EFFECTS OF HIGH-IMPACT EXERCISE ON THE PHYSICAL PROPERTIES OF BONES OF OVARIECTOMIZED RATS FED TO A HIGH-PROTEIN DIET

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ABSTRACT

The aim of this study was to evaluate the effects of high-impact physical exercise as a prophylactic and therapeutic means in osteopenic bones of rats submitted to ovariectomy and protein diet intake. A total of 64 Wistar rats were divided into eight groups (n=8 each), being: OVX, ovx, standard diet and sedentary; OVXE, ovx, standard diet and jump; OVXP, ovx, high-protein diet and sedentary; and OVXEP, ovx, high-protein diet and jump; SH, sham, standard diet and sedentary; SHE, sham, standard diet and jump; SHP, sham, high-protein diet and sedentary; and SHEP, sham, high-protein-diet and jump. OVX surgery consists of ovariectomy, and sham was the control surgery. The jumping protocol consisted of 20 jumps/day, five days/week. The bone structure was evaluated by densitometry, mechanical tests, histomorphometric and immunohistochemical analyses. A high-protein diet resulted in increased bone mineral density ($p=0.049$), but decreased maximal load ($p=0.026$) and bone volume fraction ($p=0.023$). The benefits of physical exercise were demonstrated by higher values of the maximal load in the trained groups compared to the sedentary groups ($p<0.001$). The sham groups had decreased immunostaining of osteocalcin ($p=0.004$) and osteopontin ($p=0.010$) compared to ovx groups. However, the high-protein diet ($p=0.005$) and jump exercise ($p=0.017$) resulted in lower immunostaining of osteopontin compared to the standard diet and sedentary groups, respectively. In this experimental model it was concluded that ovariectomy and a high-fat diet can negatively affect bone tissue and the high-impact exercise was not enough to suppress the deleterious effects caused by the protein diet and ovariectomy.

Keywords: Bone Diseases, Metabolic, Ovariectomy, Jump and Nutrition

INTRODUCTION

The use of high-protein diets is gaining popularity. This diet has been

recommended in strategies for weight loss and to control the increase in lean mass and post-exercise recovery (Pichon, 2008; Nikander, 2010); however, there is great controversy about the consequences of this high-protein intake. Balanced nutrition is fundamental to maintaining overall health, especially in postmenopausal women. Unbalanced nutrition is a risk factor for the development of diseases prevalent in this phase of life (Macedo et al., 2016).

At menopause, bone mineral density (BMD) declines rapidly resulting from estrogen deficiency declines, increasing the risk of women developing osteoporosis. In addition to a balanced diet, with the aim of treatment and prevention, physical exercise is exploited by its low cost and lack of side effects compared to drug treatments (Shimano et al., 2014).

Protein intake may exert both positive and adverse effects on calcium balance, generating effects on bone mass and fracture risk, dependent on the concomitant intake of calcium (Ju et al., 2008). For many years a high-protein intake was associated with an increase in urinary calcium excretion, resulting in decreased bone mass (Morais, Burgos, 2007). However, as the research progresses, it has been possible to find a positive relationship between protein-rich diets and bone tissue, especially when associated with exercise (Chevalley et al., 2014).

Several treatments have been tested and designed to prevent osteometabolic diseases. Among them is the regular practice of physical exercise, in which the benefits in bone tissue are already evident (Bonnet et al., 2007). High-impact exercise, especially jumping, generates substantial hardness with a high rate of deformation (Nikander et al., 2010).

Although some studies associate a high-protein diet with physical exercise (Takeda et al., 2012; Abizanda et al., 2015), there are no reports of the association in experimental models with estrogen deficiency. Thus, the need for additional studies relating the practice of physical exercise with high-protein diets at menopause is evident. Hence, the main aim of this study was to evaluate the effects of high-impact physical exercise (jumping) as a prophylactic and therapeutic means in osteopenic bones of rats submitted to ovariectomy and a high-protein diet.

