UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA
CÂMPUS DE ARAÇATUBA

LUIS CARLOS NOBRE DE OLIVEIRA

Consequências do desuso por imobilização na resistência mecânica óssea de ratos adultos sedentários ou submetidos ao treinamento resistido

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2020

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Tese apresentada à Faculdade de Medicina Veterinária – Unesp, Campus de Araçatuba, como parte das exigências para a obtenção do título de Doutor em Ciência Animal (Fisiopatologia Médica e Cirúrgica)
Orientadora: Profª. Associado Mary Marcondes
Coorientador: Prof. Dr. Mário Jefferson Quirino Louzada

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DEDICATÓRIA

À Luciana Ruas Esgalha de Oliveira, minha esposa, e meus dois filhos Eduardo e Elisa, com amor, admiração e gratidão pela compreensão, carinho, presença e incansável apoio ao longo do período de elaboração deste trabalho.

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“Verás que um filho teu não foge à luta”
Joaquim Osório Duque Estrada

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RESUMO

Tendo em vista a alta incidência de pacientes adultos com fraturas ósseas decorrentes do desuso é que o presente estudo teve como objetivos analisar o efeito do desuso por imobilização na resistência mecânica óssea de ratos adultos sedentários ou submetidos ao treinamento resistido, como um possível modelo experimental para os seres humanos. Foram utilizados 40 ratos (*Rattus novergicus albinus*), linhagem Wistar, machos, adultos (14 meses), divididos em quatro grupos de 10 animais cada; mantidos em gaiolas (controle), sedentarismo seguido de imobilização gessada (controle imobilizado), exercício físico que realizou treinamento resistido (EF) e exercício físico que realizou treinamento resistido seguido de imobilização gessada (EFI). Avaliou-se a microarquitetura óssea, o conteúdo, área e densidade mineral óssea, a rigidez extrínseca, energia e a força máxima do colo femoral. Os dados foram analisados por meio de análises de variância (ANOVA) *TWO-WAY*, *THREE-WAY*. O nível de significância considerado foi $p<0,05$. Baseados nos resultados obtidos nas condições do presente estudo, o exercício físico resistido foi benéfico para a densidade mineral óssea mesmo após o período de desuso por imobilização gessada, porém, a dose reposta do exercício resistido com a carga pré estimulada, após o período do desuso, não preveniu a osteopenia.

Palavras-Chave: Densidade Óssea. Imobilização. Microtomografia por raio-X. Treinamento físico.

OLIVEIRA, L.C.N. **Consequences of disuse by immobilization on bone mechanical resistance of adult rats sedentary or submitted to resistance training.** 2020. 63 f. Thesis (Doctorate) - Faculty of Veterinary Medicine, Universidade Estadual Paulista, Araçatuba, 2020.

ABSTRACT

In view of the high incidence of adult patients with bone fractures resulting from disuse, the present study aimed to analyze the effect of disuse by immobilization in mechanical resistance of adult rats sedentary or submitted to resisted physical exercise, as a possible experimental model for humans. 40 rats (*Rattus novergicus albinus*), Wistar lineage, male, adults (14 months) were used, divided into four groups of 10 animals each; kept in cages (control), physical inactivity followed by cast immobilization (immobilized control), physical exercise that performed resistance training (PE) and physical exercise that performed resistance training followed by cast immobilization (IPE). The bone microarchitecture, bone mineral content, area and density, extrinsic stiffness, energy and maximum strength of the femoral neck were evaluated. The data were analyzed using analysis of variance (ANOVA) TWO-WAY, THREE-WAY. The level of significance considered was $p < 0.05$. Based on the results obtained in the conditions of the present study, resistance physical exercise was beneficial for bone mineral density even after the period of disuse due to plaster immobilization, however, the replaced dose of resistance exercise with the pre-stimulated load, after the period of disuse, did not prevent osteopenia.

Keywords: Bone Density. Immobilization. X-ray Microtomography. Physical training.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

με – microstraing/tensão
ANOVA= análises de variância
Área= área mineral óssea
BMD= bone mineral density
BV/TV= fração do volume ósseo
C= controle
CEUA= Ethics Committee on Animal Experiment
CI= controle imobilizado
cm^2= centímetro quadrado
CMO= conteúdo mineral ósseo
CMV= capacidade máxima de geração de força voluntária
COBEA= Brazilian College of Animal Experimentation
Ct.Ar= área óssea cortical
Ct.Th= espessura cortical média
DEXA= densitômetro de dupla emissão de raios-X
DMO= densidade mineral óssea
DMOa= densidade mineral óssea areal
EF= exercício físico
EFI= exercício físico imobilizado
g/cm^2= grama por centímetro quadrado
g= grama
mg/Kg= miligrama por quilo
mm/min= milímetro por minuto
mm= milímetro
mm^2= milímetro quadrado
N/mm= newton por milímetro
N= newton
NaCl= cloreto de sódio
p= nível de significância
SEM= standard error of the mean
SMI= índice do modelo de estrutural
Tb.N= número médio de trabécula

Tb.Sp= distância média entre trabécula

Tb.Th= espessura média das trabéculas

TR= treinamento resistido

vs= versus

μm= micrômetro

SUMÁRIO

1. INTRODUÇÃO GERAL

O tecido ósseo constitui um sistema vivo, em constante atividade cuja principal função é a formação e manutenção do esqueleto, proporcionando locomoção, proteção e reserva mineral (CIVITELLI, 2008).

Sob o aspecto morfológico o osso pode ser dividido em osso cortical, representando 85% de tecido ósseo, e osso trabecular, representando os demais 15% do tecido ósseo. O osso cortical é predominante nas diáfises dos ossos longos e em menor proporção recobrindo a superfície do esqueleto axial. Sua distribuição é concêntrica, formada por vasos sanguíneos, linfáticos, nervos e tecido conjuntivo (DOBLARÉ et al., 2004). O osso trabecular compreende a microarquitetura interna do tecido ósseo, marcada por inúmeras espículas ósseas orientadas de acordo com a tensão em que o osso é submetido. É predominante em corpos vertebrais e pelve, mas também pode ser encontrado nas epífises dos ossos longos (MARTIN; BOARDMAN, 1993). Metabolicamente o osso trabecular é mais ativo que o osso cortical (KEAVENY et al., 2001).

No panorama histológico, o osso é um tecido conjuntivo mineralizado, altamente vascularizado e inervado, estruturado em lamelas de matriz osteóide calcificada. A disposição dessas lamelas determina se o osso é cortical ou esponjoso. O osso cortical ou compacto, contendo osteócitos, é organizado concentricamente em torno dos canais de Havers. O osso esponjoso ou trabecular é formado por uma rede de lamelas ósseas, delimitando cavidades areolares dentro das quais a medula óssea é encontrada. Tanto o osso cortical como o trabecular contêm células especializadas, matriz orgânica e fase mineral (HERNÁNDEZ-GIL et al., 2006).

O tecido ósseo, constituído por duas linhagens celulares, osteoblástica e osteoclástica, é uma forma especializada de tecido conjuntivo composto por uma matriz extracelular, que possui como característica a mineralização, que lhe confere extrema aspereza, permitindo-lhe representar importantes funções de sustentação e proteção (KHAN et al., 2007). As células da linhagem osteoblástica são responsáveis pelo processo de formação da matriz óssea, enquanto aquelas da linhagem osteoclástica estão relacionadas com a sua reabsorção (BONEWALD; JOHNSON, 2009). As funções de suporte estrutural e de reserva metabólica encontram-se, em condições fisiológicas, em um equilíbrio estável. Porém, havendo um desequilíbrio, a função estrutural é sempre danificada (KHAN et al., 2007).

A densidade mineral óssea (DMO) na idade adulta depende predominantemente do crescimento e mineralização do esqueleto. Em indivíduos saudáveis, 80% da massa óssea é acumulada até os 18 anos de idade, e um pico de DMO reduzida na infância e adolescência é considerado como um dos fatores predisponentes mais importantes para a ocorrência de fraturas em adultos e idosos. Um osso saudável é metabolicamente ativo e sofre contínua remodelação para manter o equilíbrio entre a formação e a reabsorção óssea. Se este processo é alterado, pode ocorrer osteopenia (WOOD; STENSON; EMBLETON, 2015; WOOD; STRAUB, 2018).

