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High-fat diet disrupts bone remodeling by inducing local and systemic alterations

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Abstract

A high-fat (HF) diet leads to detrimental effects on alveolar bone (AB); however, the mechanisms linking adiposity to bone loss are poorly understood. This study investigated if AB resorption induced by an HF diet is associated with the regulation of inflammatory gene expression and if adipocytes can directly interfere with osteoclastogenesis. We also evaluated the effects of diet restriction (DR) on bone phenotype. C57BL6/J mice were fed normal chow or an HF diet for 12 weeks. Samples of maxillae, femur, blood and white adipose tissue were analyzed. *In vitro* co-culture of bone marrow-derived osteoclasts and mature adipocytes was carried out. The results revealed an increased number of osteoclasts and fewer osteoblasts in animals fed the HF diet, which led to the disruption of trabecular bone and horizontal AB loss. Similar effects were observed in the femur. The metabolic parameters and the deleterious effects of the HF diet on AB and the femur were reversed after DR. The HF diet modulated the expression of 30 inflammatory genes in AB such as Fam3c, InhBa, Tnfs11, Ackr2, Pxmp2 and Chil3, which are related to the inflammatory response and bone remodeling. *In vitro*, mature adipocytes produced increased levels of adipokines, and co-culture with osteoclasts resulted in augmented osteoclastogenesis. The results indicate that the mechanisms by which an HF diet affects bone involve induction of osteoclastogenesis and inflammatory gene expression. Adipokines apparently are key molecules in this process. Strategies to control diet-induced bone loss might be beneficial in patients with preexisting bone inflammatory conditions.

Key words: Bone remodeling; Obesity; High-fat diet; Alveolar bone loss; Diet restriction

1. Introduction

Adipose tissue expansion generated by a high-fat (HF) diet is known to cause several systemic disorders such as cardiovascular disease, type 2 diabetes, insulin resistance and metabolic syndrome [1–3]. However, the effects of an HF diet and obesity in bone remodeling are less defined [4–6]. While some results suggested that mechanical loading caused by excess weight has a positive impact on bone mass maintenance [7], recent studies showed that body fat accumulation increases bone resorption [5,6].

Bone remodeling disorders caused by HF consumption have been studied in different experimental models of steady-state conditions [8], periodontal disease [9], ovariectomy [10] and bone healing [11]. The majority of the reports investigated the effects of HF on femurs, tibiae and vertebrae [12–14], while few studies evaluated the impact of HF consumption on alveolar bone (AB) [8,15,16].

AB preservation is essential for tooth support. AB loss can be induced or aggravated by systemic inflammatory conditions as demonstrated by experimental [17,18] and clinical studies [19,20]. Several potential mechanisms have linked adipose tissue accumulation and AB deterioration, including (1) increased gingival oxidative stress [21], (2) adipocyte differentiation in detrimental osteoblast formation, (3) local dysbiosis favoring periodontopathogens [22], (4) local and/or systemic increase of receptor activation of NF-kappaB ligand (RankL) [23], (5) release of proinflammatory cytokines and adipokines [24], and (6) the direct influence of bone marrow-driven adipocytes on osteoclast formation via RankL cell-to-cell contact [25]. However, the pathways linking obesity/adipose tissue expansion with AB alteration have not yet been defined [26]. Furthermore, there is little evidence that osteoclast differentiation and activity can be influenced by mature adipocytes. The aims of this study were to investigate the effects of HF diet and diet restriction on AB remodeling and to explore the mechanisms by which

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adiposity negatively impacts bone quality. Herein, we hypothesized that HF intake causes AB and femur deterioration by activating local and systemic pathways that favor bone loss.

2. Materials and methods

2.1. Experimental animals and diet

C57BL6/J mice, at 6–8 weeks of age, were obtained from the animal care center of the Universidade Federal de Minas Gerais (CEBIO-UFMG) and were treated following Institutional Ethics Committee regulations (257/2014). Animals were placed on either a regular diet (n=10) consisting of 60% carbohydrate, 11% fat and 23% protein (Labina) or an HF (n=10) consisting of 37.06% carbohydrate, 41.1% fat and 21.83% protein. Mice were weighed once each week and food intake was measured twice each week. After 12 weeks, animals were euthanized and samples of maxilla, femur, blood, liver, epididymal tissue (EAT), retroperitoneal tissue (RAT), inguinal adipose tissue (IAT) and mesenteric white adipose tissue (MAT) were collected. Visceral adipose tissue samples (EAT, RAT and MAT) were weighed. The adiposity index was calculated using the following formula: [(EAT+RAT+MAT)/body weight in grams]×100 [27].

