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LUAN PEREIRA DA MACENA

Long-term of a diet rich in saturated fats induced obesity, insulin resistance and microstructural alterations of the femurs in rat

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Em longo prazo, dieta rica em gordura saturada induz obesidade, resistência à insulina e alterações macro e microestruturais em fêmures de ratos.

Dissertação constituída por artigo apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral.

Orientador: Prof. Dr. Gerson Francisco de Assis.

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DEDICATÓRIA

Dedico essa dissertação...

À minha irmã Emmely Vitória Pereira da Macena (in memorian)

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ABSTRACT

In rodents and humans, dietary intake of large amounts of fat (HFD) has adverse effects on insulin sensitivity and the development of glucose intolerance and diabetes due to the type of fat consumed, the amount and time of consumption. Despite the indisputable role of obesity and insulin resistance over bone tissue, it is still not really known how much they interfere in the process of bone degradation, and what their relation is in the progression of structural changes. The purpose of the current work was to evaluate changes in body mass, insulin resistance and structural changes in rat femurs that received a high-fat diet (HFD). 6-week-old Wistar rats were divided into two groups: the group that consumed a standard diet (SD, n=16) and the group that received high fat (HFD, n=16). After 120 days of the diet, the animals' femurs were subjected to X-ray microtomography and the condylar metaphyseal region evaluated in the CTAn program (SkyScan, Bruker). The data were submitted to the evaluation of the Gaussian distribution and homogeneity of variances for subsequent contrast of the averages by the "t" or Mann-Whitney test ($p<0.05$). HFD animals showed greater body mass compared to SD (926g vs. 670g, $p<0.001$) and insulin resistance with glucose tolerance (greater area on the curve in HFD), insulin tolerance (longer glucose decay time in DH). The X-ray microtomographic evaluation of the total volume (TV) of the evaluated metaphyseal region was similar between groups ($p=0.3933$), nevertheless the total volume of cortical was higher in HFD compared to SD (47.8mm^3 vs. 33.61mm3, respectively, $p<0.0001$) and the TV of trabecular region was minor (73.1mm3 vs. 63.4mm3, respectively, $p=0.0181$), leading to the highest percentage of the cortical region (43.2% vs 30, 8%, respectively, $p<0.0001$). In the trabecular region, bone volume (BV) and bone surface (BS) were smaller in HFD than in SD (BV of mm^3 18.6mm^3 vs. 5.9mm^3 $p<0.0001$) and BS of 178.5mm vs 314mm, respectively, 0=0.0021). Based on the results, the long-term consumption of a diet rich in saturated fat induce to obesity and insulin resistance which lead to structural changes in rat femurs similar to osteoporosis.

Keywords: X-Ray Microtomography, Obesity, Insulin Resistance, Osteoporosis.

RESUMO

Em roedores e humanos, a ingestão dietética de grandes quantidades de gordura (HFD) tem efeitos adversos na sensibilidade à insulina e no desenvolvimento de intolerância à glicose e diabetes devido ao tipo de gordura consumida, a quantidade e o tempo de consumo. Apesar do indiscutível papel da obesidade e resistência à insulina sobre o tecido ósseo, ainda não se sabe realmente o grau de interferência delas no processo de degradação óssea, e qual a relação destas na progressão de alterações estruturais. O proposto do atual trabalho foi avaliar as alterações na massa corporal, quadro de resistência à insulina e alterações estruturais nos fêmures de rato que receberam uma dieta rica em gordura (HFD). Ratos Wistar de 6 semanas de idade foram divididos em dois grupos: grupo consumiram dieta padrão (SD, n=16) e o grupo que recebeu hiperlipídica (HFD, n=16). Após 120 dias da dieta, os fêmures dos animais foram submetidos a microtomografia computadorizada e a região metafisária condilar (RMc) avaliados no programa CTAn (SkayScan, Bruker). Os dados foram submetidos a avaliação da distribuição gaussiana e homogeneidade das variâncias para subsequente contraste das medias pelo teste "t" ou Mann-Whitney ($p<0.05$). Os animais HFD apresentaram maior massa corporal comparado aos SD (926g vs. 670 g, $p<0.001$) e quadro de resistência à insulina com tolerância à glicose (maior área sobre a curva nos HFD), tolerância à insulina (maior tempo de decaimento da glicose nos DH). Pela avaliação microtomográfica volume total (TV) da região metafisária avaliada foi similar entre os grupos ($p=0,3933$), porém o TV da cortical foi maior nos HFD comparado aos SD (47,8mm^3 vs. 33.61mm^3, respectivamente, $p<0,0001$) e a da região trabecular menor (73,1mm^3 vs. 63,4mm3, respectivamente, $p=0,0181$), levando ao maior percentual da região cortical (43.2% vs 30,8%, respectivamente, $p<0,0001$). Na região trabecular, o volume ósseo (BV) e a superfície óssea (BS) foi menor nos HFD que nos SD (BV de mm^3 18,6mm^3 vs. 5.9mm^3 $p<0,0001$)e BS de 178.5mm vs 314mm, respectivamente, 0=0,0021). Com base nos resultados o consumo em longo prazo de uma dieta rica em gordura saturada leva a obesidade e o quadro à resistência à insulina os quais levam a alterações estruturais nos fêmures de rato similar ao quadro de osteoporose.

Palavras-chave: Microtomografia por Raio-X, Obesidade, Resistência à Insulina, Osteoporose

LIST OF ABREVIATIONS AND ACRONYMS

μA	Microampère
μm	Micrometer
2D	Two-Dimensional
3D	Three-Dimensional
BV	Bone volume
BV/TV	Bone volume/Total Volume
CEUA	Ethics Committee on Animal Education and Research at FOB-USP
cm	Centimeter
EDTA	Ethylenediamine tetraacetic acid
FEI	Feed efficiency index
FBW	Final body weight
IBW	Initial body weight
TF	Total food
WGPCI	Weight gain Per caloric intake Index
ipGTT	Intraperitonial glucose tolerance test
ipITT	Insulin tolerance test
FL	Femoral lenght
IED	Inter-epicondylar distance
FGL	Fasting glucose levels
FHD	Femur head distance
FNSA	Femur neck shaft angle
Tb.BV	Trabecular bone volume
Tb.BV/TV	Trabecular bone percentage
Tb.Th	Trabecular Thickness
Tb.N	Trabecular number
Tb.Sp	Trabecular separtion
Ct.BV/TV	Cortical bone percentage
Ct.Ar	Cortical surface
Ct.P	Cortical perimeter
Ct.Th	Cortical thickness

MC	Medulary cavity
BMD	Bone mineral density
Fig.	Figure
IL-10	Interleukin-10
kg	Kilogram
mg	Milligram
Micro-CT	Microcomputed tomography
mL	Milliliter
mm	Millimeter
mm^3	Cubic millimeter
BMI	Body Mass Index
Hb1Ac	Glysoylated Hemoglobin
TNF-α	Tumor necrosis factor
IL 1	Interleukin-1 family
IL 6	Interleukin 6
RANK	Receptor activator of nuclear factor κ B
RANKL-L	Receptor activator of nuclear factor kappa-B ligand
OPG	Osteoprotegerin
TAM	Marrow Adipose Tissue
WHO	World Health Organization

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

Overweight and obesity are chronic non-communicable diseases of multifactorial etiologies resulting from the prolonged positive energy balance between food consumption and energy expenditure that leads to abnormal or excessive accumulation of fat. In long-term, both are risk factors for several diseases with high mortality and morbidity, such as diabetes, cancer, arthritis, hypertension, stroke and myocardial infarction (1–4)

Diet is the main factor for the development of obesity; data show that countries that follow a healthier diet have a lower prevalence in the number of overweight and obese people (5). Healthy weight control is favored when an adequate diet is associated with regular physical activities. Since, the maintenance of lean mass (muscles) is greater in people who practice some regular physical activity when compared to sedentary or with little physical activity (6).

Recently, evidences indicate that obesity affects bone quality by increasing the risk of fractures (7–9), through several routes that can be intensified with aging such as: deregulation of the progenitor stem cell, degenerative inflammation through the action of local cytokines and systemic changes through the effects of central adipokines and other metabolic abnormalities associated with obesity compromise bone health by increasing adipogenesis at the expense of osteoblastogenesis, increasing bone resorption and reducing bone mass (10).

It is important to mention that obesity associated with sedentary lifestyle is one of the main causes of glucose intolerance (11,12) by increasing the levels of circulating free fatty acids derived from adipocytes. These free fatty acids inhibit the glucose uptake, the glycogen synthesis and the glycolysis. In many obese individuals, the insulin resistance is compensated by an increase of insulin production due to the inability of the insulin to perform its functions properly (13).

In parallel with the reduction of insulin action, since the pancreatic β cells compensate for the state of insulin resistance by increasing the secretion of this hormone, then β-cell dysfunction occurs, an inappropriate response to glucose, which leads to the development of intolerance glucose and then diabetes (14). In rodents and humans, dietary intake of large amounts of fat (HFD) has adverse effects on insulin sensitivity and contributes to the development of glucose intolerance and overt diabetes (15).

Fat intake ranging from 40 to 75% of total kilocalories, usually as saturated fat, safflower oil or corn oil (both rich in n-6 polyunsaturated fatty acids) reduces the uptake of insulin-stimulated glucose in skeletal muscles (16–24) and adipose tissue (16,24,25) together with increased hepatic glucose production (18,24,26).

Anize et al (2017) recently reported the importance of the type of fat consumed in overweight/obese adults with normal glucose tolerance. Individuals that consumed a high-fat diet for 10 days (HFD, n=10: 55 % fat being 25 % saturated fat and 27% carbohydrate) showed a significantly decreased insulin sensitivity, while a low fat diet (LFD, n=10: 20% fat / 8% saturated fat / 62% carbohydrate) did not promote any changes, emphasizing that the diets were eucalyptus. Thus, the high intake of saturated fat in the diet alters the regulation of glucose transport in insulin-sensitive tissues.

