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Effects of Vitamin D and Calcium Treatment on Morphological and Metabolic Changes in Bone Tissue After Ovariectomy

Efectos del tratamiento con vitamina D y calcio sobre los cambios morfológicos y metabólicos del tejido óseo tras la ovariectomía

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SUMMARY

Introduction: *Osteoporosis is a major public health issue, especially among postmenopausal women.* **Objective:** *This study seeks to explore metabolic and morphological alterations in bone tissue following experimental ovariectomy and to evaluate the roles of vitamin D and active calcium in correcting these changes.* **Method:** *26 sexually mature female rats were used, which, after ovariectomy, were divided into two cohorts: one underwent pharmacological treatment. Morphological examination of tissues and biochemical tests were used to assess changes in hormone concentration in blood plasma.* **Result:** *The data demonstrate that on the 30th day after rats' ovariectomy, there was a marked thinning of trabecular (bone beam) structures and the presence of inflammatory foci within Haversian canals. This lead to an increase in free hydroxyproline (FHOP) (0.79 ± 0.07 µmoL/g) and reverses the trend for peptide-linked hydroxyproline (PBHOP) (3.29 ± 0.09 µmoL/g) compared with the control group. After 2 months of pharmacological treatment with vitamin D and active calcium, hydroxyproline decreased to 0.67±0.02 µmol/g and PBHOP increased to 3.74±0.06 µmol/g, indicating suppression of collagen catabolism and activation of bone formation. There was also a decrease in cortisol (201±10.6 ng/mL) and parathyroid hormone (253±11.3 pg/mL), indicating a stabilisation of the hormonal background. The findings from this study offer important insights that could help in designing effective strategies for the treatment and prevention of osteoporosis, especially in postmenopausal women. These results are of considerable value for both scientific inquiry and practical implementation in the fields of orthopaedics and gynaecological endocrinology.*

Keywords: *Estrogen, steroid hormones, mineral metabolism, menopause, osteoporosis.*

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RESUMEN

Introducción: *La osteoporosis es un importante problema de salud pública, especialmente entre las mujeres posmenopáusicas.* **Objetivo:** *Este estudio busca explorar las alteraciones metabólicas y morfológicas del tejido óseo tras la ovariectomía experimental y evaluar el papel de la vitamina D y el calcio activo en la corrección de estos cambios.* **Método:** *Se utilizaron 26 ratas hembra sexualmente maduras que, tras la ovariectomía, se dividieron en dos cohortes: una se sometió a tratamiento farmacológico. Se utilizaron el examen morfológico de los tejidos y las pruebas bioquímicas para evaluar los cambios en la concentración hormonal en el plasma sanguíneo.* **Resultado:** *Los datos demuestran que, a los 30 días de la ovariectomía de las ratas, se observó un adelgazamiento marcado de las estructuras trabeculares (haz óseo) y la presencia de focos inflamatorios en los canales de Havers. Esto condujo a un aumento en la hidroxiprolina libre (FHOP) (0,79 ± 0,07 µmoL/g) e invirtió la tendencia de la hidroxiprolina ligada a péptidos (PBHOP) (3,29 ± 0,09 µmoL/g) en comparación con el grupo control. Después de 2 meses de tratamiento farmacológico con vitamina D y calcio activo, la hidroxiprolina disminuyó a 0,67 ± 0,02 µmol/g y la PBHOP aumentó a 3,74 ± 0,06 µmol/g, lo que indica la supresión del catabolismo del colágeno y la activación de la formación ósea. También hubo una disminución del cortisol (201 ± 10,6 ng/mL) y de la hormona paratiroidea (253 ± 11,3 pg/mL), lo que indica una estabilización del fondo hormonal. Los hallazgos de este estudio ofrecen información importante que podría ayudar a diseñar estrategias eficaces para el tratamiento y la prevención de la osteoporosis, especialmente en mujeres posmenopáusicas. Estos resultados son de gran valor tanto para la investigación científica como para su aplicación práctica en los campos de la ortopedia y la endocrinología ginecológica.*

Palabras clave: *Estrógenos, hormonas esteroides, metabolismo mineral, menopausia, osteoporosis.*

INTRODUCTION

Osteoporosis (OP) is a multifactorial systemic metabolic disease of the musculoskeletal system, characterized by a long, slowly progressive course. In OP, bone remodelling is disrupted, mineral density decreases, and microarchitecture is altered, leading to an increase in traumatic incidents. The medical importance of this disease stems from its widespread prevalence. The population of people suffering from OP numbers more than 200 million and continues to grow. Socially, OP is a particular problem due to gender dimorphism and the ongoing trend of population ageing. Women are 1.5-2 times more vulnerable to this disease than men, which leads to about 40 % of women and 20 % of men experiencing OP-related fractures. Estrogen is a crucial regulator of bone metabolism and the structural integrity of the skeleton. With a decrease in estrogen levels characteristic of the postmenopausal period, the balance between bone destruction and bone formation is substantially disrupted, favoring bone destruction. The current study examines the therapeutic treatment of estrogen-deficient OP. Despite the extensive body of research on various pharmacological agents, the current state of knowledge in this field is characterized by fragmentation and chaotic systematization, especially regarding the potential for combined use of preparations containing bioavailable forms of calcium and vitamin D.

