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Joint immobilization: effects on bone tissue of obese and malnourished animals

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ABSTRACT

Introduction: The immobilization induces an unbalanced bone metabolism, followed by rapid bone loss and consequent loss of the mechanical function of the bone. In general, the obesity and protein malnutrition conditions affect a large number of people worldwide, and both morbidities have specific characteristics that may cause deleterious effects on bone tissues of patients by different mechanisms. **Objective**: the present study aimed to verify experimentally if the joint immobilization protocol causes bone tissue atrophy in obese and undernourished animals. **Method**: 20 adult male mice (C57/BL6) were used, divided into four groups (N=5): Control Group (CG), Immobilized Control Group (ICG), Immobilized Obese Group (IOG) and Immobilized Malnourished Group (IMG). The histomorphometric analysis of the tibia quantified the number of osteocytes, cortical thickness and diameter of the medullary canal. **Results**: The study involving the tibia of the animals showed statistical differences in variables analysis. All immobilized groups showed lower amount of osteocytes in the evaluated tissue and increase in diameter of the spinal canal when compared to the CG. The cortical thickness was reduced in ICG and IMG groups when compared to the CG. **Conclusion**: The used joint immobilization protocol caused bone atrophy in the studied animals. The association between obesity, malnutrition and joint immobilization conditions cause increase in bone tissue atrophy.

Keywords: Immobilization; Obesity; Protein Malnutrition; Atrophy.

INTRODUCTION

The bone tissue is a specialized type of connective tissue made up of cells and calcified extracellular material, exhibiting constant remodeling. According to Wolff's law, in addition to changes in bone function, also occur alterations in the internal architecture and external bone conformation. Therefore, the mechanical load is an essential particularity for the normal functioning of the bone tissue. [1, 2]

The performance of active exercise may increase or decrease the weight, length and bone stiffness, depending on age, gender and the characteristics of the individual. However, the lack of physical activity, immobility, weakness and neuromuscular injury, negatively affect the metabolism of bone tissue. [1]

In this sense, the immobilization induces an unbalanced bone metabolism, followed by rapid bone loss and consequent loss of the mechanical function of the bone.[2] Bone loss and its properties, due to a long period of immobilization, tend to occur in stages: the first occurs after a few days of immobilization; the second, later, with a slow bone loss and with long duration; and the last one named stabilization period can reduce bone mass between 40% and 70% of the original. [3] Should also be emphasized that the time required for recovery from bone atrophy is considerably higher compared to the time in which it is developed in immobilized patients. [2]

Obesity can be defined as an abnormal and/or excessive accumulation of fat in the tissues, which can be harmful to health. Considered to be a multifactorial disease, is carried out principally by a positive energy balance, i.e. food intake is higher than the energy spent of the individual. [4]

Overweight and obesity are considered the fifth major cause of death in adults. The World Health Organization [4] noted that about 1.9 billion adults in the world are overweight and among these, over 600 million are belong to category of obese people. In Brazil obesity levels reach the rate of 17.4% in the national population. [5] In the field of public health, obesity becomes worrying for being an important risk factor for morbidities such as cardiovascular disease, metabolic syndrome and disorders of the locomotor system. [6]

While malnutrition is characterized as a disease which arises from the insufficient food intake of energy and

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nutrients, or even with some frequency, inadequate biological utilization of food ingested. [7] The report of food insecurity in the world [8] showed that 805 million people are within the limits of malnutrition and that practically half of the affected individuals are children. The report also reveals that Brazil reduced expressively malnutrition and reached level below 5% of the population.

Protein malnutrition condition generates as result a number of changes in body composition and functioning of the organism. Therefore, among the main consequences are: slowing, stopping and involution in tissue growth; bone abnormalities such as fractures and malformations; great muscle loss and fat deposits, which causes physical weakness; decline in immune function and physical, mental and psychological changes.[9]

In general, the obesity and protein malnutrition conditions affect a large number of people worldwide, and both morbidities have specific characteristics that may cause deleterious effects on bone tissues of patients by different mechanisms. [10-12]

Knowing that both obesity and malnutrition are likely to present interactions that enhance metabolic disorders in the bone tissue of these animals, was decided to evaluated if the joint immobilization protocol causes bone tissue atrophy in these experimental models.