MATERIALS AND METHODS

This experimental study was performed according to the National Institute of Health guidelines for the use of experimental animals and was approved by the local

Ethics Committee for Animal Experimentation, under process number 001/2014.

A total of 64 female Wistar rats of the variety Rattus Norvegicus Albinus, being eight weeks old and approximately 200 g in weight were studied. The rats were divided into eight experimental groups, with eight animals per group: OVX, ovariectomized, standard diet and sedentary; OVXE, ovariectomized, standard diet and jump; OVXP, ovariectomized, high-protein diet and sedentary; OVXEP, ovariectomized, high-protein diet and jump, SH; simulated surgery, standard diet and sedentary; SHE, simulated surgery, standard diet and jump; SHP, simulated surgery, high-protein diet and sedentary; and SHEP, simulated surgery, high-protein diet and jump.

The surgery was performed according to the ovariectomy or sham groups. After 72 hours from surgery the training started, with jumping in the OVXE, OVXEP, SHE, and SHEP groups. The training protocol consisted of 20 jumps/day, five days/week for 12 weeks. The initial height was 25 cm and it was gradually increased to 40 cm (Falcai et al., 2014). In order to carry out the high-impact physical training, a wooden box measuring 40.0 x 40.0 x 40.0 cm was used, with steel wires in the bottom to support the animals, and to allow the transmission of electric current (shock generator scrambler - Insight, Ribeirão Preto/SP, Brazil). Initially the rats jumped with electrical stimulation, however after a few days, they jumped without stimulation. On the other hand, sedentary animals were considered as those that were not submitted to jump training.

The amount of feed offered was always higher than the estimated consumption so that the animals had no food restriction. They were fed either a standard or high protein diet feed according to each experimental group (Table 1). The standard diet used was the Rhosterlab maintenance feed, and the high protein diet was provided by the company Rhoster (Araçoiaba da Serra, Brazil), modified to contain approximately 40 % protein (Amanzadeh et al., 2003) (casein).

At the end of the 12 week experiment, the animals were euthanized by an overdose of anesthetic (xylazine at 30 mg/kg and ketamine at 150 mg/kg). The femurs were removed and cleaned of soft tissue. The left femurs were stored at – 20 ºC and later used for densitometry and mechanical testing, and the right femurs were prepared for histological analysis.

FOLLICLE STIMULATING HORMONE (FSH)

The plasma concentration of FSH was determined to confirm the efficacy of ovariectomy surgery. The analysis was performed using the double antibody radioimmunoassay method using iodination hormone. Both the FSH specific antibody and standard reference hormone were obtained from the National Hormone and Peptic Program (Harbor UCLA Medical Center, USA).

BONE MINERAL DENSITY ANALYSIS

The femurs were placed in a plastic vessel containing saline at a depth of 2.0 cm, aligned, and scanned by a dual energy X ray absorptiometry (DXA), DPX IQ Lunar® model (USA). The images acquired were analyzed in the region of interest (ROI) that was manually selected in the femoral neck (ROI size of 0.029 cm^2), using DPX software (version 4.7) which was specifically developed for small animals.

BIOMECHANICAL ANALYSIS

After densitometry, the bone mechanical properties of maximal load (N) and relative stiffness (N/mm) were determined by a flexion compression test using a Universal Testing Machine (EMIC DL10000, São José dos Pinhais, Brazil) and a load cell of 500 N. The load was vertically applied at a constant speed of 0.5 mm/min at the top of the femoral head until failure, with the bone maintained in a vertical position. A pre load of 5 N for 30 seconds was used.

HISTOLOGICAL PROTOCOL

The femurs were fixed in 10 % formaldehyde for 24 hours, decalcified in 10 % ETDA solution, dehydrated in an ascending series of alcohols, diaphanized in xylene and embedded in paraffin. The blocks were sectioned into 5 μm thickness longitudinal sections. Fifteen of these sections were stained by the Masson Trichrome method, and five were destined to immunohistochemistry.