In the context of an in-depth analysis of the effects of various factors on bone matrix parameters, experimental protocols have been implemented using model organisms to monitor alterations at the submolecular and ultrastructural levels. Yousefzadeh et al. (1) described the model to the greatest extent, without delving into areas beyond modelling, and provided valuable assistance to colleagues working in OP studies. A more comprehensive study on the effects of OP on rodents was proposed by Wong et al. (2), who conducted a simulation of fractures in experimental animals suffering from OP, while analysing the features of bone structure regeneration at the microlevel. Ozasa et al. (3) conducted a multiparametric study on the effects of ovarian ablation and nutritional deprivation on the spatial architecture of collagen fibrils and hydroxyapatite crystals in the cortical layer of vertebral osteons in laboratory rodents, demonstrating that osteopenic conditions induce heterogeneous transformations, determined by an aetiological factor, which manifest as anisotropic fluctuations in osteohistological architecture and modulation of elastic constants. In parallel, Harrison et al. (4) focused on intracortical porosity, investigating macroscopic manifestations of osteopenia. Nevertheless, substantial gaps were also identified in

understanding the complex interactions among different structural levels of bone tissue in pathological remodelling conditions, as well as in treating these conditions. In this regard, Wang et al. (5) assessed the therapeutic potential of indolamine derived from epiphyseal tissue in experimentally induced osteopenia. Their study demonstrated melatonin's beneficial effects on the microarchitecture of the osseous matrix, inhibition of osteoclastogenesis, and stimulation of osteoblastic activity, providing a convincing argument for its potential effectiveness in treating osteoporotic conditions. In addition, Wang et al. (6) used a biphosphonate in a similar animal model and did not obtain convincing results regarding its effectiveness in correcting OP. Huang et al. (7) investigated the efficacy of quercetin and its derivatives on bone pathology, bone-related parameters under imageology, bone maximum load, and serum bone metabolism indexes in an animal model of osteoporosis. They demonstrated that quercetin and its derivatives partially reverse osteopenia, probably via antioxidant, anti-inflammatory effects, promotion of osteogenesis, inhibition of osteoclasts, and their estrogen-like effects. Meanwhile, Besschetnova et al. (8) assessed the effects of abaloparatide on bone formation, bone mass, and bone strength in androgen-deficient orchiectomized (ORX) rats, a male osteoporosis model, providing preclinical support for evaluating abaloparatide as an investigational treatment for male osteoporosis.

Current evidence suggests that calcium and vitamin D may modestly improve bone mineral density and support osteoblast function. Still, their interactions with other osteoporosis treatments and optimal dosing regimens remain inconclusive. Their effects on bone homeostasis, including their roles in regulating osteoblast activation and modulating parathyroid hormone secretion, suggest potential synergy with several more expensive agents. Thus, this study aimed to assess morpho-functional and metabolic dysregulation in the osseous matrix induced by experimental ovarian ablation. This study assumes a description of the pathophysiological mechanisms underlying estrogen-deficient OP and an assessment of the potential of targeted pharmacotherapy to restore bone homeostasis. The following postulates were our working hypotheses: ovariectomy induces substantial structural alterations in bone architecture, manifested as thinning, reduced mineral density, and a reduced trabecular apparatus; exogenous administration of vitamin D, in combination with bioavailable forms of calcium, inhibits catabolic processes in bone tissue and stimulates osteogenesis.

MATERIALS AND METHODS

This is an experimental study conducted in the vivarium of the Department of Anatomy and Pathological Anatomy at the Tashkent Pediatric Medical Institute. 36 sexually mature female Wistar rats were used, with an initial weight of 0.18-0.23 kg, kept under standard conditions of light and temperature. The animals were randomly divided into three groups: an ovariectomy group, an ovariectomy group with subsequent pharmacological treatment, and a control sham-operated group, comprising 14, 12, and 10 individuals, respectively. The protocol was approved by the ethics committee, which confirmed its compliance with the basic ethical requirements of the European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes.

Surgical intervention was performed using inhalation anaesthesia. In animals of the experimental groups, bilateral ovariectomy was performed by means of median laparotomy, followed by ligation and excision of the ovaries along with adjacent sections of the fallopian tubes. The control group underwent pseudo-surgery (sham operation), including laparotomy and manipulation of the ovaries without removing them, to create comparable surgical stress.

30 days after the surgical interventions, pharmacological treatment was initiated in one of the ovariohysterectomized groups. The therapy regimen consisted of daily oral administration of Vigantol (contains cholecalciferol (vitamin D3)), a fat-soluble vitamin essential for calcium and phosphorus metabolism), diluted 1:2 with vegetable oil and a suspension of micronized calcium carbonate, all administered using an atraumatic gastric sonda, once a day for 60 days. Afterward, the experimental animals were euthanised by decapitation under deep

ether anaesthesia. For subsequent histological examination, samples of long tubular bones of the hind limbs (femur and tibia) were taken. Bone tissue samples were fixed in a solution of (10 %) neutral formalin for the next two days. This was followed by a twenty-day elimination of calcium in a solution of (10 %) ethylenediamine tetra acetic acid with a pH of 7.4 at a temperature of 4 °C.