Insulin resistance brings harm to the body mainly through systemic inflammation, mediated by pro-inflammatory cytokines and, through mitochondrial degeneration, which can increase the number of fat cells due to lower energy expenditure (13). In addition to the release of pro-inflammatory cytokines, the fat cell also shows itself as an active endocrine agent, being responsible for the production of leptin and adiponectin (14). Both hormones actively participate in bone metabolism; the association between these hormones and obesity has recently brought into the literature a possible deleterious effect on bone when found in inadequate concentrations, due to its greater or lesser expression (27,28).

Obese individuals with insulin resistance are more prone to the risk of fractures, as well as to a late post-trauma recovery due to the bone structure that, in many cases, is in a fragile state. In the case of the femur, the risk of fracture in these cases seems to be more likely to occur in the proximal region (extremities); which, in addition to the decrease in bone quality, goes through a continuous process of decreasing its vascularization, closely linked to the decrease in the trabecular region (29,30).

The literature also suggests that obese individuals have a higher prevalence and incidence for acetabular dysplasia, a strong predictor for hip and knee osteoarthritis (31,32). In rats or mice, there are no reports in the literature between the correlation of dysplasia and obesity; however, recent studies show that, in animal models using rats, diet-induced obesity, negative influence of the quality of cartilage tissue, leading to the development of osteoarthritis in women, hip and knee joints (33,34).

Despite the indisputable role of obesity and insulin resistance over bone tissue, it is still not really known how much they interfere in the bone degradation process and what their relationship is in the progression of biomechanical changes. The analysis of such relationships in the human clinic is a costly and difficult process, due to the complex control of variables that can interfere in the results, such as: weight, gender, adequate control of hyperglycemia, socioeconomic conditions, as well as, the use of medications before or during the study, turning difficult an assertive correlation between cause and effect (35).

In order to facilitate the understanding of the effects of obesity and insulin resistance on bone tissue, animal models have been widely used with success, since it is easier to control variables. Being the rodent model, which presents conditions (development and physiological response) that are closer to that of humans when the metabolic syndrome (Obesity / Diabetes and Hypertension), is induced, its use in this experimental model is plausible (36). In addition, Wistar rats have relatively low costs (37) and, according to previous experience of our research team, they are easy to handle.

Due to the complex factors that are associated with nutritional habits and physical inactivity in health, our research group has proposed the hypothesis that the ingestion of a diet rich in saturated fats (HFD) in young adolescent and sedentary rats leads to obesity and the scenario of insulin resistance in adulthood, which can cause macro changes and microstructures in the bone structure, especially in the long bones of the hind limbs. Thus, our study aims at a better understanding of how the inadequate diet associated with a sedentary lifestyle leads to metabolic and bone structure changes.

2 PROPOSITION

2 PROPOSITION

The aim of this study was to evaluate whether, during adolescence into adulthood, consumption of a diet rich in fat (HFD) ad libitum leads to insulin resistance and structural bone changes in the femurs of male Wistar rats evaluated by microtomographic and histological analysis.

3 ARTICLE

3 ARTICLE

Experimental and Therapeutic Medicine

LONG-TERM OF RICH SATURATED FAT DIET INDUCES OBESITY, INSULIN RESISTENCE AND MICROSTRUCTURAL ALTERATIONS IN RATS FEMURS

Luan Pereira da Macena[1], (https://orcid.org/0000-0002-9798-9390)
Ever Mena Laura[1], (https://orcid.org/0000-0002-5494-6569)
Ana Carolina Cestari Bighetti[1], (https://orcid.org/0000-0001-9177-368X)
Daniela Pereira Catanzaro[1], (https://orcid.org/0000-0002-9902-5464)
Tania Mary Cestari[1], (https://orcid.org/0000-0003-1287-5974)
Rumio Taga[1] (https://orcid.org/0000-0002-5593-0999)
Gerson Francisco de Assis[1] (https://orcid.org/0000-0001-8225-3164)

[1] *Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, São Paulo, Brazil*

* To whom correspondence should be addressed Luan Pereira Macena, Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Av. Dr. Octavio Pinheiro Brisolla, 9-75, Vila Universitária, Bauru SP, Brazil. Tel: +55 1498833-8090; Email: macena@usp.br

Running Title: MACENA *et al*: Long-term of HFD-induced femoral bone loss.

keywords: High-fat diet, Obesity, Insulin resistance, Femur bone anatomy, Microtomography, Rat

ABSTRACT

Overconsumption of highly palatable, energy dense foods increased incidences of obesity and its accompanying metabolic dysregulation and systemic inflammation. We hypothesized that that long-term of a high-fat diet (HFD) induced to obesity, insulin resistance and bone structural changes in rat femur. Twenty 6-wk-old male Wistar rats consumed a standard diet (SD, 11% of energy as fat) or an HFD (60% of energy as fat) *ad libitum* for 120 days. The femurs of the animals were submitted to micro-CT and histological analysis. In HFD rat a higher increase of body mass and fasting glucose levels compared to SD was observed after 45 days and 60 days, respectively. The reduction of insulin sensitivity and compensatory hyperinsulinemia was observed at 120 days. Although no differences was observed in femoral length and femur head diameter, an higher inter-epicondylar distance and smaller femur neck shaft angle than SD femurs were verified. In the metaphysis, the volume of the medullary cavity and bone trabeculae were, respectively, 13.36% and 71.02% smaller in HFD than SD group. In HFD, an enlargement of diaphysis of 12.29% compared to the SD also were observed. Morphologically, 30% of HFD and 70% SD femurs sowed an active but thin epiphyseal growth plate located between the epiphysis and metaphysis, while 70% of HFD and 30% SD a discontinued and inactive/senescent growth plate with areas of fusion of the epiphysis with the metaphysis was observed. Based on the results, the long-term feeding of a HFD leads to gradual picture of obesity-induced insulin resistance in the rats. In the femurs, HFD diet promoted a faster femoral epiphyseal closure or growth plate fusion with decreases cancellous bone mass and compensatory increase of cortical bone mass resulting of increase in mechanical loading of metabolic impairment.

INTRODUCTION

In recent decades, the prevalence of overweight and obesity has become increasingly common such that it is now the major nutritional problem worldwide. In according to World Health Organization over 340 million children and adolescents aged 5-19 (18% of girls and 19% of boys) were overweight and more than 1.9 billion adults, 18 years and older (39% of world population), were overweight or obese (1). Adolescence is a particularly vulnerable time for the development of obesity because it is marked by a slowing of growth and corresponding decrease in physical activity levels (2). The risk for adult obesity was more accurately assessed in adolescents rather than younger age groups (3,4). In according to study of Hales (2018) adolescents aged 12–19 years had the highest prevalence of obesity (20.6%) compared to youth aged 6–11 years (18.4%) and children aged 2–5 years (13.9%). Overweight and obesity are linked to more deaths worldwide than underweight.

The fundamental cause of obesity and overweight is an energy imbalance between calories consumed and calories expended (6). Overconsumption of high fat diet (HFD) can lead to weight gain relatively quickly since dietary fat is metabolized to free fatty acids, and subsequently, lipid synthesis. Thus, the excess fat can contribute to obesity, systemic inflammation, and impaired glucose homeostasis leading to decrease in insulin sensitivity and picture of insulin (7,8). However, more important than total fat for the management of obesity is monitoring the type of fat ingested. Saturated fat is significantly more inflammatory compared to unsaturated fat (9,10) primarily through its potent ability to activate multiple inflammatory mechanisms including macrophage infiltration and/or proinflammatory activation, and the c-Jun-N terminal kinase and TLR4 signaling pathways (6,10–12). Saturated fat is also known to directly interfere with the insulin signaling cascade at multiple steps (13,14). For these reasons, numerous agencies, including the American Heart Association, American Diabetes Association and USDA, recommend low saturated fat intake. However, data from the National Health and Nutrition Examination Survey (NHANES), which surveys representative samples from varying age groups of the United States showed that adolescents generally consume higher levels of saturated fatty acids in comparison to unsaturated fatty acids (15).

Childhood and adolescence are characterized by rapid and significant longitudinal bone growth, areal bone expansion, and bone mineral accrual (16). Ninety percent of the peak bone

mass is achieved in late teenage and the amount of bone mass reached by this age predicts BMD in adulthood (17,18).

Clinical investigations have established that a high body weight and obesity are positively correlated with bone mass and its reduction may cause bone loss and fracture risk (19,20). However, other clinical studies, overweight/obesity adolescents with a sedentary lifestyle have several risk factors for low bone density (21,22). In according to Zao et al (2008) the body fat mass is negatively correlated with bone mass when the mechanical loading effect of body weight is statistically removed. Accrual of adipose tissue within the bone marrow space lead to an inflammatory state (24), which increases bone resorption and disrupts differentiation of mesenchymal and haematopoietic stem cells (25), that impairs the regenerative and immune responses (26,27).

Childhood and adolescence are characterized by rapid and significant longitudinal bone growth, area bone expansion, and bone mineral accrual (16). Approximately 40 to 45% of adult bone mass is acquired during adolescence (28), influenced by anabolic hormones such as growth hormone (GH), insulin like growth factor 1 (IGF-1) and insulin (29,30). Insulin enhances osteoblast development, promotes OCN expression and reduces bone reabsorption (31). At puberty, insulin secretion increases physiologically but the rise is higher in adolescents with obesity who develop mild to severe hyperinsulinemia. In mice, hyperinsulinemia has been associated with higher trabecular and cortical bone mineral content, reduced bone formation but also decreased number of osteoclasts and markers of bone resorption. These findings suggest that hyperinsulinemia is associated with reduced bone turnover and, consequently, poor bone quality (32).

The interaction of diet, obesity, insulin resistance and bone metabolism is complex and not as yet fully elucidated. In the current study, we hypothesized that long term of high fat diet HFD in juvenile rat leads to obesity and insulin resistance affecting femoral bone structures. We first evaluated the body mass gain/obesity and presence or not of insulin resistance in the animals that received HFD and control diets. After 16 weeks, two-dimensional and three-dimensional morphometric evaluations of trabecular and cortical bone of the proximal, mid-diaphyseal, and distal femur by micotomographic image was determined. Finally we looked for a link between HFD/obesity/insulin resistance with alterations in the femoral micro and microstructure in rats.