METHODS

Experimental design

This study followed the ethical and legal requirements for animal research, and was approved by the Ethics Committee on Animal Use of Universidade Estadual de Campinas (CEUA / Unicamp), protocol number 2872-1 from 10/04/2012.

The research was characterized as a controlled study of experimental and analytical type in which were used 20 male mice (C57/BL6), adults (105 days) from the Multidisciplinary Center for Biological Research (CEMIB/Unicamp), kept in standard plastic cages on a temperature of 22° Celsius with light/dark cycle of 12 hours and receiving water and specific food to each group *ad libitum*.

The animals divided into four groups: Control Group (CG, n=5), Immobilized Control Group (ICG, n=5), Immobilized Obese Group (IOG, n=5) and Immobilized Malnourished Group (IMG, n=5). The immobilization of the hindlimb was performed on the 91st day of life of the animals and maintained until the end of the experiments (105th day).

To induce obesity, soon after weaning, the animals received a normoproteic (14% protein) and normolipidic diet until they reach 45 days of life. Then, until the end of the experiment (105th day) the animals received a hyperlipidemic diet (34% lipids and 14% protein). To induce protein malnutrition, soon after weaning, the animals received a hypoproteic diet containing 6% protein (Appendix). The intake of this diet remained until the end of the experimental period (105th day).

The animals were weighed in an analytical balance (Shimadzu Corporation, AUW220D model, 0.00001g precision, Kyoto, Japan). The body weight of animals, weight of the retroperitoneal and perigonadal adipose tissues were used as variables for evaluation and characterization of the obese and malnourished groups.

To perform the immobilization protocol, the following steps were followed: Previously, the animals were anesthetized with Ketamine 50mg/kg and xylazine 8 mg/kg of body weight. Subsequently, the right hindlimb was trichotomized and immobilized of prior form with hypoallergenic microporous (Cremer S.A, Sao Paulo, SP, Brazil). Then immobilization was performed by waterproof tape strips with 3 cm in width (Cremer S.A, Sao Paulo, SP, Brazil), in the pelvis, hip, knee (extension) and ankle (in plantar flexion). Further, a plaster strip 2 cm wide and 10 cm length (Cremer S.A, Sao Paulo, SP, Brazil), was moistened and applied without much torque pressure around the limb of the animal, in order to prevent that the mouse destroy the immobilization made with the tape. Immobilization was checked daily, replaced when damaged and maintained for two weeks (14 days).

At the end of treatment, the animals were anesthetized by carbon dioxide inhalation and sacrificed by decapitation, according to the resolution of the Federal Council of Veterinary Medicine (CFMV) number 714, 06/20/2002, for further dissection of the tibia.

After dissection, the distal tibial periarticular segment was fixed in 10% buffered formalin solution for 24 hours at 22ºC. Then, the fabric underwent a preliminary procedure for decalcification using ethylenediaminetetraacetic acid (EDTA) for 20 days. Subsequently, samples were washed and followed to dehydration in alcohol baths, further clarification with xylol baths and inclusion in paraffin process.

After these steps were performed transverse cuts of 7cm thickness with a microtome (Leica Biosystems Nussloch GmbH, RM2125RT model, Nubloch, Germany). Staining of sections was performed through hematoxylin and eosin technique and mounted with coverslips in Canada balsam.

For the histomorphometric study, the images were captured by a camera (Olympus America Inc, QCOLLOR5 model, Center Valley, Pennsylvania, USA) coupled to an optical microscope (Olympus America Inc, BX53 model, Center Valley, Pennsylvania, USA). All images were captured in the 4X objective.

The histomorphometric study of the tibia quantified the cortical thickness, diameter of the medullary canal and number of osteocytes by Image J Software (Image_J, Bethesda Softwork, Rockville, Maryland, USA). Four cuts were made in each sample and was measured in each cut five different points for the thickness of the cortical layer, five random visual fields for counting the osteocytes, besides the diameter of the

medullary canal measured at its entirety. The variables were quantified in five animals per group.

