

Research Article

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Effects of zoledronic acid on bone structure and organization of nanocomposites in rats with obesity and limited mobility

<https://doi.org/10.1515/ersc-2021-0002>

received November 4, 2021; accepted March 20, 2022

Abstract: Background: Some investigations show that obesity is associated with increase in bone mass due to excessive mechanical exertion. However, these data are contradictory as loss of mineral density of bone tissue and, respectively, the risk of fractures in this population group is higher. The aim of the research was to investigate impact of drug therapy with zoledronic acid on nanostructure of bones in rats with limited mobility and high-calorie diet.

Methods: Rats (n = 56) were distributed into three groups: control (n = 18) – standard vivarium conditions, I experimental group (n = 18) – rats, which were on a high-calorie diet with limited mobility (HCD+LM), II experimental group (n = 18) – HCD+LM+zoledronic acid. Zoledronic acid was injected at the dose 0.025 mg/kg intramuscularly every four weeks for six months. X-ray structure analysis, scanning electron microscopy and atomic absorption spectrometry were used for investigation of ultrastructure and quantitative assessment of mineral component loss in the femoral neck.

Results: Obesity and limited mobility reduced the level of the mineral component in the femoral neck (–31.5%) compared with control. It is significant that zoledronic acid did not permit decrease in mineral component of the bone throughout the entire experiment compared with group I (+41.8%), and all parameters were higher than in control group (+15%).

Conclusions: Obesity and limited mobility negatively affect mineral bone mass. Zoledronic acid induces increase in the mineral component as a result of remod-

eling inhibition under conditions of obesity and limited mobility modeling.

Keywords: bone remodeling, bone mineral density, osteoporosis, X-ray diffraction, field emission scanning electron microscopy

1 Introduction

Pathological disorders of locomotive apparatus in humans are increasing every year worldwide, which is associated with intake of steroid hormones, menopause, obesity, sedentary lifestyle etc. [1-3]. It was considered that excessive body weight commonly has a beneficial effect on condition and remodeling of bones due to established positive effect of intensive mechanical load. However, this mechanism remains contradictory. Pathophysiological association between obesity and bone is complex and remains an active domain for investigations [4, 5]. However, there are investigations that patients with obesity and sedentary lifestyle are at risk of developing pathologies of locomotive apparatus and loss of mineral mass of the bones. Such changes can result in osteoporosis and other diseases, which are characterized by low density of bone tissue and microarchitecture changes that lead to an increase in bone fracture risks. In literature sources, it is reported that nutrition and sedentary lifestyle are among many factors, which affect bone mass and are significant for bone tissue condition [6, 7]. There are also records that loss of mineral density of bone tissue is frequently observed in elderly and women in postmenopausal period [2]. Since risk factors of pathological fractures depend not only on mineral mass of its trabecular and cortical layers, but also compositional peculiarities – microarchitecture of the bone [12], these indices should be taken into consideration for assessment of bone tissue condition. In some cases, there is a need to administer pharmacotherapy additionally for such patients to correct bone tissue condition [8, 9]. Some researchers compare the anti-os-

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teoporotic property of propranolol with zoledronic acid and alfacalcidol. This study shows that the bone resorption induced by immobilization/disuse in rats can be suppressed by treatment with propranolol. Treatment with propranolol also improved the microarchitecture of cortical bones when compared with immobilized control, as indicated by scanning electron microscopy [10, 11]. In our study, we used one of the most common drugs in clinical use – bisphosphonate, zoledronic acid, which can inhibit osteoclasts [12, 13]. In our experiment on rats, assessment of bone tissue condition and its correction with this medicine was investigated by X-ray structure analysis and electron microscopy [14-16].

Aim. To study influence of zoledronic acid on remodeling process and microstructure of bone tissue in rats' femoral neck under conditions of long-lasting modeling of obesity and limited mobility.