A osteopenia caracteriza-se pela perda da DMO em seu estágio inicial, podendo ser precursora de um quadro de osteoporose, doença esquelética caracterizada por uma redução significativa da massa óssea, com destruição da microestrutura óssea, podendo levar a um aumento da fragilidade óssea com propensão à fraturas. Trata-se de uma doença silenciosa até que seja complicada por fraturas, que podem ocorrer mesmo após um trauma mínimo (KHAN et al., 2007; WILHELM et al., 2012). As fraturas ósseas são um problema de saúde, social e econômico, e vários estudos têm sido realizados na tentativa de reduzir sua ocorrência (LOURES et al., 2017).

A carga mecânica é fundamental para a manutenção do tecido ósseo. Uma imobilização ou repouso prolongados cessam o metabolismo ósseo e o equilíbrio entre a formação e a reabsorção óssea, resultando em perda de mineral ósseo, interrupção da arquitetura óssea e comprometimento das propriedades mecânicas ósseas (KHAJURIA et al., 2015). Os músculos e os ossos estão intrinsecamente ligados e, portanto, não é surpreendente que muitas distrofias musculares estejam associadas a problemas de saúde óssea e ao aumento do risco de osteoporose (WOOD; STENSON; EMBLETON, 2015).

Estudos têm demonstrado que os exercícios físicos de alto impacto, ou que exijam alta produção de força, possuem um efeito benéfico sobre a densidade mineral óssea areal (DMOa), em vista da deformação desse tecido ocorrida durante os exercícios (CADORE et al., 2005; LODBERG et al., 2018). O exercício físico pode aperfeiçoar a execução das tarefas diárias por meio do aumento da força e da resistência muscular, aptidão aeróbia e flexibilidade, além de abster ou minimizar o aumento da gordura visceral e a sarcopenia associadas à síndrome metabólica e à incapacidade funcional. O treinamento resistido (TR) permite aumentar a força, a hipertrofia, a potência e a resistência muscular, entretanto, dependendo dos objetivos

e das contestações individuais, os padrões de prescrição podem variar bastante (ACSM, 2009).

Se, por um lado, a prática de exercícios físicos fomenta benefícios morfológicos, fisiológicos e funcionais, o tempo necessário para que essas melhorias sejam mantidas após a interrupção no treinamento permanece controverso (KRAEMER; RATAMESS, 2004). Essa interrupção, também conhecida como destreinamento, leva à perda parcial ou completa das adaptações anatômicas, fisiológicas e de desempenho induzidas pelo treinamento, e variam quantitativa e qualitativamente dependendo do período de pausa (MICHELIN et al., 2008).

A preocupação acerca da dose apropriada de exercício necessária para que seja obtido um efeito (resposta) desejado se assemelha à necessidade do médico em determinar o tipo e a dose de um medicamento, bem como o tempo de seu uso, para obter a cura de uma doença (POWERS; HOWLEY, 2017). Dentro dessa linha de raciocínio, não há dúvidas de que o exercício físico necessário para obter um desfecho relacionado à saúde reflete a interação entre intensidade, frequência e duração deste. A resposta a uma intervenção de exercícios pode incluir mudanças funcionais e desfechos na saúde, independentes um do outro. A dose de exercício necessária para que exista resultado com efeito desejado é crucial no momento da prescrição dos exercícios, tanto em termos de prevenção como de reabilitação. Ao longo das últimas três décadas, compreendeu-se que a dose apropriada varia enormemente, dependendo do resultado desejado (POWERS; HOWLEY, 2017).

Em reconhecimento ao potencial dos exercícios como forma de tratamento, o objetivo do presente estudo foi investigar os efeitos do desuso por imobilização na resistência mecânica óssea de ratos adultos sedentários ou submetidos ao treinamento resistido, como um possível modelo experimental para os seres humanos.

2. CAPÍTULO 1 – CONSEQUENCES OF DISUSE BY IMMOBILIZATION ON BONE MECHANICAL RESISTANCE IN ADULT RATS SEDENTARY OR SUBMITTED TO RESISTANCE TRAINING

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2.1 Resumo

Tendo em vista a alta incidência de pacientes adultos com fraturas ósseas decorrentes do desuso é que o objetivo do presente estudo foi investigar o efeito do desuso por imobilização na resistência mecânica óssea de ratos adultos sedentários ou submetidos ao treinamento resistido, como um possível modelo experimental para os seres humanos. Foram utilizados 40 ratos (*Rattus novergicus albinus*), linhagem Wistar, machos, adultos (14 meses), divididos em quatro grupos de 10 animais cada; mantidos em gaiolas (controle), sedentarismo seguido de imobilização gessada (controle imobilizado), exercício físico que realizou treinamento resistido (EF) e exercício físico que realizou treinamento resistido seguido de imobilização gessada (EFI). Avaliou-se a microarquitetura ósseo; o conteúdo, área e densidade mineral óssea, a rigidez extrínseca, energia e a força máxima do colo femoral. Os dados foram analisados por meio de ANOVA, *TWO-WAY*, *THREE-WAY*. O nível de significância considerado foi $p<0,05$. Baseados nos resultados obtidos nas condições do presente estudo, apontaram que o exercício físico resistido foi benéfico para a densidade mineral óssea mesmo após o período de desuso por imobilização gessada, porém, a dose reposta do exercício resistido com a carga pré estimulada após o período do desuso não preveniu a osteopenia.

Palavras-Chave: Densidade Óssea. Imobilização. Microtomografia por raio-X. Treinamento físico.

2.2 Abstract

In view of the high incidence of adult patients with bone fractures resulting from disuse, the present study aimed to analyze the effect of disuse by immobilization in mechanical resistance of adult rats, sedentary or submitted to resisted physical exercise, as a possible experimental model for humans. 40 rats (*Rattus novergicus albinus*), Wistar lineage, male, adults (14 months) were used, divided into four groups of 10 animals each; kept in cages (control), physical inactivity followed by cast immobilization (immobilized control), physical exercise that performed resistance training (PE) and physical exercise that performed resistance training followed by cast immobilization (IPE). Bone microarchitecture was evaluated; bone mineral content, area and density, extrinsic stiffness, energy and maximum strength of the femoral neck. The data were analyzed using ANOVA, TWO-WAY, THREE-WAY. The level of significance considered was $p < 0.05$. Based on the results obtained in the conditions of the present study, they pointed out that resistance physical exercise was beneficial for bone mineral density even after the period of disuse due to plaster immobilization, however, the replaced dose of resistance exercise with the pre-stimulated load after the period of disuse did not prevent osteopenia.

Keywords: Bone Density. Immobilization. X-ray Microtomography. Physical training.

2.3 Introduction[1]

Bone mineral density (BMD) in adulthood depends predominantly on skeletal growth and mineralization. In healthy individuals, 80% of bone mass is accumulated at 18 years of age, and a reduced peak of BMD in childhood and adolescence is considered as one of the most important predisposing factors for the occurrence of fractures in adults and the elderly. A healthy bone is metabolically active and undergoes continuous remodeling to maintain the balance between bone formation and resorption. If this fine-tuned process is altered, osteopenia can occur [1, 2].

Osteopenia is characterized by the loss of BMD in its initial stage, and may be a precursor of osteoporosis, a skeletal disease characterized by a significant reduction in bone mass, with destruction of the bone microstructure, which can lead to an increase in bone fragility with a propensity to fractures [3, 4]. It is a silent disease until it is complicated by fractures, which can occur even after minimal trauma [4, 5]. Bone fractures are a health, social and economic problem, and several studies have been conducted in an attempt to reduce their occurrence [6].

Mechanical load is fundamental for the maintenance of bone tissue. A prolonged immobilization or rest ceases bone metabolism and the balance between bone formation and resorption, resulting in loss of bone mineral, disruption of bone architecture and impairment of bone mechanical properties [7]. Muscles and bones are intrinsically linked and, therefore, it is not surprising that many muscular dystrophies are associated with bone health problems and an increased risk of osteoporosis [1].

Studies have shown that high-impact physical exercises, or that require high strength production, have a beneficial effect on sandy bone mineral density (DMOa), in view of the deformation of this tissue that occurred during exercises [8, 9]. Physical exercise can improve

[1] Journal of Osteoporosis (anexo II)

the performance of daily tasks by increasing muscle strength and endurance, aerobic fitness and flexibility, in addition to abstaining or minimizing the increase in visceral fat and sarcopenia associated with metabolic syndrome and functional disability. Resistance training (RT) allows you to increase strength, hypertrophy, and power and muscle endurance; however, depending on individual goals and challenges, prescription patterns can vary widely [10].