2.2. Diet restriction

Animals were fed an HF diet for 8 weeks as described above. For caloric restriction, daily food intake was calculated. During caloric restriction, each mouse from the HF diet group received 70% of its *ad libitum* consumption (70% of average food intake/average weight) for 7 (n=5) or 42 days (n=5). Mice were weighed once each week and food intake was measured daily. At the end of the experiment, samples of adipose tissue, serum, maxilla and femur were collected.

2.3. Serum parameters

Fasting triglycerides, cholesterol and glucose levels were analyzed using enzymatic kits (KATAL, Belo Horizonte, MG, Brazil). Adiponectin, resistin, leptin and chemerin serum levels were determined by ELISA (R&D Systems Europe Ltd., Abington, UK).

2.4. Micro-CT analysis

Maxillary AB and femurs were fixed in 10% neutral-buffered formalin for 48 h and scanned using a micro-CT system (Skyscan 1172 X-ray microtomography; Skyscan, Aartselaar, Belgium). Calibration was carried out with known-density calcium hydroxyapatite phantom (Skyscan). High-resolution scans with an isotropic voxel size of 18 μm were acquired (50 kV, 0.5 mm aluminum filter, 0.5° rotation angle). Scan time was approximately 30 min per bone. The scans were reconstructed using NRecon software (Skyscan, Belgium). Contouring methods were used to delineate the region of interest to be analyzed [17]. Trabecular morphometry was measured within the furcation area of the first molar root or in the metaphyseal region of proximal femurs. The contouring method on femurs was used to delineate the trabecular bone region with an irregular, anatomic region of interest drawn manually a few voxels away from the endocortical surface. The base of the growth plate was used as a standard reference point. The region of interest was defined just after the growth plate with a further 40 slices below in the direction of the diaphysis. The tissue was analyzed to determine bone mineral density (BMD; g/cm^{-3}), bone volume (BV; mm^3), percent BV/tissue volume (BV/TV; %), trabecular thickness (Tb.Th; mm), trabecular number (Tb.n; mm^{-1}) and trabecular separation (Tb.sp; mm). Alveolar bone crest (ABC) loss was measured by determining the area between the cemento-enamel junction (CEJ) and the ABC (CEJ–ACB) in three-dimensional images (Fiji—National Institutes of Health, Bethesda, MD, USA) of the first, second and third molars [28].

2.5. Histopathological analysis

Maxillae were decalcified in 14% ethylenediaminetetraacetic acid (pH 7.4) for 21 days and embedded in paraffin. The samples were cut into sagittal sections (5 μm thick). The sections were stained with Masson's trichrome, and the osteoblasts were counted on the distal side of the first molar distal–buccal root. Osteoclasts were identified as tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells and counted on the mesial side of the first molar distal–buccal root by immunohistochemistry (Santa Cruz Biotechnology Inc., Dallas, TX, USA).

2.6. Mechanical analysis

Mechanical properties of maximum load (Lmax) and stiffness (St) were determined by testing right femoral fractures in a universal testing machine (EMICs, DL 10000, Brazil) equipped with a load cell of 500 N using TESC software, version 13.4 (EMIC). The bones were tested by the three-point bending flexural test with force applied at a speed of 1.0 mm/min in the anterior–posterior direction. The gap between the two points was 8 mm, and a 2-N preload was used for 30 s [29].

2.7. RNA extraction

Total RNA was isolated from 16 samples of periodontal ligaments and surrounding AB from control (N=8) and HF diet animals (N=8) using Trizol followed by column purification (RNeasy Mini Kit; Qiagen Inc., Valencia, CA, USA). RNA was treated for complete digestion of DNA using the TURBO DNA-free Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Subsequently, 2 μg of RNA from each sample was used for the reverse transcription reaction using SuperScript VILO cDNA Synthesis Master Mix (Invitrogen Life Technologies).