2 Materials and methods

2.1 Experimental design

Experimental investigation was performed on 54 Wistar male rats weighing 180-200 g. Animals were kept at constant 12-hour cycle of light and darkness, air temperature 21-23° C and relative humidity 60 ± 10%. All animals were kept in hanging cages with mesh bottom to prevent coprophagy. Experimental rats were distributed into three groups with 18 animals in each: control group – rats, which were kept in standard vivarium conditions and followed a standard diet and water ad libitum; experimental group I – rats on a high-calorie diet (C 11024, Research Diets, New Brunswick, NJ), which were kept in cages with limited mobility (HCD+LM) [17], II experimental group – HCD+LM + injection of zoledronic acid (HCD+LM+Zol.). Rats in the control group were injected 0.3 ml of physiological solution 0.9 % NaCl subcutaneously. Zoledronic acid was administered at a dose of 0.025 mg/kg (0.1 ml ZOLTA® + 0.9 ml, 0.9% NaCl) intramuscularly (quadriceps femoris) [14]. In 8th, 16th and 24th weeks, six rats were euthanized from each group by decapitation under general intraperitoneal urethane anesthesia at a dose 0.3 g/kg.

Ethical approval: The research related to animal use has been complied with bioethical principles of The European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes as well as approved by Ethics committee of Danylo Halytsky Lviv

National Medical University (report of the Ethics committee № 10, dated 16.12.2019).

2.2 X-ray diffraction

The bones was dried at 110 °C. The X-ray diffraction spectra of the samples were obtained on an automated X-ray diffractometer in Cu K α radiation ($\lambda=1.5418$ Å), monochromatized by reflection from a plane (002) of a single pyrographite crystal, mounted on a diffracted beam, Bragg-Brentano focusing scheme ($\theta - 2\theta$) [15 - 17]. The diffraction patterns were recorded in the continuous movement mode of the detector with an angular velocity of 2°/min, a constant value of the integration time $\tau = 1$ s., x-ray tube voltage at $U = 26$ kV, and anode current at $I = 15$ mA. The volume fraction of the crystalline phase represented by hydroxyapatite was determined through the following formula: $X=I_a/I$, where I_a is the integral intensity of hydroxyapatite, and I is the total integral intensity. The average crystallite size was determined by the Debye-Scherrer formula [18, 19] per the diffraction maximum (002) extension: $L=\lambda/(\beta \cos(\theta))$, where $\lambda=0.15148$ nm is the wavelength of the x-ray radiation, β is the physical half-width of the maximum (002), 2θ is the reflection diffraction angle (002) of the crystalline phase.

2.3 Atomic Absorption Spectrometric Investigation

To determine calcium in bone tissue, samples were prior mineralized by dry ashing method with further acid extraction. Element composition was determined in prepared samples by the method of atom-absorption spectroscopy on a device C-115PC using acetyl/air mixture.

2.4 Field emission scanning electron microscopy

For the investigations of the microstructure of the bone samples a field emission scanning electron microscopy (FESEM) analysis was performed using a Hitachi S-4100 microscope with a secondary electron detector. The scanning of the sample surface was carried out by electron beam operating at 15 kV and 10 μ A with the spatial resolution of 100 nm in the secondary electron image regime [20-22].

2.5 Statistical analysis

Statistical analysis of the data was performed in StatSoft STATISTICA 8.0.360. In the STATISTICA package, the comparison of two average samples of normally distributed features (Student's t-criterion) was implemented in the *Basic Statistics/Tables* module. The t-test, independent, by variable submodule, was used for two different general summations.

3 Results

Weight of laboratory rats in group I (HCD+LM) increased from 194.6 ± 6.1 g to 340.8 ± 8.6 g by the 24th week, which indicates a statistically significant increase in weight that constituted 20 % compared with control group ($p < 0.05$). In the group HCD+LM+Zol. weight increased from 198.3 ± 12.5 g to 329.6 ± 17.4 g by the 24th week, increase in body

mass constituted approximately +17.2 % ($p < 0.05$) compared with control.

