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Effect of 8-Week Exercise Program on Bone Biomarker Osteocalcin and Bone Histomorphometry Features in Male Rats

Alaa M. Musleh[1] *MSc*, **Zainab H. Hashim**[2] *PhD*, **Haider A. Jaafar**[3] *PhD*

[1]Dept. of Physiology, College of Medicine, Tikrit University, Saladin, Iraq, [2]Dept. of Physiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [3]Dept. of Anatomy, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background The anabolic effect of physical exercise on osseous tissue is related to mechanical effort, leading to the osteogenic response by causing dynamic changes, which stimulate osteocytes through fluid shifts in their canalicular network. They produce signaling molecules that regulate bone formation and absorption by osteoblast and osteoclasts.

Objective To study the effect of 8-week exercise training programs on the histomorphometry of male rat femur bone including weight, length, thickness and the bone formation biomarker (osteocalcin).

Methods The study was done in the labs of College of Medicine, Al-Nahrain University, from September 2019 to February 2020. A thirty adult healthy male rat (albino rat), were selected and divided into three groups; the group (A) of rats with exercise training programs of treadmill running, for 8 weeks. The control group, (B) were kept under free movements without exercise. The group (C) was kept under restricted movements in small cages. Tail blood sample were obtained twice from all animals; at zero day and after 8 weeks, for measurement of osteocalcin. Then after 8 weeks all animals were sacrificed and dissected for extraction of femoral bone for measuring bone length, weight, thickness and bone ultrastructure under light microscope by staining with hematoxylin and eosin.

Results The osteocalcin, femoral bone weight, length, thickness, haversian thickness and lamellar thickness showed significant increase in value of group A in comparison to group B and group C which show a significant decrease in femoral bone thickness, haversian thickness, lamellar thickness, and osteocalcin level.

Conclusion Exercise training has an anabolic effect on bone, in contrast to restriction movement that cause catabolic effect on bone. Osteocalcin increases with exercise and could be used as a marker in monitoring the exercise program therapy.

Keywords Femoral bone weight, length, thickness, haversian thickness, lamellar thickness, osteocalcin

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List of abbreviations: DEXA = Dual-energy X-ray absorptiometry, ELISA = Enzyme linked immunosorbent assay, RE = Resistant Exercise, TNF = Tumor necrotic factor

Introduction

Bone is a specialized form of connective tissue that, like other connective tissues, consists of cells and extracellular matrix. The feature that distinguishes bone from other connective tissues is the mineralization of its matrix, which produces an extremely hard tissue capable of providing support and protection. The mineral is calcium phosphate in the form of apatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. By virtue of its mineral content, bone also serves as a storage site for calcium and phosphate [(1)].

Both calcium and phosphate can be mobilized from the bone matrix and taken up by the blood as needed to maintain appropriate levels throughout the body. Thus, in addition to support and protection, bone plays an important secondary role in the homeostatic regulation of blood calcium levels. Bone matrix contains mainly type I collagen along with other matrix (non-collagenous) proteins. The major structural component of bone matrix is type I collagen and, to a lesser extent, type V collagen. Trace amounts of other types such as type III, XI, and XIII collagens have also been found in the matrix [(2)]. All collagen molecules constitute about 90% of the total weight of the bone matrix proteins. The matrix also contains other matrix (non-collagenous) proteins that constitute the ground substance of bone. As a minor component of bone, constituting only 10% of the total weight of bone matrix proteins, they are essential to bone development, growth, remodeling, and repair [(3)]. Bone matrix contains lacunae connected by a network of canaliculi. Within the bone matrix are spaces called lacunae (sing., lacuna), each of which contains a bone cell, or osteocyte [(4)].

The osteocyte extends numerous processes into small tunnels called canaliculi. Canaliculi course through the mineralized matrix, connecting adjacent lacunae and allowing contact between the cell processes of neighboring osteocytes. In this manner, a continuous network of canaliculi and lacunae-containing cells and their processes is formed throughout the entire mass of mineralized tissue. Bone tissue depends on the osteocytes to maintain viability. In addition to osteocytes, four other cell types are associated with bone [(5)]. Osteoprogenitor cells are cells derived from mesenchymal stem cells; they give rise to osteoblasts. Osteoblasts are cells that secrete the extracellular matrix of bone; once the cell is surrounded with its secreted matrix, it is referred to as an osteocyte. Bone-lining cells are cells that remain on the bone surface when there is no active growth. They are derived from those osteoblasts that remain after bone deposition ceases [(6)]. Osteoclasts are bone-resorbing cells present on bone surfaces where bone is being removed or remodeled (reorganized) or where bone has been damaged. Osteoprogenitor cells and osteoblasts are developmental precursors of the osteocyte. Osteoclasts are phagocytotic cells derived from fusion of hemopoietic progenitor cells in bone marrow that give rise to the neutrophilic granulocyte and monocyte lineages.