After complete demineralization, the samples were subjected to standard histological treatment, including embedding in paraffin. Microtomic tissue sections with a total thickness of 5 microns were treated with hematoxylin and eosin (H&E) to assess the structure of bone tissue, as well as picrofuchsin using the Van Gieson method for visualisation of collagen fibrils. Additionally, histochemical staining by Masson was performed to differentiate between mineralized and non-mineralized bone matrix.

As part of the biochemical analysis of bone metabolism, studies were conducted on bone tissue and blood plasma homogenates from experimental animals.

Assessment of the content of glycosaminoglycans (GAG), using spectrophotometry through a reaction involving the conversion of 5-carboxyfurfural in the interaction of GAG with a combination of carbazole and undiluted H_2SO_4. The colour intensity, proportional to the GAG concentration, was measured at 525 nm.

Determination of hydroxyproline fractions. Free hydroxyproline (FHOP) and peptide-bound hydroxyproline (PBHOP) were quantified using the colorimetric method with Ehrlich reagents. The light absorption levels of the formed chromogen was determined at a wavelength of 558 nm.

Hormonal profile analysis. The concentrations of the following hormones were determined by enzyme immunoassay: estradiol, parathyroid hormone, prolactin, cortisol, and iodised thyroid hormones (T4 and T3).

Equipment setup and quality verification were conducted in strict accordance with the manufacturer's instructions for the reagents. Each measurement was performed in three repetitions. The analysis of all samples obtained from a single experimental animal was conducted during a single analytical session to minimize inter-series fluctuations.

The Statistica 6.0 software package was used to analyze the data obtained. A p-value of <0.05 was considered statistically significant.

RESULTS

Histological examination of bone tissue in experimental animals identified progressive changes in structure and cellular composition throughout the entire follow-up period after ovariectomy. On the 30th day after surgery, pronounced bone resorption was detected, which manifested itself by substantial thinning of the trabeculae and uneven destruction of both the periosteal and endosteal surfaces. Bone beams exhibited heterogeneity in thickness and increased basophilia during staining, indicating a violation of matrix mineralization, a phenomenon described in several other studies. In the periosteal zone of bone tissue, chondroid metaplasia of osteoid tissue was observed. In contrast, on the endosteal surface, a zone with reduced optical density, practically devoid of cellular elements, was visualised (9). The medullary space was characterised by pronounced oedema, decreased cellularity, and disorganisation of the stromal component. In addition, dilation of the Haversian channels was observed, accompanied by the accumulation of lipid inclusions and the formation of foci of granulation tissue, within which an inflammatory infiltrate consisting of both polymorphonuclear leukocytes and mononuclear cells prevailed (Figure 1).

At the end of the initial stage of the study, on its thirtieth day, architectural changes in the bone trabeculae intensified, which was manifested by further deformation and unevenness of their contours. Microfractures were observed in some areas due to the critical thinning of bone beams and their replacement with connective tissue elements, which is a typical sign of OP (10,11). Foci of increased basophilia were detected intrabecularly, indicating a disturbance of calcium homeostasis and focal chondroid transformation. The peripheral zones of trabeculae showed a dominance of osteoclasts over osteoblasts, and

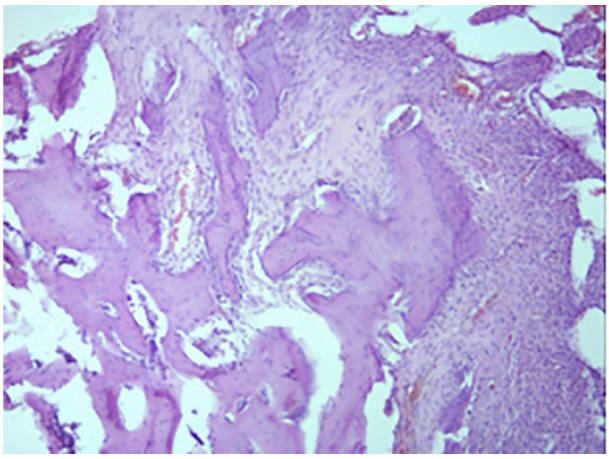

Figure 1. 30 days after surgery. Thinning of bone beams, loosening of the bone marrow.
Note: color: H&E. Magnification: 10. Lens 40. Source: created by the authors.

osteoclasts were characterized by hypertrophy of both cytoplasmic and nuclear components, indicating increased functional activity. The lumen of the Haversian canals was substantially expanded and filled with adipocytes, granulation tissue elements, and inflammatory infiltrate, which was dominated by lymphocytes and macrophages, including cells of both monocytic and osteoclastic origin (Figure 2).

At the sixtieth day of the experiment, the morphological picture of the bone tissue became even more diverse: in some areas, pronounced deformation and unevenness of the trabeculae were observed, while in other areas, progressive thinning of the bone beams continued. Foci of destruction and lysis of the mineralized matrix were observed in some areas, manifested by defects and zones of enlightenment in the trabecular bone structure, reflecting the increasing imbalance of mineralization and the intensification of chondroid metaplasia, as noted by other authors. Calcium precipitates and fragments of mineralised bone matrix destruction were identified at the boundary between bone beams and intertrabecular soft tissue elements, which confirms the activation of the pathological process. Osteoclasts localised along the periphery of bone trabeculae showed morphological signs of increased resorptive activity. A substantial increase in the number of inflammatory cells, mainly of lymphoid and histiocytic origin, was observed in the expanded lumen of the Haversian canals. Macrophages of both monocytic and osteoclastic origin predominated in the inflammatory infiltrate, indicating high bone remodelling activity (12,13) (Figure 3).

