Influence of high-fat diet on bone tissue: An experimental study in growing rats

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ABSTRACT

Background: The relationship between obesity and bone tissue remains contradictory, especially when the effect of high-fat diet is assessed in experimental models. The aim of this study was to evaluate the effects of high-fat diet on bone metabolism of growing rats. **Methods**: Twenty weaned female Wistar rats were equally divided into two groups: SD (standard diet) and HFD (high-fat diet with 60 % of energy as fat). After five weeks of the two diets, the rats were euthanized, and the liver, blood and bones extracted. The liver was analysed for malondialdehyde (MDA) and reduced glutathione (GSH) concentrations. Blood was analysed by the ELISA method for osteoprotegerin (OPG) and tumour necrosis factor ligand superfamily member 11 (TNFSF11/RANKL). The bone tissue was analysed for bone mineral density (BMD), mechanical strength by computed microtomography, histological quantitative analysis, scanning electron microscopy and the gene expressions of PPAR-γ Runx-2, RANKL and Cathepsin-K were also evaluated. **Results:** HFD caused an increase in the MDA concentration, indicating oxidative stress. It also increased the expression of PPAR-γ, which is the gene that is related to adipocyte differentiation. There was an increase in BMD of the tibia of animals fed with the HFD, but other microstructural and mechanical properties were maintained unaltered. In addition, there were no changes in the gene expressions related to the differentiation of osteoblasts and osteoclasts, as well as no changes to the biochemical markers of bone formation and bone resorption. **Conclusion**: Liver and gene parameters are changed in response to the HFD. However, although there was an increase in BMD, the microstructure and function of the bone did not change after a 5-week HFD.

Keywords: Bone mineral density; obesity; osteoporosis; bone quality

INTRODUCTION

A western style diet provides fast and cheap food and, today, is found in many parts of the world [1-3]. With this change in eating patterns, the incidence of obesity as of childhood has increased, and the complications resulting from this disease represent a major clinical and financial impact on national health systems [4, 5], as it affects more than 300 million people worldwide.

Despite being considered a risk factor for many chronic diseases [6], obesity has been considered beneficial for bones and may represent a protective factor against osteoporosis [4, 7, 8]. The association between obesity and bone mass has been under investigation for some time now. The alleged beneficial effects for bones are based primarily on the well-established correlation between body weight and Bone Mineral Density (BMD) [9]. The mechanical load stimulates bone formation by reducing apoptosis and increases proliferation and differentiation of osteoblasts and osteocytes [4, 6]. Although this suggests a beneficial effect of adipose tissue on bone maintenance, other more recent data argue against this effect. Some studies have shown that this alleged positive relationship is not only non-existent [10-13] but it can actually represent a negative relationship [14-16]. Therefore, the present worldwide theory is that obesity can harm the bones and often speeds up the process of bone loss. But despite all the scientific advances in the field, there are still conflicting reports in the literature concerning the relationship between high-fat diet and bone tissue because the effects of this diet have not yet been fully clarified.

The clinical functional evaluation of bone tissue does not usually explore minimal metabolic changes. However, animal models may allow a detailed review of

this system. The need for studies using growing animals is justified due to the conflicting reports on the effects of increased fat mass and body mass index related to the risk of childhood fractures. There is however, evidence that child obesity is also related to an increased risk of fracture but it is not clear why these fractures occur since BMD is frequently higher in obese children [6]. Individuals with higher peak bone mass after adolescence have advantages in bone protection when there is bone mass decline due to age. Peak bone mass is strongly influenced by genetic factors. However, often the genetic potential for bone mass is reached only if nutrition, physical activity and other lifestyle factors are optimized [17].

The incidence of childhood obesity is high. Current estimates of the World Health Organization indicate that over 42 million children are overweight or obese [18], and bone mass achieved during growth is a critical determinant for the risk of osteoporosis later in life [6, 17]. Although some studies have shown that there is some relationship between fat and bone, further bone tissue analyses are needed to fully understand this relationship. Therefore, the aim of this study was to investigate the effects of a high-fat diet on bone tissue in order to add to our current knowledge concerning the fat-mass and bone metabolism relationship.

MATERIALS AND METHODS

This study was approved by the Local Ethics Committee for Animal Experimentation, under process number 188/2013, that follows the International rules for experimentation with animals.

Experimental Design

Twenty (20) weaned (three weeks old) female Wistar rats *(Rattus Norvegicus Albinus),* were used in this study. They were kept under standard laboratory conditions (room temperature 22 ± 2 °C, 55 ± 5 % humidity and a 12 h light-dark cycle) and equally divided into two groups:

1. Standard Diet (SD): rats fed with a standard diet;
2. High-fat Diet (HFD): rats fed with a high-fat diet (60 % Kcal of saturated fat).

The SD group were fed the AIN-93G diet recommended by the American Institute of Nutrition [19]. While, the HFD group were fed a modified AIN-93G diet containing 60 % Kcal saturated fat. Food and water were provided *ad libitum* and the weekly consumption for each group was recorded. The body mass of the animals was measured weekly.