To investigate impact of high-calorie diet, limited mobility and zoledronic acid on bone tissue condition we assessed microarchitecture of the femoral neck by means of X-ray structure analysis. Diffraction patterns of samples are presented in figure 1B compared with theoretical diffraction pattern of chemical solution $\text{Ca}_{10}(\text{PO}_4)_2(\text{OH})_2$ (hexagonal crystal family, space group P 63/m, parameters of lattice cell $a=9.42$ Å, $c=6.88$ Å) fig. 1A [23]. Significant blurring of diffraction maximums of the crystalline phase indicates low degree of compound crystallinity, which is caused by a small size of the regions of coherent diffraction (size of crystallites did not exceed 12 µm, table 1). Besides, a wide diffuse halo is observed on diffraction patterns in the region of diffraction angle $2\theta \approx 21^\circ$, which demonstrates presence of the amorphous phase (of collagen) in samples.

In a series of femoral neck fragments, the highest level of the amorphous phase is observed in samples of

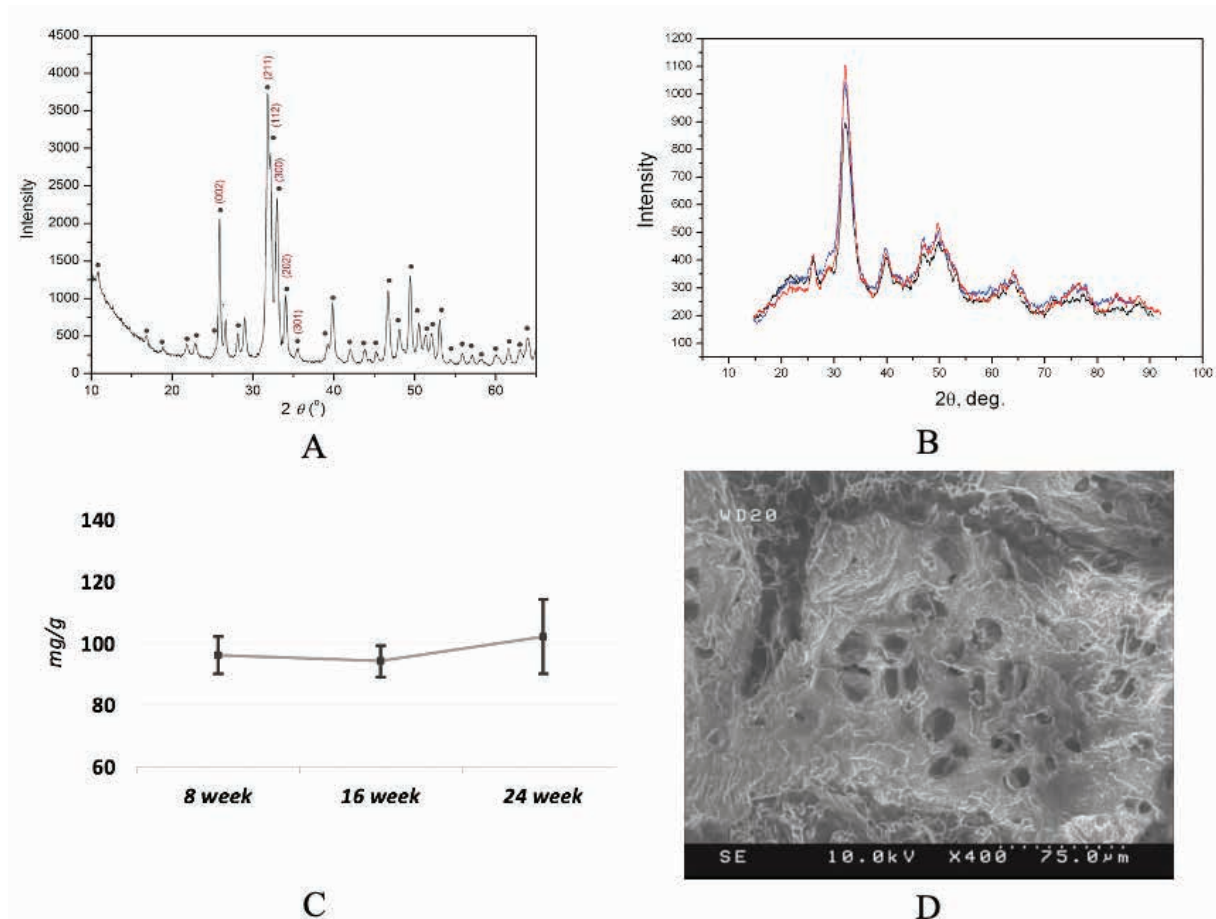


Figure 1: Typical experimental XRD spectrum of hydroxyapatite (Hap.) (1A) sample [23], on the spectra of samples (1B) in the sequence on - 8th; - 16th; - 24th week of investigation, the volume fraction of the crystalline phase does not change in rats of the control group. Content of calcium (mg/g) in fragments of the femoral neck in rats (1C). FESEM micrograph showing the spherical morphology of 15,8±0,5 nm Hap. particles (1D).

the first group during the 8th week of the experiment. Decrease in intensity of diffuse maximum of these samples demonstrates reduction of the amorphous phase level and increase in crystallinity. Meanwhile, decrease in both diffuse maximum and intensity of maximums of the crystalline phase is observed on the 24th day, which is particularly evident in the region of the most intensive lines (211), (121), (112) and (300) of the hydroxyapatite phase.