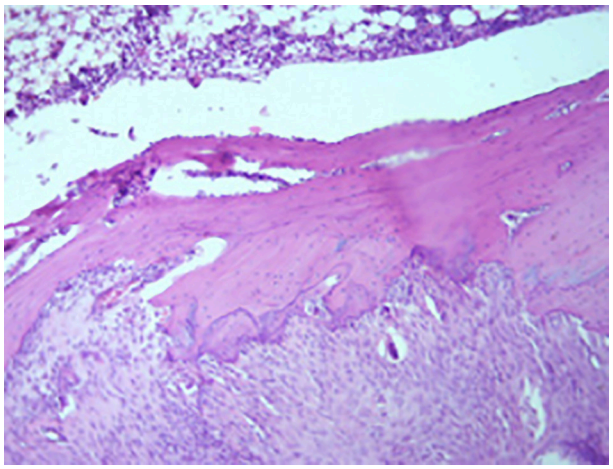

Figure 2. Day 30 of the experiment. Deformation of bone beams due to OP, lipomatosis of Leeuwenhoek channels, formation of an array of granulation tissue and progression of inflammatory infiltrate growth. Note: color: H&E. Magnification: 10. Lens 40.
Source: created by the authors.

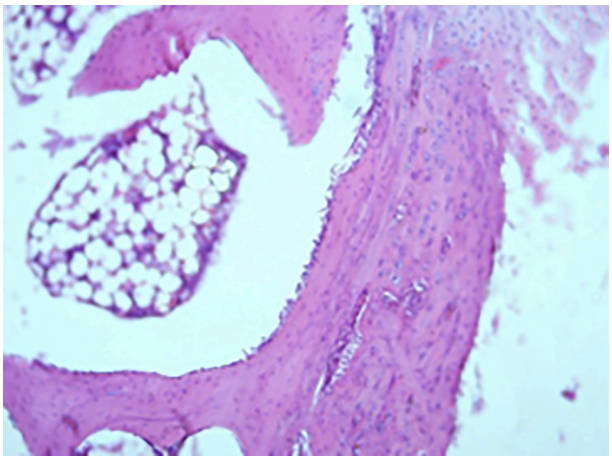

Figure 3. Monocytic macrophages and osteoclasts are among the inflammatory infiltrates.
Note: colour according to Van Gieson; Magnification: 10; Lens: 40. Source: created by the authors.

During the final stage of the study, at the ninetieth day of the experiment, bone tissue degradation increased, resulting in combined atrophic and destructive changes. The pathological process spreads in a gradient from the proximal to the distal bone, apparently due to polymorphisms in mechanical stress levels and vascularization of these segments. Compact bone tissue was characterised by uneven mineralisation, with a particularly noticeable weakening of the structure at the periphery, where osteoclasts were present, closely associated with the bone surface. In some areas, osteoid layers were observed on bone trabeculae, which may reflect compensatory osteoblast activity in response to increased resorption (14,15). Loose connective tissue prevailed in the intertrabecular spaces, in which foci of myxomatous transformation were noted. Areas of cell-free enlightenment containing bone fragments and calcifications were observed along the bone plates, indicating a disruption of remodelling and mineralisation. The interplate spaces were mainly filled with loose fibrous connective tissue. In contrast to the earlier stages of the experiment, there was a negative trend regarding the cellularity in the soft-tissue components. Residual cellular elements in the stroma demonstrated morphological characteristics typical of chondroblasts or macrophages. The fibrous structures of connective tissue were arranged randomly, forming relatively thick bundles, which is confirmed by other studies (Figure 4).

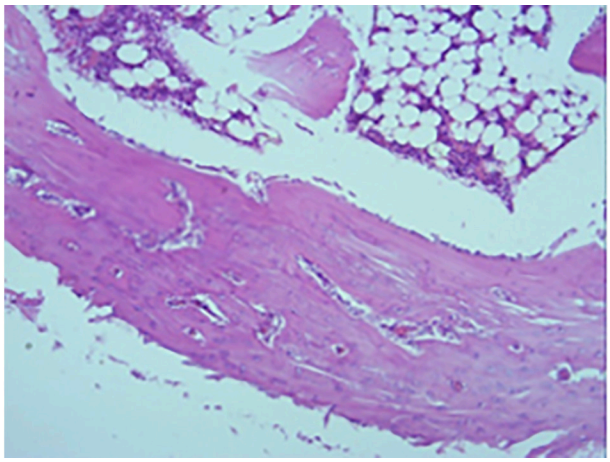

Figure 4. Day 90 of the experiment. Substantial atrophy of compact bone plates, replacement by fibrous connective tissue. Note: colour according to Van Gieson; Magnification: 10; Lens: 40.
Source: created by the authors.