MATERIALS AND METHODS

Animal model and design experimental

Twenty male 6-week‑old *Wistar* rats (*Rattus norvegicus*) provided by the Central Animal House of Bauru School of Dentistry, University of São Paulo that received 2 weeks of a standard rodent chow (Nuvilab CR1-irradiada, Nuvital Nutrientes SA. Colombo, PR). The experimental protocol followed "The Guiding Principles for the Care and Use of Animals", according to the principles of the Declaration of Helsinki, and was approved by the Institutional Review Board for Animal Research of Bauru School of Dentistry – University of São Paulo (Process n°. Ceua-Proc nº 008/2019). The rats were randomly divided into a standard diet (SD) and HFD groups (n=16 per group) and maintained in singly caged with free access to water and their corresponding diets with the room temperature at 22±1˚C, humidity at 60‑70% and under a 12‑h light/dark cycle.

Diet composition

The animals of the SD group had access to standard chow diet (11% fat, 63% carbohydrate and 26% protein) HFD group were fed a high-energy or high-fat diet as instructed by Correia-Santos et al,. (2012) containing a 60% fat, 26% carbohydrate and 14% proteins, and the remaining nutrients met the AIN‑93 criterion (18). The energy values are 17 kJ/g (4.0 kcal/g) for protein, 37 kJ/g (9.0 kcal/g) for fat and 17 kJ/g (4.0 kcal/g) for carbohydrates. Hence, energy concentrations were higher in the HFD (5.40 Kcal/g) than SD (3.39 Kcal/g), but the micronutrient concentrations were the same in the SD and HFD diets. Body weight and blood glucose level (FGL) were assessed at 0, 10, 20, 30, 60, 90 and 120 days of administration of the diet.

The Feed efficiency index (FEI) or amount of food consumed that can promote body weight gain was calculated in according to Cardoso et al (2020) using equation: FEI = FBW − IBW/TF, where FBW is the final body weight in grams, IBW is the initial body weight in grams, and TF is the total amount of food ingested in grams. Furthermore, the weight gain per caloric intake index (WGPCI) was calculated to evaluate the animal's capacity to convert consumed energy into body weight, following equation: WGPCI = FBW − IBW/kcal consumed

Intraperitoneal glucose tolerance test (ipGTT) and insulin tolerance test (ipITT)

After 16 weeks of administration of the diets, ipGTT and ipITT tests were performed in the morning, after an overnight fast of 12 hours. In according to Bighetti *et al* (2014), the animals were

anesthetized with sodium thiopental (60mg/kg of body weight) and a drop of blood was collected from the tail for measurement of blood glucose levels with a glucometer (Accu-Check® Active, Roche Diagnostics GmbH, Mannheim, Germany) for establishment of a stable baseline glucose level. Subsequently, to perform the ipITT and subsequent calculation of the decay constant of the glucose (Kit) in the rats HFD (n=10) and SD (n=10) groups were injected intra-peritoneally with regular insulin solution diluted in saline solution (10UI: 1mL) at a dosage of 2UI/Kg of body mass and whole-blood glucose levels were then monitored every 10 min for 30 min. For ipGTT test other animals (HSFD n=10 and CD n=10) were injected with a solution containing glucose (marca) diluted in saline solution (1g: 2mL) in the dosage of 2g/kg of body mass and whole-blood glucose levels were then monitored at 15, 30, 60, 90 e 120 min.

Femurs collection and micro-CT procedures and analysis:

After 16 weeks, the animals were killed with an overdose of a ketamine/xylazine mixture. After confirmation of death the femurs were collected and fixed in 10% phosphate-buffered formalin for 48 hours. The femurs were scanned using a commercially available microcomputed tomography system (SkyScan 1176; Bruker icroCT, Kontich, Belgium). Each femur was submitted to follow pattern scanning: number of connected scans was of 3 (**proximal** extremity, middle of the bone, and **distal**-extremity), X-rays tube potential of 50kV, beam current of 500μA, exposure of 380ms and camera Pixel Size of 12.45um. 0.5mm Aluminum filter was used and the sample rotated 180 ° with a "rotation step" of 0.5 ° Of the three files generated, a single three-dimensional image containing 2496 slices was reconstructed using NRecon software (SkyScan N. V. Belgium). For 2D and 3D analysis, the femurs were aligned from the sagittal, transversal and coronal anatomical axes using the SkyScan DataViewer software ((SkyScan N. V. Belgium).

Linear and angular measurements of femurs

The linear and angular measurements of the femurs in the sagittal plane were determined in the CTAn program (Bruker, Kontich, Belgium). Femoral length (FL) was measured from femoral head midpoint to the base of the femoral condyle (Fig. 1a). Inter-epicondylar distance (IED) was obtained from the longest distance between the body of the medial and lateral condyles (Fig. 1b). The femur head diameter (FHD) was obtained was illustrated in the Fig. 1c. The femoral axis or femur neck shaft angle (FNSA) formed between long axis of neck of femur and long axis of the shaft of femur was determined as suggested by Joe Hauptman et al (1979) and illustrated in the Figure 1d.

Volumetric determination of trabecular and cortical bone microarchitecture

Cortical and trabecular bone microarchitecture was evaluated in the shaft and distal metaphysis of the femurs. In the CTAn software, a VOI (350x115x340) representing 18% of the total length of the femur (Fig.2a and 2c) was positioned/selected at 6000μm or 173pixels of the growth plate in the distal metaphysis (Fig. 2a-b) and other similar VOI in the central region of the diaphysis (staft or body), as a showed in the Fig. 2c-d. The trabecular bone variables assessed were as follows: trabecular bone volume (Tb.BV), trabecular bone percentage (Tb.BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp). The cortical bone variables assessed were as follows: cortical bone percentage (Ct.BV/TV), cortical surface (Ct.Ar), cortical perimeter (Ct.P) and cortical thickness (Ct.Th).

Histological procedures for morphological evaluation of bone structures in the distal portion of femures

For histological evaluation of bone structure in the distal portion, the femurs were demineralized in EDTA a solution containing 4.13% tritriplex III (Merck KGaA, Darmstadt, Germany) and 0.44% sodium hydroxide, for a period of approximately 40 days. The femurs were sectioned in the central region of diaphysis. The distal portion were histologically processed for embedding in Histosec® (paraffin enriched with polymers of the EMD Millipore - division of Merck KGaA, Darmstadt, Germany). Latero-lateral semi-serial 5-μm thickness sections (intervals of 100 μm) of all defects were obtained and stained with Hematoxilin-Eosin (HE).

Statistical analysis

All experimental data were compiled as means and standard deviations (mean ± standard deviation) and reported separately for each group of rats. Micro-CT data were tested for normal distribution by D' Agostino & Pearson and homogeneity of variance by F-test subsequently the mean comparisons between HFD and SD groups were performed by two-tailed Student's t-test or Mann-Whitney U-test using GraphPad Prism 7 at level of significance of 5%.

RESULTS

Laboratorial data

The data laboratorial of feed intake (Table 1) showed that the daily Feed consumption in the HFD group was 16.16% lower than SD. Although the initial body weight was similar between HFD and SD groups (average of 212,95g). After 120 days of consumption of diets, the

total weight gain, FEI, and WGPCI were 53.05%, 82.48% and 18.42% higher in HFD rats compared to SD.

The graphs of the evolution of the body weight (BW, Fig. 3A) and fasting glucose levels (FGL, Fig.3B) did not show statistical differences between groups at 0, 15 and 30 days ($p>0.05$). In the HFD group After 45 days and 60 days of diet consumption a significant increase in the BW and FGL, respectively, in HFD group compared to SD was observed, which remained until 120 days of diets consumption.

Table 1. Laboratorial dada of initial and final weight, weight gain and Feed intake of rats fed with SD and HFD

Parameter	SD (mean±DPM)	HFD(mean±DPM)	"t" test "p" values
IBW (g)	207.4±11.07	218.5±26.61	0.2390
FBW (g)	670,0±42.15	926.52±78.17	<0.0001
Total weight gain (g)	462.6±33.96	708.0± 62.69	<0.0001
Daily weight gain (g)	3,86±0,28	5,9±0.52	<0.0001
Daily feed consumption (g)	24.13±1.98	20.23±1.325	<0.0001
FEI	0.1598±0.0117	0.2917±0,0258	<0.0001
WGPCI	0.0456±0.0033	0.0540±0.0048	<0.0002

Intraperitoneal glucose tolerance test (ipGTT) and Intraperitoneal insulin tolerance test (ipITT)

After 120 days of HFD, the rats exhibited reduced peripheral insulin sensitivity, as indicated by a smaller constant glucose disappearance rate (KITT of 1.31 ± 0.11% per min, $p< 0.001$) compared to SD rats (KITT of 1.71 ± 0.14% per min) obtained during the ipITT experiment (see Figs. 3C and D). A glucose tolerance test (ipGTT) was performed to verify the presence of glucose intolerance in the insulin-resistant rats. As expected, the HFD rats showed higher blood glucose values after a D-glucose challenge at 60, 90 and 120 min compared with the SD animals (Fig. 3E). In HFD rats a higher area under the curve (AUC, Fig. 3F) than SD was observed, pointing to a reduction of ability to metabolize glucose in the HFD rats ($p<0.004$). These observations demonstrate the negative impact of dexamethasone on peripheral insulin sensitivity and glucose tolerance.

