

A high-fat diet induces obesity and impairs bone acquisition in young male mice

XIAO-MEI LU¹, HONG ZHAO² and EN-HUA WANG²

¹Department of Pathophysiology, College of Basic Medicine; ²Institute of Pathology and Pathophysiology, China Medical University, Shenyang, Liaoning 110001, P.R. China

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Abstract. The postnatal development of obesity is highly associated with the excessive consumption of a high-calorie, high-fat diet (HFD). However, the correlation between HFD-induced pediatric obesity and skeletal development remains to be elucidated. In the present study, postnatal day 17 (PND17) mice were weaned on a HFD for eight weeks *ad libitum* to induce obesity. The HFD mice showed a significant increase in the total body weight and gonadal and abdominal fat mass compared with the control animals. Peripheral quantitative (pQ) CT scans of the tibial bone revealed that the bone mineral density (BMD), including the total, trabecular and cortical BMD, was unchanged between the HFD and control diet groups, but that it was inversely associated with body fat. By contrast, the bone mineral content (BMC) and trabecular area were significantly decreased in the HFD group compared with the control. RNA and protein were isolated from the femur. qPCR and western blot analyses showed a significant downregulation in the gene expression of the key canonical Wnt signaling molecule β -catenin, the osteoblastic cell differentiation marker Runt-related transcription factor 2 (Runx2) and also in the β -catenin gene encoded protein levels of the HFD mice when compared with the controls. Consistent with the increased fat mass in the HFD-induced obese animals, the expression of the adipogenic genes and aP2 was increased compared with the controls. Bone marrow cells were aspirated and the *ex vivo* bone marrow cell cultures showed that the number of colony-forming unit osteoblasts (CFU-OBs) per bone was significantly decreased in the samples from the HFD mice compared with those from the controls. These observations suggested that HFD-induced obesity in growing animals may affect the total available osteoblastic cell differentiation progenitors in the bone, while increasing adipogenesis. This

may result in negative consequences for the bone later on in adult life.

Introduction

The prevalence of obesity or being overweight is on the increase, particularly in Western countries (1). In children, the prevalence of obesity or being overweight has nearly doubled in the last 2 decades (2). In total, 31% of children aged 6-19 years are either overweight or at risk of being overweight (NHANES, 1999-2002) (3). It has been well documented that obesity is a risk factor for the onset of metabolic disorders, including type 2 diabetes, cardiovascular disease, osteoporosis and numerous other chronic diseases (4,5). Clinically, significant health care costs are generated as a result of obesity-related bone fractures. With burgeoning medical, social and health care costs, obesity is rapidly becoming the Western world's number one health problem.

However, when compared with previously published data, when genetic factors were precluded, the incidence of hip fractures was much lower in countries such as China, where less high-fat diets (HFDs) are consumed compared with Western countries which consume HFDs on a regular basis (6). Although there is no published statistical data, the fact that HFDs are increasingly consumed by children in China has recently become a cause of public alarm. A substantial number of children are estimated to be becoming obese in China and therefore the incidence of metabolic syndromes associated with obesity or being overweight is expected to grow more rapidly in the country.

With regard to obesity, low bone mass osteopenia or osteoporosis is considered another disease with an altered body composition. Osteoporosis and obesity have multifactorial etiologies, including genetic and environmental factors, with potential interaction between these two factors. Earlier epidemiological data showed that a high body weight or BMI was correlated with a high bone mass and that reductions in body weight may cause bone loss (7). The basic mechanism underlying this correlation remains unclear, although several explanations have been proposed. It is generally accepted that a larger body mass imposes greater mechanical loading on the bone and that the bone mass increases to accommodate this greater load. Body weight and lean body mass are strong determinants of bone mass that reflect the adaptations of skeletal

Correspondence to: Professor Hong Zhao, Institute of Pathology and Pathophysiology, College of Basic Medicine, China Medical University, No. 92, Beier Road, Heping, Shenyang, Liaoning 110001, P.R. China
E-mail: zhaohong1966@hotmail.com