Summarizing morphological changes of bone tissue at various stages of experimental modeling of OP, it can be noted that, before reaching atrophy, the tissues underwent dystrophy and destruction, with successive changes. The pathological process initiated in the proximal part of the bones, spreading distally, and the most pronounced changes were observed in the diaphyseal segment. During the initial stage of the experimental period, intensive bone plate resorption was dominant, accompanied by expansion of the Haversian channels, followed by the formation of granulation tissue and the development of inflammatory infiltration. As the experiment progressed, atrophic and destructive changes in bone plates intensified, characterised by chondroid metaplasia and uneven calcification. An overgrowth of fibrous connective tissue morphologically corresponding to fibrous bone dysplasia was observed in the Haversian canals.

An analysis of the effect of combined therapy with vigantol and active calcium on osteoreparation processes in experimental OP revealed substantial changes at the 60th day of drug administration. The first signs of reparative regeneration activation were observed in the vascular-stromal elements of the endost. The vessels of the Haversian canals showed expansion and fullness, accompanied by activation of cellular elements, as evidenced by hypertrophy of both endothelial cells and pericytes. Cellular elements of the connective tissue within the Haversian canals were characterized by increased metabolic activity, as evidenced by hyperchromasia and hypertrophy of nuclear structures. Osteoblasts with a characteristic eosinophilic cytoplasm were concentrated along the inner surface of the bone plates, forming new osteoid structures. In parallel, signs of osteogenesis activation were observed in the compact bone, including osteoid hyperchromasia and osteocyte hypertrophy (Figure 5).

An assessment of morphological changes on the 60th day after initiation of complex therapy revealed substantial modifications of the cellular composition in bone tissue that had

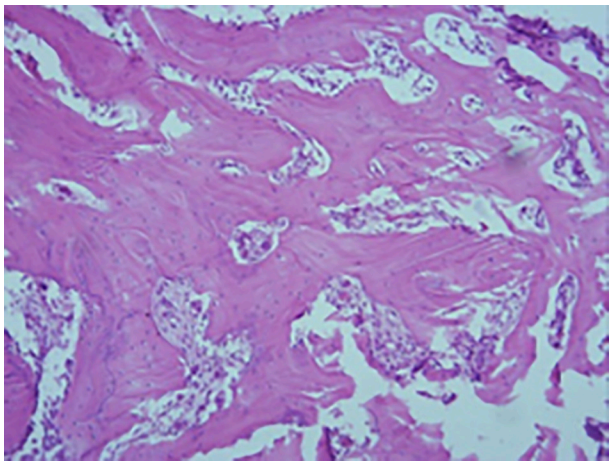

Figure 5. Day 60 of the second experimental group. Vasodilation, hyperchromasia, and hypertrophy of connective tissue cellular elements, and the appearance of primary osteoid. Note: Color: H&E, Magnification: 10. Lens 40. Source: created by the authors.

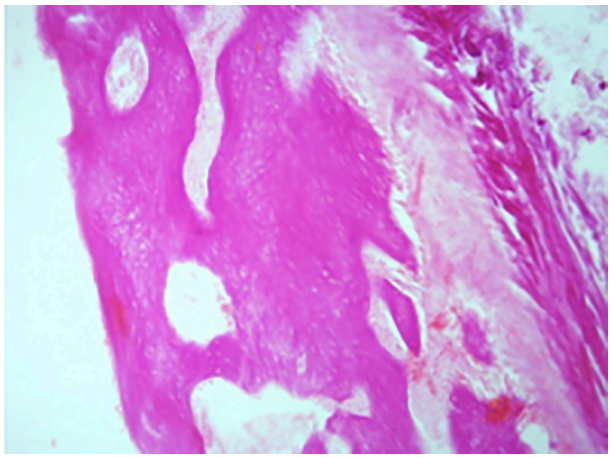

Figure 6. Day 60 after the start of treatment. Visualisation of large osteoblasts and osteocytes in the connective tissue of the Haversian channels. Note: colour according to Van Gieson; Magnification: 10; Lens: 40. Source: created by the authors.

undergone osteoporotic changes. There was a noticeable increase in the population of large hyperchromic cells identified as osteoblasts and osteoclasts, localised mainly in the connective tissue component of the Haversian canals. The cytoplasm of these cells was characterised by hematoxylin-positive inclusions, indicating calcification. Osteoblasts tended to adhere to a compact bone plate, forming de novo osteoid structures, suggesting the activation of osteogenesis processes (Figure 6).

The progression of changes induced by therapy was characterized mainly by the development of signs of reparative bone consolidation. This manifested as an intense proliferation of fibroblasts in both the periosteal zone and the Haversian canal area, a characteristic of osteoreparation processes (16,17). Fibroblasts were characterised by hypertrophy, increased metabolic activity, and hyperchromasia of the nuclei, and a substantial part of these cells showed morphological signs of differentiation towards osteoblasts and osteocytes. The cytoplasmic compartment of these cells contained both eosinophilic protein masses and hematoxylin-positive inclusions, indicating mineralisation processes.