HISTOMORPHOMETRY ANALYSIS

The histological slides were examined under the AxioImager® Z2 microscope (ZeissTM, Oberkochen, Germany) using Axiovision software (ZeissTM), which identifies the trabecular bone based on the color, and quantifies the trabecular bone volume/total volume (BV/TV) (%) in the femoral neck (Parfitt et al., 1987).

IMMUNOHISTOCHEMISTRY ANALYSIS

The histological sections were deparaffinized in xylene and hydrated in a decreasing ethanol series. Antigen retrieval was performed in Diva Decloaker® buffer (Biocare Medical, CA, USA) in a pressurized Decloaking Chamber® (Biocare Medical, CA, USA) at 95 °C for 10 minutes. After washing in 0.1 M saline phosphate buffer solution (PBS), pH 7.4, the histological slides were immersed in 3 % hydrogen peroxide for 1 hour for endogenous peroxidase blocking. After washing with PBS, the histological sections were treated in 3 % bovine serum albumin for 12 hours.

The histological slides containing samples from all experimental groups were incubated with one of the following primary antibodies: rabbit-generated anti-OC (osteocalcin) (1: 100; Santa Cruz Biotechnology, CA, USA); anti-OPN (Osteopontin) from rabbit-generated mouse (1: 100; Santa Cruz Biotechnology, CA, USA). Primary antibodies were diluted in PBS plus 0.1 % Triton X-100 (PBS-TX) for 24 h in a humid chamber. In subsequent steps, the Dako Labeled (HRP) Streptavidin-Biotin Kit® (Dako Laboratories, CA, USA) was used. After washing, the histological sections were incubated with the biotinylated secondary antibody for 2 h, washed and treated with streptavidin conjugated with strong root peroxidase (HRP) for 1 h. After three washes in PBS-TX the development was carried out using 3,3'-diaminobenzidine tetrachloride (DAB Chromogen Kit®, Dako Laboratories, CA, USA) as the chromogen. At the end of a series of PBS washes, the histological sections were stained with Harris hematoxylin. As a negative control, the samples were subjected to the procedures previously described, suppressing the use of the primary antibodies.

IMMUNOLABELING ANALYSIS

Positive areas of immunoblotting (OC-positive and OPN-positive) were expressed in a brownish appearance. The proximal regions of the femurs were evaluated using an Axio Imager Z2® optical microscope (Zeiss, Göttingen, Germany) at a magnification of 100x, and AxioVision software version 4.8 (Zeiss). The areas of interest were identified based on the color and then quantified. The proportions of positive areas of immunolabeling per total area of interest were assessed (Yamanaka et al., 2017).

STATISTICAL ANALYSES

Statistical analyses were performed using IBM SPSS (version 20, IBM Corporation, Armonk, NY, USA). Generalized linear models were used to analyze the influence of the factors on the fixed variables. All comparisons were performed with Bonferroni adjustment, and a significance level of 5 % ($p \leq 0.05$) was adopted. Values are presented as mean ± standard deviation.

RESULTS

BODY WEIGHT

All animals gained weight during the experiment. Analyzing the effect of the types of surgery, exercise, and diet it was possible to observe that the kind of surgery interfered in the weight gain, with greater gain for the groups submitted to ovariectomy ($p < 0.001$). The high-protein diet also resulted in more significant weight gain when compared to the standard diet groups ($p < 0.001$) (Fig. 1).

DOSAGE OF FOLLICLE STIMULATING HORMONE (FSH)

After 12 weeks of surgery, there was a significant increase in FSH levels in ovariectomized animals (13.61 ± 9.49) ($p < 0.001$) in comparison to the sham groups (1.04 ± 0.052).

BONE MINERAL DENSITY ANALYSIS

BMD analysis showed statistical differences between diets ($p = 0.049$), the high

protein diet (0.184 ± 0.052) being higher than the standard diet (0.165 ± 0.036), and between surgeries ($p < 0.001$), with ovariectomized (0.156 ± 0.036) being lower than sham (0.195 ± 0.047). When assessing the diet*exercise interaction the sedentary groups with the protein diet (0.199 ± 0.057) were higher than sedentary with a standard diet (0.160 ± 0.030), and the animals with the high protein diet with jumping (0.169 ± 0.042) were lower than the sedentary ($p = 0.025$) (Table 2).