The animals in both groups were euthanized after five weeks by an intraperitoneal lethal dose of sodium thiopental (Vetec, Brazil). Livers, blood and bones (femurs and tibiae) were collected. The livers were analysed for malondialdehyde (MDA) and reduced glutathione (GSH) concentrations. The blood was analysed for osteoprotegerin (OPG) and tumour necrosis factor ligand superfamily member 11 (TNFSF11/RANKL) by the enzyme-linked immunosorbent assay (ELISA method). The tibiae were used for BMD analysis, mechanical testing, Micro-CT, scanning electron microscopy (SEM) and histological quantitative analysis. The femurs were used for gene expression analysis.

Liver Assessment Testing

The livers were analysed for MDA following the method proposed by Gerard-Monnier and colleagues in 1998 [20]. MDA concentration was calculated by comparing it to a 1,1,3,3-tetramethoxypropane (TMP) hydrolysate curve Analysis of GSH following the method proposed by Sedlack and Lindsay in 1968 [21].

Biochemical Analyses

For the bone formation evaluation, the serum levels of OPG were assessed by the enzyme-linked immunoabsorbent assay, using the Osteoprotegerin BioAssay™ ELISA kit – Rat (US Biological Life Science, USA). For the bone resorption evaluation, TNFSF11/RANKL was assessed by the enzyme-linked immunoabsorbent assay, using the RatTNSF11/RANKL/TRANCE ELISA Kit for Rat (LifeSpan BioSciences, Inc, USA).

BMD Analysis

For the BMD analysis, the bones were submerged in a plastic vessel containing saline at a depth of 2.0 cm, aligned and scanned by a dual-energy X-ray absorptiometry (DXA) with a DPX-IQ densitometer (Lunar, USA). The region of interest (ROI) was selected manually in the proximal metaphysis. The squared ROI used in the analysis had an area of 0.09 cm^2. BMD was determined using high resolution DPX software (version 4.7E, Lunar, USA) specifically developed for small animals.

Mechanical Testing

The mechanical properties of maximal load (N) and stiffness (N/mm) were determined using a low speed mechanical test with a Universal Testing Machine (EMIC DL10000, São José dos Pinhais, Brazil). The machine is equipped with a load cell of 500 N and TESC software (version 13.0, EMIC, Brazil). The three-point bending test consisted of a force applied at a speed of 1.0 mm/min in the posterior-anterior direction of the tibia. The distance between the points was 25 mm, and a pre-load of 5 N for 30 seconds was used based on the recommendations found in [22-24].

Micro-CT Analysis: Microstructure Assessment

Five bones of each group (n=5) were scanned using a SkyScan scanner 1176 (Bruker MicroCt Skycan, Kontich, Belgium) from the Graduate Dentistry Department at Ribeirão Preto University, Brazil. The proximal region of the bones was scanned at 65 kVp, and 318 μA, using a 1 mm aluminium filter and a cubic voxel of 18 μm^3. Three dimensional (3D) image reconstructions were made using NRecon software (version 1.6.9, Bruker, Kontich, Belgium) which provided axial cross-sections of the inner structures of the samples. The reconstruction of the metaphysis was selected manually, starting next to the proximal growth plate for an extension of 3.0 mm and the evaluations were performed in this region. Cortical and trabecular bone were isolated using manually drawn contouring. CTAn software (version 1.14.4, Bruker, Kontich, Belgium) was used to determine the optimal threshold from the image histograms. However, only the trabecular region was evaluated. The trabecular architecture structures were characterized by determining the trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and porosity (Po.T). All bone morphometric measurements and nomenclature were in accordance with the recommendations of the American Society of Bone and Mineral Research [25].

Scanning Electron Microscopy and Image Analysis

The same bones used for the Micro-CT were analysed by SEM. The bones were dehydrated in 99 % alcohol for 7 days. Then, they were cut in the coronal plane using a Sierra precision sectioning cutter (Isomet 1000, Lake Bluff, USA) with a diamond-coated blade. The thickness of each slice was 1.7 mm. The samples were subjected to ultrasound for 10 minutes using an Alt Sonic Clean (model 3P, Alt, Ribeirão Preto, Brazil) to remove any waste material. Subsequently, they were stored over silica gel for drying. After drying, they were sputter-coated with 24 karat gold (Coater 220 VAC, Ted

Pella, Redding, USA). Microscopic images were acquired by a scanning electron microscope (Zeiss™ Evo-MA10, Göttingen, Germany) using 200x and 500x magnification.

As far as authors know, there are no automatic or even semi-automatic methods to assess bone quality from SEM images. Briefly, due to the irregular arrangement of bone trabeculae associated with the high focusing power used in SEM, the simple two-dimensional image based assessment is not appropriate. Therefore, the quantitative analysis of the SEM images was performed using the QIMEV computer program [26], which was developed and patented by authors of this study. This program takes into account the three-dimensional information presented in the SEM images by using the grey level of the image pixels to estimate the depth of the imaged structures. Thus, it is possible to calculate the surface area expressed in pixels. The ROI analysis was standardized across all images and the dimensions of the ROI were defined as 700, 700 and 50 pixels in terms of height, width and depth, respectively.