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modeling to loading (8). However, clinically, obese individuals usually perform fewer physical activities and the bone mass is therefore not accommodated. Zhao *et al* previously demonstrated that body fat mass is negatively correlated with bone mass when the mechanical loading effect of body weight is statistically removed (9). It has consistently been observed that if the confounding factor of body weight is adjusted, a strong but inverse association between percent fat mass and bone mass is evident (10). Moreover, children who have increased body fat or are obese have a significantly increased fracture risk (11). The exact correlation and mechanisms behind obesity and bone acquisition and the increased fracture risk in obese children remain poorly understood.

Bone marrow surrounds the trabecular elements of the skeleton and is composed of pluripotent stromal cells. Stromal cells are regulated by a number of factors. When osteoblast differentiation signals, including Runt-related transcription factor 2 (Runx2) and Wnt/ β -catenin, are activated, stromal cells enter into the osteoblast lineage (12). By contrast, entry of the stromal cells into the adipocyte lineage occurs through activation of the nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR γ). Since bone and fat cells share a common origin, a switching mechanism in the mesenchymal stromal cells may explain a few of the previous observations in which factors enhanced adipogenesis at the expense of osteoblast differentiation (13). It is not known how dietary-induced obesity affects this process or osteoblast and adipocyte differentiation.

In the present study, a HFD-induced pediatric obesity mouse model was utilized to show that a reduced bone quality occurs in HFD mice. The model also showed that HFD-induced obesity may favor the activation of adipogenic genes, but suppress osteoblastic cell differentiation. *Ex vivo* bone marrow cell cultures showed that the number of colony-forming unit osteoblasts (CFU-OBs) per bone was significantly reduced in the samples from the HFD mice compared with those from the controls. These observations suggested that HFD-induced obesity in growing animals may affect the total available osteoblastic cell differentiation progenitors in the bone, while increasing adipogenesis. This may result in negative consequences for the bone later on in life.

Materials and methods

Animal experiments. Male C57BL mice (23–24 g) were provided by the animal center of China Medical University, Shenyang, China. Mice (n=10/group) were randomly assigned to one of two diets containing either a high-fat (45% total calories, HFD) or low-fat (14%, chow diet control) mixture. The diet of the HFD group contained 25% protein, 45% fat (corn oil) and 30% carbohydrate and was fed at 375 kcal/kg^{3/4} days. The control low-fat chow diet group was fed *ad libitum* with the standard AIN-93G diet made with casein as the sole protein source and 14% fat (14). The body weights were monitored for 8 weeks. At the completion of the experiment the mice were anesthetized with an injection of 100 mg Nembutal/kg body weight, followed by decapitation and collection of the serum, left tibia, femur and gonadal and abdominal fat. Peripheral quantitative computerized tomography (pQCT) was performed on the formalin-fixed left tibia for the bone mineral density

measurement using a previously well-established method (15). Animal procedures were approved by the Animal Care and Use Committee of the China Medical University.

Bone pQCT scanning and three-point bending. The body compositions of the mice were evaluated according to a previously published method (15). Indices of the percent fat mass, including abdominal and gonadal fat, were derived using this procedure. pQCT scans were performed on individual bones (left tibia) from each mouse. The scanning was performed with a XCT unit (XCT Research SA, Stratec Biomedical Systems, Birkenfeld, Germany) specifically configured for small bone specimens. Software version 5.4 was used and the threshold of 570 mg/cm³ was used to distinguish the cortical bone, while 214 mg/cm³ was used to distinguish the trabecular from the cortical and sub-cortical bone. *Ex vivo* pQCT analysis was conducted on each bone. The tibial bone mineral density (BMD) and bone mineral content (BMC) were automatically calculated by the software and color images were generated. The coefficient of variation (CV) for these measurements were <2%. The position for the pQCT scan was defined as the distance from the proximal tibia growth plate corresponding to ~7% of the total length of the tibia. The distance between each scan was 1 mm and a total of 5 scans (5 slices) were carried out. Three-point bending of the left femur was performed at room temperature using a miniature bending apparatus with the posterior femoral surface lying on the lower supports (7 mm apart) and the left support immediately proximal to the distal condyles. A load was applied to the anterior femoral surface by an actuator midway between the two supports and moving at a constant rate of 3 mm/min to produce a physiological *in vivo* strain rate of 1% to mimic the average mouse femur. The mechanical properties, including ultimate strength/stress and stiffness, were recorded with a digital caliper.

