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Aerobic Exercise Increases the Damage to the Femoral Properties of Growing Rats with Protein-Based Malnutrition

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HIGHLIGHTS

- Malnutrition damages the biomechanical and densitometric properties of the femur.
- Aerobic exercise reduces maximum femoral load until fracture in malnutrition.
- Aerobic exercise increases the damage to BMC and BMD established by malnutrition.

Abstract: The present study investigated the effects of aerobic physical training on the femoral morphological, densitometric and biomechanical properties in growing male rats subjected to protein-based malnutrition. Four-week-old male Wistar rats were randomized into groups of 10 animals: Control Sedentary (CS), Control Trained (CT), Malnourished Sedentary (MS) and Malnourished Trained (MT). Control and malnourished animals received diets with 12% protein and 6% protein, respectively. The trained groups were submitted to a treadmill running program for 8 weeks. Total proteins and albumin were analyzed in the animals' blood plasma. Histological, densitometric and biomechanical analyzes were performed on the animals' femur. Body mass gain, physical performance, biochemical markers and the femoral morphological, densitometric and biomechanical properties were determined. Exercise tolerance increased in trained groups. Malnourished animals exhibited lower serum protein and albumin levels than controls. Porosity and trabecular bone density were not different between groups. The femoral maximum load, maximum load until fracture, resilience, stiffness, tenacity and densitometric properties were reduced by malnutrition. Physical training associated with malnutrition exacerbated the impairment in the femoral maximum load, maximum load until fracture, bone mineral content and density. Aerobic physical training worsens the damages induced by protein-based malnutrition in the femoral biomechanical and densitometric properties of growing male rats.

Keywords: protein malnutrition; trabecular bone; fracture; aerobic exercise; bone mineral density.

INTRODUCTION

A balanced and healthy diet is essential to fight malnutrition, besides protecting the body against noncommunicable diseases, such as osteoporosis, diabetes, heart disease and cancer [1,2]. The World Health Organization recommends using a high-quality diet, containing more fruits and vegetables, less salt and sugar, and well-established values of proteins, vitamins and other necessary macromolecules [2].

Malnutrition can be defined as a state resulting from lack of intake or absorption of nutrients, leading to altered body composition, and decreasing physical and mental functions [3]. In this sense, protein malnutrition is a nutritional disorder that affects a large part of people worldwide, commonly seen in children from countries in Latin America, Africa, and Asia [4]. This disease is characterized by inadequate protein intake, which may or may not be associated with poor consumption of other energy macromolecules [5]. This condition damages the human body and has been associated with the development of structural, functional and metabolic modifications in body tissues, including the bone tissue [6-8].

Physical exercise has been indicated for the prevention and non-pharmacological treatment of osteopenia and osteoporosis, as it generates necessary mechanical osteogenic stimuli with few side effects 9. Studies have demonstrated that systematic physical exercises can improve bone properties such as strength, bone mineral content and density [9,10]. Furthermore, it is indicated that physical activities performed in the childhood and adolescence favor the accumulation of minerals in the bones and contribute to the bone health in the adulthood [11].

Regarding malnutrition, running physical exercise is reported to cause beneficial effects on the contractility of cardiomyocytes of rats subjected to protein-based malnutrition 6. However, the effects of physical exercise on the bone health of individuals with protein-based malnutrition are still not well understood. For example, the study by Takeda and coauthors [12] identified negative effects of the association of physical training with low protein diet (10%) on bone mass gain and bone strength. On the other hand, Huang and coauthors [13] evaluated ovariectomized rats that were treated with methionine restriction and / or aerobic exercise. The authors show that both methionine restriction and combination with aerobic exercise improved cortical bone properties, but only the combination of treatments preserved cancellous bone.

Therefore, the objective of the current study was to evaluate the effects of aerobic physical training on the femoral morphological, densitometric and biomechanical properties in growing male rats subjected to protein-based malnutrition.

MATERIAL AND METHODS

Four-week male Wistar rats were kept in a room with controlled temperature (22ºC ± 2), 12/12 hours light/dark cycle and ad libitum food and water intake. The animals were divided into four groups: Control Sedentary (CS; n = 8); Control Trained (CT; n = 8); Malnourished Sedentary (MS; n = 8); and Malnourished Trained (TM; n = 8). All ethical procedures were approved by the Ethics Committee on the use of animals at the Federal University of Viçosa under protocol number 39/2010.