Histological Quantitative Analysis

After dissection the bones were immersed in 10 % formaldehyde for 24 h, decalcified (0.5 M EDTA), dehydrated in a sequence of alcohols and diaphonized in xylene. After this, they were embedded in paraffin. Then, 5 μm thick serial sections of the paraffin specimens were prepared from the frontal plane using a Leica RM 2165 microtome (Leica, Houston, USA). The sections were stained following the Masson Trichrome protocol [27] and observed under an optical microscope. The histological slides were captured with a 5x objective lense using the Axio Imager Z2® optical microscope (Zeiss, Göttingen, Germany). The analysis was performed using AxioVision 4.8 software

(Zeiss, Göttingen, Germany). The trabecular bone in the proximal tibial region was recognized by its stain and quantified by bone volume per total volume [27].

Gene Expressions

After dissection the femurs were stored at -80 ° C to maintain the integrity of the RNA. The procedures were performed in an RNAase free environment. The samples were homogenized using Trizol reagent (Sigma-Aldrich, Sintra, Portugal) in a large quantity, according to the manufacturer's specifications. Total RNA was extracted from bone marrow cells with an extraction kit (Promega, São Paulo, Brazil) following the manufacturer's instructions. To generate the template for PCR amplification, 1 μg of RNA was reverse transcribed into cDNA using a high capacity RNA-to-cDNA kit (Applied Biosystems, USA). The quantitative measurement of mRNA expression was performed by RT-PCRq in real time using a Taqman® RT-PCR System (Life Technologies, São Paulo, Brazil) on a thermal cycler and detection system (Step One Plus – Applied Biosystems, Foster City, USA). Gene-specific primers and probes for PPAR-γ (NM_001145366.1), RANKL (NM_057149.1), Runx-2 (NM_001278483.1) and Cathepsin-K (NM_031560.2) were assessed using the comparative cycle threshold method, and normalized against the detected expression of the house-keeping gene, ß-actin (NM_001270548.1). The relative expression of the target gene was calculated based on the threshold cycle (Ct) and was analysed by the $2^{-\Delta\Delta Ct}$ equation [28], using:

$$\Delta Ct = Ct_{gene} - Ct_{gene\ reference}, \text{ and}$$

$$\Delta\Delta Ct = \Delta Ct_{HFD} - \Delta Ct_{SD}$$

Statistical Analysis

All data are expressed here as means ± standard deviations. All statistical analyses were performed using IBM SPSS (version 20, Armonk, NY, USA). The Shapiro-Wilk test was used to test the normality of the data. Comparisons among the groups were statistically assessed by the parametric Student's t-test and non-parametric Mann-Whitney test. The level of statistical significance was set at $p<0.05$.

RESULTS

The protocols used in our study did not suffer cause any of the complications commonly found in experiments involving surgery, physical training or drug intake. Therefore, the set of animals (n = 20) studied here was the same throughout the experiment.

Body Mass and feed intake

Figure 1 represents the animal body mass and the weekly feed intake The body mass between the two groups was similar at the beginning of the experiment (p=0.257), and after the first week of the experiment (p=0.185). However, after two weeks, the HFD group had a higher body mass than the SD group (+12.64 %, p=0.004). After three weeks, the body mass was again similar between the groups (p=0.380). After four weeks, the HFD group had a higher body mass than the SD group (+8.06 %, p=0.022). Finally, at the end of the experiment, the HFD group had a higher body mass than the SD group (+8.30 %, p=0.022). The feed intake was higher in the HFD group in the first weeks, but declined in comparison with the SD group as of the third week.

Liver Assessment

Results obtained by liver assessment are shown in Figure 2. MDA of the HFD group was higher than the SD group (+22.60 %, p=0.023). GSH concentration was similar between the two groups (p=0.962).

Biochemical Analyses

The biochemical analyses showed that there was no statistical differences between the HFD and SD groups for OPG ($p=0.505$) and RANKL ($p=0.592$), Table 1.

Bone Mineral Density Analysis

BMD of the HFD group was significantly higher than the SD group (+18.85 %, $p<0.02$), as shown in Figure 2.

Mechanical Testing

Mechanical testing showed that there was no statistical difference between the groups for maximal load ($p=0.348$) or for stiffness ($p=0.680$), Table 1.

Micro-CT

The microstructure analysis using Micro-CT showed that the trabecular microstructure was similar between the two groups. There was no statistical difference for BV/TV ($p=0.668$), Tb.Th ($p=0.802$), Tb.N ($p=0.374$), Tb.Sp ($p=0.360$) and Po.T ($p=0.668$), as shown in Figure 2. Examples of the 3D images reconstructed from the 2D micro-CT images are shown in Figure 4a.