If, on the one hand, the practice of physical exercises promotes morphological, physiological and functional benefits, the time necessary for these improvements to be maintained after the interruption in training remains controversial [11]. This interruption, also known as detraining, leads to partial or complete loss of anatomical, physiological and performance adaptations induced by training, and vary quantitatively and qualitatively depending on the pause period [12].

The concern about the appropriate dose of exercise necessary to obtain a desired effect (response) is similar to the need of the doctor to determine the type and dose of a drug, as well as the time of its use, to obtain the cure of a disease [13]. Within this line of reasoning, there is no doubt that the physical exercise necessary to obtain a health-related outcome reflects the interaction between its intensity, frequency and duration. The response to an exercise intervention can include functional changes and health outcomes, independent of each other. The amount of exercise required for the desired effect to be effective is crucial when prescribing the exercises, both in terms of prevention and rehabilitation. Over the past three decades, it has been understood that the appropriate dose varies enormously, depending on the desired result [13].

In recognition of the potential of exercise as a form of treatment, the present study aimed to analyze the effects of resisted physical exercise and disuse by plastered immobilization on the trabecular and cortical bone tissue of adult rats, as a possible experimental model for humans.

2.4 Materials and Methods

This study was approved by the Ethics Committee on Animal Experiment (CEUA - annex A) of the Faculty of Veterinary Medicine of Universidade Estadual Paulista Júlio Mesquita Filho, Campus of Aracatuba, under protocol number 00337-2016. All procedures performed are in accordance with the standards described by the Brazilian College of Animal Experimentation (COBEA).

2.5 Experimental design

2.5.1 Animals and experimental groups

Forty adult male Wistar rats (*Rattus novergicus albinus*), with approximately 14 months of life were used. The rats came from the bioterium of the Department of Basic Sciences, Faculty of Dentistry, São Paulo State University, Brazil. All animals were kept in boxes with four animals in each box, under controlled conditions of temperature (22ºC - 24ºC), humidity (65%) and air filtration. In addition, they were subjected to a 12-hour light and 12-hour dark regime, commercial feed (Purina - Labina®, Paulínia, Brazil) and water *ad libitum*. All rats were monitored daily to assess possible clinical changes.

Before the beginning of the experiment, the rats, already 14 months old, were kept in their respective boxes for a period of seven days to adapt to the new environment. On the first day after adaptation, bone densitometry was performed on animals in all groups. Then, the experimental protocol started, with 50% of the animals (IPE and PE groups) submitted to the beginning of resistance training (RT). After a period of eight weeks, the animals belonging to the IPE and FC groups were submitted to 14 days of plastered immobilization of the right pelvic limb. Those belonging to the PE group remained in physical training. Two days after the immobilization period, the rats in the IPE group were again subjected to eight weeks of physical training, returning to their exercises with the same load that was used before the immobilization,

while the FC group returned to the cages. The schematic diagram of the experimental test is shown in figure 1.

2.6 Plaster cast design

For immobilization, a plaster bandage with quick drying (Polar Fix, Mauá, Brazil) was used. The immobilization model was based on the one proposed by Booth and Kelso [14], with adaptation for only one right member (figure 2B).

2.7 Strength training protocol and measurement of maximum voluntary force generation capacity (CMV)

Initially, the animals were familiarized (adapted) to the stairs, used to perform the RT protocol. The adaptation period lasted three sessions on non-consecutive days, without the use of overload [15]. After the adaptation period, the maximum capacity for voluntary force generation (CMV) was assessed. The CMV test was performed with the use of apparatus and steel balls used in the proximal part of the animals' tail. The initial imposed overload corresponded to 75% of the animals' body mass (individual), being increased by 30 grams for each session performed in a complete way (complete climb up the stairs). At each attempt, the animals rested for 120 seconds and then performed a new series until failure to climb the ladder (incomplete climb) occurred. When the failure occurred, the overload prior to the failure was considered as CMV, used to control the intensity of the RT and to induce correct overload to each animal, respecting the principle of individuality in the training. The CMV test was performed every 30 days in order to maintain sufficient intensity to obtain benefits to the bone tissue, since the animals adapt quickly to the imposed overloads. During the first week of the training protocol, the animals performed the RT with an overload of 65% compared to the initial CMV test, in the second week with 75% and in the third 85%. The intensity of 85% was

maintained until the end of the experimental period, that is, 18 weeks. The animals performed three weekly sessions of RT on non-consecutive days and each session consisted of eight to 12 series (ascents), and the CMV was redone every four weeks [15].

2.8 Assessment of body mass

The body mass (g) of all animals in the four groups was determined at four times, namely, at the beginning of the experiment, before and after immobilization, and at the end of the experiment, at 18 months of age. For this purpose, a precision digital scale (Model 9094C / 5, Toledo do Brazil, São Paulo, Brazil) was used.

2.9 Outline of euthanasia

At the end of 134 days, all rats were euthanized by beheading by guillotine, following ethical principles in animal research Concea [16]. The right femurs were disarticulated, dissected and stored in cryogenic tubes containing saline solution (NaCl 0.9%) and kept at -20ºC until the analysis of bone mineral densitometry, microtomography and mechanical testing on the femoral neck.

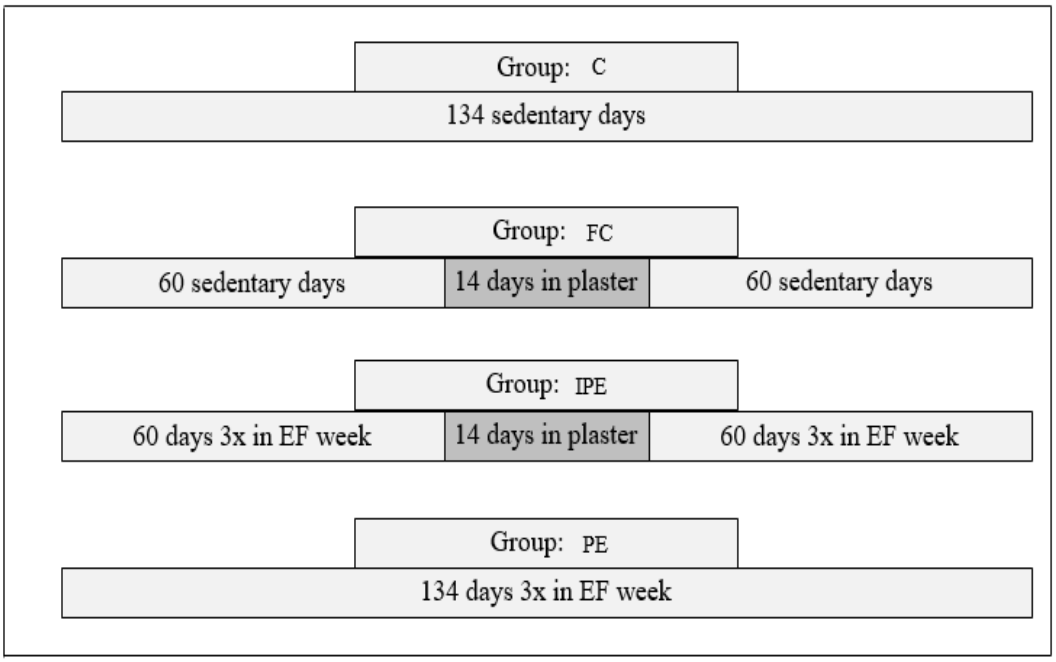

Figure 1. Schematic diagram of resistance training imposed on four different groups of rats over a period of 134 days, according to the experimental groups. C: control, FC: immobilized control, PE: physical exercise, IPE: immobilized physical exercise.

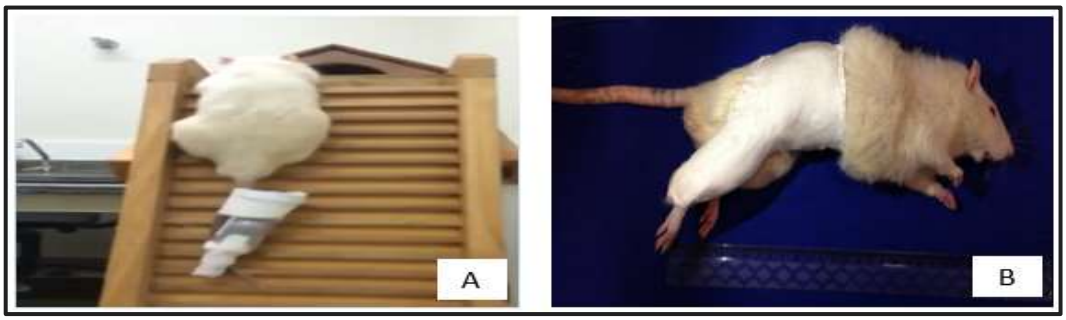

Figure 2. A = Rat performing the climbing with an apparatus attached to the tail. B = Anesthetized rat submitted to the application of the plastered immobilization technique.