Forty-eight hours after the intervention period, the animals were euthanized (ketamine 10 mg / kg and xylazine 2 mg / kg, i.p.). Blood samples were collected by aortic puncture to assess serum levels of total protein and albumin (Roche diagnostics, Switzerland). In addition, femurs were collected for densitometric, histological and biomechanical analyzes.

All animals were submitted to a protocol for adaptation to the treadmill (Insight Instrumentos – Ribeirão Preto, SP), for four consecutive days, at a constant speed of 10 m/min for 10 min/day, according to Moraes-Silva and coauthors [14]. To determine the physical training intensity (i.e. running velocity), after adaptation the animals were submitted to a maximal running velocity (MRV) test, based on the total exercise time until fatigue (TTF) test, as previously described [15]. The mean MRV obtained by the animals in the TTF test was calculated and established as a reference (i.e. 100% of MRV) to determine the exercise intensity during the physical training sessions. Then, animals of the TC and TM groups were submitted to a gradual running program, starting at 50% of the MRV (11 m/min, 5 times a week). The treadmill speed increased by 1 m/min per week for 7 weeks (final running velocity 18 m/min; 82% of the MRV) [14]. The TTF test was used also as an index of tolerance, thus it was performed at the end of the experimental period. The difference in distance traveled between the end and the beginning of the experimental period (Δ distance) was calculated in order to monitor the influence of treatments in the exercise tolerance.

Thirty days after birth, CS and CT animals received standard feed (AIN-93, 12% protein) [16], and animals MS and MT received a diet containing 6% protein (casein) [17]. The diets were isocaloric (360 kcal / 100 g). Salts and vitamins were administered in similar concentrations in both groups (Table 1).

Table 1. Chemical composition of the diets.

	Standard diet	**Low protein diet**
Protein (casein)	15	7.5
Corn starch	61	68
Sucrose	10	10
Corn oil	4	4
DL-methionine	0.3	0.3
Salts mix	3.4	3.4
Vitamin mix	1	1
Choline hydrochloride	0.3	0.3
Fiber (cellulose)	5	5
Caloric value	**360 Kcal**	**360 Kcal**

Standard diet, 12 % protein. Low protein diet; 6 % protein. Values are expressed in g/100 g of chow.

Forty-eight hours after the last training session, the animals were euthanized by cervical dislocation under anesthesia (ketamine 10 mg/kg and xylazine 2 mg/kg, i.p.). Blood samples were collected by aortic puncture, centrifuged at 3,000 rpm for 10min, and the serum was stored at -80ºC. The serum concentrations of total proteins and albumin in the control and malnourished groups was performed using the colorimetric method (Cobas Mira – EUA).

The right femur was dissected and submerged in a 5.5% EDTA solution (Sigma-Aldrich) dissolved in 10% formaldehyde solution, for a period of two to three weeks, in order to guarantee complete decalcification [18]. Afterwards, the proximal epiphysis was processed and embedded in paraffin blocks. Thereafter, 4.0 µm-thick sections were cut using a manual microtome (Leica 2065, Germany). The slides were diaphanized, hydrated and subjected to Hematoxylin and Eosin dyes [19] and, then, assembled in synthetic Entellan®. Images (10x increase) were obtained using a microscope (Leica DM5000B, Germany) equipped with a camera and the Leica Application Suite program (2.4.0 R1 version, Leica Microsystems). The images were analyzed using the ImageJ software (National Institute of Health, USA). The average of 6 visual fields for each animal were analyzed. Of these, porosity and bone trabecular density were evaluated. The porosity calculation (POR) was performed using the formula [POR = number of points in cavity × 100/total number of points] [20, 21]. The bone trabecular density (BTD) was determined using the formula [BTD = number of points on the trabeculae × 100/total number of points] [20].

The left femur was dissected and frozen in saline solution at -20°C until the day of mechanical assays. The mechanical properties were tested using the three-point bending test in the universal testing machine INSTRON model 4444 (São José dos Pinhais, Brazil) equipped with a loading cell of 100 kgf maximum capacity (approximately 1 kN) 22. The test results were recorded by the Instron Series IX software that generated a load x deformation (strain) curve. From the analysis of the curves, the following biomechanical properties were obtained: maximum deformation (displacement), deformation (displacement) until fracture, maximum load (force) and maximum load until fracture (force), resilience, tenacity, and stiffness.