Micro-CT evaluation of femurs

Linear and angular measurements of femurs

In the Figure 4 showed representative images of the femurs of the SD and HFD group, as well as the graphs of the averages and DPM of the linear and angular measurements. The FL and FHD were similar between the groups, with an average of 39.31 ± 2.64mm and 3.28 ± 0.47mm, respectively. However, in the femurs of HFD group, the IED (7.52 ± 0.48mm) and FNSA (141.25 ± 9.35) were 13.64% and 17.03% higher, respectively, than SD groups (6.62 ± 0.77mm and 120.7 ± 7.44, respectively)

Volumetric determination of trabecular and cortical bone microarchitecture

2D Cortical and trabecular bone microarchitecture and graphs of volumetric evaluations obtained by micro-CT morphometry were represented in the Figure 5. The region of the epiphysis and distal metaphysis of the femurs (Fig. 5A) of the HFD group exhibited a larger medullary cavity with a greater quantity trabecular bone and less bone cortical thickness. Morphometrically, the evaluated segment (VOI) obtained from the metaphysis region (Fig. 5B1) showed a similar total volume (mean of 108.85mm^3). However, in the HFD group, the volume of the medullary cavity and bone trabeculae (Fig. 5B1) were 13.36% and 71.02% smaller, respectively, than SD group, while the cortical volume was 42.13% higher. The same was observed regarding the percentage of these regions (Fig. 5B2). Due to the small volume of bone trabeculae (Fig. 5B3) in the HFD group, small values of BS and BS / TV (Fig 5B4) than SD all were observed. BS/BV (Fig.5B4) in HFD was significantly higher than in SD group, suggesting that bone trabeculae in HFD were less thick than in SD metaphysis. In the region of the femoral diaphysis, the evaluated segment or VOI presented a volume (Fig. 5B5) 12.29% higher in the HFD group compared to the SD due to the enlargement of this region (Fig. 5A). The increase in total volume in HFD occurred due to increase in the volume of the medullary region in 9.71% and of the cortical region in 14.67% compared to SD. Thus, the percentage of each region of the diaphysis was similar between the groups (Figure 5F).

Morphological evaluation of bone structures in the distal portion of femures

Histological representative images of distal femurs of rats after 120 days of diet consumption represented in the Figure 6 showed higher bone maturation in HFD than SD. The distal femurs were composed by epiphyseal region containing the condyles, epiphyseal plate, metaphysis and diaphysis (Panoramic images in Fig. 6). A thin thickness epiphyseal growth plate

located between the epiphysis and metaphysis was present in 6 animals of SD (Figs. 6A-B) and 3 in HFD (Fig. 6D). The epiphyseal growth plate was composed of three zones (resting, proliferative, and hypertrophic zone). Each zone contains chondrocytes in different stages of differentiation (Figs. 6A-B and D). In 3 animals of SD (Fig. 6C) and 7 of HFD (Figs. 6E-F), the femur showed inactive/senescent growth plate with absence of resting, proliferative and hypertrophic zones. In this case, the epiphyseal plate exhibited a disorganized distribution of chondrocytes and cartilage mineralization surrounded by dense cortical-like bone (see Figs. 6E-F). Besides, the epiphyseal plate was discontinued, forming points of fusion of the epiphysis with the metaphysis. This process is known as epiphyseal closure or growth plate fusion

Metaphysis is the region between the epiphyseal plate and the diaphysis. In rat femures with active longitudinal growth (Figs. 6A-B and D) showed a thinner cortical and high quantity of trabecular bone in the medullary cavity, whereas in the diaphysis, the bone marrow occupied the entire cavity. In rat femurs with inactive longitudinal growth (Figs. 6C and E-F) the metaphysis showed a dense and thicker cortical bone as well as few trabecular bones in cavity compared to active growth plate (compare the Figs. 6A-B and D with Figs. 6A-B and D).

Discussion

The obesity has been attributed in part to the wide availability of highly palatable, calorically dense foods. Diets rich in fat not only induce obesity in humans but also make animals obese (37). In actual study, Junvenil-adult Wistar rats that consumed *ad libitum* a high fat diet containing 60% fat, 26% carbohydrate and 14% proteins spontaneously increases caloric intake, which correlates positively with higher weight and gain compared to SD animals. Besides, the rats also developed insulin resistance as shown by significant reduction in glucose decay and mild/high glucose intolerance in ipITT and ipGTT tests, respectively. Additionally, the HFD-fed excessive dietary accelerated bone maturation lead to morphological alteration of rat femurs in comparison to SD.

In this study, micro-CT investigation of femoral structures showed, similar femoral length (FL average of 39.31mm) and femoral head diameter (FHD average of 3.28mm) both groups HFD and SD. However, the inter-epicondylar distance (IED) and femoral axis/ femur neck shaft angle (FNSA) were, respectively, 13.64% and 17.03% higher in HFD than SD groups. In human, abnormal femoral neck angle (FNSA) may be associated with various clinical problems ranging from harmless in toeing gait in childhood to disabling osteoarthritis in adults.

Differences between normal weight and obese individuals in the size and shape of the femoral shaft and the medial side of the knee joint are associated to mechanical loading on bone(38). This influence is greater during childhood compared with adulthood (39,40). Thus, individuals that were obese as children or adolescent will have greater skeletal changes than those who become obese as adults (38). The age-dependence of HFD effects on femoral bone also were observed in mice. The skeletally immature animals (5 weeks old) that received HFD (60% kcal fat) for 12 weeks demonstrated a significantly reduced femoral trabecular bone volume compared to femur of skeletally mature mice (20 weeks old) (41).

During the long bone growth, the bone formation and growth, the pubertal growth spurt, epiphyseal senescence, and fusion, are stimulated by nutritional, cellular, paracrine, and endocrine factors. In human, the closures of epiphyseal plates are an indicator of the adolescent. Epiphyseal closure lower limbs (femur and tibia) are found during 18-25 years of age. In rats, a significant longitudinal growth enters a decay phase at approximately 64 days of age, lagging slightly with sexual maturity (male at 45–55 days and female at 35–40 days) (42). However, the epiphyseal cartilage persists until one year old (43) and only after 20-weeks of age, the growth drastically declined to insignificant gains (43). In actual work, the administration of HFD initiated at 6-weeks old when the skeletal structure of the animals was still "immature". After 120 days of diets, 30% of HFD and 70% SD femurs sowed an active but thin epiphyseal growth plate located between the epiphysis and metaphysis. The epiphyseal plate was composed of three zones (resting, proliferative, and hypertrophic zone) containing chondrocytes in different stages of differentiation. Metaphysis region, the medullar cavity was filled by a high quantity of trabecular bone involved by a thin cortical. On the other hand, in 70% of HFD and 30% SD femurs discontinued and inactive/senescent growth plate with areas of fusion of the epiphysis with the metaphysis (epiphyseal closure or growth plate fusion) were observed. In these cases, the metaphysis showed a more dense and thicker cortical bone as well as few trabecular bones in cavity compared to active growth plate. Thus, morphometrically, the volume of the medullary cavity and bone trabeculae were, respectively, 13.36% and 71.02% smaller in HFD than SD group, while the cortical volume was 42.13% higher. Although, several studies have showed that excessive dietary fat is detrimental to bone homeostasis and has a greater effect on trabecular than cortical bone (44–49), our study point to an enlargement of diaphysis of 12.29% compared to the SD accompanied by increase of 9.71% in the volume of medullary region (endocortical resorption) and 14.67% in cortical region (periosteal bone formation) also were detected. Comparatively, four-week old mice that consumed HFD (60% fat) for 19 weeks the parameters of femoral cortical bone obtained of micro-CT images were significantly higher

compared to SD group (10.5% for cortical wall thickness; 6.1% outer cortical radius, 4.8% inner cortical radius) (50). In other study, young mice (3-week-old) that consumed HFD showed a larger femoral diameter compared to SD young, while adult mice (15-week-old) a smaller femoral thickness than SD adults was observed (51).

In rats that consumed the HFD a higher increase of body mass and fasting glucose levels compared to SD was observed after 45 days and 60 days, respectively, of diet consumption. The reduction of insulin sensitivity and compensatory hyperinsulinemia (Increased insulin levels compensate for the reduced responsiveness of target cells) in the rats were determinate at 120 days of diet consumption. Differences in bone response associated to HFD diet may be related to diet consumption time. Study with shorter duration (5 weeks) of HFD feeding demonstrated no alterations of bone remodeling, unchanged bone architecture, liver function, and adipogenesis, but elevated BMD was observed (52). Moreover, 12 weeks of HFD feeding impaired bone architecture, decreased bone strength, and reduced bone formation with compensatory rising of serum osteogenic markers in young male mice (Picke et al., 2018c). In parallel with adipose tissue accumulation, serum insulin levels were greater in these mice due to lower uptake of insulin into the bone marrow, while bone resorption was not altered (53). After 14–24 weeks of HFD consumption, led to higher bone resorption, lower bone formation, poor quality of bone architecture, and loss of bone strength (54,55).

Insulin signaling in osteoblasts stimulated bone formation and increased peak bone mass during development (56). In addition, the insulin downregulates the expression of osteoprotegerin, thereby indirectly up-regulating bone resorption and the release of matrix-bound OC, particularly in its uncarboxylated form (57). Dysfunction of insulin signaling in obesity is a result of the impairment of IGF-1 binding with insulin receptors on osteoblasts, resulting in a negative impact on bone remodeling (58–61). Similar to Lecka-Czernik et al. (2015), ours results showed that the obesity induced by HFD induced to high cortical bone mass probably caused by an increase in mechanical loading and/or increased production of bone anabolic adipokines and/or nutritional effect of fatty acids. This was followed by a second phase characterized by decreased trabecular bone formation/remodeling resulting of development of metabolic impairment with insulin resistance and hyperinsulemy and Diabetes.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Macena LP contributed to study conception, methodology design, acquisition of data, analysis and interpretation of data and reviewing the manuscript.

Laura EM performed the acquisition of data and critical review of manuscript.

Bighetti ACC performed the acquisition of data and critical review of manuscript.

Catanzaro DP performed the acquisition of data and critical review of manuscript.

Cestari TM was involved with study conception, performed the analysis and interpretation of data and critical review of manuscript.

Taga R performed the critical review.

Assis GF contributed to study conception, methodology design and critical review of manuscript.

All authors read and approve the final manuscript.

Ethics approval

The protocol of the experiment was approved by the Institutional Animal Care Committee (CEUA protocol 008/2019) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). In this study, ARRIVE guidelines for the reporting of animal studies was followed.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interest in this study.