Scanning Electron Microscopy and Image Analysis

The images obtained by SEM using 200x and 500x magnification are shown in Figure 3. Analysing the results of the quantitative analysis shown in Figure 2, one can conclude that no differences were found between the two groups for the surface area ($p=0.997$). Examples of the 3D images reconstructed from the 2D SEM images are shown in Figure 4b.

Histological Analysis

The histological analysis showed that HFD did not influence trabecular bone because there was no statistical difference between the groups (P=0.606). Figure 5 presents photomicrographs showing the histological appearance of trabecular bone in the groups as given in Table 1.

Gene Expressions

Gene expression of PPAR- γ of the HFD group was statistically higher than the SD group (+39.87 %, p=0.0277). However, there was no statistical difference between groups for the Runx-2 (p=0.952), RANKL (p=0.505) and Cathepsin-K (p=0.9867) genes. Figure 6 depicts these results.

DISCUSSION

The present study evaluated and compared the bone quality of growing rats fed with a high-fat diet and a standard diet. The results of the study show a positive influence of HFD on BMD, but without affecting bone microarchitecture and bone strength. The high-fat diet resulted in increased liver MDA concentration and increased PPAR-γ gene expression; however, there was no change in expression for the other genes evaluated, and there were no changes in any of the biochemical markers evaluated compared with the standard diet.

The skeleton has until recently been considered as an independent system with specific functions. However, with the advances in science, today it is known that this system has direct and indirect connections with other body systems, especially the endocrine and metabolic systems [29]. The relationship between fat mass and BMD was reported many years ago, but not all the mechanisms that explain this relationship are known [18].

Some authors have shown that obesity may be considered a protective factor against osteoporosis [7, 8], because body weight has positive effects on bone mass and strength [30-32]. In 1998, a retrospective study showed that bone mass and fat mass are under strong genetic regulation [30]. In addition to genetics, other factors possibly explain this relationship; first, the increase in body mass index of an individual results in an increase in mechanical overload, and mechanical stress is known that one crucial mechanism for bone formation [4]. Another explanation is that adipocytes and osteoblasts share the same precursors of mesenchymal stem cells, leading us to understand that the greater number of adipocytes increases the number of osteoblasts. A third theory is that leptin (a hormone synthesized by adipocytes) is strongly related to the increase of BMD [33-36]. However, these hypotheses have been contested [13]. A research group noted that the risk of osteoporosis and spine fractures are significantly higher in individuals with a higher fat percentage, independent of their body weight, level of physical activity and age [14]. Looker et al. showed that excessive weight gain in elderly women in the US seems unlikely to be accompanied by a significant reduction in osteoporosis [11]. In addition, BMD alone, although currently considered the strongest single factor correlated to the risk of fracture, does not define a clear cut fracture risk [9]. Thus, after many studies, the theory now adopted worldwide is that there is competition between fat cells and bone formation cells and that fat can actually harm the bones, often speeding up the process of bone loss. Such results can be found in experimental studies [37-39].

The main objective of this study, considering the high epidemic of childhood obesity [18], was to analyse bones of growing rats in order to better understand the effect of obesity on BMD during growth, which has not yet been fully explained. However, some evidences in a paediatric clinical study [9] showed an association

between obesity and fracture. Furthermore, it is necessary to understand the early physiological effects of obesity or fat mass.

The choice to use saturated fat in the fat diet was made based on the work of Wang et al. (2016). Different types of fat have different effects on bone tissue, and these authors reported deleterious effects of saturated fat on rat bones [40]. However, these authors only studied old rats. Moreover, the literature states that an unbalanced nutrition in early childhood can cause harm to bones later in life. For this reason, we chose to study the possible effects of saturated fats on bones in growing rats. Furthermore, our choice was also based on the remarkable increase of saturated fats being consumed by the global population in general.

In our study, we observed that although the BMD increased in rats fed with a HFD, this was not enough to increase mechanical strength. This may be due to no change in the bone microarchitecture. The tissue volume, trabecular number, trabecular thickness, trabecular spacing and porosity - values used for determining bone quality - were similar between the two groups. In addition, we did not observe a difference in the percentage of trabecular bone in the histological analysis. This similarity was also observed in the microstructural images obtained by SEM. Probably, the trabecular bone microstructure did not change because there were no gene and biochemical marker modifications reported in our study. In 2016, Macedo and colleagues showed the same results: an HFD influenced the bone mineral content (BMC), but did not influence the mechanical properties [41]. Clinically, similar to observations among overweight and obese adults, obese children have a higher BMD compared to eutrophic children, but that does not mean stronger bones [6].

Although some authors have shown that obese children have low bone mass for their body weight, without full compensation of BMD for excessive weight [42, 43], our findings are consistent with experimental studies found in the literature. Fied et al. (2012) evaluated male rats subjected to a HFD for 8 weeks and found no differences in the morphometric parameters of cortical bone or in the mechanical strength of the tibia subjected to a three-point bending test [44]. Bone volume/total volume % of tibia observed by histologic analysis was not influenced by the high-fat diet in some studies that used rats [41, 45].