The left femur was used also to determine bone mineral content and density after the mechanical assays. The analyzes were performed using the X-ray Bone Densitometer (DPX-Alpha Lunar, USA), equipped with software for small animals.

Data were analyzed using the GraphPad Prism 8.2.1® software and presented as mean and standard deviation. Data distribution was analyzed using the Shapiro-Wilk test. Student's t-test was used to compare protein and albumin data between Control and Malnourished animals. Comparisons between groups were performed using two-way ANOVA followed by Tukey post hoc test. The level of significance was set at $p < 0.05$.

RESULTS

The weight of the animals was recorded throughout the experiment, showing an exponential weight gain. The walking pattern of the animals was not changed.

There was a decrease in the levels of total proteins and albumin in the malnourished groups. The animals submitted to protein restriction had lower plasma levels of protein and albumin ($p < 0,0001$) (Figure 1a&quot and 1b&quot).

Protein-based malnutrition reduced weight gain in experimental animals. This significant reduction was found in the MS and MT groups ($p < 0,0001$) (Figure 1c&quot).

Aerobic physical training was able to increase the distance covered by the animals at the end of the experiment. The CT and MT groups had greater distances covered, in relation to their sedentary peers ($p < 0,0001$) (Figure 1d&quot).

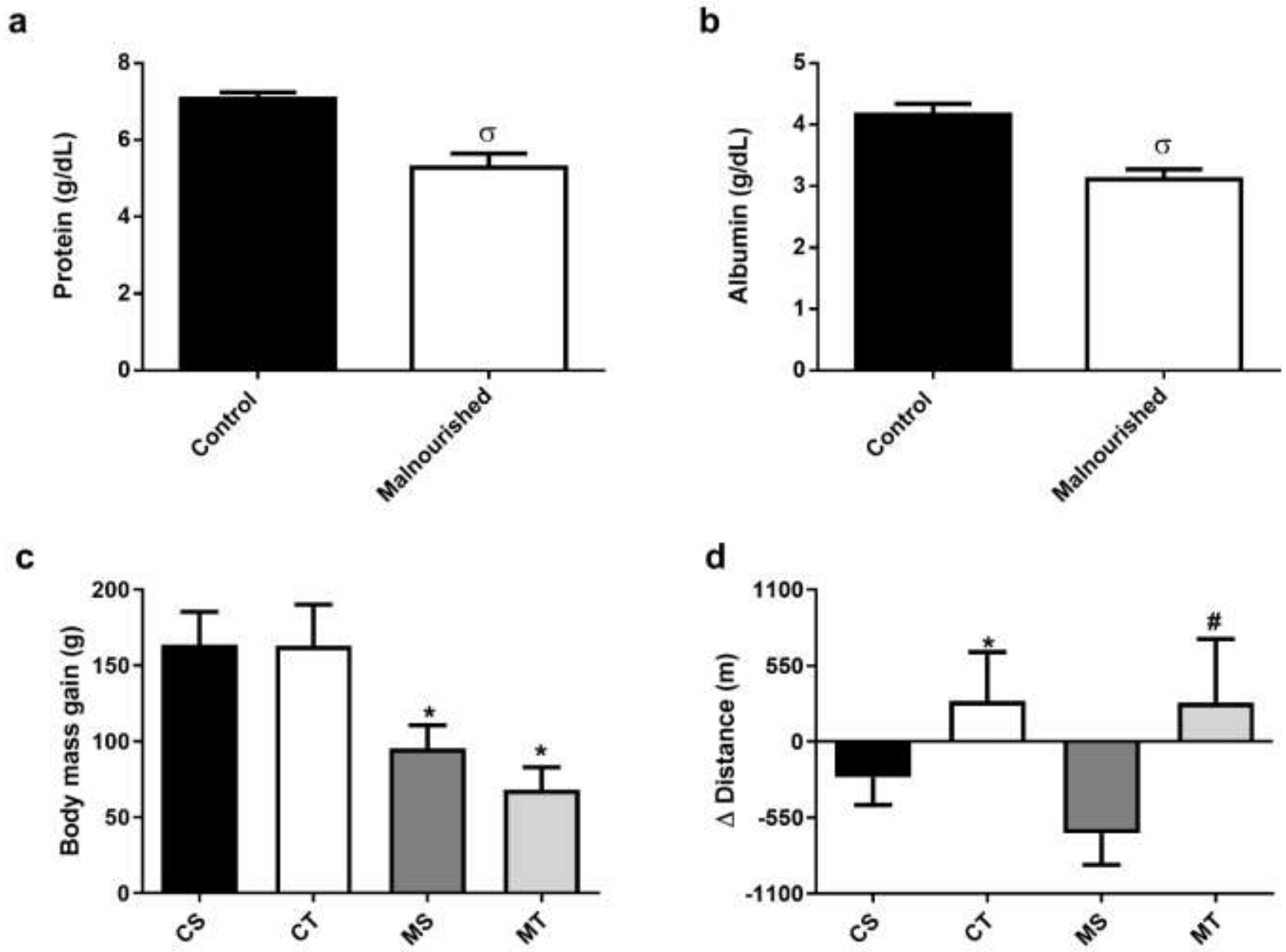

Figure 1. Plasma concentrations of protein (a) and albumin (b), body mass gain (c) and exercise tolerance (d) of experimental groups. CS, Control Sedentary. CT, Control Trained. MS, Malnourished Sedentary. MT, Malnourished Trained. σ, Significantly different from Control group. *, Significantly different from CS group. #, Significantly different from MS group.

Porosity and trabecular density assessed by histology were not influenced by aerobic physical training or malnutrition, and no statistical differences were found ($p > 0,05$) (Figure 2b&quot and 2c&quot).