2.10 Determination of bone mineral content, bone mineral area and bone mineral density

The determinations of bone mineral content (CMO), bone mineral area (Area) and femoral areal bone mineral density (DMOa) were performed in four moments; before the beginning of the experiment, before the plastered immobilization, immediately after the

plastered immobilization, and after the euthanasia of the animals. To obtain these parameters, the rats were initially anesthetized, intraperitoneally, with ketamine hydrochloride (Cetamin®, Syntec, Santana de Paraíba, Brazil) at a dose of 80mg/kg and xylazine hydrochloride (Anasedan®, Ceva, Paulínia, Brazil) at a dose of 15mg/kg. After reaching the anesthetic plane, the animals were placed in prone position and subjected to CMO (g), area (cm^2) and DMOa (g/cm^2) analyzes, by means of densitometry performed in a dual emission X-ray densitometer (DEXA, model DPX-Alpha, Lunar®, WI, USA), with special software for small animals coupled to a computer (Lunar Excellence in Image of X-rays with Smartscan, DPX. Version 4.7).

2.11 Determination of bone tissue microarchitecture

The determination of bone tissue quality was performed by means of computerized microtomography, with a Skyscan 1272 model microtomograph (Skyscan, Aartselaar, Belgium). After euthanasia, the femurs on the right side of six animals in each group were chosen randomly and immediately stored in saline at -20°C. Twenty-four hours before the analysis, the bones were transferred to a refrigerator at a temperature of 7°C, and kept at room temperature during the performance of the bone microtomography. For the non-destructive three-dimensional evaluation of the bone architecture, the femur was positioned craniocaudally to obtain "slices" with a 12x12x12μm resolution [17]. The spongy and cortical bone region of interest was manually inserted for the analysis of 30 slices in the femoral neck region, 2 mm below the first scanned bone image. The parameters analyzed in the trabecular bone included: the fraction of bone volume, that is, the proportion of segmented bone volume with the total volume of the region of interest (BV/TV) (%); the average thickness of the trabeculae evaluated by direct 3D methods (Tb.Th) (mm); the measurement of the average number of trabeculae per unit length (Tb.N); the average distance between trabeculae evaluated by direct 3D methods

(Tb.Sp) (mm); and the structure model index (SMI) [18, 19]. In the analysis of the cortical bone, the cortical bone area (Ct.Ar) (mm2) [19, 20], and the average cortical thickness (Ct.Th) (mm) [21], were analyzed.

2.12 Analysis of maximum strength, extrinsic stiffness and bone energy

The biomechanical behavior of the bones was evaluated using the mechanical compression test of the femoral head. After densitometry, the right femurs were subjected to mechanical testing to obtain maximum strength (N) and extrinsic stiffness (N/mm), using a universal testing machine, brand EMIC® - model DL 3000 São José dos Pinhais - Brazil). For this test, each femur was fixed in a device that kept it in a perpendicular position, as shown in figure 3. The compression point was the femur head and the 2000 N load cell used at a speed of 2 mm/min was applied until the moment of bone fracture [22]. The applied force and the traverse displacement of the machine were monitored and registered using the equipment's own software.

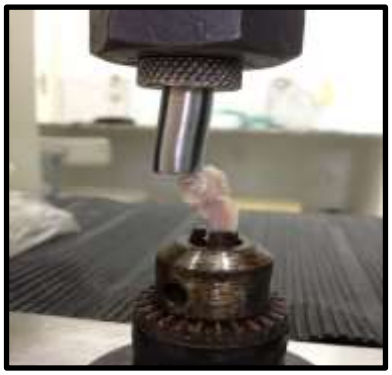

Figure 3. Mechanical compression test on the femoral head.

2.13 Statistical analysis

The results were evaluated as mean ± standard error of the mean (SEM). The data were submitted to analysis of variance (ANOVA) TWO-WAY, THREE-WAY, the interaction that represents the combined effects of factors in the dependent measure and followed by the Tukey post-test for multiple comparisons. The computer program GraphPad Prisma®, version 7.0, was used. The level of significance considered was $p < 0.05$.

2.14 Results

During the experimental period, eight animals died; two rats in group C right after bone densitometry; two animals in the FC group during the immobilization period; two animals from the IPE group during the immobilization period and, finally, two rats from the PE group immediately after bone densitometry. For this reason, the experimental groups were composed, at the end of the study, by eight animals each.

2.15 Assessment of body mass

The analyzes of the average body mass of the animals of the four experimental groups during the 134 days of the experiment are shown in figures 4A and 4B. At the beginning of the experiment, there was no significant difference between the body mass of the animals in the four groups. In the pre-immobilization moment, that is, 60 days after the beginning of the experiment, there was a significant increase in the body mass of all groups, and there was a significant difference between the FC, PE and IPE groups with group C ($p<0.0001$), with the latter presenting the lowest body mass values. At the moment post immobilization, that is, 74 days after the beginning of the experiment, the FC and IPE groups had the lowest body mass values of the four groups, with a sharp drop in body mass to values close to those observed when the study started. There was a statistically significant difference between the FC and IPE

groups ($p<0.001$) at this time in relation to the previous moment, with a significant difference between the FC and IPE groups and the other two groups at this time ($p<0$, 0001). At the end of the experiment (euthanasia), there was an increase in the body mass of all groups in relation to the previous moments, which was more pronounced in the animals of the FC and IPE groups in relation to the previous moment (post-immobilization). In-group C there was a significant difference ($p<0.001$) between the body mass of the FC, PE and IPE groups at the end of the study.

2.16 Bone mineral content

The analyzes of the average femoral bone mineral content of the animals of the four experimental groups during the 134 days of the experiment are shown in figures 5A and 5B. At the beginning of the experiment, there was no significant difference between the determination of bone mineral content of animals in the four groups. At the time of pre-immobilization, that is, 60 days of experiment, there was an increase in bone mineral content in the four groups in relation to the initial moment, with no significant difference between the PE and IPE groups, nor between the groups. C and FC. However, the bone mineral content was higher in the groups that performed physical exercise in relation to the control groups. At 74 days of experiment, at the moment after immobilization, there was a decrease in bone mineral content in the animals of the FC and IPE groups in relation to the pre immobilization moment. On the other hand, the rats in groups C and PE showed an increase in bone mineral content in relation to the previous moment. There was a statistically significant difference ($p<0.001$) between all groups evaluated. At the end of the experiment, at the time of euthanasia, there was an increase in the average bone mineral content in the four experimental groups when compared to the previous moment and the beginning of the study. There was no significant difference between the means of groups C and IPE, but there was a difference

between the means of these groups with the PE groups (whose bone mineral content was the highest) and the animals of the FC group (whose bone mineral content was the smaller).

2.17 Femoral bone area

The analysis of the mean total femoral bone area of the animals in the four experimental groups during the 134 days of the experiment are shown in figures 6A and 6B. At the beginning of the experiment, there was no significant difference between the four groups. In the moment after immobilization, there was an increase in the average values of the four groups, with a significant difference between the FC group and the others, which was the one with the lowest values. At the moment after immobilization, at 74 days of experiment, there was a decrease in the femoral bone area of the FC and IPE groups and an increase in the average values of the C and PE groups in relation to the previous moment. In post immobilization, there was a statistically significant difference between all groups, with the highest values being observed in the PE group, followed by group C, IPE and finally FC, whose values were the lowest of all. Finally, at the time of euthanasia, all groups showed an increase in values compared to previous moments, maintaining the relationship of the previous moment, that is, the highest values observed in the PE group and the lowest values in the FC group, with a difference statistically significant between the four groups.

2.18 Areal bone mineral density

The analyzes of the average femoral areal bone mineral density of the animals of the four experimental groups during the 134 days of the experiment are shown in figures 7A and 7B. At the beginning of the experiment, there was no statistically significant difference between the mean values of femoral areal bone mineral density in the FFC, PE and IPE groups; however, animals in group C showed significantly lower values compared to others. In the pre-immobilization moment, there was a significant increase in the average values of the four

groups in relation to the beginning of the study, with no difference between groups C and FC, both presenting lower values in relation to the PE and IPE groups. After immobilization, there was a decrease in the values of femoral areal bone mineral density in relation to the previous moment in all groups analyzed, with the largest falls observed in the FC and IPE groups. At this time, the PE group showed significantly higher values than the other groups. Finally, at the time of euthanasia, there was a maintenance in the average values of the group C, a significant drop in the average values of the group FC ($p<0.001$), a decrease in the values of the group PE ($p<0.0001$), and an increase in the values of the IPE group ($p<0.001$), in relation to the previous moment. When euthanizing the animals, there was a statistically significant difference between the PE group and the others, which had the highest values. Still, there was no significant difference between groups C and IPE, and between the group FC and the others, which had the lowest values.