To determine the sample size was used the equation $n = 1 + [2C.(s/d)^2]$ in which C= $(z\alpha + z\beta)^2$. *C* is dependent on the values chosen for strength or power test (β = 90%) and significance level (α = 0.05). ***s*** is the acceptable standard deviation according to the projection of the researcher (20%) and ***d*** is the expected difference between the groups (50%). [13, 14] However, the result obtained is n = 4.36 which was approximate to five animals per group.

For statistical analysis was used initially the Shapiro-Wilk test. After verify the normality of the data, we used analysis of variance (ANOVA) followed by the Tukey's post hoc test for multiple comparisons. Statistical significance was set at p <0.05 with 95% confidence interval, using as analysis feature the GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Characterization of the experimental groups

The result of the comparison of total body weight between the groups showed a statistically significant difference (p<0.05) with test power of 90%. In comparison made on the day of sacrifice (105th day), the ICG and IMG groups presented, respectively, decreased by 8.1% and 17.5% in body weight (g) when compared to the CG (p<0.05). But the IOG group showed weight 14.3% higher when compared to the CG (p<0.05). When comparing the immobilized groups, can be observed that the IOG showed weight 25.4% higher when compared to ICG (p<0.05) and IMG showed a 10.3% reduction in body weight when compared to ICG (p<0.05).

The data relating to the retroperitoneal adipose tissue showed statistically significant difference (p<0.05) with test power of 90%. The values showed a quantity 42.3% higher in fat reserves of IOG when compared to CG (p <0.05) and a quantity 67.93% greater when compared to ICG (p <0.05). The IMG presented reduction in retroperitoneal fat reserves in relation to CG and ICG, however, with no significant difference.

The results related to perigonadal adipose tissue showed statistically significant difference (p<0.05) with test power of 90%. The IOG presented fat reserves 23.1% higher than the CG and 27.3% higher than the ICG (p<0.05). However, was observed in the IMG a 32% lower rate of the amount of adipose tissue in relation to CG (p <0.05) and 29.6% lower in relation to the ICG (p <0.05). All data are shown in absolute values in Table 1.

Histomorphometric analysis of the bone tissue

The study involving the tibia of the animals showed statistical differences in all variables (p<0.05) with test power of 90%. Quantifying the number of osteocytes is observed that the ICG, IOG and IMG presented, respectively, quantities 7.6%, 7.7% and 7.9% lower when compared to CG (p<0.05). The comparison between the immobilized groups showed no statistical difference.

The values regarding the thickness of the cortical bone layer demonstrated that the ICG and IMG showed lower values when compared to CG (p<0.05). However, was observed that the IOG showed no statistical difference when compared to the CG and, in immobilized groups, the IMG showed a significant reduction compared to ICG (p<0.05).

The values found relating to the diameter of the medullary canal showed higher values in all groups compared to CG (p <0.05). When comparing only the immobilized groups, the IOG and IMG presented, respectively, values 20.6% and 20.7% lower when compared to ICG (p <0.05). The absolute values of the morphometric analysis of the tibia are shown in Table 2.

DISCUSSION

In the context of orthopedics, the joint immobilization is an effective and widely therapeutic approach used in situations involving bone fractures. However, this procedure may lead to hemostatic and morphofunctional disorders in bone tissue, with a consequent decrease of their mechanical properties. [2, 15]

In this study, the used immobilization generated a reduction in the number of osteocytes and a reduction in the diameter of the medullary canal (μm) in all immobilized groups when compared to CG. Nonetheless, the cortical thickness was significantly reduced only in the ICG and IMG when compared to the CG. Thus, the results suggest that the used joint immobilization procedure adversely affect the bone tissue of the evaluated animals.

Table 1. Body weight (g), weight of the retroperitoneal and perigonadal adipose tissue (mg) evaluated according to the specific diet consumed by each group. CG= Control; ICG= Immobilized Control; IOG= Immobilized Obese; IMG= Immobilized Malnourished.