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LIST OF FIGURES

Figure 1. micro-CT linear measurements of the femur: a) FL (green line) distance between the base of the femoral condyle (dashed line in red) and the highest plane of the femoral head (dashed blue line); b) FDI (green line) between the medial (red dotted line) and lateral (blue dotted line) condyles c) FHD; and d) FNSA angle formed between a red dashed line along the femoral length and the blue dashed line that divides the head of the femur and its body in half.

Figure 2. Micro-CT volumetric measurements of femoral bone microstructure: a) VOI in distal metaphysis; b) 3D (b1) VOI in the distal metaphysis region and one 2D (b2) image of selected VOI showing the trabecular area evaluated after binarization (b3); c) VOI in central region of diaphysis; and b) 3D VOI (d1) in the diaphysis region and one 2D image (d2) of the selected VOI showing the cortical area evaluated after binarization

Figure 3. Laboratorial data: A) Graph of Body mass show higher weight gain in HFD than SD during 120 days of consumption of diets; B) Graph of fasting blood glucose level show mean values compatible with normal glucose level (110mg/dL), but significantly higher in the HFD than SD at 90 and 120 days. C-D) Graphs of Insulin tolerance test (ipITT) and the area under glucose curve (AUC) show reduction in the insulin sensitivity in the HFD compared with the SD rats; and D-E) Graphs of glucose tolerance test (ipGTT) and AUC also show that HFD resulted in mild glucose intolerance in the HFD rats. Data are presented as the means ± SEM *significantly different vs. control group using unpaired Student's t-test ($P < 0.05$; $n = 10$). **$p<0.01$ and ***$p<0.001$

Figura 4. 3D-microCT images of the femurs (A) show greater thickness of the femurs of the HFD group compared to the SD. Graphs of Linear and angular evaluations (B): bar graphs of mean ± DPM show similar FL (B1) and FHD (B3) between groups and higher IED (B2) and FHD (B4) in HFD femurs than SD. "t" test *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Figura 5. 2D-microCT images of the femurs (A) show few bone trabeculae in the medullary region of the distal metaphysis and greater thickness of the cortical bone in HFD compared to SD. 3D-MicroCT morphometry (B) show bar graphs of mean ± DPM obtained for volume (B1), percentage (B2), bone surface/BS (B3) and ratio of trabecular bone (B4) in the distal metaphisis, as well as volume (B5) and percentage in the diaphysis. "t" test *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Figure 6. Histological views of distal femurs: Panoramic views of femurs show lateral condyle epiphysis (CO), epiphyseal plate (red arrow), metaphysis (between blue and black arrows) and diaphysis. Note a higher thickness of cortical bone (black arrow) in HFD (D,E,F) than SD (A,B,C). Details show the epiphyseal plate (area surrounded with black dashed line) and metaphysis region (area surrounded with blue dashed line) corresponds to the calcification zone/layer of the plate (red arrow) together with the interdigitating bone (blue arrow) and bone marrow (BM). Details also show different degrees of ossification and trabecular bone in the medullary cavity (MC) in SD and HFD groups. In SD groups details show presence of thin thickness epiphyseal growth plate with high (A, n=2) or moderate (B, n=4) trabecular bone, and epiphyseal line (C, n= 4) with lamellar trabecular bone. In HFD show active growth plate with moderate trabecular bone (D, n=3) and inactive or senescent epiphyseal plate with mature/lamellar trabecular bone (E, n= 3) or bone marrow fill a large part of the medullary cavity (F, n=4). HE

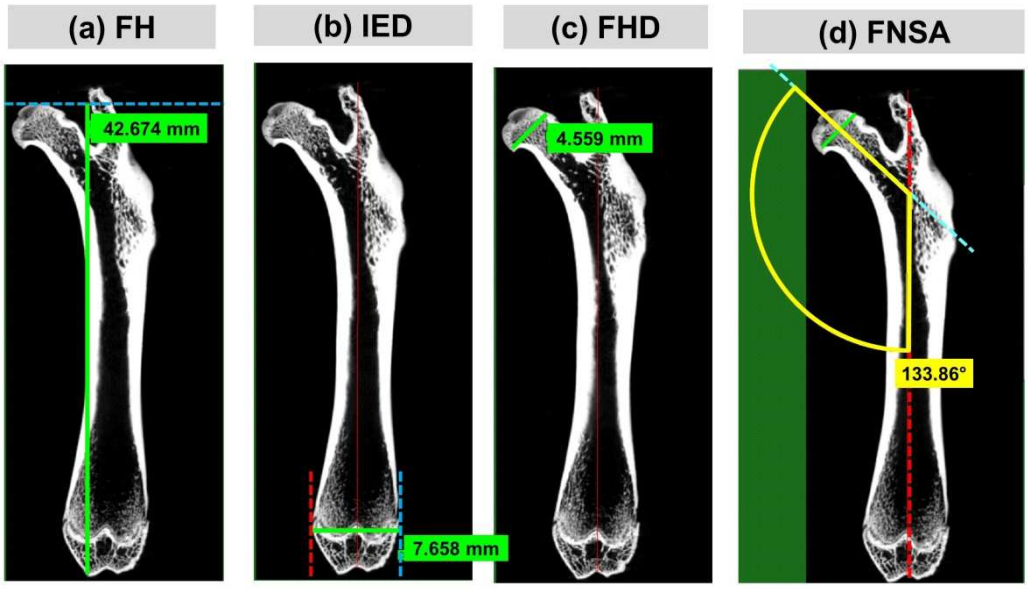

Figure 1

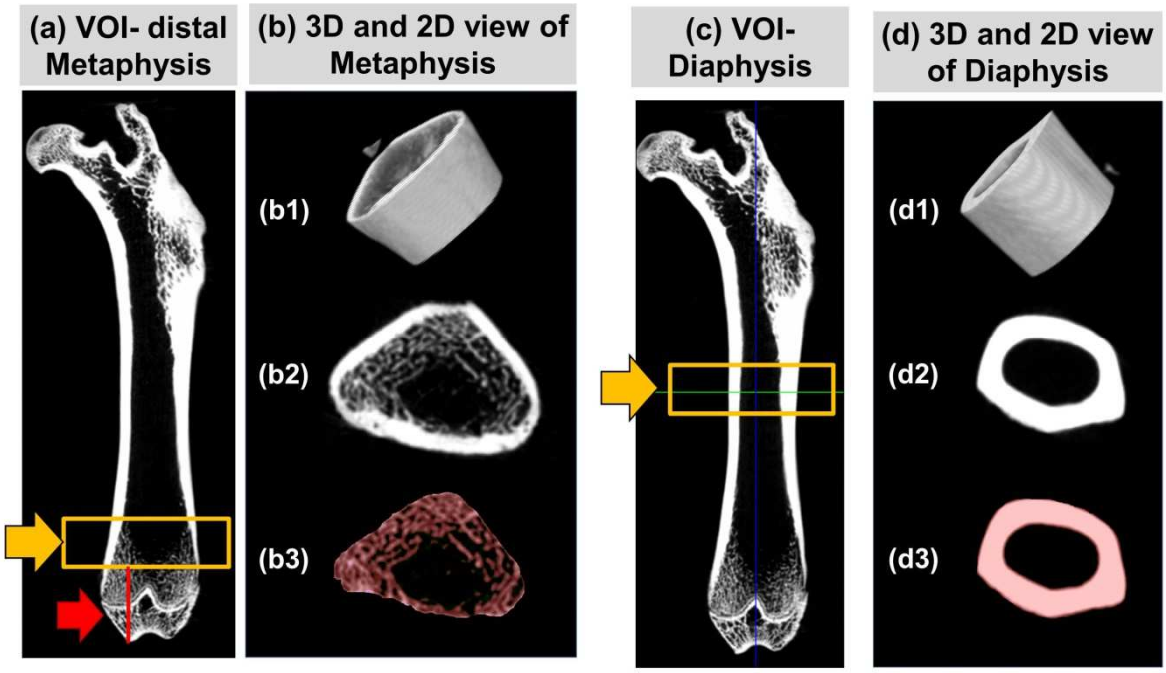
(a) VOI- distal Metaphysis
(b) 3D and 2D view of Metaphysis
(c) VOI- Diaphysis
(d) 3D and 2D view of Diaphysis
(b1)
(b2)
(b3)
(d1)
(d2)
(d3)