2.19 Trabecular bone microtomography

The evaluation of the average femoral bone volume fraction (BV/TV) of the animals in the four experimental groups demonstrated a statistically significant difference between the IPE group and the C ($p=0.0003$), FC ($p=0.0016$) and PE ($p=0.0123$), with lower mean values observed in the IPE group. There was no interaction between physical exercise and immobilization ($p=0.0707$; $F=(1.20)= 3.645$) between groups C, FC and PE (figures 8A and 8F).

When the average trabecular separations (Tb.Sp) were compared, there was a statistically significant difference between the IPE group and the C ($p=0.0407$) and PE ($p=0.0008$) groups. On the other hand, animals in the IPE group showed higher values when compared with group C ($p<0.0003$). There was an interaction between the PE and IPE groups ($p=0.0119$; $F = (1.20) = 7.664$) (figures 8B and 8F).

The evaluation of the mean thickness of the trabeculae of the femoral neck (Tb.Th) showed a statistically significant difference between the IPE group and the C (p=0.0004) and FC (p=0.0321) groups, with lower values observed in the animals in the IPE group, and there was a statistically significant difference between the PE group and the C group (p=0.0144). There was no interaction between the PE and IPE groups (p = 0.7961; F = (1.20) = 0.0685) (figures 8C and 8F).

The evaluation of the average number of trabeculae (Tb.N) showed a statistically significant difference between the IPE group and the PE group (p=0.0138), with higher values observed in the PE group. There was an interaction between the PE and IPE groups (p=0.0019; F = (1.20) = 12.7520.02) (figures 8D and 8F).

The results of bone microtomography of the femoral neck trabeculate show that the mean values of the structure model index (SMI) were statistically significantly lower in the IPE group when compared with groups C (p=0.0189) and FC (p=0,0049), with the highest values observed in the FC group. There was no interaction between the PE and IPE groups (p=0.1511; F = (1.20) = 2.228572) (figures 8E and 8F).

2.20 Cortical bone microtomography

The evaluation of the parameters of the cortical bone microtomography of the femoral neck of the experimental animals identified a lower mean cortical area (Ct.Ar.) in the CI group (p=0.0133) and in the IPE group (p=0.0156), when compared to group C, with no statistically significant difference when compared to the PE group (pFC vs.PE = 0.9207; pPE vs.IPE = 0.9431; pC vs.PE = 0.0510, pFC vs.PE = 0.9999). There was no interaction between the PE and IPE groups (p=0.0566; F (1.20) = 4.096) (figures 9A and 9C). Regarding the mean cortical thickness (Ct.Th.), there was no statistically significant difference between the groups (pC vs.PE = 0.5691; (pPE vs.IPE = 0.8282; pFC vs.PE = 0.9981; pC vs. IPE = 0.1685; pCvs.FC =

0.6727; pFC vs.IPE = 0.7367) and no interaction between the PE and IPE groups (p = 0.8441; F (1.20) = 0.0396) (figures 9B and 9C).

2.21 Mechanical test of the femoral neck

During the mechanical test of the femoral neck, in the assessment of extrinsic stiffness (N/mm) there was a statistically significant difference between the IPE group and the FC group (p<0.0058) (figures 10A and 10D). There was no interaction between the PE and IPE groups (p=0.0.0993; F = (1.31) = 2,888). At the time of the energy absorption capacity (mJ), there was a statistically significant difference between the IPE group and the FC group (p=0.0365). There was no interaction between the PE and IPE groups, (p=0.6972; F = (1.26) = 0.1548) (figures 10B and 10D). In the evaluation of the maximum force (N) applied to the femur, there was no statistically significant difference between the groups (pC vs.PE = 0.2505; pPE vs.IPE = 0.2188; pFC vs.PE = 0.2076; pC vs.IPE = 0, 9999; pC vs.FC = 0.9999; pFC vs.IPE = 0.9978) (Figures 10C and 10D). There was no interaction between the PE and IPE groups (p=0.0993; F = (1.31) = 2,888).

2.22 Discussion

All animals in the present study had a similar body mass at the beginning of the experiment and, at the end of the study, there was an increase in all groups studied (figures 4A and 4B). The increase in body mass in rats in the PE and IPE groups is probably due to a response to physical exercise, as observed by Irving [23]. On the other hand, in the animals in the control groups, the average increase in body mass is probably due to a sedentary lifestyle, as reported in previous studies [24, 25].

During the experiment, it was decided to immobilize only one of the pelvic limbs with the objective of preventing the performance of exercises and, thus, not causing bone changes that could interfere with the results. Immobilization processes because bone changes that follow even after a first moment of remobilization, making the bone weaker, despite the restoration of normal activity [24, 26]. The animals belonging to the groups that were immobilized showed a significant loss of body mass at the moment after immobilization (figures 4A and 4B), a fact that was also reported by Booth and Kelso [14] and Trebacz [24]. The increase or decrease in body mass can cause interference in aspects of bone quantity and quality, since this is closely correlated with different properties of bone [27, 28].

The determinations of bone mineral content (CMO) (figures 5A and 5B), bone mineral area (Area) (figures 6A and 6B) and areal bone mineral density (DMOa) (figures 7A and 7B) of the animals in the present study showed an increase in animals submitted to physical exercise (PE), in agreement with the findings of studies carried out in rats [29, 30] and in humans Kirchner [31]. The load imposed on the bone structure, especially during physical exercise, triggers osteogenic effects [32].

In the EFI group, areal bone mineral density returned to normal conditions in terms of determining bone mineral content over a period of 60 days (figures 7A and 7B). This was expected, since the bones, when submitted to pressure changes and stresses caused by external

stimuli, activate cellular mechanisms that trigger their remodeling, resulting in bone adaptation to this new stimulus [33]. Bone characteristics may vary according to the time of immobilization, remobilization and the methodology used, as well as the species under study. Kaneps [34], evaluating adult dogs immobilized for 16 weeks, found complete recovery after 32 weeks of remobilization.

The reduction in the total bone mineral area (figures 6A and 6B) of the femur of the CI group rats occurred, probably, due to the lack of stimulus caused by immobilization, hindering their growth, a fact also verified by Osaki [25].

The results of trabeculated bone microtomography of the femoral neck showed values below the averages in the IPE group, data that corroborate the idea that the results of the exercise relationship and its dose response do not improve bone quality in BV/TV, Tb. Sp, Tb.Th, Tb.N (figure 8A, 8B, 8C and 8D and figure F) after high intensity exercises after disuse, as in Frost's mechanostatic concept, (2003) [35], where bone formation (F) and bone resorption (R) are the remodeling phenomena that alter the shape and/or shape of the bone, however where the fatigue damage can quickly accumulate in more than 4,000 με and change the bone to a fatigue collapse, as the formation bone is greater than resorption.

In this study, it was found, through mechanical testing, that the groups that performed the TR obtained the best results when compared to groups C and FC (figures 10 A and 10B). Morey and Baylink [36], Convertino et al. [37], who found that the mechanical elimination caused by the decrease or stop of the physical exercise triggers a reduction in the resistance and in the bone mass, observed similar results. This is due to the decline in the number of osteoblasts, causing a decrease in bone formation, which also leads to an increase in the incidence of bone fragility. The mechanical tests reflect, in part, the bone quality, since the clinical fracture is a biomechanical fact. Therefore, any marked change in bone quality can change the biomechanical performance of this tissue Astur et al., [38].

A limitation of the present study was the fact that all the rats evaluated were male, which made it difficult to discuss the results since most of the studies previously carried out used females.

One of the limitations was the intake of feed and water. Another limitation was not having analyzed the adipose tissue.

2.23 Conclusion

The results obtained under the conditions of the present study allowed us to conclude that resistance physical exercise was beneficial for bone mineral density, even after the period of disuse due to plastered immobilization. However, the response to resistance exercise after the period of disuse with the dose used did not prevent the occurrence of osteopenia.