MECHANICAL TESTING

RELATIVE STIFFNESS

In the relative stiffness values, no statistical difference was observed between the variables ($p = 0.200$) (Table 2).

MAXIMAL LOAD

For maximal load, statistical differences between diets ($p = 0.026$) were found, with the high protein diet (122.20 ± 18.67) being lower than the standard diet (182.69 ± 27.84). In addition, a difference between exercise ($p < 0.001$) was also found with the highest trained groups (140.63 ± 25.13) compared to sedentary (115.40 ± 15.14) (Table 2).

HISTOLOGICAL ANALYSIS

The quantification of trabecular bone showed statistical differences between the diets ($p = 0.023$), with the high protein diet (25.59 ± 6.07) being lower than the standard diet (27.00 ± 7.81) and between surgeries ($p = 0.001$), with the ovariectomized groups (22.10 ± 5.46) being lower than the sham groups (30.44 ± 5.65). The diet*exercise interaction ($p = 0.017$) for the sedentary groups with the standard diet (29.11 ± 5.09) was greater relative to the sedentary groups with the high protein diet (23.87 ± 6.33) (Table 2) (Figure 2).

IMMUNOHISTOCHEMISTRY ANALYSIS

Positive immunolabeling showed statistical differences in the surgery variable ($p = 0.004$), with the ovariectomized groups (50.30 ± 37.15) having the highest values compared to the sham groups (110.68 ± 129.28). The diet*exercise interaction was significant ($p = 0.001$), with the sedentary groups with the standard diet (46.69 ± 39,39) being smaller than sedentary groups with the high protein diet (151.52 ± 155.50) ($p = 0.026$), and the groups with the high protein diet and exercise (42.22 ± 35.59) were smaller than the sedentary with the high protein diet ($p=0.020$). The diet*exercise*surgery interaction was also significant ($p = 0.001$), the SHP group (260.23 ± 170.45) was higher than SH ($p = 0.021$) (23.79 ± 0.01), SHEP ($p = 0.028$) (28.65 ± 19,85), and OVXE ($p = 0.029$) (29.00 ± 14.25) (Table 2).

As to the osteopontin marking, statistical differences regarding diet were found ($p = 0.005$), the standard diet groups (65.47 ± 63.39) being smaller than the high protein diet groups (115.47 ± 96.27); the exercise variable was also significant ($p = 0.017$), with the exercise groups (69.29 ± 72.33) being less than the sedentary groups (111.65 ± 91.79). Surgery was significant ($p = 0.010$), with the ovariectomized groups (113.28 ± 107.22) being greater than the sham groups (107.67 ± 44.85). In the

diet*surgery interaction, the ovariectomized groups with the standard diet (63.22 ± 82.48) were smaller than ovariectomized groups with the high protein diet (163.34 ± 109.07) ($p = 0.008$), the sham groups with the standard diet (67.73 ± 40.87) being smaller than the ovariectomized groups with the high protein diet ($p = 0.011$), and the ovariectomized groups with the high protein diet higher than the sham groups with the high protein diet (67.60 ± 50.76) ($p = 0.010$) (Table 2).

DISCUSSION

In the present study, the effects of physical exercise (jumping) on biomechanical properties and bone microarchitecture of ovariectomized rats submitted to a high-protein diet were evaluated. As a result, it was possible to observe an adverse influence of the high-protein diet on bone tissue, and although useful, physical exercise alone did not supply the deficits caused by the high-protein diet and ovariectomy.

To induce osteopenia, ovariectomy was performed, which is a model widely used to study osteometabolic diseases (Thompson et al., 1995; Macedo et al., 2016). The literature indicates that after ovariectomy, osteopenia is present within a month (Thompson et al., 1995). The experiment started with the animals having a weight between 150 and 180 grams, with the animals clearly classified as young. According to Thompson et al. (1995), age is a major factor in bone loss research, stating that the bone decrease that occurs after ovariectomy depends on the age of the animal, being faster in young animals and slower in older animals.

