl function inhibition reduces bone resorption and increases bone tissue regrowth (Figure 3).

Skl expression in osteocytes is primarily regulated by bone tissue metabolic hormones, such as parathyroid hormone, calcitonin, and glucocorticoids [36]. Skl levels in serum were shown to be inversely proportional to the estrogen levels and significantly higher in postmenopausal women [35].

Figure 3 shows the dependence of the dynamics of the Skl level on bilateral ovariectomy, after which a dramatic decrease and subsequent growth occur as OP is formed, which correlates with the dynamics of estrogen levels in the blood. In addition, the phosphorus level represents the dynamics of OP and its pharmacotherapy: the decrease, increase, and subsequent normalization of P and Ca to the intact group level. Such dynamics demonstrate the effectiveness of the anti-osteoporosis therapy. Moreover, the P level in peripheral blood is affected by the intensity of bone tissue resorption and the interaction of vitamin D_3 with FGF-23 expression, thus forming negative feedback in regulating tubule phosphate reabsorption.

Thus, the administration of anti-osteoporosis drug X3, according to the scheme of monotherapy and in combination with vitamin D_3, leads to a significant increase in serum concentrations of bone remodeling markers, indicating an increase in osteogenesis and osteoblastogenesis.

CONCLUSIONS

Unlike other tested drugs, the developed drug X3 increased the effectiveness of prevention and treatment of postmenopausal OP on the experimental pathology model. Due to the increased bioavailability of succinic acid, the use of acidic salts of natural conformer of succinic acid salts in the composition of the new drug affects signaling systems and bioavailability and assimilation of macronutrients (Ca, Zn, and Mg) from the drug's composition. The drug has a pronounced anti-osteoporosis effect when administered 30 times during 30 days in the therapeutic dose.

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Bone remodeling is the process of renewing bone to preserve its strength and mineral homeostasis. Bone remodeling involves the continuous removal of discrete areas of old bone and the replacement of these areas with a newly synthesized protein matrix, which is then mineralized. The findings of the present study evaluating the processes of bone remodeling in OP and anti-osteoporosis therapy show that the effectiveness of the X3 drug is significantly enhanced when combined with vitamin D_3.

The dynamics of bone remodeling markers in serum revealed that the change in these markers reflects the processes of osteoclastogenesis and osteoblastogenesis determined by other methods, notably in terms of macronutrient concentrations and collagen type 1 in bone tissue. The formation of OP and subsequent pharmacotherapy with the anti-osteoporosis drug X3 alone or in combination with vitamin D_3 leads to a significant change in the level of osteoblastogenesis indicators — OC and OPG — in the blood, indicating the potentialization of osteogenesis and osteoblastogenesis.

During therapy with drug X3, there was a decrease in the concentration of RANKL and a significant increase in the level of OPG in blood serum, indicating activation of osteoblasts and a decrease in the potency of osteoclasts due to a decrease in RANKL synthesis. During OP formation, when bone resorption is most pronounced due to osteoclast activation, there is a significant decrease in OPG. Significant and repeated reductions in OPG levels immediately after ovariectomy indicate the hormone-dependent nature of this process.

Thus, administering the anti-osteoporosis drug X3 as monotherapy or combined with vitamin D_3 causes a significant increase in serum concentrations of bone remodeling markers, indicating an increase in osteogenesis and osteoblastogenesis. The proposed anti-osteoporosis drug X3 may contribute to more effective treatment and prevention of age-related and postmenopausal OP and young-onset OP. According to the obtained data, the developing technology of OP treatment may be more effective than the currently accepted schemes associated with calcium overloading when the increase in bone tissue density is primarily caused by a relatively sharp increase in mineralization and subsequent brittleness of bone tissue, rather than by enrichment of the organic component with calcium. The risk of disabling fractures will be reduced with the selected treatment method. Consequently, the financial costs of prevention, treatment, and life support for postmenopausal OP patients will be reduced.

ADDITIONAL INFORMATION

Authors' contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study. The contribution of each author: N.Sh. Mamina, D.A. Lisovskiy, N.A. Fedorov, T.L. Karonova — manuscript drafting, writing and pilot data analyses; A.A. Bairamov, P.D. Shabanov — paper reconceptualization and general concept discussion.

Competing interests. The authors declare that they have no competing interests.

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Ethics approval. The present study protocol was approved by the local Ethics Committee of the Institute of Experimental Medicine (09/06/2022, protocol No. 6).

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