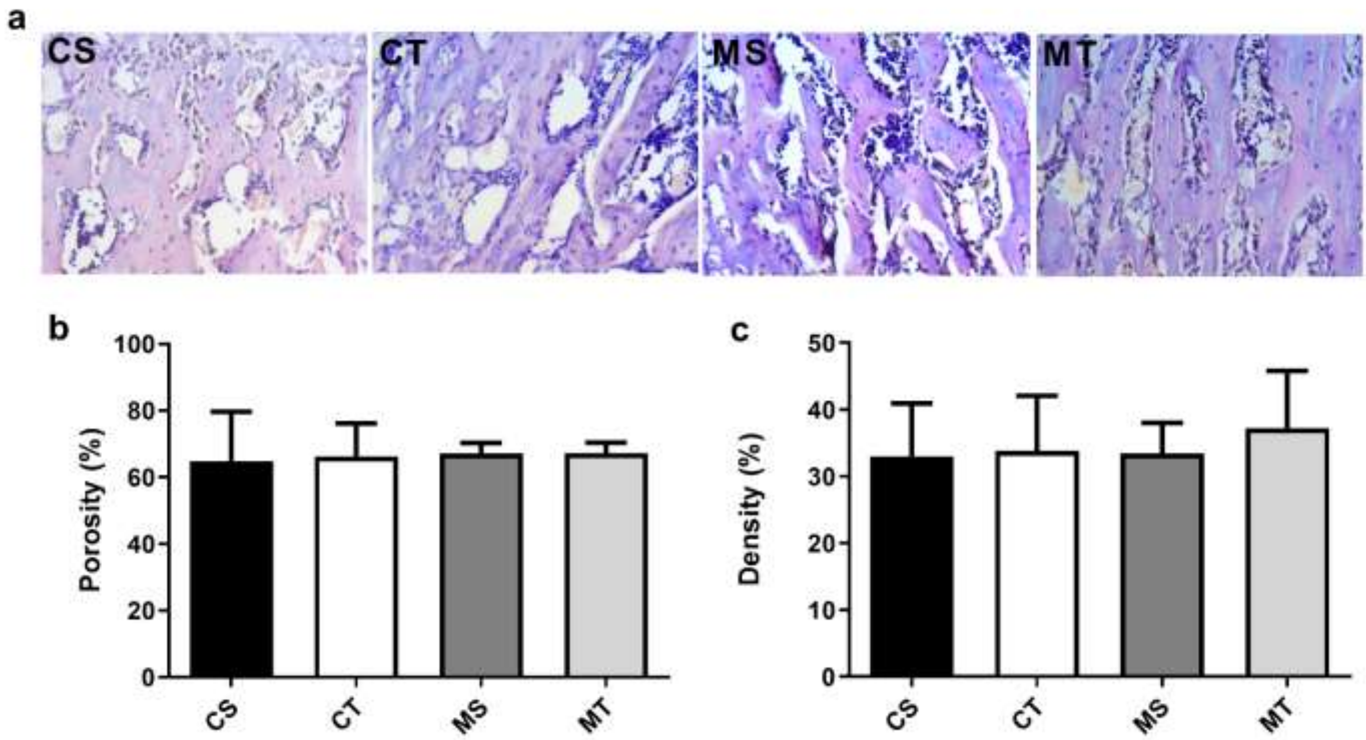

Figure 2. Representative photomicrographs of femoral epiphyses stained with H&E (a). Porosity (b) and Bone Trabecular Density (c) evaluated in the femurs of experimental animals. CS, Control Sedentary. CT, Control Trained. MS, Malnourished Sedentary. MT, Malnourished Trained. Images photographed at 100× magnification.

Data for bone biomechanical properties are presented in Table 2. Protein-based malnutrition reduced femur weight, maximum load, maximum load until fracture, resilience, stiffness, and tenacity. It was noted that the MS group obtained reduced results for all these parameters, compared to the CS group. The addition of protein-based malnutrition with aerobic physical training worsened the maximum load until fracture. The MT group reduced the maximum load until fracture values, compared to the MS group.

Table 2. Femoral biomechanical properties of the experimental groups.

	Groups						
	CS	**CT**	**MS**	**MT**	**Diet**	**Ex.**	**Inter.**
F. weight	1.02 ± 0.08	1.08 ± 0.13	0.70 ± 0.07*	0.63 ± 0.04	< 0.0001	0.9651	0.0619
M. load (N)	164.1 ± 21.38	154.0 ± 27.32	108.5 ± 12.10*	85.65 ± 16.57	< 0.0001	0.0320	0.3880
M. load until fracture (N)	144.9 ± 24.13	130.6 ± 13.13	98.01 ± 9.91*	76.93 ± 19.60#	< 0.0001	0.6060	0.0115
Displa. (mm)	0.72 ± 0.14	0.70 ± 0.08	0.74 ± 0.09	0.76 ± 0.27	0.5599	0.9772	0.7245
Displa. until fracture (mm)	0.97 ± 0.23	1.13 ± 0.34	0.96 ± 0.14	0.77 ± 0.30	0.0813	0.8741	0.0975
Res. (J)	0.08 ± 0.02	0.06 ± 0.02	0.04 ± 0.02*	0.03 ± 0.01	< 0.0001	0.1210	0.8449