Groups	Body weight (g)	Retroperitoneal adipose tissue (mg)	Perigonadal adipose tissue (mg)
CG	25.7±0.3	85.57±5.8	254.0±7.8
ICG	23.6±0.3*	72.53±1.9	245.6±12.1
IOG	29.4±0.3*#	121.8±8.1*#	312.7±14.7*#
IMG	21.2±0.3*#	67.90±4.6	172.8±14.1*#

NOTE: * statistically significant values in relation to the CG (p <0.05); # statistically significant values in relation to the ICG (p <0.05). Values expressed as mean ± standard error, Anova One Way with Tukey's post hoc test for multiple comparisons with p<0.05, test power of 90% and confidence interval of 95%.

The mechanical stimulation can justify such results, because when reduced, whether through sedentary habits or disuse caused by systemic or regional conditions (e.g. immobilization), entails a process of adaptation with increased bone resorption and consequently bone weakening. [15] This result seems to be associated with the decrease in bone metabolism, which causes a decrease in the cells number of the maintenance of this tissue. [15-18]

According to studies by Yeh et al., [16] the immobilization protocol allows the reabsorption and depression of the bone formation, results in decreased bone mass relative to its volume and simultaneously changes the material and geometric properties of the bone. It is estimated that about 30% of the bone loss induced by immobilization experiment in rats is caused by increase of bone resorption and about 70% by decreased bone formation. [17]

Reinforcing our results, Kiratli [18] showed that joint immobilization resulted in an imbalance in bone metabolism, followed by a rapid loss of tissue and impairment of mechanical function. According to the theory of structural adjustment proposed by Frost,[19] the decrease in the mechanical use reduces the bone mass gain, depresses the longitudinal growth of the bone and stimulates loss bone dependent of the resorption.

On the other hand, Portinho et al. [20] found that two weeks of immobilization caused no significant changes in the diameter of the medullary canal and bone thickness of the femur of *Wistar* rats; however, there was a significant decrease in the number of osteocytes in these animals. These findings were justified in previous study, in which the author found that the period of detention, analyzed bone, age and species of animal directly influenced the data relating to the bone tissue. [2]

Analyzing specifically the immobilized groups, it can be noticed that the number of osteocytes showed no statistical difference in the comparison between groups. However, the result of the evaluation of cortical thickness presented a statistical difference, showing a decrease of 15.8% in the IMG when compared to the ICG. In the evaluation of the diameter of the medullary canal, the IOG and IMG had significantly higher values when compared to the ICG. Guided by these results, was observed that the animals of the IOG and IMG presented, in general, a greater loss in bone tissue when compared to the ICG.

Regarding the results found for the IOG, can be observed that the values relating to the count of osteocytes and cortical thickness showed no statistical differences in relation to the ICG. In contrast, the diameter the medullary canal presented high values in relation to ICG. Therefore, it is suggested that obese animals (IOG) have a disadvantage in bone metabolism when compared to animals of ICG.

According to Brandalize and Leite, [21] bone tissue has the capability to reshape itself according to the load exerted on it. Studies conducted in adults and children have shown that bone mass is directly related to the body weight of the individual, although there is controversy regarding the fact whether the fat or lean mass is the most important determinant of bone density. [21, 22] The positive association (direct) between body weight and bone mass can be attributed to the increased mechanical load on the skeleton, particularly in the cortical elements. [21, 22]

However, the bone tissue seems extremely sensitive to reduction in body weight. Recent studies have shown that a 10% reduction in body weight may adversely affect the bone tissue, which may reduce 2% the mass. [10, 11] The impact can be even greater in individuals exposed to rapid and severe weight loss [23] when compared to those who have moderate reduction in long periods. [24]

Based on these findings, it was suggested the hypothesis that the IOG showed an increase in diameter of the medullary canal in relation to the ICG due to a structural change in the bone tissue as a result of the rapid loss of body weight. This phenomenon was not measured in this study, but the literature indicates that the joint immobilization protocol generates weight loss in animals imposed to such condition. [2, 25] Nonetheless, for better understanding of what happened, are necessary depth studies in the area of interest in order to elucidate the mechanisms involved.