RNA isolation and real-time PCR array. The mouse femurs were harvested, followed by removal of the marrow cells by aspiration according to the methods previously described (16). RNA from the femur tissue was extracted using TRIzol reagent (MRC Inc., Cincinnati, OH, USA) according to the manufacturer's instructions, followed by DNase digestion and column cleanup using Qiagen mini columns (Qiagen GmbH, Hilden, Germany) using a previously described procedure (16). Reverse transcription (RT) was performed using an iScript cDNA Synthesis kit purchased from Bio-Rad (Hercules, CA, USA). Primers for the real-time PCR analysis used in the present study were designed using Primer Express software 2.0.0 (Applied Biosystems, Foster City, CA, USA) and are listed in Table I.

Cell cultures. The bone marrow cells were aspirated and harvested from each mouse femur at the end of each experiment as previously described (17). For quantification of the CFU-OBs, the cells were seeded in six-well cell culture plates at a density of 1.5x10⁶ cells per well. The cell cultures were maintained in the presence of minimal essential medium (Invitrogen, Calsbad, CA, USA) with 15% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), 1 mM ascorbyl-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 4 mM L-glutamine and 100 U/ml each of penicillin and streptomycin (Sigma-Aldrich).

Table I. Real-time reverse transcription polymerase chain reaction (RT-PCR) primer sequences.

Gene	Forward primer	Reverse primer
Runx2	CCGTGGCCTTCAAGGTTGTA	ATTTCGTAGCTCGGCAGAGTAGTT
aP2	CCAAGCCCAACTTGATCATCAG	TGGTCGACTTTCCATCCCCT
PPAR γ	GCTTCCACTATGGAGTTCATGCT	CCGGCAGTTAAGATCACACCTAT
β -catenin	GATATTGACGGGCAGTATGCAA	AACTGCGTGGATGGGATCTG
GAPDH	GTATGACTCCACTCACGGCAA	GGTCTCGCTCCTGGAAGATG

PPAR- γ , peroxisome proliferator-activated receptor- γ ; Runx2, Runt-related transcription factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

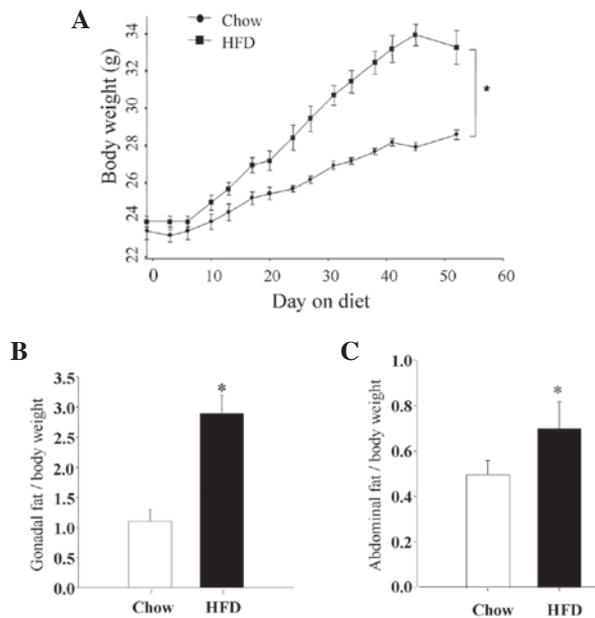


Figure 1. Body weight and composition in HFD male mice. (A) Growth curves from the two diet groups. (B) Visceral (gonadal fat) and (C) abdominal fat composition from the two diet groups presented with their relative values to body weight. The values are presented as the mean \pm SEM (n=9). Chow, chow diet group; HFD, high-fat diet group. *P<0.05, HFD versus Chow.