Figure 2

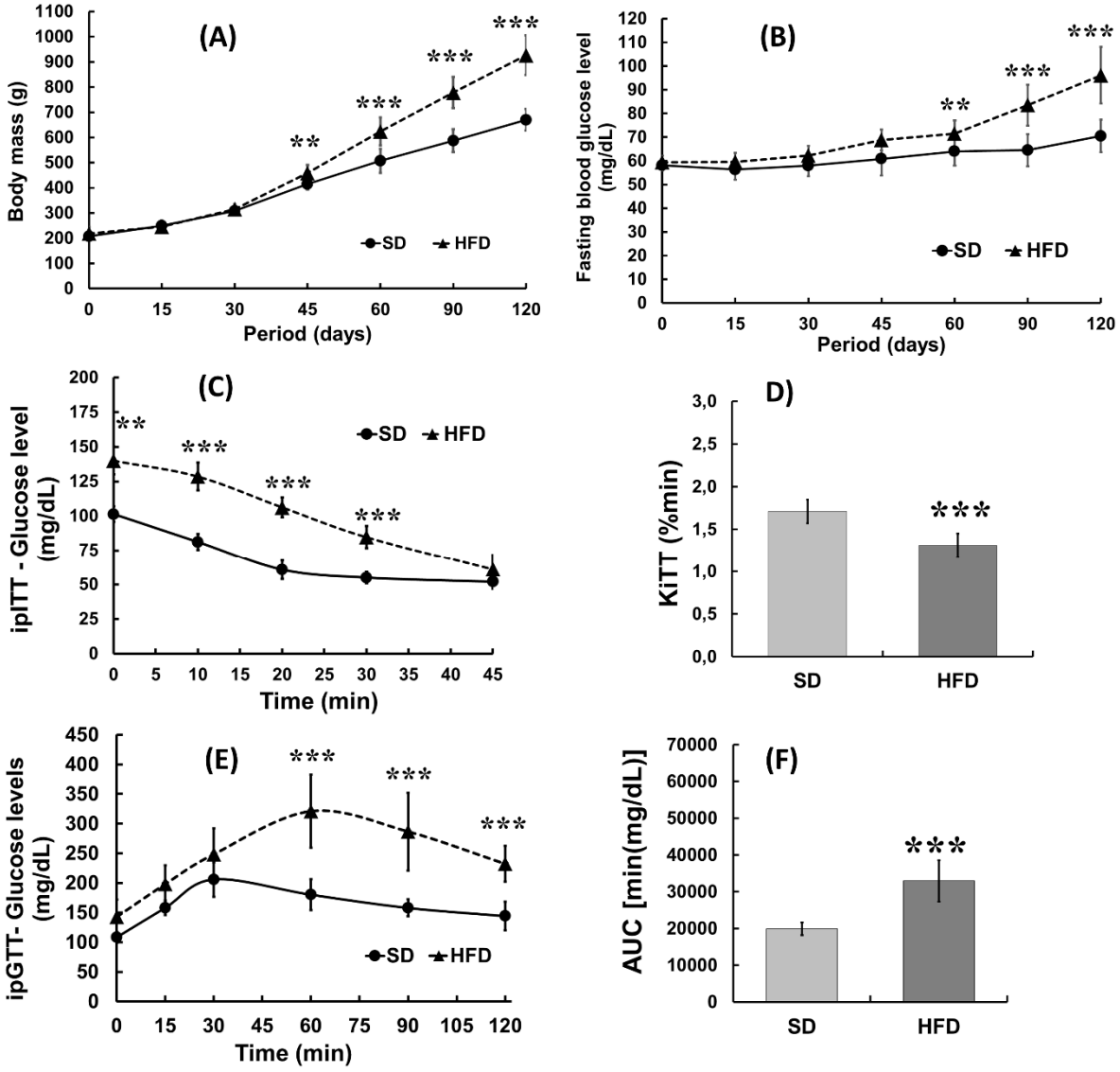
(A)
Body mass (g)
Period (days)
SD
HFD
(B)
Fasting blood glucose level (mg/dL)
Period (days)
SD
HFD
(C)
ipITT - Glucose level (mg/dL)
Time (min)
SD
HFD
D)
KiTT (%min)
SD
HFD
(E)
ipGTT- Glucose levels (mg/dL)
Time (min)
SD
HFD
(F)
AUC [min(mg/dL)]
SD
HFD

Figure 3

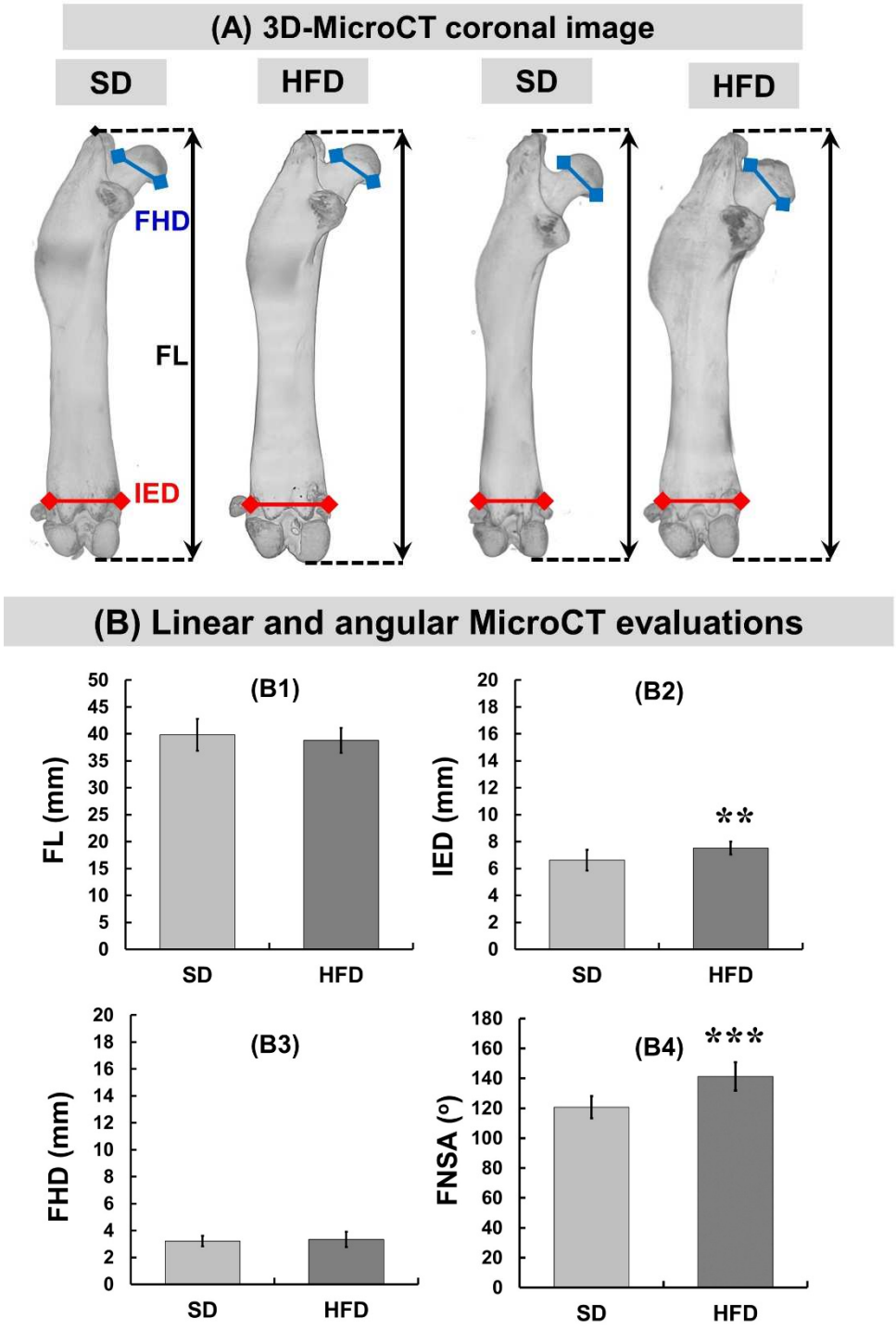
(A) 3D-MicroCT coronal image
SD
HFD
SD
HFD
FHD
FL
IED
(B) Linear and angular MicroCT evaluations
(B1)
FL (mm)
SD
HFD
(B2)
IED (mm)
**
SD
HFD
(B3)
FHD (mm)
SD
HFD
(B4)
***
FNSA (°)
SD
HFD

Figure 4

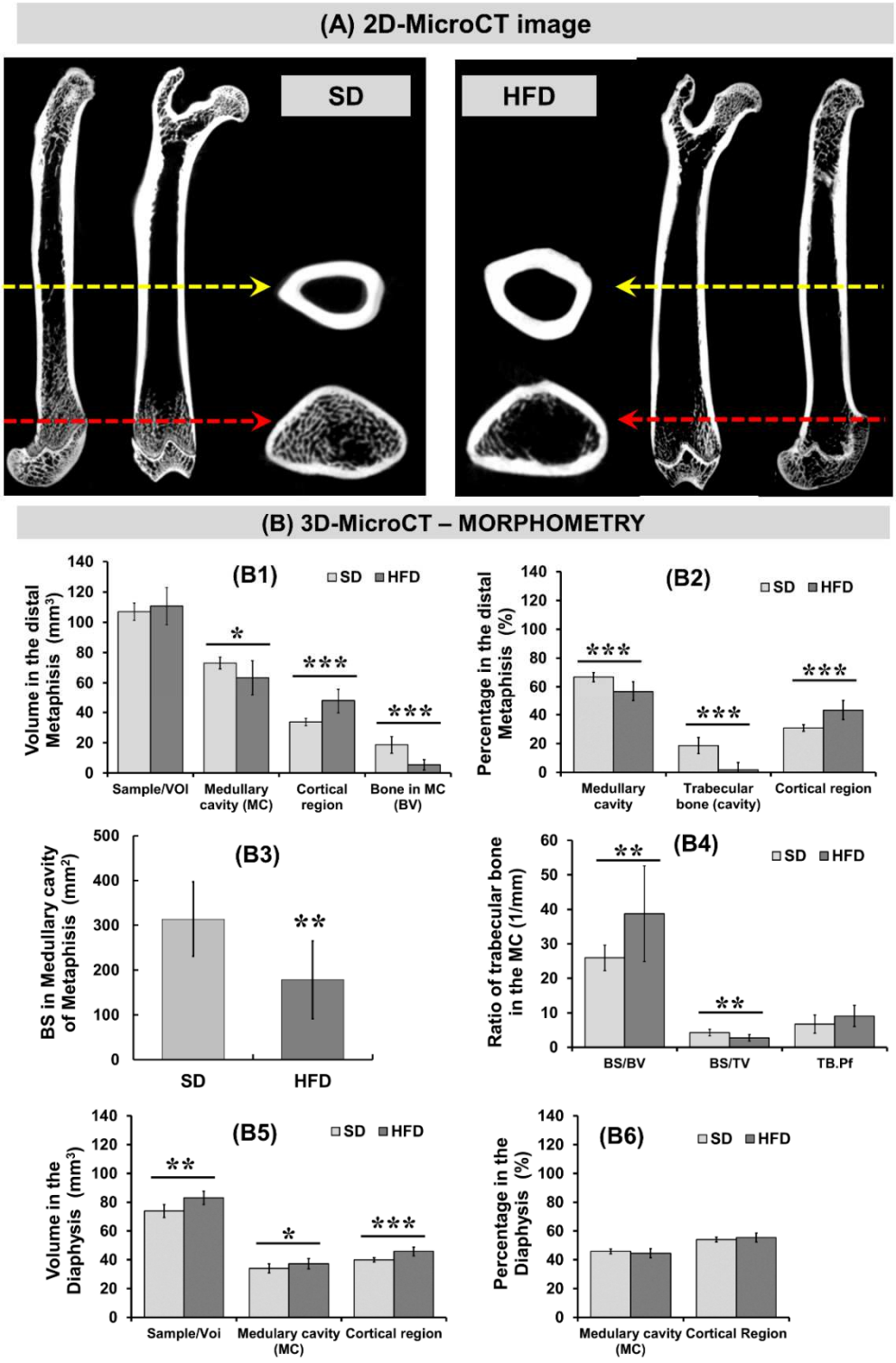
(A) 2D-MicroCT image
SD
HFD
(B) 3D-MicroCT – MORPHOMETRY
(B1)
SD HFD
Volume in the distal Metaphisis (mm³)
Sample/VOI
Medullary cavity (MC)
Cortical region
Bone in MC (BV)
(B2)
SD HFD
Percentage in the distal Metaphisis (%)
Medullary cavity
Trabecular bone (cavity)
Cortical region
(B3)
BS in Medullary cavity of Metaphisis (mm²)
SD
HFD
(B4)
SD HFD
Ratio of trabecular bone in the MC (1/mm)
BS/BV
BS/TV
TB.Pf
(B5)
SD HFD
Volume in the Diaphysis (mm³)
Sample/Voi
Medulary cavity (MC)
Cortical region
(B6)
SD HFD
Percentage in the Diaphysis (%)
Medulary cavity (MC)
Cortical Region