On the 90th day of the treatment period, as shown in Figure 7, the examined bone tissue samples showed a decrease in the severity of osteodystrophic and osteoporotic changes. Proliferative processes characteristic of the intensified reparative regeneration of bone tissue have become dominant. Pronounced fibroblastic and osteoblastic reactions were observed in the Haversian canals, accompanied by the formation of newly formed osteoid structures both on the surface of compact structures and in the lumens of the Haversian canals. On the surface of compact bone plates, osteoblasts formed monolayer palisade-like structures showing signs of active osteogenesis. In the lumen of the Haversian canals, osteoblasts were organised into parallel-oriented osteoid colonies, which in some areas were anastomosed with compact bone tissue, contributing to its thickening. Similar morphological transformations were observed in the periosteal zone, with the formation of new osteoid bone layers. These changes indicate complex osteogenic activation, affecting both the endosteal and periosteal compartments of the bone, suggesting the systemic nature of the treatment combination's consequences.

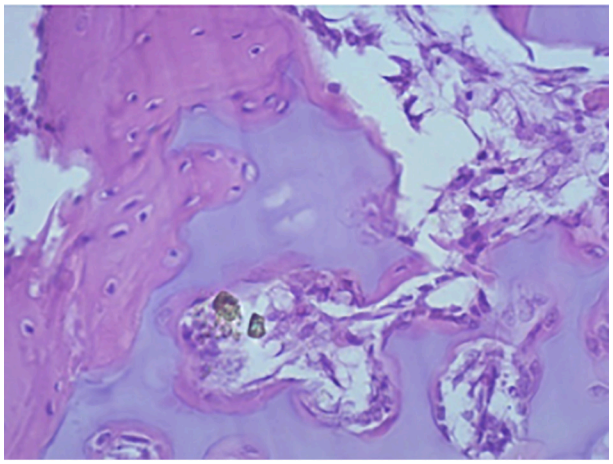

Figure 7. Day 90 of treatment. The appearance of fibroblasts and osteoblasts on the surface of the plate and in the lumen of the osteon. Note: Color: H&E, Magnification: 10, Lens 40. Source: created by the authors.

Thus, the dynamics of morphological changes against the background of ongoing therapy demonstrate a consistent activation of reparative processes in bone tissue, characterised by an intensification of proliferation and differentiation of osteogenic cell populations, increased osteosynthesis, and the formation of new bone structures. These data suggest the potential of combining vigantol with active calcium to restore osteoporotic changes and stimulate osteoreparation.

On the 90th day after ovariectomy, biochemical analysis of bone homogenates from experimental animals revealed an increase in FHOP concentration, a marker of increased degradation of type I collagen. These changes were accompanied by a decrease in the concentrations of PBHOP and GAG, indicating increased resorption and catabolism of these structures. In rats receiving combination therapy with vitamin D and active calcium for two months, activation of regenerative processes in bone tissue was observed, which was characterized by a decrease in the intensity of collagen catabolism, as confirmed by an increase in PBHOP levels, increased synthesis of proteoglycans, and a reduction in the concentration of FHOP (Table 1).

Table 1. The concentration of FHOP, PBHOP, and GAG in bone tissue homogenates of the examined animals after surgery.

Group of animals	**Analyzed indicators, *μ*moL/g**		
	FHOP	**PBHOP**	**GAG**
Control sham, n=10	0.49±0.03	4.08±0.13	1621±14.6
Ovariectomy group, n=12	0.79±0.07**	3.29±0.09**	959±11.3***
Ovariectomy group and treatment, n=14	0.67±0.02*	3.74±0.06**	1121±21.7*

Note: *** – p≤0.005; ** – p≤0.01; * – p≤0.05: differences relative to the control group.

A detailed study was conducted to assess bone tissue metabolic activity by examining the incorporation of radioactively labelled amino acids into bone tissue protein fractions. Proline and tyrosine were chosen as labels since they differ in their content in collagen and non-collagen bone proteins: proline and its derivative, hydroxyproline, constitute up to 19 % of the amino acid composition of the key protein forming the bone matrix, while tyrosine makes up an insubstantial part (about 2 %) of the total amino acid pool (18,19). Accordingly, the level of radioactive proline incorporation directly reflects the intensity of collagen protein synthesis. In contrast, the incorporation of labelled tyrosine mainly characterises the synthesis of non-collagen proteins, which is especially important for assessing the general state of bone metabolism.

The analysis of the hormonal profile conducted at the final stage of the experiment revealed substantial changes in the experimental animals' hormonal profile. There was a noticeable decrease in estradiol and iodothyronines, hormones with osteogenic effects and important for maintaining the normal state of bone tissue. Synchronously, increases in prolactin, parathyroid hormone, and cortisol concentrations were recorded, which enhance catabolic processes and increase bone resorption, thereby worsening bone structure (Table 2).

Table 2. Levels of blood plasma hormones of experimental animals during ovariectomy with and without treatment

Analysed indicators	**Control-sham, n=10**	**Females with ovariectomy**	
		Without treatment, n=12	**With treatment, n=14**
Estradiol, pg/mL	25.2±1.22	13.6±1.02***	18.6±1.12***
Prolactin, mLME/L	13.3±1.02	16.1±0.62** p=0.0261	14.2±0.14**
Total T3, nmol/L	2.32±0.06	1.92±0.04** p=0.0425	2.21±0.06**
Total T4, nmol/L	52.5±2.11	47.4±2.24** p=0.0173	49.8±2.08*
Parathyroid hormone, pg/mL	17.8±1.08	29.9±1.32** p=0.0271	25.3±1.13*
Cortisol, ng/mL	16.6±1.03	23.4±1.12***	20.1±1.06**

Note: *** – p≤0.005; ** – p≤0.01; * – p≤0.05: differences relative to the control group.