Fisher and colleagues compared BMC and BMD in obese and normal children through a cross sectional study of case-control. They found that obese children have higher total body BMC, but there is no statistical difference in femoral and spine BMD [46]. Hasanoglu and colleagues determined the relationship of childhood obesity with BMD, showing that BMD is not affected by obesity in children [47]. A study in 2004 showed that obesity in childhood and adolescence is associated with increased vertebral bone density and increased bone dimensions [17]. However, higher BMD values for rats fed with a high-fat diet found in this study can be possibly explained by increased body weight in these animals.

The systemic effect of HFD found here was mainly related to the increased PPAR-γ gene expression. Additionally, liver assessment of the experimental groups showed higher MDA values for rats fed with HFD, which suggests increased oxidative stress in the animals. This was already expected since the fat diet has 60 % of its energy presented in the form of animal fat. Despite these changes, gene expression of bone formation and bone resorption genes showed no statistically significant differences between groups in our study. The mRNA analysis is an interpretative analysis that examines the "message sent" by the RNA. The RT-PCR results do not mean that in fact

the studied protein will be different, but indicate that there is the possibility of more or less cellular differentiation of the studied gene, which probably happened in our study. We observed this in the biochemical analyses performed, which showed no statistical differences between the groups.

Leptin is a hormone synthesized and secreted primarily by fat cells and plays an important role in regulating food intake. In obesity, leptin levels are increased [48, 49]. Mohiti-Arkedani and colleagues studied the relationship between adipocytokines (adiponectin, leptin and resistin) and BMD in patients with and without osteoporosis [50]. In the present study, we did not measure hormones secreted by adipocytes, which may represent a limitation. However, we believe that the findings concerning this hormone would be inconclusive to the observed results.

This study provides novel insights into the relationship between metabolic changes induced by HFD, especially those that are related to bone quality and function. While future studies involving hormonal analysis are needed to better understand the influence of high-fat diet on hormones related to adiposity and possible late changes in bone tissue, the results presented here suggest that, although there is an increase in BMD in response to HFD, there is no influence on bone quality and function.

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Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

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FIGURE CAPTIONS

Figure 1. Comparison between the two groups for body mass shows similar body mass at the beginning of experiment ($p>0.257$), and a higher body mass in the HFD group after the 2nd ($p=0.004$), and 4th week ($p=0.02$) and at end of experiment ($p=0.02$). The feed intake was higher in the HFD group in the first weeks, but declined in comparison with the SD group starting during the 3rd week. (*$p<0.05$)

Figure 2. Comparison between groups shows an increased MDA for the HFD group ($p=0.023$), similar GSH concentration ($p=0.962$), increased BMD for the HFD group ($p=0.02$), similar bone volume fraction ($p=0.668$), trabecular thickness ($p=0.802$), trabecular number ($p=0.364$), trabecular separation ($p=0.376$), percentage of porosity ($p=0.668$) and surface area between groups ($p=0.997$). (*$p<0.05$)

Figure 3. SEM images (200x and 500x magnification).

Figure 4. Microarchitectural images of the trabecular region in proximal tibial metaphysis. The microarchitecture was similar between the two groups.

Figure 5. Photomicrographs showing the histological appearance of trabecular bone of the experimental groups (5x magnification).

Figure 6. Relative Gene Expression (Gene/Reference Gene). Comparison among groups for PPAR- γ shows increased fat cell expression in the HFD group ($p=0.027$). (*$p<0.05$)

TABLE CAPTIONS

Table 1: Results of the biochemical analyses (OPG and RANKL), mechanical testing (maximal load and stiffness) and histological analysis (trabecular bone / total area)

TABLE

Table 1: Results of the biochemical analysis (OPG and RANKL), mechanical testing (maximal load and stiffness) and histological analysis (trabecular bone / total area).

	Analysis - Mean±Standard deviation				
	Biochemical Analysis		Mechanical Testing		Histological Analysis
Groups	OPG (ng/ml)	RANKL (ng/ml)	Maximal Load (N)	Stiffness (N/mm)	Trabecular Bone / total area (%)
SD	0.2286±0.0011	0.2484±0.0077	53.18±2.69	69.93±2.94	24.93±2.89
HFD	0.2264±0.0029	0.2412±0.0370	49.15±3.18	67.59±4.76	22.94±2.38