The anabolic effect of physical exercise on osseous tissue is related to mechanical effort, although the osteogenic response may also be influenced by other factors [(7)]. Physical loads associated with exercise impact bone mass and structure by causing dynamic changes to local mechanical conditions, which stimulate resident osteocytes through fluid shifts in their canalicular network. These osteocytes then produce signaling molecules that regulate bone formation and absorption by osteoblast and osteoclasts [(8)]. Bone tissue has an intrinsic "mechanostat" that regulates functional adaptation to stresses [(9)].

Bone mass can be measured well by densitometry; however, it is more difficult to accurately examine bone structure and strength in live tissue. Some substances produced during bone remodeling are specific biochemical markers of bone metabolism. Products of active osteoblasts can serve as markers of bone formation; serum concentrations of these markers reflect osteoblast function during specific phases of bone formation [(10)]. Coordination, self-assurance, and appropriate muscular strength help to prevent falls and preserve bone mass by stimulating bone formation and reducing bone resorption [(11)]. Training programs aimed at preserving bone health should incorporate three basic components: 1) impact exercise, such as brisk walking or jogging; 2) strength training with weights; and 3) balance training, while lower-impact exercises, such as walking, have minimal effects on density [(12)]. In contrast to aerobic exercise training, resistance training may have more profound site-specific effects on bone,

and progressive resistance training has further advantages in patients with osteoporosis due to the resulting improvements in muscle strength, mass, and balance [12]. The bone matrix proteins produced by the osteoblast include calcium-binding proteins such as osteocalcin. Circulating levels of osteocalcin are used clinically as markers of osteoblast activity.

The objective of this study is to study the effect of 8-week exercise training programs on the histomorphometry of femur bone including weight, length, and thickness, in addition to study the effect of 8-week exercise training programs on the bone formation biomarker (osteocalcin).

Methods

The study was done in the labs of College of Medicine, Al-Nahrain University, from September 2019 to February 2020. A thirty adult healthy male rat (albino rat), were selected, weighing 200-220 g whom age (2-4) months from animal house.

Animals were divided into three groups; the first group of 10 rats (group A) were the exercise sample with exercise training program of treadmill running, 1200 cm/min training for one hour per day, five days per week and for 8 weeks (by the treadmill shown in the figure 1). The second 10 rats (group B) were the control group whom kept under free movements without exercise. The third 10 rats (group C) were having restricted movements. The rats were housed in clean polypropylene cages having five rats per cage except third group where were housed in five small cages; two rats per cage to restrict their movement. The rats were given standard diet and water throughout the experimental period. At zero-day blood samples were obtained from the tail of all groups for serum separation by centrifuge and stored, this sample was regarded as A1, B1 and C1. Then after 8 weeks, another tail blood sample were obtained for serum separation and storage, thus the A1, B1 and C1 groups were regarded as A2, B2 and C2 groups respectively. The animals were euthanized by inhalation of chloroform in soaked cotton piece in airtight chamber for 3-5 min.

Figure 1. The treadmill used in the exercise training [13]

Then, the animal was set on anatomical stage on dorsal position and fixed the four limbs on dissecting table. Median incision through thigh was done by using scalpel and scissor (figure 2).

With a fine scissor was used to dissecting the whole femoral bone from the thigh (figure 3). Both femurs were extracted and immersed immediately in to 10% neutral buffered formalin. After cleaning of the femur from all soft tissues, measurement of the weight, length and thickness were done. Then further histological procedure was done so that to obtain paraffin block of specimens for histological assessment.

Figure 2. Dissection and extraction of femoral bone from the thigh

Figure 3. Dissection and extraction of femoral bone from the thigh

The effect of 8-week exercise training programs on the histomorphometry of femur bone including weight which was measured by electronic balance, length and thickness, which were measured by vernier caliper (figure 4), in addition to lamellar thickness, haversian thickness which were measured histologically using light microscope and Digital Image Analysis Software. Osteocalcin was measured using serum for enzyme linked immunosorbent assay (ELISA) method.