After ovariectomy, one of the factors that can be changed is body mass gain (Macedo et al., 2016). In the present study, all animals increased body mass during the experiment. However, the ovariectomized groups showed higher gain when compared to sham animals, corroborating with some authors who also studied ovariectomized rats (Lespessailles et al., 2009; Macedo et al., 2016).

The high-protein diet groups presented greater body mass gain when compared to the standard diet, justified by Silva et al., (2014), who affirmed that weight loss with the high-protein diet is related to a dietary restriction, defining that it is not the high-protein diet that leads to weight loss, but the restriction imposed on animals. In addition, some studies indicate that this diet can lead to weight loss only after extended periods of time, based on the change in metabolism leading to satiety (Halton, Hu, 2004).

There is debate by many authors on increasing protein intake on bone, imposed as harmful by associating the acid load to consequent hypercalciuria. In 1992, Abelow et al. found a positive correlation between protein intake and hip fracture rates. However, this theory was contested in the following years. Studies by Munger et al. (1999) focused on hip fractures, and showed that the high fracture rates were related to low protein intake in the diet.

According to some authors, a high-protein diet increases intestinal calcium absorption benefiting bone homeostasis, bone remodeling and production of insulin-like growth factor 1 (IGF-1) (Kerstetter et al., 2005, O'Connell et al., 2005). Most of the epidemiological studies positively associate protein intake and BMD (Munger et al., 1999), corroborating our findings that the high-protein diet was able to sensitize bone tissue by improving BMD. On the other hand, many studies use BMD as a measure of fragility, and their accuracy in measuring bone strength is already questioned. Also, another disadvantage is the variation of bone mass and geometry, depending on the region analyzed (Yanagihara et al., 2016).

Divergences were found in our study between BMD and biomechanical properties. According to Jamsa et al., 2002, in general, BMD is associated with mechanical properties, but depending on the conditions to which the bone tissue is exposed, the increase in bone mass is not accompanied by an increase in strength.

In the histomorphometric analysis, the results also contrasted with densitometry. However, according to Kalak, Dempster (2010), histological quantification is one of the most powerful analyzes, since it encompasses the quantification of trabecular bone with structural parameters of the imbalance of bone in an altered metabolic condition, providing information in a manner no other investigative approach can.

Zerwekh et al. (2009) evaluated diets with high and low protein concentrations supplemented with potassium chloride or potassium citrate, to assess the effects of alkaline therapy on acid-induced changes in bone turnover in rats. Some authors have demonstrated that the acidic effect generated by a diet rich in proteins can lead to bone loss related to aging, which may justify our findings, in which the high-protein diet was significantly detrimental to bone tissue.

When associating a high-protein diet with women in menopause, this gap in the literature is even greater, generating extensive discussions. For Sellmeyer et al. (2001), older women with a high protein intake have a more significant bone loss

from the femoral neck. However, evaluations in young and premenopausal women after consuming a high-protein diet showed a positive relationship with BMD (Beasley et al., 2010).

In addition to the food issue in menopause, much discussed is the effective way of prevention and treatment for osteopenia/osteoporosis. In general, an ideal diet is not sufficient to supply the hormonal deficit caused by menopause, especially regarding bone tissue. Physical exercises are considered a good strategy for maintaining bone mass; however, although the effectiveness of different types of exercise has already been widely discussed, there is a significant gap on the ideal modality (Shimano et al., 2014; Paliologo et al., 2015).

Some authors affirm that aerobic exercise of medium intensity and with overload is more efficient (Tromp et al., 2006; Bonnet et al., 2007). However, other authors argue that the most valuable exercise for the prevention of bone loss is high-impact (et al., 2003; Nagasawa et al., 2008). According to Martin (2007), bone remodeling is determined by the way in which it requested, that is, the bones that suffer the greatest deformations, impacts or compression present better adaptation, and consequently greater bone mass.