The cell cultures were stopped at day 12 and then the cells were fixed and stained with alkaline phosphatase for the CFU-OBs.

Western blot analysis. The tibia bone tissue proteins were extracted using a cell lysate buffer as previously described (18). β -catenin, PPAR γ and β -actin expression in the bone tissue was assessed by western immunoblotting using goat polyclonal antibodies recognizing β -catenin (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal antibodies recognizing PPAR γ (Cell Signaling Technology) and mouse polyclonal antibodies recognizing β -actin (Sigma-Aldrich Co., Oakville, ON, Canada), followed by incubation with either an anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase (Santa Cruz Inc., Santa Cruz, CA, USA). A SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) was used for developing the blots. Quantification of the intensity of the bands in the autoradiograms was performed using a ChemiDoc XRS imaging system (Bio-Rad).

Statistical analysis. Data were presented as the mean \pm standard error. A one-way or two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc analysis was used to compare the treatment groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Body weight and body fat mass in the HFD-induced obese mice. To examine the effect of diet-induced obesity on bone, the majority of the previously studied *ad libitum* diet-based animal models of obesity either focused on adults or utilized long-term (3- to 4-month) diets started following puberty. In the present study, postnatal day 17 (PND17) mice were weaned on a HFD or control diet for only 8 weeks. During the period of the experiment, a diet with 45% fat was provided to the HFD group every day to induce obesity. The chow diet group was defined as the control group. As the data show in Fig. 1A, the mean body weight gains were significantly higher after 2 weeks and thereafter in the HFD animals. At sacrifice, the mean body weight in the HFD group was 33.6 ± 0.6 g versus 28.3 ± 0.5 g in the control animals (P<0.01). This was accompanied by a significantly increased retroperitoneal adipose tissue mass (percent body weight; gonadal adipose) of 2.9 ± 0.4 g in the HFD group versus 1.1 ± 0.3 g in the control diet animals (Fig. 1B; P<0.01). Similarly, the mean abdominal fat mass in the HFD group was 0.78 ± 0.02 g versus 0.5 ± 0.01 g in the control diet animals (P<0.01; Fig. 1C). These data clearly indicated that obesity was induced in the young mice by feeding them *ad libitum* with a diet containing 45% fat for 8 weeks.

Bone quality. To obtain a better idea of the bone quality, pQCT was first used to measure the tibial BMD and BMC in the two groups. The total, trabecular and cortical BMDs showed no significant changes between the two groups (Fig. 2A-C). However, other parameters from the pQCT measurement, including the total and trabecular BMCs and the total bone and trabecular areas were all significantly decreased (P<0.05) in the HFD group compared with the control group (Fig. 2G-J). Notably, when the BMD was normalized by body weight, the total, trabecular and cortical BMDs were significantly lower in the HFD mice compared with those from the control mice (Fig. 2D-F). Moreover, in the HFD group, the trabecular BMD in particular was inversely (negatively) correlated with the observed retroperitoneal/gonadal fat accumulation (Fig. 2K),