Statistical analysis

The statistical package for social sciences (SPSS) version 19 were used for all statistical analyses. The data are expressed as mean and standard deviation. P value at ≤ 0.05 was regarded as significant.

Figure 4. Vernier caliper used for measuring the femoral bone length and thickness

Results

The mean level of weight of group A2 (0.68±0.06) g was higher than group B2 (0.54±0.11) g, this difference was statistically significant (P value ≤ 0.05). The mean level of length among group A2 (3.53±0.23) cm was higher than group B2 (3.21±0.1) cm, this relation was statistically significant (P value ≤ 0.05). The mean level of bone thickness among group A2 (3.05±0.22) mm was higher than group B2 (2.74±0.2) mm, this difference was statistically significant (P value ≤ 0.05). Histologically, the mean level of lamellar thickness among A2 group (164.66±8.5) µm was higher than group B2 (158.53±3.48) µm, this difference was statistically significant. The mean level of Haversian thickness among A2 group (113.35±12.17) µm was higher than group B2 (93.6±26.26) µm, this relation was statistically significant (Table 1).

Table 1. Comparison of morphometric and histomorphometric measurements between group A2 and group B2

Parameter	Study groups	Mean	Std. Deviation	P value
Weight (g)	A2	0.68	0.06	0.002
	B2	0.54	0.11	
Length (cm)	A2	3.53	0.23	0.001
	B2	3.21	0.10	
Thickness (mm)	A2	3.05	0.22	0.004
	B2	2.74	0.20	
Lamellar thickness (µm)	A2	164.66	8.50	0.04
	B2	158.53	3.48	
Haversian thickness (µm)	A2	113.35	12.17	0.045
	B2	93.60	26.26	

P value is significant at ≤ 0.05, g=gram, cm=centimeter, mm=milliliter, µm=microliter

The mean level of femoral bone length among group A2 (3.53±0.23) cm was higher than group C2 (3.12±0.11) cm, this difference was statistically significant. The mean level of femoral bone thickness among group A2 (3.05±0.22) mm was higher than group C2 (2.39±0.38) mm, this difference was statistically significant. The mean level of lamellar thickness among A2 group (164.66±8.5) µm was higher than group C2 (145.36±10.6) µm, this difference was statistically significant. The mean level of Haversian thickness among A2 group

(113.35±12.17) µm was higher than group C2 (55.01±21.26) µm, this difference was statistically significant (Table 2).

Table 2. Comparison of morphometric and histomorphometric measurements between group A2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Weight (g)	A2	0.68	0.06	0.0001
	C2	0.54	0.04	
Length (cm)	A2	3.53	0.24	0.0001
	C2	3.12	0.11	
Thickness (mm)	A2	3.05	0.22	0.0001
	C2	2.39	0.38	
Lamellar thickness (µm)	A2	164.66	8.5	0.0001
	C2	145.36	10.6	
Haversian thickness (µm)	A2	113.35	12.17	0.0001
	C2	55.01	21.26	

P value is significant at ≤ 0.05, g=gram, cm=centimeter, mm=milliliter, µm=microliter

The mean level of weight of group B2 (0.54±0.11) g was almost equal to group C2 (0.54±0.04) g. The mean level of length among group B2 (3.21±0.1) cm was higher insignificantly than group C2 (3.12±0.1) cm, (P value > 0.05). The mean level of bone thickness among group B2 (2.74±0.2) mm was higher than group C2 (2.39±0.38) mm, this difference was statistically significant (P value ≤0.05). Histologically, the mean level of lamellar thickness among B2 group (159.33±433) µm was higher than group C2 (145.36±10.6) µm, this difference was statistically significant (P value ≤0.05). The mean level of Haversian thickness among B2 group (93.60±26.26) µm was significantly higher than group B2 (55.01±21.26) µm, (P value ≤0.05) (Table 3).