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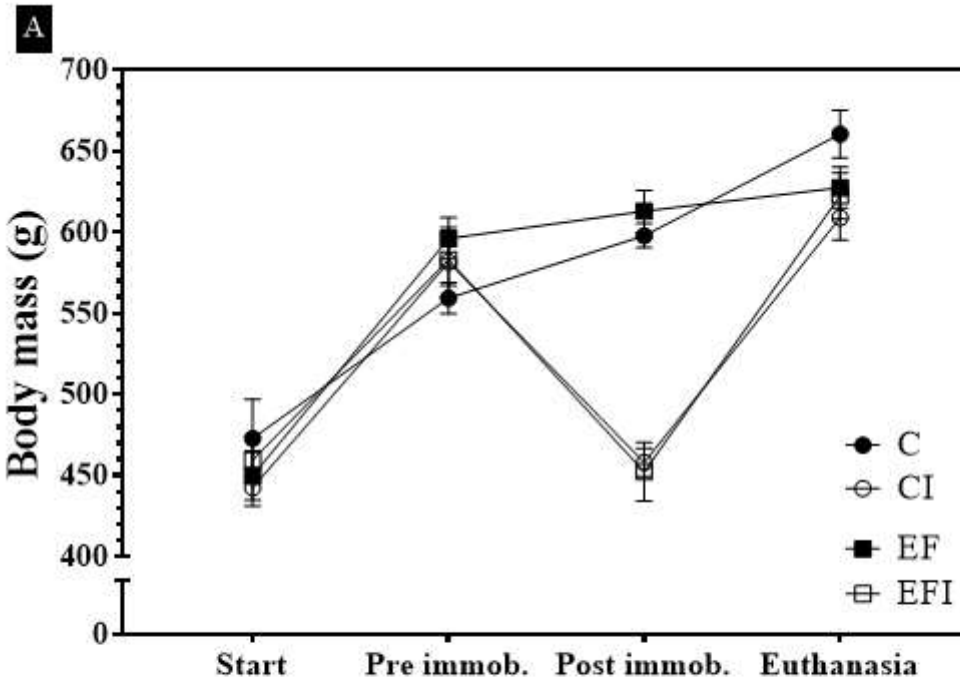
Figure 4. (A) Graph of the evolution of body mass (mean±standard error of mean); (B) summary table of the evolution of body mass of rats from the four experimental groups (n=8 rats/group); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).

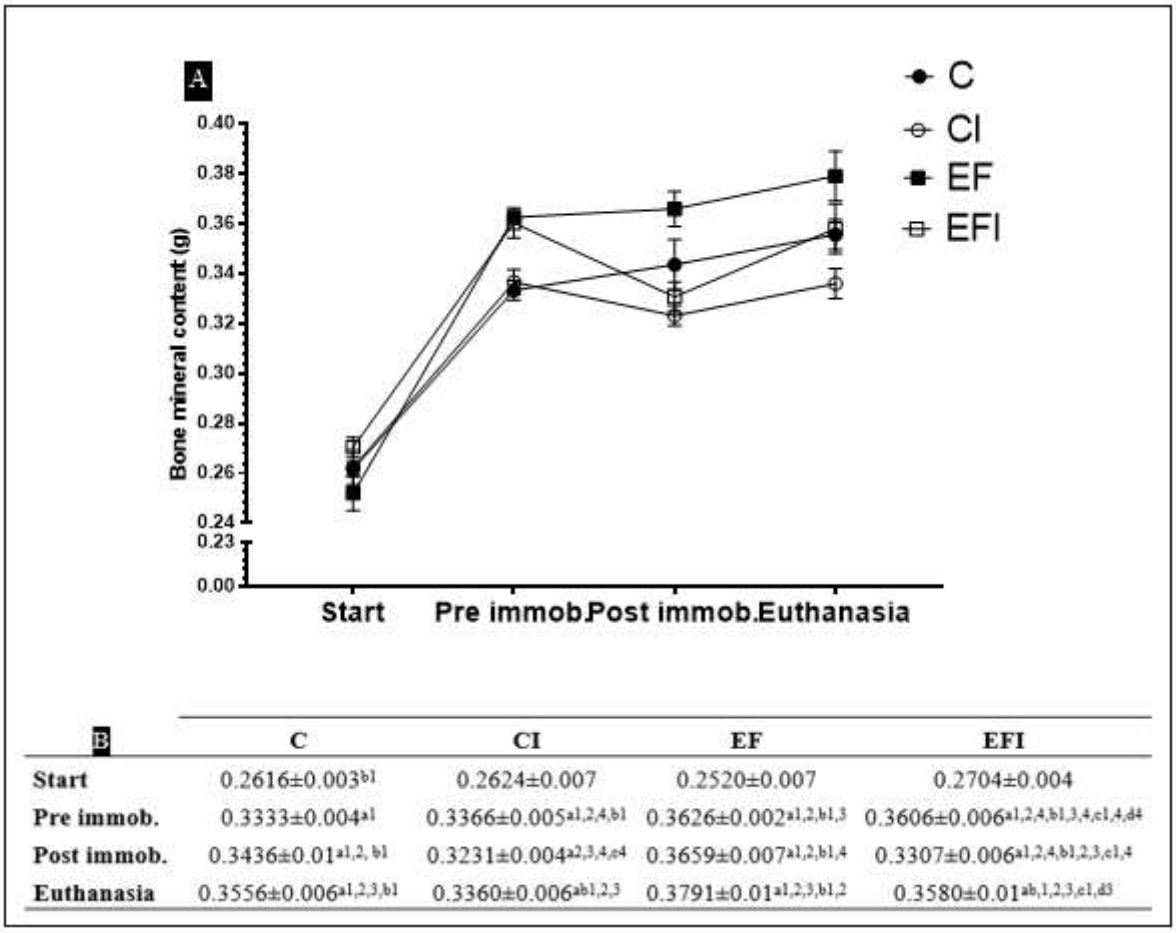


B

	C	CI	EF	EFI
Start	472.8±24.16	442.5±11.38	449.9±15.19	459.5±14.27
Pre immob.	559.3±9.5^{ab1}	581.5±21.41^{a,b,c,d,1,a4}	596.1±12.76^{a,b,c,d,1}	583.0±16.13^{a,b,c,d,1,b2}
Post immob.	597.8±7.41^{a,b,c,d,1}	458.1±8.64^{b1,ab2,a3,a4}	612.9±12.98^{cd1,b3}	452.3±1797^{a1,3,4,bd2,c3,4}
Euthanasia	660.5±14.6^{a,b,c,d1,ab2}	609.0±13.89^{a,b,c,d,1,b3}	627.5±12.66^{a,b,c,d,1,b3}	622.5±14.13^{a,b,c,d,1,b3}

Different letters mean significant differences with $p<0.05$ performed with the three-way ANOVA test followed by the Tukey test. Lower case letters represent the groups, with ^{a} <0.05 vs. C; ^{b} <0.05 vs. FC; ^{c} <0.05 vs. PE; ^{d} <0.05 vs. IPE. Numbers represent the periods of the interventions, being 1 = beginning (zero day), 2 = pre-immobilization (60 days), 3 = post-immobilization (74 days), 4 = euthanasia (134 days).

Figure 5. (A) Evolution of femoral bone mineral content (mean±standard error of mean); (B) Determination of femoral bone mineral content (mean±standard error of mean) of rats from the four experimental groups (n=8 rats/group); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).



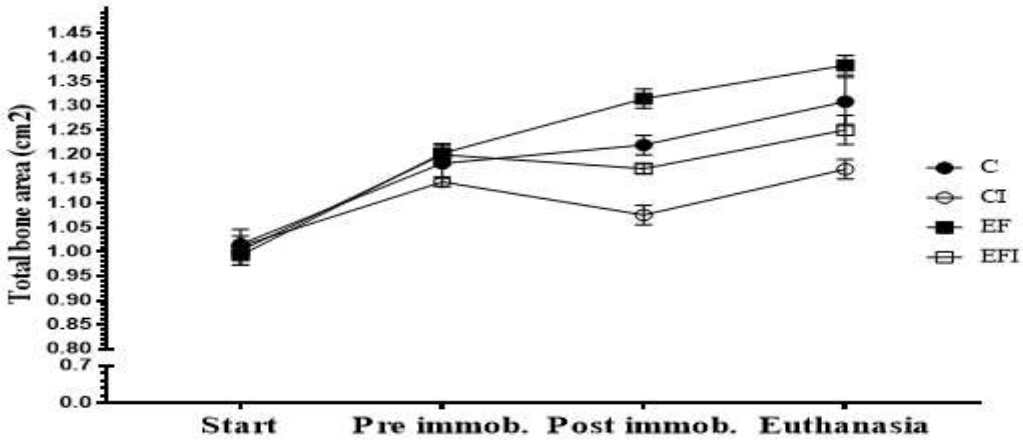
B

	C	CI	EF	EFI
Start	0.2616±0.003^{b1}	0.2624±0.007	0.2520±0.007	0.2704±0.004
Pre immob.	0.3333±0.004^{a1}	0.3366±0.005^{a1,2,4,b1}	0.3626±0.002^{a1,2,b1,3}	0.3606±0.006^{a1,2,4,b1,3,4,c1,4,d4}
Post immob.	0.3436±0.01^{a1,2,b1}	0.3231±0.004^{a2,3,4,c4}	0.3659±0.007^{a1,2,b1,4}	0.3307±0.006^{a1,2,4,b1,2,3,c1,4}
Euthanasia	0.3556±0.006^{a1,2,3,b1}	0.3360±0.006^{ab1,2,3}	0.3791±0.01^{a1,2,3,b1,2}	0.3580±0.01^{ab,1,2,3,c1,d3}