Figure 2 demonstrates a significant increase in intensity of the crystalline phase line in diffraction patterns of the femoral neck in rats with obesity and limited mobility during the 8th week (Fig. 2A). Besides, there is a noticeable decrease in intensity of diffuse background of the amorphous phase in rats of this group starting from the 16th week, which can indicate accelerated bone remodeling and further loss of a mineral component. The results of assessment of both phases (table 1) showed that maximum increase in the crystalline phase is observed in samples of group II during the 16th and 24th weeks of the investigation (fig. 3A). Assessment of changes in hydroxyapatite and collagen amounts was conducted by ratio of integral intensities of reflex and hydroxyapatite, halo and collagen for each diffraction pattern according to *Inorganic Crystal*

Structure Database (ICSD) [24]. Table 1 presents parameters of a crystal lattice of investigated samples.

Scanning electron microscopy permitted to assess microstructure of samples from the point of view of morphological visualization of osteocyte lacunae and geometry of their canals (fig. 1D). Figure 2C demonstrates sections of the trabecular layer with osteocyte lacunae and trabeculae. Microstructure of the femoral neck in group I on the 24th day of the experiment indicates dilation of bone canals and thinning of bone trabeculae. Multiple lacunae of bone resorption and extension of nervous and vascular canals are detected on the surface of the trabecular bone in experimental group I. Microstructure of the femoral neck in group HCD+LM+Zol. on the 24th day of the experiment indicates arrangement of the structure with common direction of collagen fibers, which are located in parallel layers, forming bone plates (fig. 3C). On the surface of bone section, there are the areas of bone remodeling, where fewer lacunae of resorption are detected compared with experimental group I. Some nervous and vascular canals are slightly dilated.

Figures 2B and 3B present quantitative characteristics of calcium in examined samples of the femoral bone. On the 8th day of the experiment, we observed reduction of