Table 3. Comparison of morphometric and histomorphometric measurements between group B2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Weight (g)	B2	0.54	0.11	0.9
	C2	0.54	0.04	
Length (cm)	B2	3.21	0.10	0.09
	C2	3.12	0.10	
Thickness (mm)	B2	2.74	0.20	0.01
	C2	2.39	0.38	
Lamellar thickness (µm)	B2	159.33	4.33	0.001
	C2	145.36	10.60	
Haversian thickness (µm)	B2	93.60	26.26	0.002
	C2	55.01	21.26	

P value is significant at ≤ 0.05, g=gram, cm=centimeter, mm=milliliter, µm=microliter

The means of all the morphometric and histomorphometric measurement of the three study groups are illustrated in the figures 5 and 6.

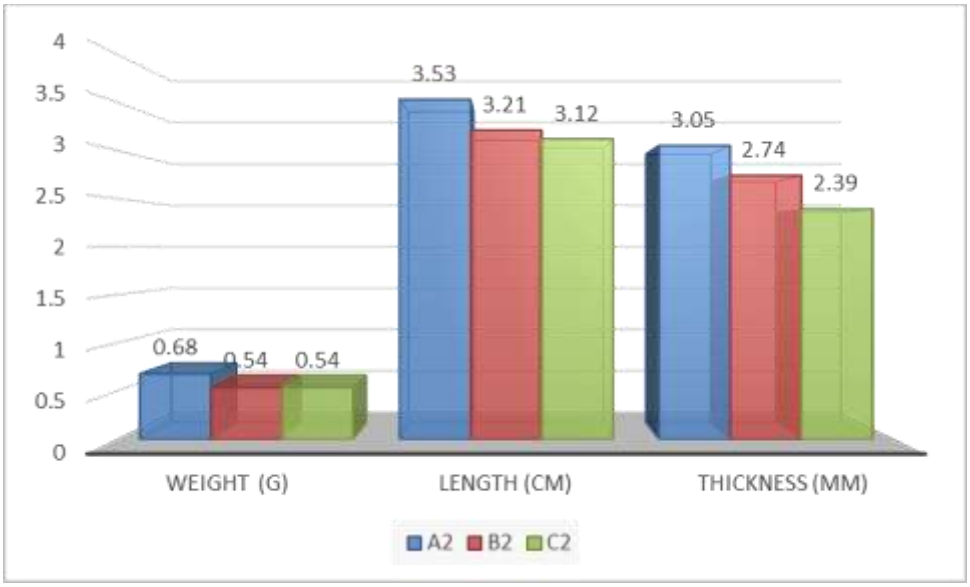

Figure 5. The morphometric measurements among group A2, B2 and C2 groups

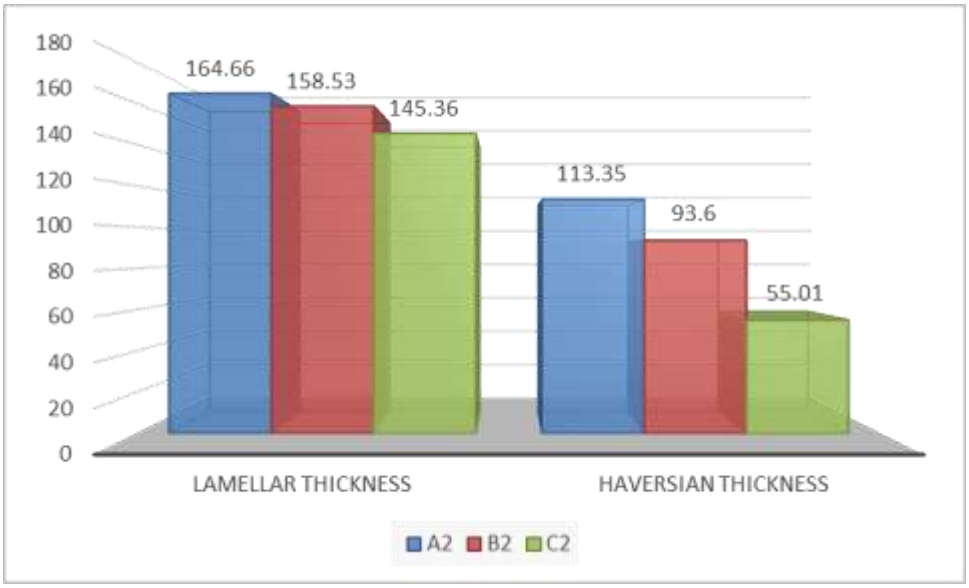

Figure 6. The histomorphometric measurements among group A2, B2 and C2 groups

The mean level of Osteocalcin (11.52±1.48) ng/ml among group A2 was higher than A1 (6.86±4.81) ng/ml, this difference was statistically significant. The mean level of

osteocalcin (6.1±1.28) ng/ml among group B2 was higher than B1 (6.92±1.49) ng/ml, this relation was statistically non-significant. The mean level of osteocalcin (5.20±0.79) ng/ml among group C2 was less than C1 (6.68±1.69) ng/ml, this relation was statistically significant. (Table 4).