In the present study, jumping was chosen as the high-impact exercise. The performance of training resulted in an increase in biomechanical properties, especially in maximal load, independent of surgery or diet. Along the same line and with similar results, Ju et al. (2012) studied the effects of jumping in tail suspended rats to induce bone mass loss. The authors concluded that skeletal adaptation occurs through mechanical stimulation since an increase in trabecular thickness was observed. In addition, Honda et al. (2003) evaluated the jump effect in ovariectomized animals, and the training had greater effects in ovariectomized rats compared to sham rats, concluding that this activity improves bone mass independently of the estrogen deficiency or osteopenia present.

Takeda et al., (2012) evaluated the growing bone tissue of the rats and concluded that a high-protein intake combined with physical exercise caused an additional effect on the resistance of bone tissue, differing from our results that did not present benefit between the association of the diet rich in protein and physical exercise. In BMD, physical exercise associated with the diet showed inferior results to the groups with only a high-protein diet, independent of the surgery.

The sham groups showed higher concentrations of osteocalcin compared to

the ovx groups and can be explained by Owen et al. (1990), who state that osteocalcin is a marker of the mature osteoblast. Osteocalcin undergoes changes in its levels throughout life and the greater the requirement of energy supply, the higher the bone remodeling to metabolic activity (Chen, 2012). As for diet, Aoe et al. (2005) studied the effects of milk protein on bone metabolism in menopausal women and, corroborating our findings, the authors also did not find statistical differences in osteocalcin concentrations between the standard diet groups and the high-protein diet.

In the marking of osteopontin, the ovariectomized groups presented higher values than the sham groups. With similar but serologically evaluated results, Wei et al. (2016) observed that in menopausal women, serum osteopontin levels are negatively related to BMD and correlated positively with bone turnover levels.

Given the increasing prevalence of osteoporosis and the impact of protein-rich diets on bone metabolism, it is imperative that a better understanding of the interaction between high-protein intake and skeletal health should be attained.

With these data, the need for additional studies to define a better standard, both for the amount of proteins capable of benefiting or not interfering in the bone tissue, as well as the type and intensity of physical exercise, is evident. From our results, one can conclude that the difference between surgeries is evident, and that the high-protein diet is capable of impairing bone microarchitecture and mechanical resistance, despite promoting an increase in BMD. In addition, one can conclude that high-impact physical exercise, although alone improving biomechanical properties, was not able to supply the deleterious effects caused the by high-protein diet and ovariectomy.

PERSPECTIVES

Ageing of the population is accompanied by a high incidence of osteopenia/osteoporosis. The experimental models come to fill the existing gap in the protocols for treatment and prevention of poor bone quality, applicable in humans. The main objective of this study was to evaluate the effects of a high-protein diet by associating high-impact physical exercise on bone microarchitecture in osteometabolic conditions. Important indicators of bone were used to assess bone

quality. Although some studies associate a protein diet and physical exercise (Takeda et al., 2002), this relationship is not clear in women with estrogen deficiency. This hormonal change affects a large part of the female population, which confirms the importance of this experimental methodology.

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Table 1. Composition of maintenance feed and 40 % protein feed

Types of diet	Rhosterlab maintenance feed		Rhosterlab 40 % protein feed	
Caloric Profile	% mass	% Kcal	% mass	% kcal
Protein	21.25	24.52	38.65	44.3
Carbohydrate	50.64	58.43	31.78	37.57
Fat	6.57	18.13	7.03	18.13