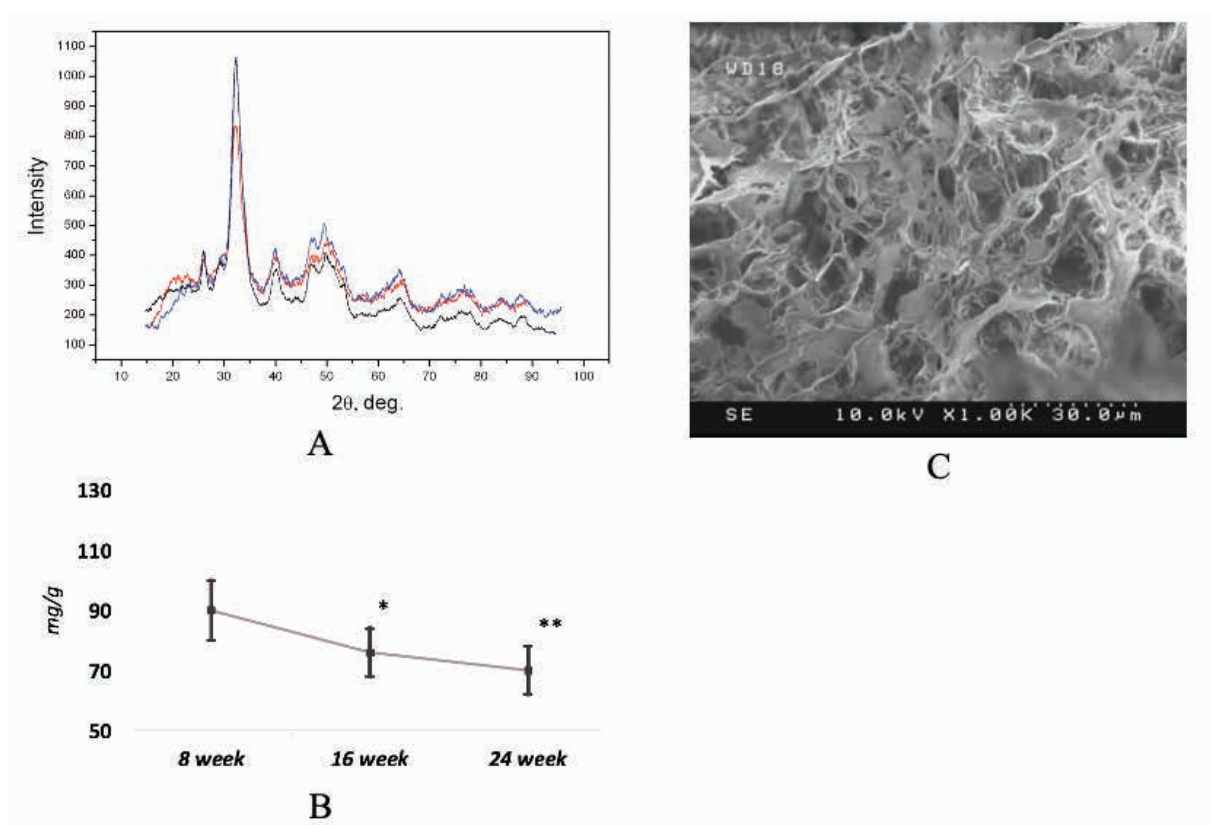


Figure 2: A, C - diffraction patterns and electron microscopy of the femoral neck in group I (HCD + LM); B - quantitative content of calcium at 8-16-24 weeks of the experiment, *p < 0.05; **p < 0.01; vs control group