Figure 5

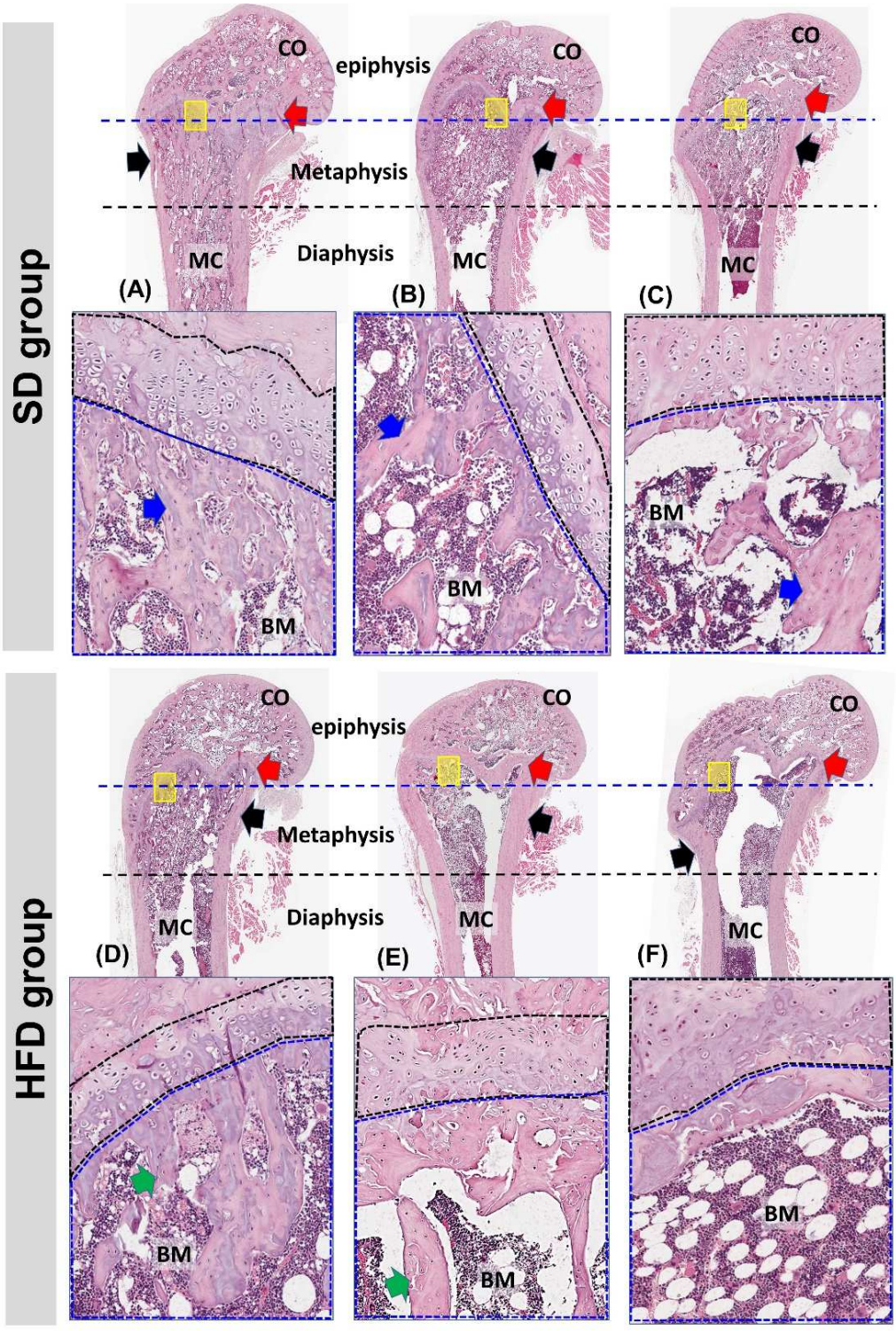
SD group
HFD group
CO
epiphysis
Metaphysis
Diaphysis
MC
BM
(A)
(B)
(C)
(D)
(E)
(F)

Figure 6

4 DISCUSSION

4 DISCUSSION

Obesity is one of the greatest public health challenges, identified as a pandemic and is responsible for 3 to 4 million deaths per year worldwide (1). Between the years 1996 and 2006, the expenditure of the North American government for the control and treatment of obesity and other chronic diseases directly linked, increased by 76 million dollars (2). In Brazil, according to study conducted by McKinsey Global Intitute in 2014, public health spending on obesity and related diseases consumes 2.4% of GDP, about 110 billion reais. By the year 2030, it is estimated that 57.8% of the world population will have obesity (4).

Obesity is mainly characterized by a body mass index ≥ 30, a condition in which adipose cells are found in number and volume beyond normal. In addition to being an aggravating factor in the recovery of patients diagnosed by the recent COVID-19 virus (5), it also influences the development and worsening of several pathologies such as; coronary heart disease, type 2 diabetes and metabolic bone diseases (6,7).

For decades, obesity and its consequences for bone health has become one of the main problems for global health, as obesity increases, consequently osteoporosis increases over the years. The interaction between obesity and its impact on bone tissue metabolism is still controversial in the scientific community is not fully understood and, in recent studies, it is possible to find a positive relationship between obesity and bone mass (8). However, several studies show negative effects related to BMD (9,10).

We know that the development and health of bone tissue does not depend exclusively on mechanical forces, several other mechanisms directly interfere with its quality, such as, for example, the excess of adipose tissue that is interconnected with the development of osteoporosis due to its proinflammatory cytokines that stimulate the process of osteoclastogenesis and consequently bone resorption (11).

Obesity also contributes to insulin resistance by raising levels of circulating free fatty acids derived from adipocytes. These free fatty acids inhibit glucose uptake, glycogen synthesis and glycolysis. In many obese individuals, insulin resistance is offset by an increase in insulin production due to the inability of insulin properly to perform its functions (12). Type II diabetes progresses in parallel with the worsening of insulin action, since pancreatic β cells compensate

for the state of insulin resistance by increasing the secretion of this hormone, then β-cell dysfunction, an inappropriate response to glucose, occurs. which leads to the development of glucose intolerance (13). Insulin resistance occurs when the tissue has its ability to respond to the hormone reduced, leading to fasting hyperinsulinemia and hyperglycemia, and high levels of glycosylated hemoglobin (Hb1Ac).

The literature points out that insulin resistance brings complications to the body, mainly through systemic inflammation, mediated by pro-inflammatory cytokines and, through mitochondrial degeneration, in which there is an increase in the number of fat cells (12). In obese individuals, an interesting metabolic interaction occurs in bone tissue, because in addition to the alteration of its metabolism by mechanical force and pro-inflammatory cytokines, there is also the action of the fat cell itself, where further studies are needed to understand its performance as an endocrine cell on bone metabolism (14). Looking at adipose tissue and its role in bone modeling, McLaughlin et al. (2011), observed in his study that the region of accumulation of adipose tissue has a direct influence on insulin resistance, where visceral fat has been shown to increase insulin resistance by up to 80% against 48% of subcutaneous adipose tissue.

This difference occurs due to the fact that visceral fat accumulates a greater number of macrophages when compared to subcutaneous fat, this increase in the number of macrophages is closely linked to the release of TNF-α, IL-6 and IL 1. This explains the existence of "benign" and "malignant" obesity, in which insulin resistance is not seen in all individuals with high BMI. In addition to proposing that insulin resistance in individuals with an ideal BMI, it may be associated with ethnic and / or personal characteristics, where an inadequate waist-to-hip ratio is found, reflecting visceral obesity (16-18). This leads to the need for extra exams for the correct assessment of the correct ratio of muscle and fat tissue to better understand this process.

In our search for literature, it was found that adipose tissue is capable of promoting the induction of osteoclastic activity through the RANK / RANL-L and OPG pathway (Fig. 1). The fat cell and the osteoblastic cell have a mesenchymal origin in common, osteoblasts are responsible for both the recruitment and activation of osteoclastic cells through the expression of RANKL that binds to its ligand in the target (pre-osteoclast) RANK cell, this connection activates the transcription factor and stimulates the differentiation of the pre-osteoclast in osteoclast, the osteoclast in turn performs bone resorption that gives space to the new tissue that is produced by the osteoblast once (19). Also of mensenquimal origin, the adipose cell is

capable of producing pro-inflammatory cytokines that induce a greater expression of RANKL (20). Macrophages are present in adipose tissue and are capable of regulating tissue regeneration as well as controlling insulin resistance. With the increase in adipose tissue, pro-inflammatory cytokines are secret and end up in turn, attracting monocytes to the region, where transcription is activated and gives rise to macrophages, the macrophages present in regions of tissue infiltration are the main responsible for development of type 2 diabetes, in addition to being able to express TNF-α, IL-6 and IL 1-ba that stimulate osteoclastic activity (17,18).