Sex steroids are the main factors controlling bone metabolism. The main effect of estrogens on bone is to inhibit the processes of resorption by direct action on osteoclast precursors and inhibition of osteoclastogenesis (20). Unlike their predecessors, mature osteoclasts do not express estrogen receptors, so the action of these hormones is mediated by osteoblasts, which, upon estrogen stimulation, reduce the secretion of proosteolytic factors. In conditions of sex steroid deficiency, typical of the postmenopausal period, an imbalance in bone remodelling is observed, with bone tissue destruction prevailing over its restoration, leading to osteopenia and, subsequently, OP.

Thus, a comprehensive analysis of molecular indicators of osteometabolism, including free and PBHOP levels, protein synthesis intensity, and hormonal status, demonstrates that ovariectomy causes profound systemic changes that disrupt the balance of bone remodelling processes, favouring increased resorption. These changes are associated with both the inhibition of osteoblast osteogenic activity and the potentiation of osteoclast activity. The use of combination therapy with vitamin D and active calcium, can partially normalise the disturbed processes (21,22). This therapy promotes the restoration of bone homeostasis by stimulating osteogenesis—the formation of new bone tissue—and suppressing excessive catabolism of the bone matrix, as evidenced by increased PBHOP levels and decreased FHOP levels (Table 1).

Due to these properties, combination therapy with vitamin D and calcium appears to be an effective approach to the prevention and treatment of OP, especially in postmenopausal estrogen deficiency, which not only slows bone tissue destruction but also promotes its repair.

DISCUSSION

Ovariectomy (OVX) in rodents is the most widely accepted experimental model for postmenopausal osteoporosis, mimicking estrogen-deficiency-induced bone loss seen in

human females. Estrogen deficiency is a key driver of postmenopausal osteoporosis. OVX surgically removes ovaries, inducing a rapid decline in estrogen levels (23). This leads to increased osteoclast activity and bone resorption, a decreased osteoblast function and bone formation, and a net loss of bone mineral density (BMD), especially in trabecular bone. The present results confirmed that ovariectomy, simulating postmenopausal estrogen deficiency, causes substantial structural and metabolic changes in bone tissue in female rats. The identified changes include progressive bone resorption, significant thinning of trabeculae, and a decrease in bone mineral density, characterised by increased osteoclast activity and a disruption of the balance of bone remodelling.

In the context of experimental modelling of various metabolic scenarios and their ability to provoke the development of osteoporosis (OP) or exacerbate its course, Saleh et al. (24) investigated the effect of visceral obesity on bone structure in ovariectomized rats. This study used a model of postmenopausal OP that is similar in characteristics to the one described in the present work. Still, there are minor differences between them in terms of sample size and observation duration. Both studies used experimental animals of similar size, age, and species, which makes the results comparable. However, in the present study, factors like obesity were not considered. In contrast, Saleh et al. found that obesity's impact was a key aspect of the study; however, they did not evaluate the effect of pharmacological treatment. Combining the results of both studies allows consideration of metabolic factors (in particular, obesity) and potential therapeutic approaches in the context of postmenopausal OP. Such an integrated approach opens a broader field for further experiments that could combine the analysis of the effects of metabolic disorders with the effectiveness of pharmacological interventions for osteoporosis. This complementarity of research highlights the importance of a multifaceted approach to studying OP, considering both hormonal and metabolic underlying causes of the disease. In the future, this may lead to the formation of more effective and personalised OP management strategies, especially in patients with concomitant metabolic disorders.

Ying et al. (25) examined the impact of diabetes on the course of OP, emphasising that in addition to obesity during menopause, diabetes can also be a substantial aggravating factor. This aspect is especially important because diabetes, as an endocrinological disease, increases the risk of developing OP due to complex metabolic changes associated with hyperglycemia and oxidative stress (26,27). Thus, diabetes is an important factor in developing comprehensive therapeutic strategies to prevent and treat OP.

Regarding OP treatment, our study demonstrated that 2 months of pharmacological treatment with vitamin D and active calcium promotes bone tissue regeneration, as confirmed by an upward trend in PBHOP, increased proteoglycan biosynthesis, and a downward trend in FHOP. These data indicate suppression of bone matrix catabolism and restoration of calcium homeostasis, as confirmed by morphological data demonstrating improved bone structure and decreased osteoclast activity. Lee et al. (28) investigated the anti-osteoporosis activity of the *Salvia miltiorrhiza* ethanol extract (SME) in osteoporosis-prone conditions, ovariectomized (OVX) and naturally menopaused (NM) ICR mice. They found that SME suppressed the loss of trabecular bone via suppressing bone resorption and osteoclast differentiation both in OVX and NM mice. SME is likely to be developed as a therapeutic agent for osteoporosis. This study employed similar experimental approaches to those used in this work; however, there are important differences in design, sampling, and analysis methods. The animal models in both studies are similar in size and age, allowing their results to be comparable. However, Lee et al. focused on models with various conditions of estrogen deficiency, including acute and chronic, thereby enabling the evaluation of the effectiveness of *Salvia miltiorrhiza* across a broader range of endocrine system disorders. Histological analysis methods in both studies include standard approaches, such as micro-computed tomography (Micro-CT), as well as biochemical markers of bone metabolism. Nevertheless, this study offers a more detailed analysis of the molecular mechanisms, including an assessment of gene expression related to osteoclast differentiation and activation. While this study focuses on a more extended follow-up

period, this suggests long-term effects of therapy, although the influence of genetic factors on its outcome remains unknown.