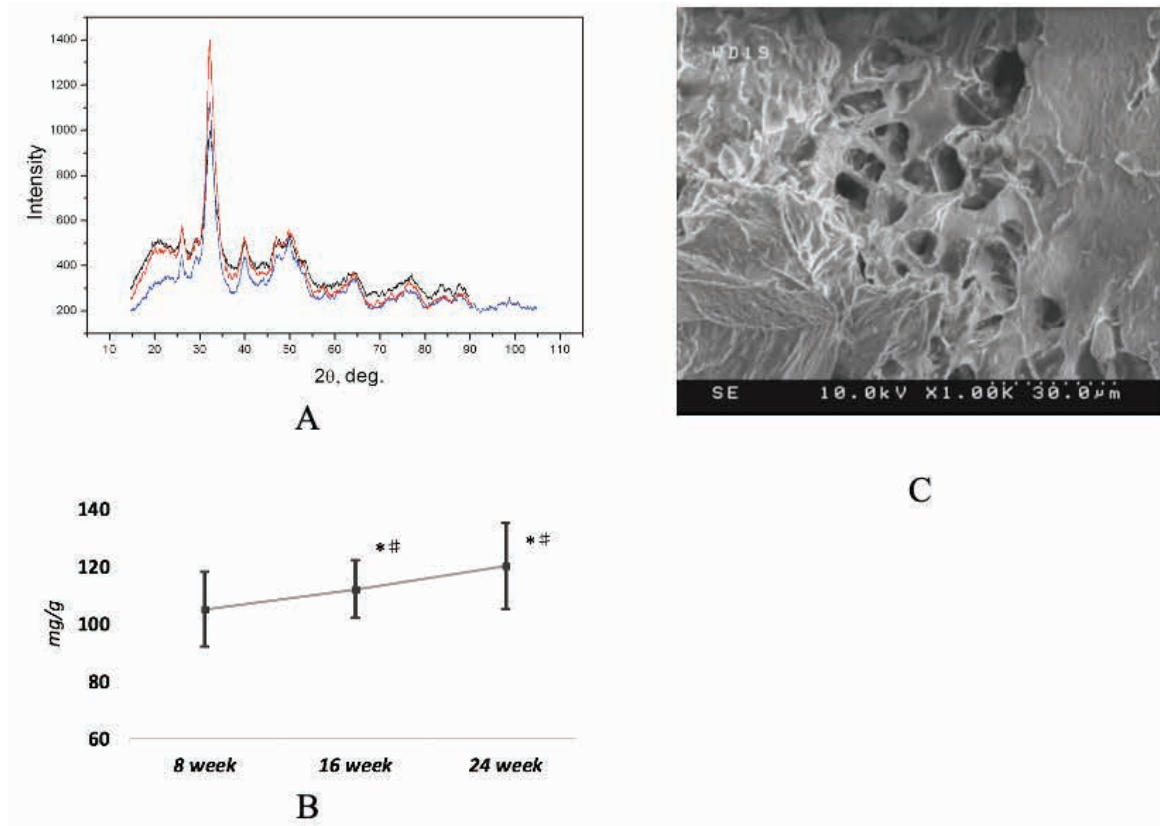


Figure 3: A, C - diffraction patterns and electron microscopy of the femoral neck in group II (HCD + LM + Zol.); B- quantitative content of calcium at 8-16-24 weeks of the experiment *p <0.05, **p <0.01 vs control group; #p <0.01 group I vs group II

Table 1: Structural parameters of femoral neck samples

Group/week	X _{am}	X _{cr}	B ₍₀₀₂₎ , °	L _{nm}
Control 8 th week	0,169	0,831	1,113±0,052	12,0±0,9
Control 16 th week	0,164	0,836	1,163±0,060	11,3±0,9
Control 24 th week	0,110	0,890	0,937±0,019	15,8±0,5
HCD+LM 8 th week	0,145	0,855	1,175±0,037	17,1±0,5
HCD+LM 16 th week	0,195*	0,805	0,762±0,009	23,1±0,5
HCD+LM 24 th week	0,212*	0,788	0,897±0,050	17,1±1,6
HCD+LM+Zol. 8 th week	0,160	0,840	1,244±0,038	10,2±0,4
HCD+LM+Zol. 16 th week	0,143**	0,857	0,899±0,038	17,0±1,2
HCD+LM+Zol. 24 th week	0,168**	0,832	1,014±0,030	13,9±0,7

*groups differ statistically, p <0.05 (Control vs HCD+LM);

**groups differ statistically, p <0.05 (HCD+LM vs HCD+LM+Zol.);

X_{am} - the volume fraction of the amorphous phase

X_{cr} - the volume fraction of the crystalline phase

B₍₀₀₂₎ - the half-width of the maximum (002) of the Ca₁₀P₆O₂₆H₂ phase

L - the average crystallite size of the Ca₁₀P₆O₂₆H₂ phase

calcium level in samples of the first group and increase in quantitative indices of the second experimental group; however, the results were statistically insignificant. During the 16th and 24th weeks of monitoring, calcium mass reduced in the first experimental group by -20 % (p < 0.05) and -31.5 % compared with control group (fig. 1C, 2B).