FIGURES

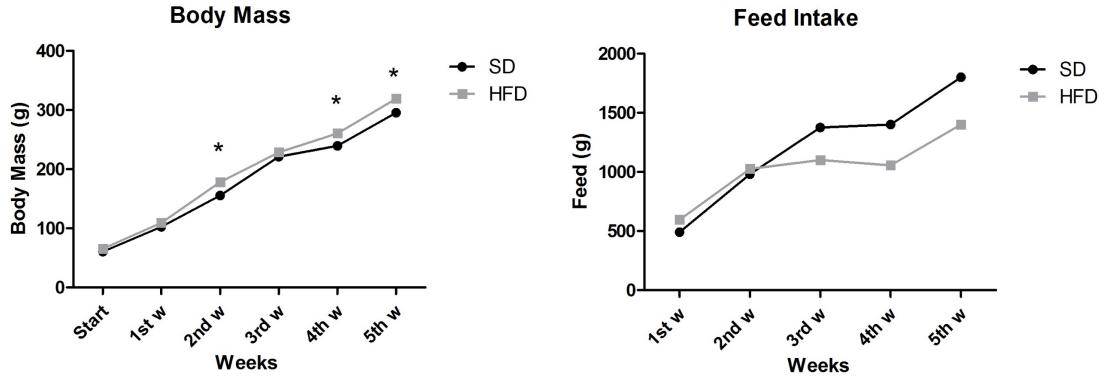


Figure 1

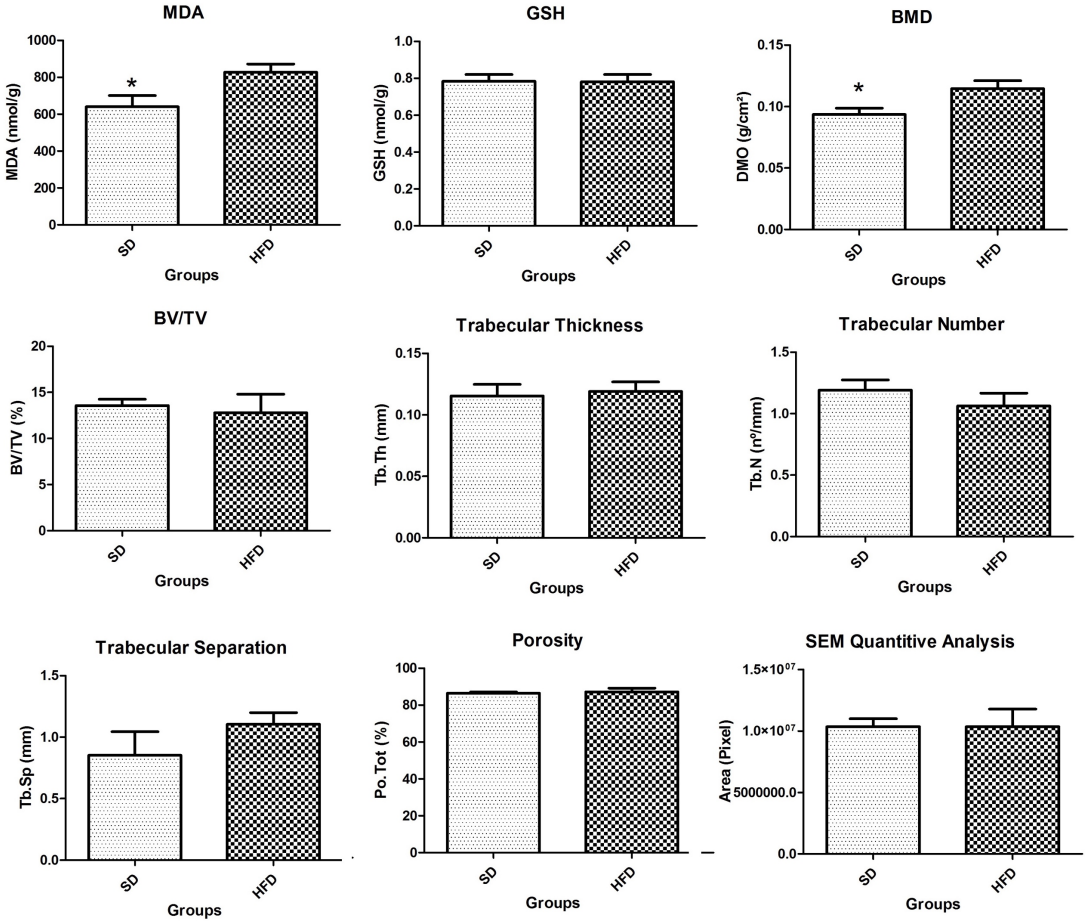
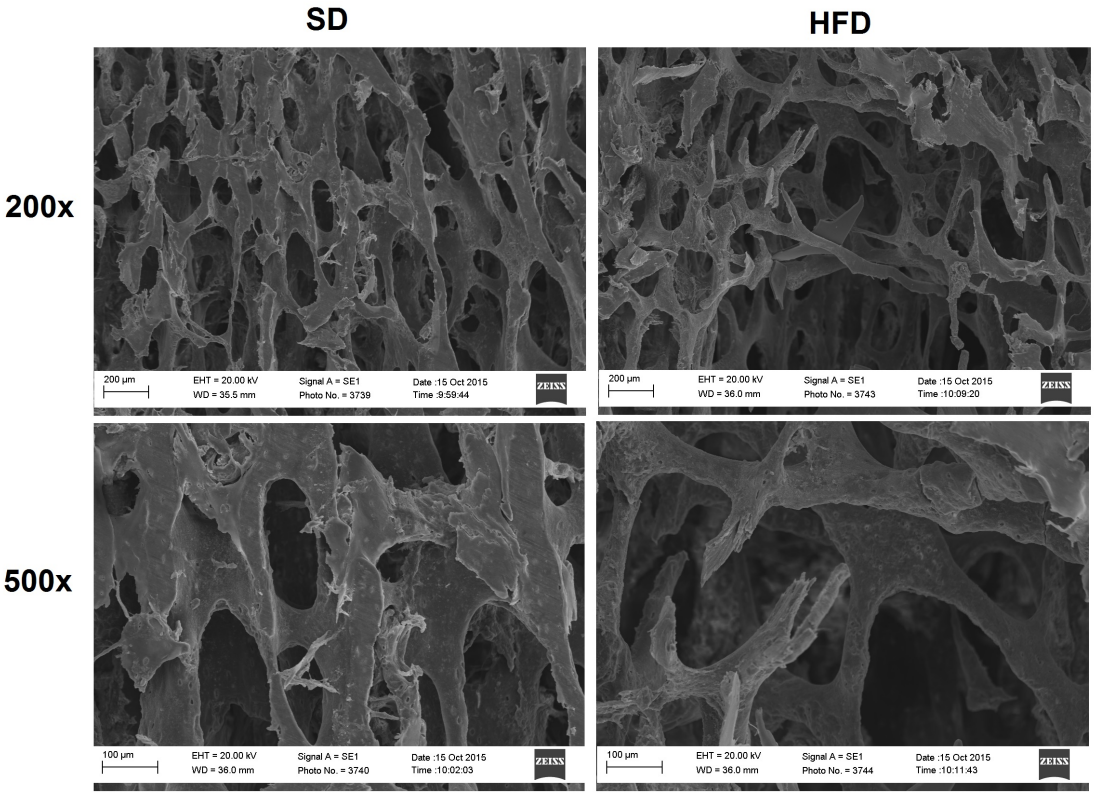