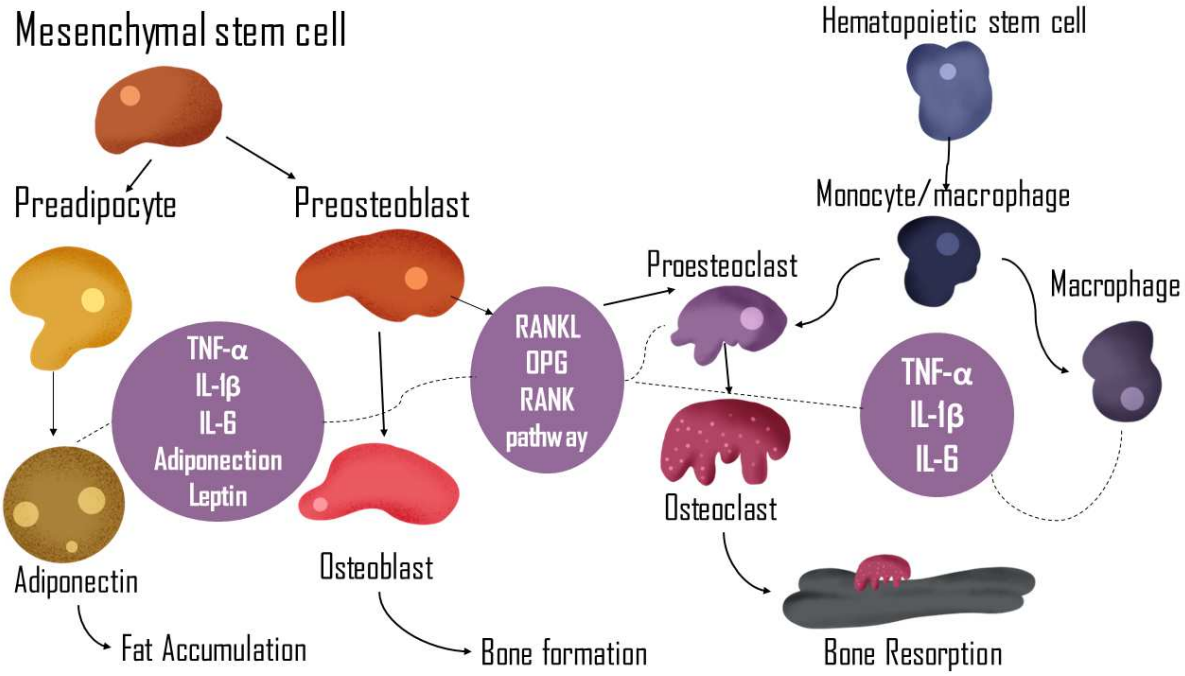

Figure (1). Fat accumulation is closely related to bone formation and resorption. Osteoblasts and adipocytes are derived from a common multipotential mesenchymal stem cell. Osteoclasts are differentiated from monocyte/macrophage precursors of hematopoietic stem cells origin. Adipocytes secrete several cytokines such as TNF-α, IL-1β, IL-6, adiponectin, and leptin which are capable of modulating osteoclastogenesis through RANKL/RANK/OPG pathway. IL, interleukin; OPG, osteoprotegerin; RANK, receptor activator of nuclear transcription factor κB; RANKL, receptor activator of nuclear transcription factor κB ligand; TNF-α, tumor necrosis factor alpha. Description by Cao, 2010.

Bone marrow is the only place in the human body where trabecular bone and fat are directly connected (21), suggesting that bone marrow adipose tissue (TAM) may be a key regulator of trabecular bone health (22). The yellow bone marrow rich in adipocytes that develops during aging and obesity, and can contribute to dysfunction of the osteogenic activity of long bones. According to recent data, the increase in TAM in obese individuals seems to be correlated with osteoporosis and increased risk of fractures (23). In the present study, our wistar

rats demonstrated a decrease in the trabecular region, which may be interconnected with the accumulation of adipose tissue in this region, inducing osteoporosis. The decrease in trabecular bone in our wistar rats was analyzed using the computerized microtomography technique, which is consistent with recent data in the literature (24). However, more studies are needed for this hypothesis to be validated.

Osteoporosis is an osteo-metabolic disorder that is characterized by the degradation of bone mass, which leads to a lower mineral density, increasing the risk of fractures (25). It is expected that there will be an increase in the number of fractures in the coming years, mainly affecting the elderly population (26). A study carried out in 2003, the World Health Organization (WHO) predicts that by the year 2050 the number of bone fractures will be around 6.56 million every year.

The BMI, despite not being the only metric to be taken into account, recent data show an important relationship between those in children, where it was more frequent in obese and overweight people. This is mainly due to the fact that children and adolescents have more fragile bones when purchased from adults, taking into account the greater presence of non-mineralized tissue (Collagen) and their growth epiphyses not yet finalized, in addition, obesity and overweight affects the entire skeletal muscle system due to the difficulty in postural stability, increasing the regional mechanical requirements, leading to structural deformities due to the high concentration of collagen in young bone tissue (28). In our study, obese wistar rats were shown to reach epiphyseal bone maturation early. This seems to have occurred due to the constant mechanical stimulus on the growth plates, forcing them to mineralize earlier. Despite reaching bone maturity, unlike humans, the long bones of Wistar rats continue to grow more slowly throughout life. In this regard, the current work is a pioneer in reporting, experimentally, the early epiphyseal maturation in obese wistar rats. Despite this growth interrupted at an early stage, we found no statistically significant difference between the size of femurs between obese and non-obese individuals. Anyway, more experimental studies are needed to better understand the mechanism of action that leads to this early mineralization, in addition to elucidating better because there was no difference between the size of the femurs.

Our results are in agreement with recent studies, where trabecular bone was lower in rats in the HFD group (29). In our study, in addition to noting the decrease in the trabecular bone and its consequent spacing in the femoral neck, the proximal and distal regions of the femur also had their trabecular region altered. As in several studies, the entire cortical region

was shown to be thicker in the HFD group, possibly due to mechanical adaptation as we mentioned earlier. Despite the visible process of the development of osteoporosis, we cannot affirm the resistance of femurs to traction force and pressure for not carrying out this type of test in our experiment, and further studies are needed to understand how much the bones became more fragile in due to obesity.

In humans, there is little research that links obesity and femoral morphometry, as well as their relationship with other systemic diseases over the long term. In our search for literature, we had no knowledge of studies that related, together with obesity, its other complications, insulin resistance and type II diabetes. When food is included, the data are not very concrete, since the creation of a control group with a controlled diet is not ethically viable in humans, being in charge of self-reported information by patients. In the present work, we demonstrated an increase in the angle of femoral inclination in our rodents. This pathological change was strongly present in obese rats, raising the hypothesis that obese rats may have acetabular dysplasia due to the change in mechanical forces in the acetabulum-femoral region. To date, we have not found studies in the literature that reported such a biomechanical change. In order for us to understand how this change in the angle of femoral inclination can influence the bone quality of the femoral head and increase the risk of dysplasia, further studies are needed.

5 CONCLUSIONS

5 CONCLUSIONS

Our study demonstrates that the adolescent-to-adult Wistar rats that consumed *ad libitum* a high fat diet containing 60% fat, 26% carbohydrate and 14% proteins spontaneously increase the caloric intake leading to high weight and gain leading to insulin resistance. In the femurs, the HFD induced to increase of cortical bone mass caused by an increase in mechanical loading and/or production of bone anabolic adipokines and/or nutritional effect of fatty acids. This was followed by a second phase characterized by decreased trabecular bone formation/remodeling resulting of development of metabolic impairment with insulin resistance and hyperinsulemy.

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APPENDIX

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION

We hereby declare that we are aware of the article *"Long-term of saturated diet induces obesity, insulin resistence and microstructoral alteratons in rats femurs"* will be included in Dissertation of the student **Luan Pereira da Macena** and can not be used in other Works of the Graduate Programs of the Faculty of Dentistry of Bauru of the University of São Paulo.

Bauru, January 21, 2021

Luan Pereira da Macena

Ever Elias Mena Laura

Ana Carolina Cestari Bighetti

Daniela Pereira Catanzaro

Tania Mary Cestari

Rumio Taga

Gerson Francisco de Assis

ANNEXS

ANNEXS

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso de Animais

CEUA-Proc. Nº 008/2019

Bauru, 25 de setembro de 2019.

Senhor Professor,

Informamos que a proposta de pesquisa intitulada ***"Obesidade e resitência à ação da insulina associado à dieta hiperlipidica e seus efeitos na microestrutura ósse femoral em ratos. Análise ao Micro-CT, histomorfometria e imunohistoquimica"***, registrada sob *CEUA-Proc. Nº 008/2019*, tendo Vossa Senhoria como Pesquisador Responsável, foi analisada e considerada APROVADA em reunião da Comissão de Ética no Uso de Animais (CEUA), realizada no dia 20 de setembro de 2019.

Finalidade	*( ) Ensino (x) Pesquisa Científica*
Vigência da autorização:	*01/10/2019 a 01/10/2020*
Espécie/Linhagem:	*Rato heterogênico / Albino Wistar (fêmures já coletados e armazenados originados do Prot. CEEPA 006/2016)*
Nº de animais:	*Não se aplica (utilização de fêmures coletados no Prot. CEEPA 006/2016)*
Peso/Idade	*200-250 / 60 dias*
Sexo:	*Macho*
Origem:	*Biotério Central da Prefeitura do Campus Ribeirão Preto-USP*

Esta CEUA solicita que ao final da pesquisa seja enviado um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Profª Drª Ana Paula Campanelli
Presidente da Comissão de Ética no Uso de Animais

Prof. Dr. Gerson Francisco de Assis
Docente do Departamento de Ciências Biológicas

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
e-mail: ceepa@fob.usp.br – Fone/FAX (0xx14) 3235-8356
http://www.fob.usp.br