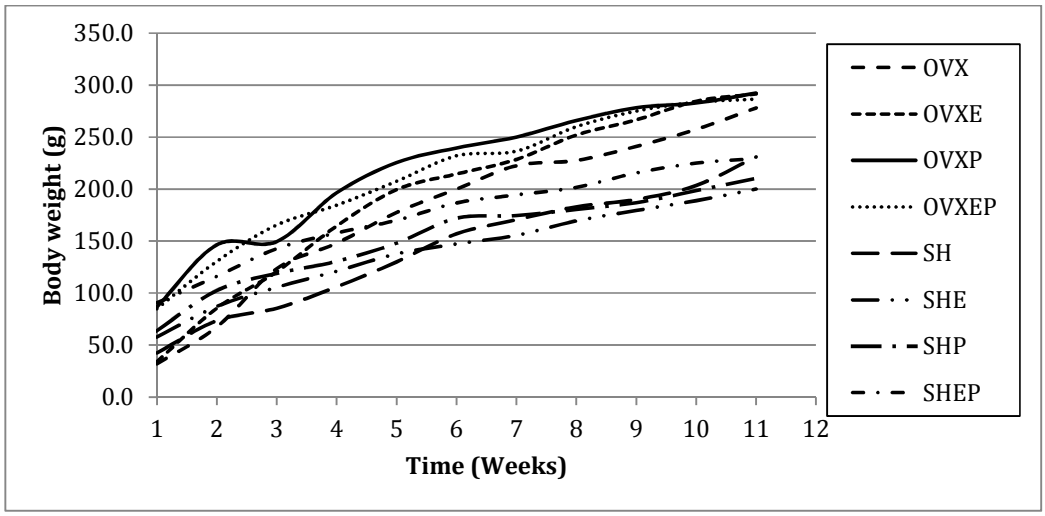

Figure 1- Graph of the weekly weight gain of the groups.