Figure 2



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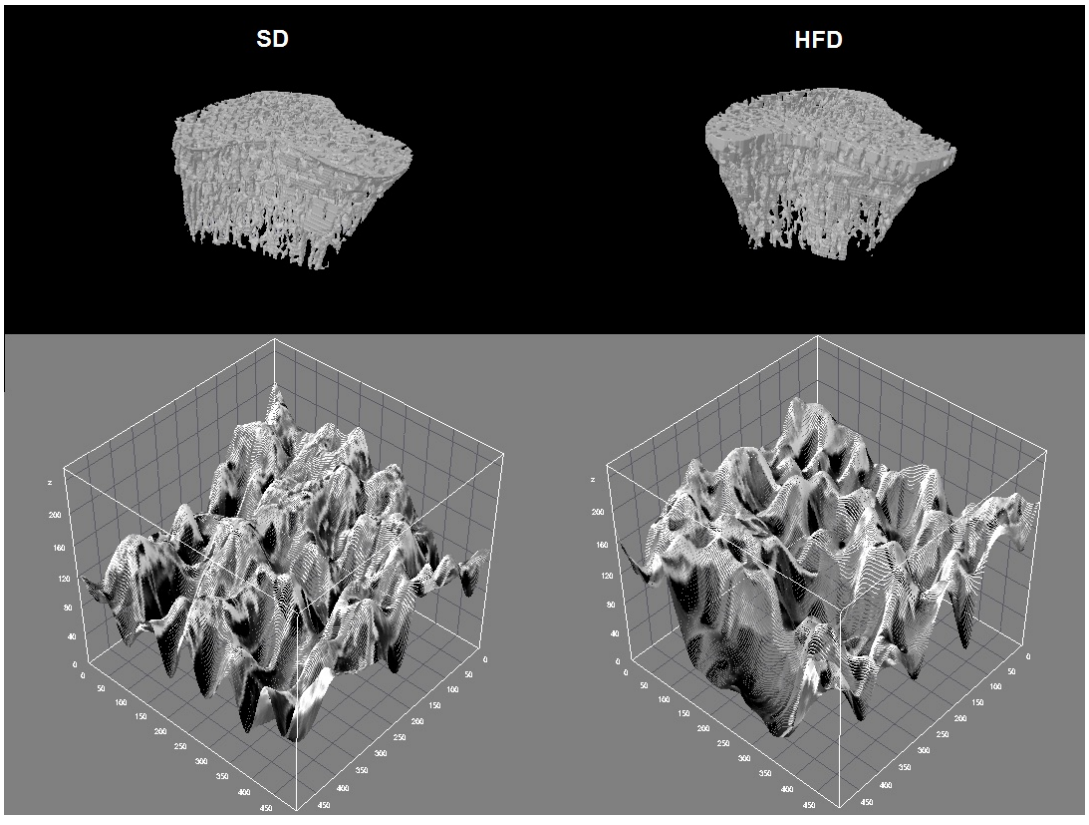