Oliveira et al. (29) examined the effect of lycopene on osteoblastic activity and bone protection in ovariectomised rats. It highlights lycopene's antioxidant properties in preventing bone loss, especially in conditions where hormonal deficiency increases the risk of OP. Unlike other approaches that focus on hormonal or pharmacological interventions, this study focuses on dietary interventions, such as antioxidant supplementation, which can play a key role in maintaining bone health. This study expands the understanding of how OP can be mitigated not only through medications but also through dietary changes. This complements the picture by suggesting that antioxidant therapy be considered as a possible addition to traditional methods for combating OP.

Wang et al. (30) made a substantial contribution to understanding the role of inflammatory mediators in the pathogenesis of OP, especially in conditions of estrogen deficiency caused by ovariectomy. Using an experimental OP model, the authors demonstrated the multifaceted effects of geranyl on bone tissue. According to the results, the effect of geranyl is not limited to strengthening the density and shape of skeletal tissue but also substantially reduces the level of inflammatory signalling molecules that function as catalysts for osteoresorption. In particular, it was determined that geranyl effectively inhibits the production of signaling molecules, including IL-6 and TNF-α. The mechanism of action of geranyl, involves reducing the inflammatory background and, consequently, slowing bone resorption (31,32). This observation is important because it allows considering geranyl as a potential component of complex OP therapy aimed not only at stimulating bone formation but also at inhibiting inflammatory reactions that contribute to bone tissue destruction. While the current study focuses on alternative aspects of the pathogenesis and therapy of OP, the inclusion of anti-inflammatory strategies, such as geranyl, can substantially enhance the effectiveness of anti-osteoporotic treatment.

Based on the evidence, the multifactorial nature of OP becomes apparent, characterised by complex interactions among endocrine, metabolic, and inflammatory processes. Studies examining the effects of visceral obesity, diabetes, oxidative stress, and inflammatory mediators on bone metabolism reveal the versatility of the pathogenetic mechanisms of OP. Each of the examined factors has the potential to become a target for one or more correction methods. The integration of these data with results from the use of pharmacological agents, phytocomponents, and nutraceuticals opens the door to the development of multi-vector therapeutic strategies. Such an integrated approach allows not only targeting specific links in the pathogenesis but also personalising treatment based on patients' individual characteristics.

CONCLUSIONS

The results of the study confirmed that ovariectomy, simulating postmenopausal estrogen deficiency, causes substantial structural and metabolic changes in bone tissue in female rats. The identified changes include progressive bone resorption, significant thinning of trabeculae, and a decrease in bone mineral density, characterised by increased osteoclast activity and a disruption of the balance of bone remodelling. Biochemical analyses showed an increase in the concentration of FHOP, which indicates an increase in the degradation of a key element of the bone matrix – type I collagen. Concurrently, the opposite observation was noted for PBHOP and GAG, confirming the intensification of catabolic processes in bone tissue. Combination therapy with vitamin D and active calcium demonstrated high effectiveness in reversing osteoporotic changes.

It was determined that this therapy promotes bone tissue regeneration, as confirmed by an upward trend in PBHOP, increased proteoglycan biosynthesis, and a downward trend in FHOP. These data indicate suppression of bone matrix catabolism and restoration of calcium homeostasis, as confirmed by morphological data demonstrating improved bone structure and decreased osteoclast activity. The results obtained emphasise the importance of a holistic, universal approach to the management of postmenopausal

OP, encompassing not only hormonal correction but also agents that restore bone structure and function. These data can be used to form effective approaches to preventing the development and correction of OP in postmenopausal women, which has substantial clinical importance in light of the threatening epidemiology of this pathology. Thus, the study substantially contributed to understanding the pathogenesis of postmenopausal OP and highlighted the potential of combined pharmacotherapy in improving clinical outcomes in this disease.

The limitations of this study primarily consisted of the inability to determine the extent of genetic factors' influence on pharmacological correction outcomes and the isolated consideration of the hormonal factor, independent of the potential impact of other variables. Subsequent research in this area should aim to expand on existing ideas about the mechanisms underlying the interaction between hormonal and metabolic factors that cause and characterise OP, as well as to optimise combination therapy for its prevention and treatment. Special attention should be paid to identifying additional biomarkers that better reflect bone metabolism in conditions of hormonal deficiency. In addition, there is a need to implement projects with longer experimental observation periods to assess the effectiveness and safety of therapeutic approaches being developed in clinical settings, thereby enabling the development of more personalised strategies for the treatment of postmenopausal OP. Solving these tasks will substantially optimise the quality of life, especially for postmenopausal women.

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