Table 4. Comparison of osteocalcin before and after 8-week exercise in the three study groups

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	A1	6.86	4.81	0.009
	A2	11.52	1.48	
	B1	6.92	1.49	0.2
	B2	6.10	1.28	
	C1	6.68	1.69	0.02
	C2	5.20	0.79	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

The mean level of osteocalcin among A2 group (11.52±1.48) ng/ml was higher than group B2 (6.1±1.28) ng/ml, this difference was statistically significant (Table 5), also significantly higher than group C2 (5.2±0.8) ng/ml (Table 6). While there is no statistical difference in the mean level of ostoecalcin between group B2 and C2 (Table 7).

Table 5. Comparison of osteocalcin between group A2 and group B2

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	A2	11.52	1.48	0.0001
	B2	6.10	1.28	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

Table 6. Comparison of osteocalcin between group A2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	A2	11.52	1.48	0.0001
	C2	5.20	0.79	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

Table 7. Comparison of osteocalcin between group B2 and group C2

Parameter	Study groups	Mean	Std. Deviation	P value
Osteocalcin (ng/ml)	B2	6.10	1.28	0.07
	C2	5.20	0.79	

P value is significant at ≤ 0.05, ng/ml=nanogram/milliliter

Discussion

The femoral bone net weights, length, bone thickness, lamellar thickness and Haversian thickness of the exercise group in the present study, revealed a significant increase in their values in comparison to other groups (control and restricted groups) as well as in control group as compared to restricted one This is in agreement with other researchers who found that exercise and training has been recommended as a promising therapeutic strategy to encounter the loss of bone and muscle mass due to osteopenia and sarcopenia. They concluded that stimulation of the osteogenesis in order to increase the bone mass, bone tissues must be exposed to mechanical load exceeding those experienced during daily living activities. The over exercise of the several exercise trainings programs, is known to be highly beneficial for the preservation of bone and muscle mass. They summarized that the mechanisms of over exercise for the preservation of bone and muscle mass and supports the clinical evidences for the use of other form of resistant exercise (RE) as a therapeutic option in osteopenia and sarcopenia [14]. certain researchers found that the long-term bedridden patients tend to had reduced bone mineral density with greater predicting for bones fractures. They concluded that unique bone metabolic abnormalities were found in patients who had been bedridden for long periods, and these metabolic abnormalities were altered by further bed confinement [15].

In the present study, found that there was a marked increase in serum osteocalcin level in the exercise group after 8-week exercise program protocol in comparison to the pre-exercise period, restricted and even the control group that recorded low value. This result agrees with other researchers who found that the with an aging population, which has little and limited diurnal movements, and there was a marked increase in prevalence of metabolic bone diseases, especially osteoporosis as a consequence of these conditions [16].

Up to now, several roles of osteocalcin have been revealed and current reports, which are mainly based on murine and in vitro [17]. Since osteocalcin has been reported to regulate glucose metabolism, which provides energy to muscles during exercise, it may be involved in the communication between these two tissues [17]. Initially, osteocalcin levels were found to increase in mice and humans during exercise [8]. Furthermore, the Karsenty group demonstrated that osteocalcin levels decline during aging, coinciding with a diminished exercise capacity and a decrease in muscle mass [19].

This study concluded that the mechanical physical effects of muscles activities have positive values on compact bone density and thickness via measurement the gross morphological and microscopically histological evaluation procedures of the bones, in contrast to restriction movement that cause catabolic effect on bone. Osteocalcin increases with exercise and could be used as a marker in monitoring the exercise program therapy.

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Author contribution

Dr. Musleh conducted the study, collected the data, performed the statistical analysis and drafting the manuscript. Dr. Hashim and Dr. Jaafar contributed in the designing, organization and finalization of manuscript.

Conflict of interest

There are no conflicts of interest.

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Correspondence to Dr. Alaa M. Musleh
E-mail: alalmu31@gmail.com
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