Figure 4

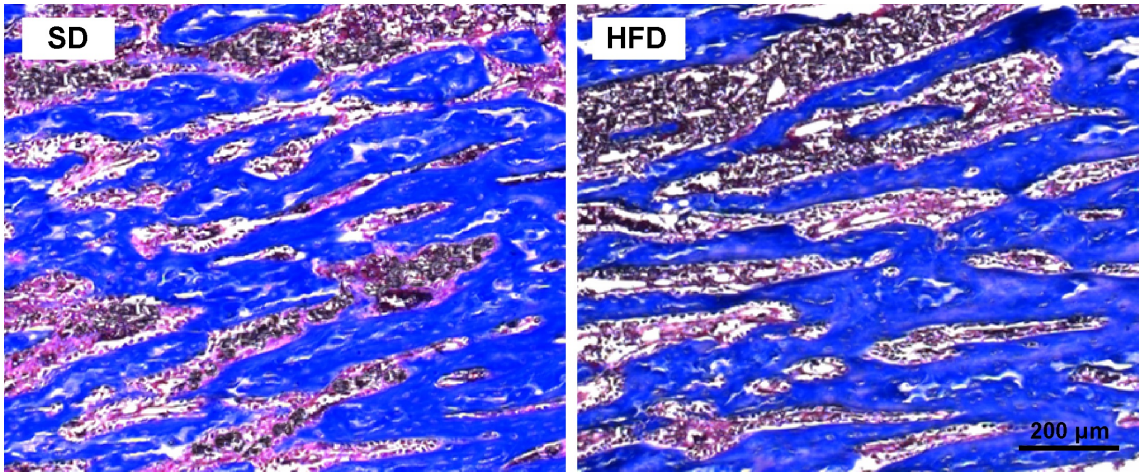

Figure 5

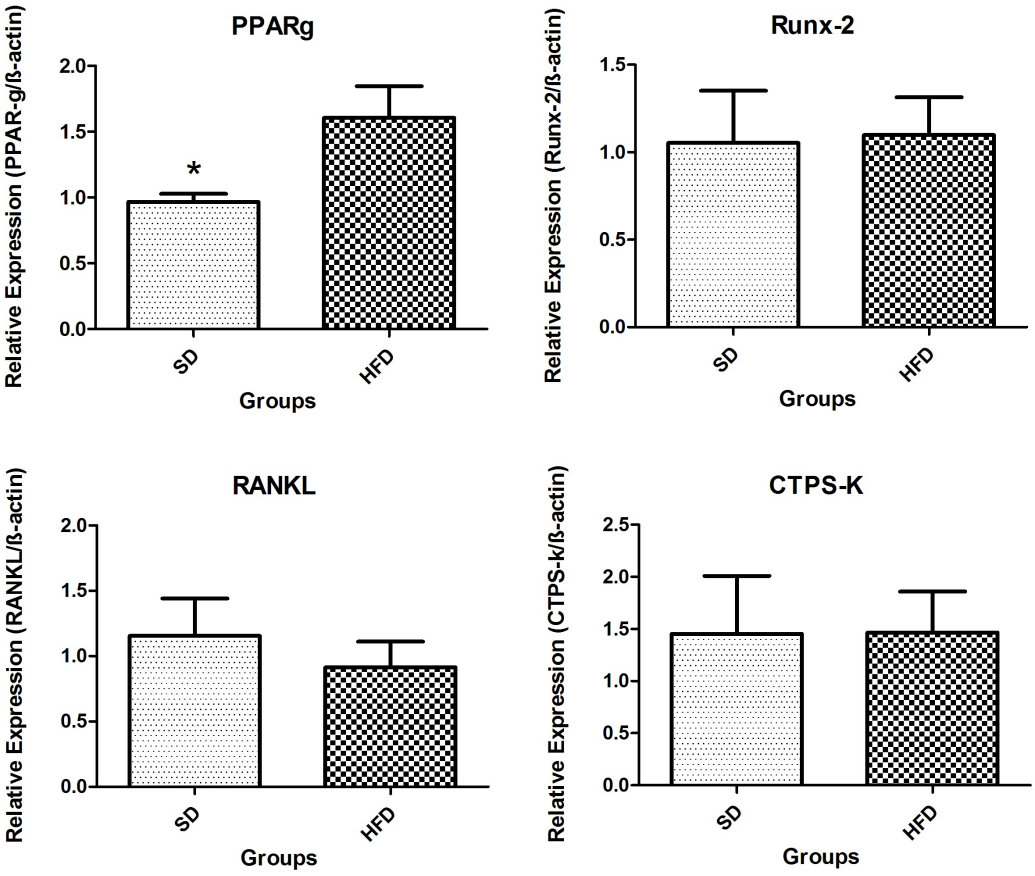

Figure 6