Stiffness (N/mm)	479.4 ± 35.44	405.1 ± 55.31	322.7 ± 41.06*	286.9 ± 58.84	< 0.0001	0.0078	0.3227
Tenacity (J)	0.12 ± 0.04	0.10 ± 0.03	0.07 ± 0.01*	0.05 ± 0.01	0.0001	0.1171	0.8127

CS, Control Sedentary. CT, Control Trained. MS, Malnourished Sedentary. MT, Malnourished Trained. F. weight, Femur weight. M. load, Maximum load. Displa., Displacement. Res., Resilience. Ex., Exercise. Inter., Interaction. * Significantly different from CS group. # Significantly different from MS group.

The densitometric data are displayed in Table 3. Protein-based malnutrition reduced the area, BMC, and BMD in the animals' femurs. Protein-based malnutrition reduced the area, BMC, and BMD in the animals' femurs. Aerobic exercise training modified the BMD. The addition of protein-based malnutrition with aerobic physical training worsened the BMC and BMD. Thus, there was a reduction in BMD and BMC in the MT group compared to the MS group.

Table 3. Bone densitometric properties of experimental groups.

	Groups						
	CS	**CT**	**MS**	**MT**	***Diet***	***Exercise***	***Interaction***
Area (cm^2)	1.56 ± 0.16	1.60 ± 0.13	1.22 ± 0.11*	1.17 ± 0.08	< 0.0001	0.8694	0.3256
BMC (g)	0.29 ± 0.04	0.30 ± 0.04	0.18 ± 0.02*	0.12 ± 0.03#	< 0.0001	0.1446	0.0181
BMD (g/cm^2)	0.18 ± 0.01	0.19 ± 0.01	0.14 ± 0.01*	0.10 ± 0.03#	< 0.0001	0.0373	0.0106

CS, Control Sedentary. CT, Control Trained. MS, Malnourished Sedentary. MT, Malnourished Trained. BMC, Bone mineral content. BMD, Bone mineral density. Significantly different from CS group. # Significantly different from MS group.