Different letters mean significant differences with $p<0.05$ performed with the three-way ANOVA test followed by the Tukey test. Lower case letters represent the groups with ^{a} <0.05 vs. C; ^{b} <0.05 vs. FC; ^{c} <0.05 vs. PE; ^{d} <0.05 vs. IPE. Numbers represent the periods of the interventions, being 1= beginning (zero day), 2= pre-immobilization (60 days), 3= post-immobilization (74 days), 4= euthanasia (134 days).

Figure 6. (A) Evolution of the total femoral bone area (mean±standard error of the mean); (B) Determination of the total femoral bone area (mean±standard error of the mean) of rats from the four experimental groups (n=8 rats/group); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).

A



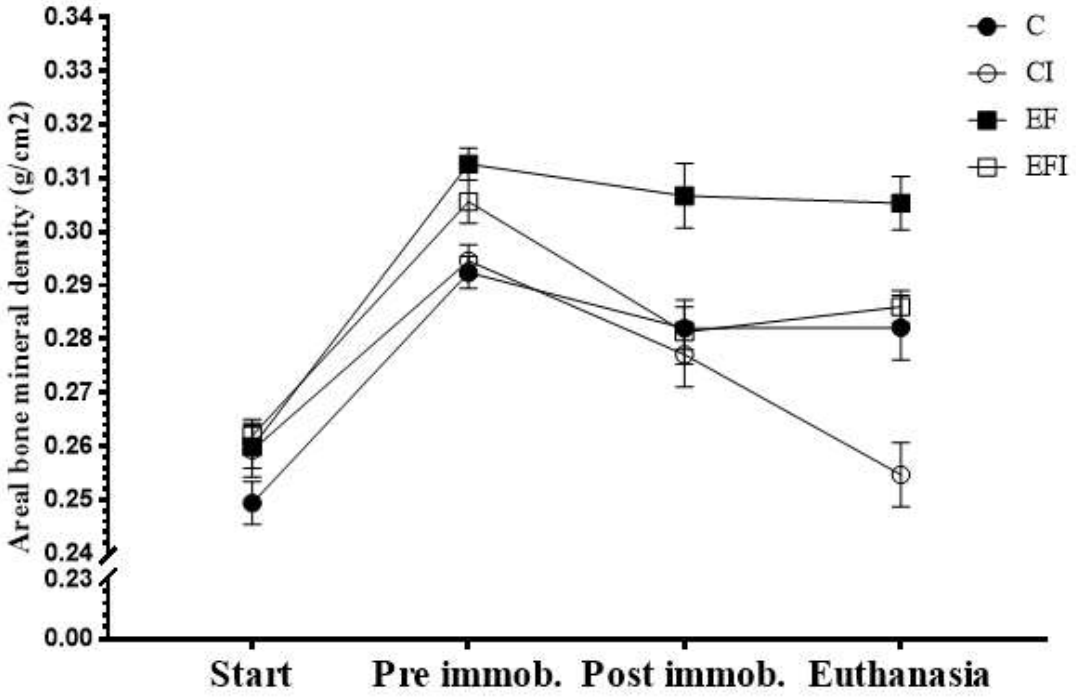
B

	C	CI	EF	EFI
Start	1.016±0.03	1.0120±0.01	0.993±0.02	1.003±0.03
Pre immob.	1.182±0.03^{a1}	1.144±0.01^{a1}	1.203±0.01^{a,b,c1,b2}	1.200±0.02^{a,b,c,d1,c4}
Post immob.	1.220±0.02	1.076±0.02^{a3,4}	1.315±0.02^{a,c,d1,b2,3,c4}	1.171±0.01 ^{a,b,c,d1,c4}
Euthanasia	1.309±0.05^{a1}	1.170±0.02^{a,b1}	1.384±0.02^{a,b,c1,2,3,b4}	1.251±0.03^{a,c,d1,b3}

Different letters mean significant differences with $p<0.05$ performed with the three-way ANOVA test followed by the Tukey test. Lower case letters represent the groups, with ^{a} <0.05 vs. C; ^{b} <0.05 vs. FC; ^{c} <0.05 vs. PE; ^{d} <0.05 vs. IPE. Numbers represent the periods of the interventions, being 1= beginning (zero day), 2= pre-immobilization (60 days), 3= post-immobilization (74 days), 4= euthanasia (134 days).

Figure 7. (A) Evolution of femoral areal bone mineral density (mean±standard error of mean); (B) Determination of the femoral areal bone mineral density (mean±standard error of the mean) of rats from the four experimental groups (n=8 rats/group); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).