Regarding the joint immobilization procedure and considering that the ICG and IMG are in similar conditions, was highlighted that the results for the IMG indicated that diet intake of low protein content accentuated bone loss and increased bone fragility through mechanisms which probably involved changes in the regulation of IGF-1 levels.

Table 2. Number of osteocytes, cortical thickness (µm) and diameter of the medullary canal (µm). CG=Control; ICG= Immobilized Control; IOG= Immobilized Obese; IMG= Immobilized Malnourished.

Groups	Number of Osteocytes	Cortical thickness µm	Diameter of the medullary canal µm
CG	23.69±0.56	203.1±1.5	663.6±9.1
ICG	21.89±0.46*	195.5±2.1*	720.7±9.9*
IOG	21.87±0.35*	196.7±1.4	869.6±5.2*#
IMG	21.82±0.36*	164.6±2.1*#	870.1±18.6*#

NOTE: * statistically significant values in relation to the CG ($p < 0.05$); # statistically significant values in relation to the ICG ($p < 0.05$). Values expressed as mean ± standard error, Anova One Way with Tukey's post hoc test for multiple comparisons with $p<0.05$, test power of 90% and confidence interval of 95%.

Clinical and experimental studies suggest that dietary proteins influence the production and action of growth factors, in particular the growth hormone (GH), which may influence bone metabolism. Moreover, another factor influenced by the consumption of proteins which also plays a key role in bone metabolism is the IGF-1 metabolism. Therefore, higher levels of IGF-1 contribute to the formation of the bone whereas reduced levels promote reabsorption. [12, 26]

According to Thissen et al., [27] a dietary protein influences on the production and action of IGF-1 and thus on bone metabolism. On the other hand, its restriction reduces plasma levels of IGF-1, because induces a resistance to the action of the GH hormone in the liver [28] and increases the metabolic clearance rate of IGF-1. [29] The increase in protein intake will prevent the decrease in IGF-1 levels observed in malnourished states and feeding the malnourished patients will increase and restore IGF-1 levels. [30]

Based on our results, we conclude that this study extended the knowledge of the events involved in bone tissue atrophy induced by immobilization, and associated with obesity and malnutrition. Though, depth studies on these issues should be conducted in a manner that provides greater scientific information on bone atrophy in periods of joint immobilization.

CONCLUSION

According to the results obtained in this study and from the comparison of these data with those in the literature, can be concluded that:

- The used joint immobilization protocol caused bone atrophy in the studied animals.
- Obese and malnourished animals showed higher loss in the bone tissue as compared to healthy animals.
- The association between obesity, malnutrition and joint immobilization conditions caused increase in bone tissue atrophy.

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AUTHORS' CONTRIBUTIONS

RR conducted the practical part of the experiments, collected and analyzed the data, wrote and edited the article. GAL, ILSP and RLC participated in the elaboration of practical experiments and edited the article. ETP participated in orientation, writing and final review of the article.

CONFLICT OF INTERESTS

We declare that there is no potential conflict of interest between the authors.

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APPENDIX

I - TABLE OF THE DIETS NUTRITIONAL COMPOSITION.

Ingredients (g/Kg)	Normoproteic and Normolipidic (AIN-93M)	Hypoproteic (6% protein)	Hyperlipidemic (34% lipids)
Casein	140	71.4	140
Corn starch	465.7	511.9	208.7
Dextrin	155	178.6	100
Sucrose	100	100	100
L-cysteine	1.8	0.6	1.8
Fiber (microcelulose)	50	50	50
Soy oil	40	40	40
Lard	-	-	312
Mixture of salts AIN93G*	35	35	35
Mixture of vitamins* AIN93G	10	10	10
Choline chloride	2.5	2.5	2.5

* Mixtures of mineral salts and vitamins were prepared according to the norm; AIN-93 (REEVES & NIELSEN *et al.*, 1993).