Obviously, it indicates continuation of bone remodeling and activity of osteoclasts and osteoblasts in the process of the experiment and results in loss of mineral mass of the femoral bone. It should be emphasized that amount of calcium increased in group II (fig. 3B) in relation to control indices starting from the 8th week, and from the 16th week

by +13.3 % ($p < 0.05$) and even +15 % during the 24th week of the experiment ($p < 0.05$). Amount of calcium in group II increased by +32.5 % ($p < 0.05$) and +41.8 % ($p < 0.01$) compared with group I during the 16th and 24th weeks of the investigation, respectively.

4 Discussion

Presented model of obesity with application of high-calorie diet and limited mobility in rats demonstrated a significant decrease in a crystalline mass of the femoral neck despite a considerable increase in body mass. Literature data emphasize that obesity can affect bone metabolism directly or indirectly via cytokines, which originate from adipocytes such as leptin and adiponectin. It is usually accompanied by a significant increase in serum leptin. High concentration of leptin in blood serum as described in our earlier work can negatively affect bone metabolism [5]. We have established that increased level of leptin in blood serum is a negative regulator of bone mass. It should be highlighted that high-fat diet, which is often the cause of obesity, inhibits absorption of calcium in the intestines. Recent investigations [6] demonstrate that probability of femoral neck fractures increases in women during menopause and in elderly men, who suffer from obesity, and is significantly higher than in individuals with normal body mass index (BMI); however, risk factor – smoking – is common. Evidence-based strategy of treatment, prophylaxis of osteoporosis and further prevention of fractures in individuals with obesity is insufficient, but important for further investigations. Some researchers [25] demonstrated a significant role of obesity in epidemiology of fractures. It has been described that women, who suffer from obesity, have a higher risk of fractures of the ankles, tibia, humerus and vertebral column as well as high risk of fractures of the wrist, femur and pelvis compared with women, who are not obese. Insufficient efficacy of antiresorptive therapy in individuals with obesity has also been established.

Cao J., 2011 investigated alterations in bone structure and cytokines of blood serum, associated with bone metabolism in mice and with obesity caused by high-calorie diet. Mice fed on diet with high level of fat (HFD) had a higher level of tartrate-resistant acid phosphatase 5b in blood serum (TRAP5b) and leptin, but lower concentrations of osteocalcin than those on a standard diet [5]. Mice fed on HFD also had lower mineral mass of the femur, which indicates that obesity, caused by HFD, increases bone resorption. Liu et al., 2021 studied safety and efficacy

of zoledronic acid (Zol., single-dose injection 5 mg, intravenously) on bone remodeling condition in patients with obesity after bariatric surgery [26]. The authors recorded slight elevation of biochemical markers C-telopeptide and Procollagen type I N-terminal propeptide during 24-week investigation. Mineral density of bone tissue was measured in the vertebral column and thighs by means of Dual-energy X-ray absorptiometry (DXA) and Quantitative computed tomography (QCT) at preoperative starting level and during the 24th week after operation. Injection of zoledronic acid can protect from significant loss of bone tissue in a trabecular region of the brain compared with control group ($+4.8 \pm 8.0$ % versus -5.9 ± 7.0 %, $p = 0.075$ between groups). Wessel et al., 2008 reported high efficacy of bisphosphonates in the treatment of bone metastases, hypercalcemia and osteoporosis. However, their influence can be associated with osteonecrosis of the jaw. The authors emphasized a 30-fold increase in risk of development of jaw osteonecrosis after a course of treatment with Zol. in patients who smoked and suffered from obesity. However, the investigation had restrictions as chemotherapy scheme and possible interaction of Zol. with other drugs was not taken into consideration [27]. Walsh and Vilaca, 2017 conducted analysis of bone tissue in patients with obesity and type 2 diabetes mellitus. The authors emphasize that body mass index is positively associated with mineral density of bone tissue and mechanisms of this association in vivo may include increased load on the bone. However, cytokines from visceral fat are proresorptive, and high body mass increases the risk of fractures. Dual-energy X-ray absorptiometry (DXA) permits to assess mineral density of bone tissue and risk of fractures. However, the bone at microstructure level should be analyzed for better understanding of bone tissue in obesity. Standard clinical risk factors cannot encompass all relative information and clinicians do not admit this risk. The authors note that timely effective antiresorptive therapy in patients with obesity gives positive clinical results and leads to improvement of mineral mass [28].