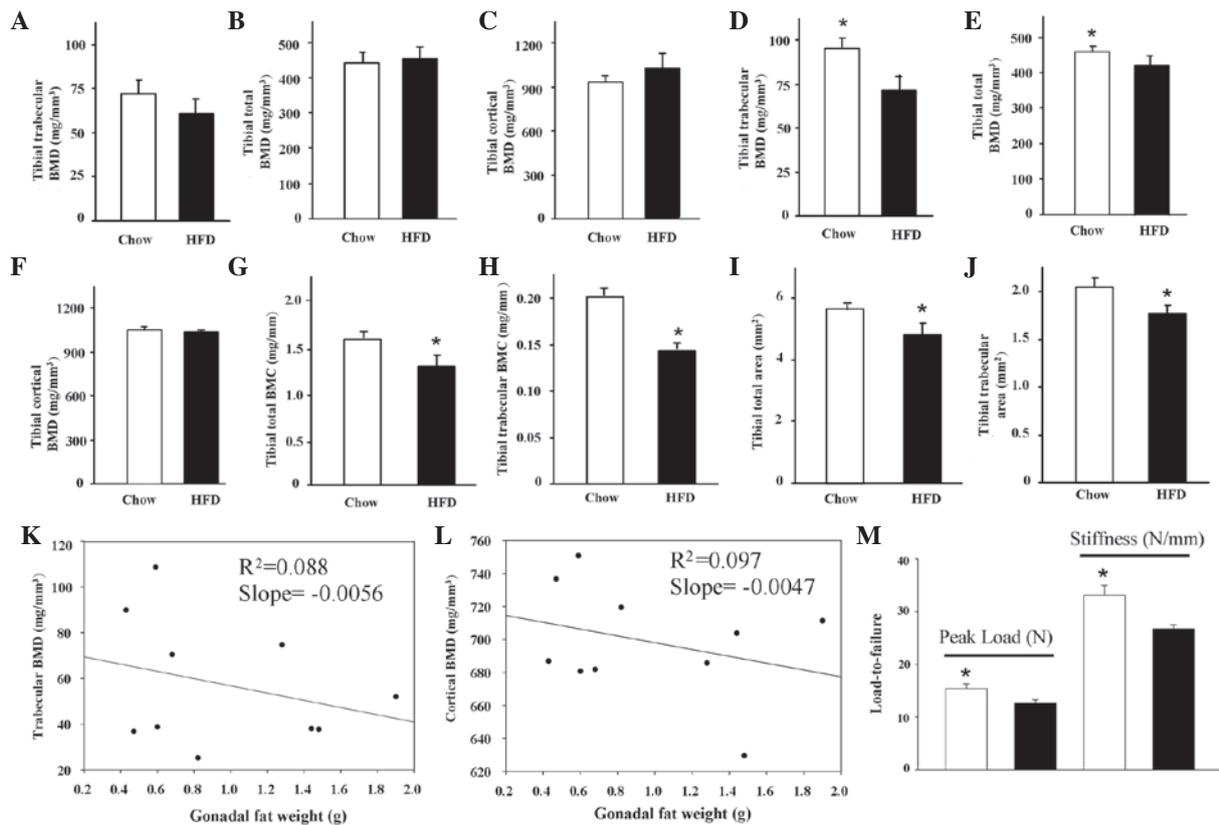


Figure 2. Bone quality in the HFD-induced mice. Peripheral quantitative CT scan (pQCT) for (A) tibial trabecular bone mineral density (BMD), (B) total tibial BMD and (C) tibial cortical BMD without weight normalization, pQCT for (D) tibial trabecular BMD, (E) total tibial BMD and (F) tibial cortical BMD following body weight normalization. pQCT parameters of (G) tibial total bone mineral content (BMC), (H) tibial trabecular BMC, (I) tibial total bone section area and (J) tibial trabecular bone area, without body weight normalization. Regression between (K) trabecular BMD and gonadal fat weight and (L) cortical BMD and gonadal fat weight in the HFD mice. (M) Three-point bending analysis, $n=9$. Chow, chow diet group; HFD, high-fat diet group. * $P<0.05$, HFD versus Chow.

while the correlation of the cortical BMD with the fat mass was not clearly observed (Fig. 2L). Most significantly, a femur bone fracture (bone strength) test was carried out using a three-point bending analysis. The peak load and stiffness were significantly lower in the HFD group compared with the control group ($P<0.05$; Fig. 2M). These data indicated for the first time that there is an inverse correlation between fat and bone mass in a short-term HFD-induced growing obese mouse model.

Changes in the expression of osteogenic and adipogenic markers in the bone.