2.8. Polymerase chain reaction array

The TaqMan OpenArray Mouse Inflammation panel (Applied Biosystems, Foster City, CA, USA) was used to evaluate the expression of 632 genes associated with inflammatory diseases and 16 endogenous control genes. Real-time polymerase chain reaction (PCR) reactions were conducted on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). cDNA was added to the TaqMan OpenArray Real-Time PCR Master Mix (Applied Biosystems) and distributed into 384-well plates. The samples with the master mix from the 384-well sample plates were loaded onto the OpenArray Plate using the OpenArray AccuFill System (Applied Biosystems). PCR was run on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). We conducted the analysis with the Applied Biosystems analysis software, v. 1.0, using global normalization to determine the amount of mRNA in each total RNA sample. We considered differentially expressed genes to be significantly different when P≤.05, adopting the correction of Benjamini and Hochberg for false-positive findings [30].

2.9. Adipocyte and osteoclast co-culture

Bone marrow cells (BMCs) were obtained from the femur and tibia. For osteoclast differentiation, BMCs were incubated in minimum essential medium alpha (with no nucleosides; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Carlsbad, CA, USA) and soluble macrophage colony-stimulating factor (30 ng/ml; R&D Systems, Minneapolis, MN, USA) for 3 days. RANKL (10 ng/ml; R&D Systems) was then added, as well as mature adipocytes (0.2×10^6), which were isolated from epididymal fat pads, as previously described [31]. Briefly, digestion with collagenase (1 mg/ml) was carried out at 37°C with constant shaking (140 cycles/min) for 40 min. Cells were filtered through nylon mesh and washed three times with buffer plus 1% bovine fatty acid-free serum albumin. A concentration of 4.4×10^6 adipocytes was obtained. Adipocytes were incubated with osteoclasts (2×10^4 cells/well) for 20 h. At the end of the incubation period, an aliquot of the infranatant was removed for adipokine and cytokine quantification. The medium was replaced and osteoclast cell culture continued for 3 days without adipocytes (Fig. 6A). Cells were then fixed with acetone, citrate and formaldehyde 37% and stained with TRAP (Sigma-Aldrich) after 7 days of total cell culture. TRAP-positive cells were counted and their areas were measured (Fiji—National Institutes of Health). To analyze the possible modifications of osteoclast function in the presence of adipocytes, the cells were also seeded in an osteoassay surface 96-well plate (Sigma-Aldrich) for a resorption pit assay. The same protocol described above was carried out. To analyze the surface of the pit formation, the media was aspirated from wells after 10 days of osteoclast differentiation, and 100 μl of 10% bleach solution was added. Cells were then incubated with a bleach solution for 5 min at room temperature. The wells were washed twice with distilled water and allowed to dry at room temperature overnight. The pits were observed and photographed under a microscope with 100× magnification. The pit areas were determined using contouring tools in Fiji software (Fiji—National Institutes of Health).

2.10. Statistical analyses

Data are presented as the mean±standard error of the mean. Statistical analyses were performed using Student's t test or one-way analysis of variance followed by Newman–Keuls posttest for multiple comparisons. P<.05 was considered statistically significant.

3. Results

3.1. HF diet affects metabolic parameters and adipokine release

To explore the mechanisms by which an HF diet could affect bone metabolism, we fed male C57BL6/J mice with regular chow or HF diet. As reported previously [27], mice fed with an HF diet gained significantly more body weight than control mice after 12 weeks (Fig. 1A). HF animals also presented increased food intake (kcal/d) compared with control group (Fig. 1B). Animals receiving the HF diet exhibited a considerable increase in the adiposity index and cholesterol levels (Fig. 1C, E). Glucose and triglyceride levels were similar for both groups (Fig. 1D, F). Serum levels of leptin increased significantly (Fig. 1I), whereas adiponectin and resistin levels were reduced in the HF diet group (Fig. 1G, H). No significant difference was observed in serum levels of chemerin between the groups (Fig. 1J).