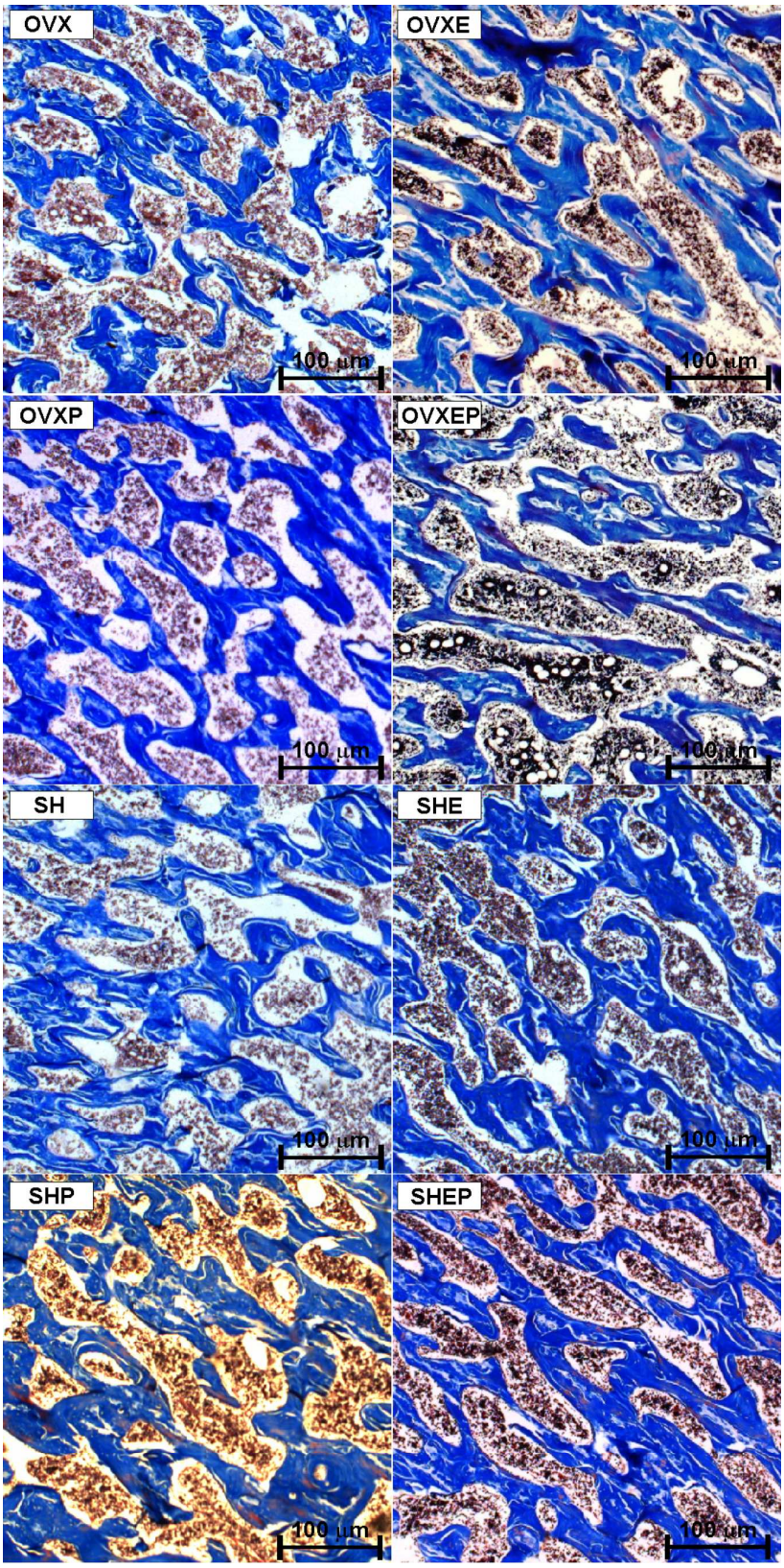

Figure 2- Photomicrographs showing the histological aspects of trabecular bone

Table 2 - Parameters and statistical results of bone mineral density, stiffness, maximal load, histology and immunohistochemistry.

	OVX Average (sd)	OVXE Average (sd)	OVXP Average (sd)	OVXEP Average (sd)	SH Average (sd)	SHE Average (sd)	SHP Average (sd)	SHEP Average (sd)	OVX (O) Effect	p	Exercise (E) Effect	p	Diet (D) Effect	p	O x E p	O x D p	E x D p	OxExD p
BMD	0.139 (0.027)	0.152 (0.027)	0.168 (0.035)	0.164 (0.049)	0.182 (0.017)	0.191 (0.045)	0.229 (0.063)	0.174 (0.034)	↓	<0.001*		0.319	↑	0.049*	0.132	0.762	0.025*	0.196
Stiffness	195.0 (41.4)	187.1 (36.6)	159.0 (40.9)	220.4 (54.0)	190.5 (40.0)	180.4 (29.1)	227.2 (50.4)	228.8 (72.4)		0.129		0.296		0.057	0.150	0.043	0.061	0.180
M. Load	116.1 (14.1)	147.7 (15.8)	116.7 (18.8)	134.9 (17.0)	116.7 (12.3)	152.2 (40.4)	111.8 (17.2)	127.6 (15.4)		0.704	↑	<0.001*	↓	0.026*	0.934	0.353	0.077	0.727
Histology	26.88 (4.06)	19.54 (6.59)	20.75 (4.97)	27.56 (7.27)	31.47 (5.34)	31.37 (6.16)	27.02 (6.41)	28.20 (5.65)	↓	0.001*		0.722	↓	0.023*	0.354	0.652	0.017	0.116
Osteocalcin	69.61 (50.67)	29.00 (14.25)	42.82 (34.15)	59.78 (37.29)	23.79 (0.00)	130.05 (54.63)	260.23 (172.45)	28.66 (19.85)	↑	0.004*		0.574		0.097	0.025	0.040	0.010*	0.001*
Osteopontin	106.42 (103)	20.03 (3.48)	170.10 (130.65)	156.58 (97.89)	92.39 (35.71)	43.07 (25.36)	77.70 (67.21)	57.51 (32.02)	↑	0.010*		0.017	↑	0.005*	0.002*	0.010*	0.001*	0.001*

* Statistically significant difference; sd = standard deviation; ↓ = decreased; ↑ = increased; BMD = bone mineral density; M. Load = maximal load; OVX = ovariectomized; OVXE = ovariectomized with exercise; OVXP = ovariectomized with high-protein diet; OVXEP = ovariectomized, exercise and high-protein diet; OVX = ovariectomized; SHE = sham with exercise; SHP = sham with high-protein diet; SHEP = sham, exercise and high-protein diet