A previous study in rats have suggested that HFD-induced obesity may favor adipogenesis whereas bone formation may be reduced (19). To determine whether obesity affected the balance between osteoblastogenesis and adipogenesis in the bone in the present mouse model, RNA and protein were isolated from the bone following aspiration of the bone marrow cells. The expression of the markers for osteoblastogenesis and adipogenesis were then measured. Consistent with the increased adiposity in the HFD mice, real-time RT-PCR analysis of the mRNA levels of the adipocyte-specific genes PPAR γ and aP2 were significantly increased in the bone (Fig. 3C and D). By contrast, the mRNA levels of the bone-forming gene β -catenin and the osteoblast differentiation transcription factor Runx2 were significantly lower in the HFD mice compared with the controls (Fig. 3A and B; $P<0.05$). The protein expression of β -catenin and PPAR γ

in the bone was further confirmed by western blotting (Fig. 3E and F). Consistent with the mRNA expression, the β -catenin protein expression was significantly lower, but the PPAR γ protein expression was significantly higher in the HFD mice compared with the control animals (Fig. 3E and F). These data suggest that increased adipogenesis but decreased osteoblast differentiation in the bone occurred in the HFD obese mice. The bone marrow cells were then aspirated and 1.5×10^6 cells per well were cultured *ex vivo* in 6 well plates. After 14 days of culture in the presence of an osteoblast differentiation medium, alkaline phosphatase activity staining was performed. There were no differences in the number of CFU-OBs between the HFD obese mice and the controls (Fig. 4A). However, the mean number of isolated total bone marrow cells per femur was significantly lower in the HFD obese mice compared with that in the control animals ($20.8 \pm 1.2 \times 10^6$ versus $30.0 \pm 1.0 \times 10^6$, respectively; $P<0.05$). Therefore, the recalculated CFU-OBs per femur were significantly reduced in the HFD obese mice compared with their controls (Fig. 4B), indicating that there were lower numbers of available osteoblast progenitor cells in bone marrow of the HFD mice compared with their controls.

Discussion

Chronic overconsumption of a HFD with fewer physical activities is well known to cause obesity. However, the effects

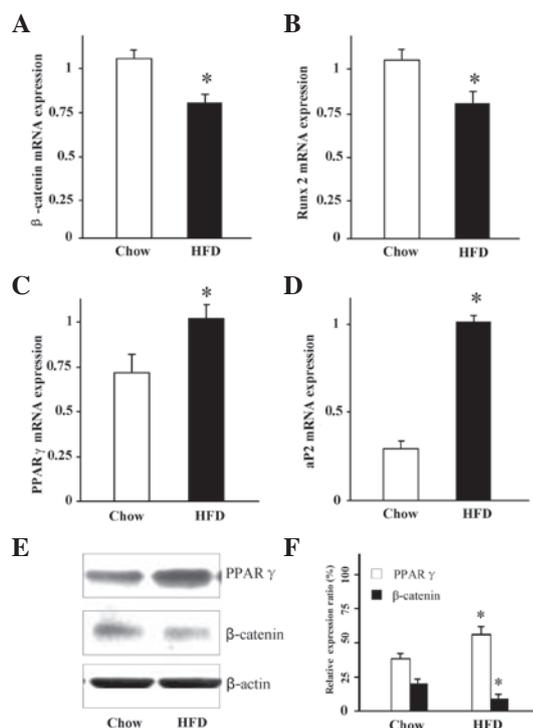


Figure 3. High-fat diet reciprocally regulating osteoblast and adipocyte differentiation-associated gene expression in mice. RNA was extracted from the femur and real-time PCR was performed. Relative (A) β -catenin, (B) Runx2, (C) PPAR γ and (D) aP2 mRNA expression. n=9. Western blot analysis for (E) PPAR γ and (F) β -catenin, n=5. Chow, chow diet group; HFD, high-fat diet group. *P<0.05, Chow versus HFD. PPAR- γ , peroxisome proliferator-activated receptor- γ ; Runx2, Runt-related transcription factor 2.