A

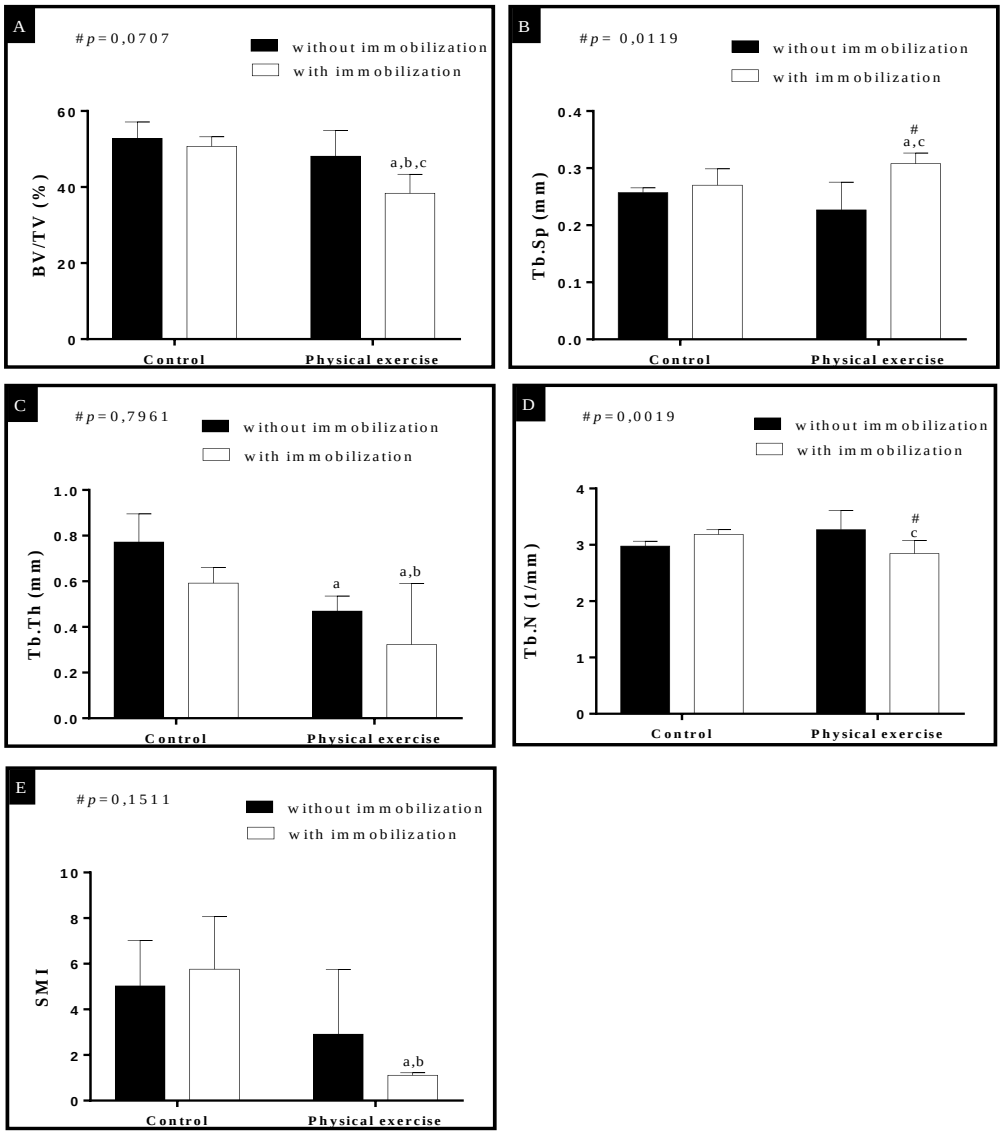


B

	C	CI	EF	EFI
Start	0.2494±0.004	0.2593±0.005	0.2599±0.004	0.2620±0.003
Pre immob.	0.2924±0.003^{a1}	0.2946±0.003^{ab1}	0.3126±0.003^{abc1,ab3,ab4}	0.3056±0.004^{a,b,c,d1,b3,4}
Post immob.	0.2820±0.004 ^{a1}	0.2771±0.006^{ab1}	0.3067±0.006^{ac1,b3,4}	0.2813±0.006^{a1,cd2,b4}
Euthanasia	0.2821±0.006 ^{a1}	0.2547±0.006^{a1,2,3,4,b2}	0.3053±0.005^{b1,3,4,c1}	0.2860±0.003^{abc1,b4}

Different letters mean significant differences with $p<0.05$ performed with the three-way ANOVA test followed by the Tukey test. Lower case letters represent the groups, with ^{a} <0.05 vs. C; ^{b} <0.05 vs. FC; ^{c} <0.05 vs. PE; ^{d} <0.05 vs. IPE. Numbers represent the periods of the interventions, being 1= beginning (zero day), 2= pre-immobilization (60 days), 3= post-immobilization (74 days), 4= euthanasia (134 days).

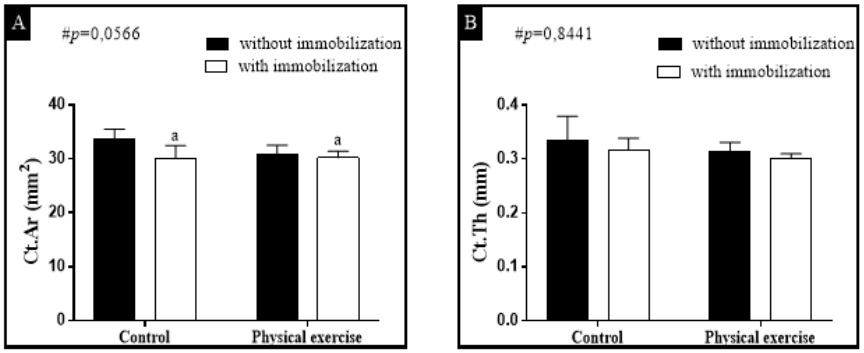
Figure 8. Graphs of the mean bone volume fraction parameters - BV/TV (A), trabecular separation - Tb.Sp (B), mean thickness of the trabeculae - Tb.Th (C), trabecular number - Tb.N (D), structure model index - SMI (E) of the femoral neck of the animal's four experimental groups. (n=6 rats/group) - summary table of bone microtomography values (F); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).



F	C	CI	EF	EFI
BV/TV	50,70±1,037	48,14±2,762	52,83±1,1752	38,40±0,380[a,b,c]
TB.Sp	0,2573±0,003	0,2702±0,011	0,2267±0,019	0,3077±0,007[a,c]
Tb.Th	0,7722±0,050	0,5924±0,027	0,4693±0,027[a]	0,3226±0,109[a,b]
Tb.N	2,979±0,034	3,184±0,0345	3,267±0,1390	2,844±0,095[c]
SMI	5,027±0,810	5,760±0,939	2,915±1,150	1,112±0,043[a,b]

Different letters mean significant differences with p<0.05 performed with the two-way ANOVA test followed by the Tukey test. #= PE VS interaction. IPE. Lower case letters represent the groups with [a] <0.05 vs. C; [b] <0.05 vs. FC; [c] <0.05 vs. PE; [d] <0.05 vs. IPE.

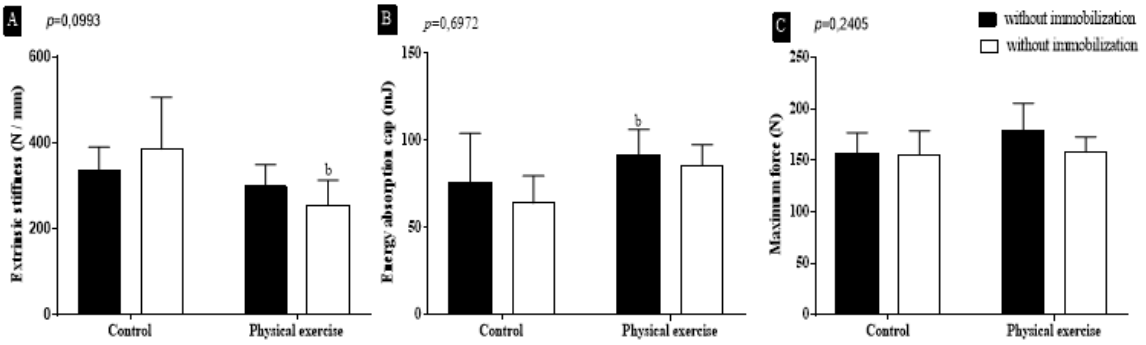
Figure 9. Graph of the average parameters of the cortical area - Ct.Ar (A), mean cortical thickness - Ct.Th (B) - summary table of the values of the cortical bone microtomography (C), obtained by microtomography of the femoral neck of animals four experimental groups. (n =6rats/group); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).



C	C	CI	EF	EFI
CT.Ar	33,723±0,662	30,130±0,875[a]	30,793±0,659	30,206±0,445[a]
Ct.Th	0,3342±0,016	0,3166±0,008	0,3139±0,006	0,3007±0,003

Different letters mean significant differences with $p<0.05$ performed with the two-way ANOVA test followed by the Tukey test. #= PE vs. Interaction IPE. Lower case letters represent the groups, with [a] <0.05 vs. C; [b] <0.05 vs. FC; [c] <0.05 vs. PE; [d] <0.05 vs. IPE.

Figure 10. Graph of the average parameters of extrinsic stiffness (A), energy absorption capacity (B), maximum strength (C), summary table of the mechanical compression test of the femoral neck (D) - four experimental groups (n=6 rats/group); control (C), immobilized control (FC), physical exercise (PE) and immobilized physical exercise (IPE) over time (beginning of the experiment, pre-immobilization, after immobilization and right after euthanasia).



D	C	CI	EF	EFI
Extrinsic stiffness (N / mm)	337.3±16.723	385.6±36.123	298.1±17.849	254.2±18.529[b]
Energy absorption cap (mJ)	75.64±9.953	63.94±5.864	91.87±4.519[b]	85.51±3.973
Maximum force (N)	156.9±7.413	155.6±8.559	178.8±8.373	157.7±4.927

Different letters mean significant differences with $p<0.05$ performed with the two-way ANOVA test followed by the Tukey test. #= interaction. N = Newton. N/mm = Newton per millimeter. Lower case letters represent the groups, with [a] <0.05 vs. C; [b] <0.05 vs. FC; [c] <0.05 vs. PE; [d] <0.05 vs. IPE.

APÊNDICE A – Referências da Introdução Geral

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ANEXO I - Comitê de Ética

unesp

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"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado "**Efeitos do exercício resistido e do destreinamento por imobilização gessada sobre o osso fêmur no tratamento da osteopenia por desuso em ratos jovens e ratos adultos**", Processo FOA nº 00337-2016, sob responsabilidade de Mary Marcondes apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 23 de junho de 2016.

VALIDADE DESTE CERTIFICADO: 30 de Novembro de 2017.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 30 de Dezembro de 2017.

CERTIFICATE

We certify that the study entitled "**Effects of resistive exercise and detraining by plastered detention on femoral bone in the treatment of osteopenia by disuse in young and adult rats**", Protocol FOA nº 00337-2016, under the supervision of Mary Marcondes presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on June 23, 2016.

VALIDITY OF THIS CERTIFICATE: November 30, 2017.
DATE OF SUBMISSION OF THE FINAL REPORT: December 30, 2017.

Profa. Ass. Dra. Maria Gisela Laranjeira
Coordenadora da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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