In our earlier investigations, we used non-physiological whole body vibration (0.3 g) for correction of bone tissue condition in rats with obesity and limited mobility. We established an increase in mineral density and slight increase in the crystalline phase level. Therefore, regular physical activity can not only improve metabolism of bone tissue, but also decrease body weight. Besides, adipose tissue decreases and bone mass increases. Our research showed that amount of mineral component was higher in control group of rats (without obesity) and in group treated with whole body vibration than in rats with obesity. These results demonstrate that obesity has a negative impact on

mineral bone mass, reducing relative amount of the crystalline component presented by hydroxyapatite. As an alternative of medication treatment for osteoporosis, we investigated whole body vertical vibration as a mechanical stimulation. This stimulation induces an increase in the mineral component due to mechanical vibration with bone microdeformation [19, 29].

Since regular physical activity has an impact on bone formation and density, this results in increase of its mechanical strength. Mechanism of action, which promotes improvement, is mainly associated with increase in bone formation. Therefore, the authors assumed that combined effects of physical exercises and application of bisphosphonates could have more benefits for alterations in the bones than individual methods of correction. Lespessailles et al., 2009 investigated impact of zoledronic acid (Zol.) on alterations in bone strength in ovariectomized rodents (OVX). OVX rats were injected single dose of Zol. intravenously (20 mcg/kg) and put on a treadmill (15 m per minute, 60 minutes a day, 5 days a week) for 12 weeks. BMD of the femur was conducted by DXA method, QCT – for studying biomechanical and trabecular parameters of the skeleton structure. In 12 weeks, volumetric fraction of the bone decreased in OVX rats whereas metabolic rate of bones and a trabecular index of the structure increased compared with the indices in control group (SHAM) ($P < 0.05$). Zoledronic acid prevented trabecular loss of bone tissue, induced by ovariectomy, and its further trabecular microarchitecture deterioration. It has been demonstrated that running on a treadmill aimed at preservation of bone strength and induction of changes in bone metabolism, was beneficial for bone formation. However, combined effects of zoledronic acid and physical exercises used simultaneously did not cause any synergistic or additive effects [30]. Although more effective drugs are known, such as denosumab, which improves bone remodeling and mineral composition and is safer. Zoledronic acid is currently a drug that is often used in the treatment of osteoporosis of various etiologies [31].

5 Conclusions

To infer results, our investigation gives new understanding of mechanisms of loss of bone tissue mineral component in obesity and prognostication of future risks of osteoporotic fractures. Zoledronic acid at the dose of 0.025 mg/kg/month (5 injections) is beneficial for prophylaxis of osteoporosis and prevents loss of bone mass in obesity and limited mobility.

Acknowledgments. Authors would like to acknowledge the University of Wurzburg for providing the equipment for the FESEM investigation.

Funding information: This work was supported by the Department of Normal Physiology of Danylo Halytsky Lviv National Medical University “Researching the role of systemic and paracrine regulatory mechanisms in providing homeostasis of functional and metabolic parameters of the organism under conditions of adaptation to extreme factors of diverse nature” (state registration number 0116U004510).

Author contributions: All authors take responsibility for the integrity of the data and the accuracy of the data analysis. Conceptualization, NMK; Methodology, NMK; Investigation, NMK; Formal Analysis, IIS, NMK.; Writing - Original Draft, NMK; Writing - Review & Editing, NMK, IIS.; Supervision, IIS; Funding Acquisition, NMK

Conflict of interest: The authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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