DISCUSSION

The present study aimed to evaluate the effects of aerobic physical training on the femoral morphological, densitometric and biomechanical properties in growing male rats subjected to protein-based malnutrition. Our main findings were that protein-based malnutrition impaired the femoral maximum load, maximum load until fracture, resilience, stiffness, tenacity, weigh, BMC, and BMD. Moreover, despite the aerobic physical training employed has improved the exercise tolerance, it potentiated the reductions in the femoral maximum load until fracture, BMC, and BMD.

In the present study, the low protein intake was confirmed by the reduced plasma concentrations of protein and albumin. The main consequence of such diet was that it impaired femoral mechanical and densitometric properties. The bone tissue is composed of around one third of proteins [23], which influences bone mineral content [9]. Indeed, while a protein-based diet has been positively associated with bone quality [24-27], the reduction of protein intake has been associated with increased risk for osteoporosis [26, 28]. The bone quality is related to micro fractures, formation of collagen cross links, turnover rate and mineralization degree [29]. Since there was no difference between groups for bone porosity and trabecular density, the reduced mechanical properties seem to be related to low BMC and BMD. In fact, low-protein diet associated with reduced BMC and BMD and mechanical properties was reported by others [30, 31]. Moreover, in this experiment we used growing male rats, and it is known that bone development relays upon modeling and remodeling, which is affected by hormones. For instance, alterations in the secretion of hormones such as procollagen type 1 N-terminal pro-peptide (P1NP), receptor activator of nuclear factor-kappa B ligand (RANKL), insulin-like growth factor 1 (IGF-1) and fibroblast growth factor-21 (FGF-21) may unbalance the bone modeling/remodeling process and thus damage bone mechanical and densitometric properties [30].

Mechanical loads are considered important factors for the regulation of skeletal homeostasis, affecting bone remodeling. Physical exercise involves an increase in mechanical tension, promoting bone mass gain [29]. In addition, physical exercise can promote a series of physiological responses involving the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes, resulting in bone tissue adaptations [32]. Therefore, running exercises can generate osteogenic actions, promoting bone mineralization [33]. Studies involving interval or continuous running have demonstrated biomechanical benefits in the bones of trained rats [33, 34]. However, in the present study, no positive adaptations promoted by aerobic physical exercise were observed in the bones of trained animals. The physical exercise applied may not have been intense enough to generate mechanical overloads that would increase bone mass gain. It has been observed that interval running offers recovery cycles favorable to recovery to strengthen bones and downhill running can increase the ground's reaction forces at each step [35].

Surprisingly, the exercise program employed here did not protect bone from the harm imposed by the protein-based restriction. It is known that physical exercise increases mechanical tensions that results in bone mass gain [29, 33, 34] and enhanced mechanical properties [33, 34]. Therefore, it is conceivable that the applied exercise had no intensity enough to generate mechanical stress to the bone to induce osteogenesis. In fact, intermittent and downhill running have promoted benefits to bone strength [35]. It is also possible that the aerobic physical exercise used have induced bone resorption without bone deposition in the growing rats with protein restriction due to an increased demand for protein by other organs. Indeed, the work by Takeda and coauthors [12] reported that the association of running training with low-protein diet resulted in reduced bone strength and mass gain in growing male rats. Moreover, Huang and coauthors [30] demonstrated that growing male rats fed methionine-restricted diet exhibited reduced femoral BMD and levels of osteocalcin that is crucial for bone mineralization. They also found diminished femoral maximum load and stiffness in these animals and that endurance exercise did not mitigate such impairments caused by the diet. It is important to note that, despite the studies having applied aerobic exercise, the duration, volume, and intensity of physical training, as well as the diet used, were different.

Our study has limitations. First, as we used growing animals an increased caloric competition involving somatic growth, protein malnutrition and aerobic physical training might have influenced the outcomes. Nevertheless, our study design included control groups to minimize such influences. Second, we applied a protocol of eight weeks of aerobic physical training, which is a relatively short intervention time. Probably, a longer intervention would produce different results.

CONCLUSION

In conclusion, aerobic physical training worsens the damages induced by protein-based malnutrition in the femoral biomechanical and densitometric properties in growing male rats. These findings are of clinical relevance inasmuch as it provides information about the prescription of physical exercise to in individuals who are under protein-based malnutrition.

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