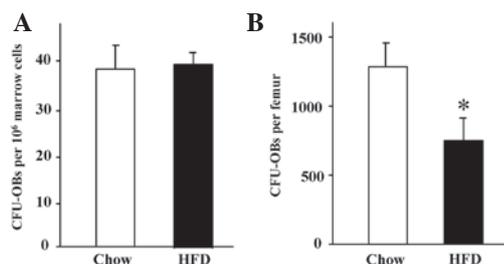


Figure 4. CFU-OB numbers in mice fed a HFD or chow diet (n=9). After eight weeks, marrow cells were obtained from their femurs for the determination of the number of CFU-OBs. Cells from individual animals was analyzed separately. The mean marrow cell yield was $30.0 \pm 1.0 \times 10^6$ cells per femur for the chow diet animals and $20.8 \pm 1.2 \times 10^6$ cells per femur for the HFD animals. Data shown are the mean (\pm SEM) number of progenitors. (A) CFU-OB numbers per 10⁶ marrow cells. (B) Total CFU-OB numbers per femur. Chow, chow diet group; HFD, high-fat diet group. *P<0.05, HFD versus Chow. CFU-OB, colony-forming unit osteoblast.

of such HFD-induced obesity on postnatal and pre-natal bone development are not well studied. The present study demonstrated that feeding mice a high-fat *ad libitum* diet for eight weeks starting from PND17 induced obesity. The HFD-induced adiposity was inversely associated with the BMD and BMC. A mechanical loading test indicated that there may be a significantly increased fracture rate in HFD-induced obese animals compared with the control diet animals. This is consistent with recent clinical data that showed there was an increased fracture risk in obese children and adolescents (4)

and a higher prevalence of obesity among diagnosed osteoporotic patients (20). Of note, the present study suggests that HFD-induced obesity in growing animals may affect the total available osteoblastic cell differentiation progenitors in bone, while increasing adipogenesis. These data provided a potential mechanistic explanation for the imbalance between adipogenesis and osteogenesis in HFD-induced obesity and further suggested HFD-induced obesity in the negative consequences that may arise in the bone later on in adult life.

It has generally been accepted that postnatal body composition is affected by diet intake, intrinsic hormonal milieu and physical activities, although genetics or pre-natal uterine conditions may also be significant. Obesity or being overweight was considered to be correlated with an increased bone mass based upon observation of the correlations between BMD and weight and body mass index, particularly at weight-bearing sites (21). The majority of these previous analyses on BMD did not consider the mechanical loading of the total body weight as one of the confounding factors, therefore, the true association between obesity and bone development is not well understood. The majority of more recent studies have emphasized a negative association between obesity and bone development or quality (22) if the confounding factors are assumed to have been removed. This observation is consistent with the data shown in the present study, which indicated that the BMD normalized by body weight was significantly lower in the HFD obese mice compared with their controls, however, the difference was not clear when the actual BMD was compared. Moreover, in the present diet-induced mouse model, the BMD was observed and confirmed to be negatively correlated with the visceral fat mass, particularly when using the trabecular BMD. Although there was no difference in the BMD without weight normalization between the groups, the mechanical loading test showed significantly decreased long bone strength in the HFD animals compared with their controls, which further indicated a poor bone quality in the HFD obese animals. Although it has been reported that rats fed on high-vegetable oil diets showed no significant effects in their bone parameters, a diet which was rich in saturated fatty acids had decreased digestibility and adversely affected energy and bone metabolism in growing, healthy male rats (23). Such a saturated fatty acid diet was used in the present study.

We particularly believe that data of this study on markers for osteoblastogenesis and adipogenesis, support our knowledge and understanding of the changes in the signaling mechanisms that lead to increased adipocyte differentiation, but also to decreased osteoblast differentiation of the mesenchymal stromal cells in the bone, as occurs in diet-induced pediatric obesity. It is therefore possible that shifting the differentiation program from osteogenesis to favor adipogenesis may lead to a deficit in bone formation. This is fully supported by previously observed clinical data, which indicated that populations with more body fat may have a lower bone mass (24) and may result in fractures more easily later in life. It also agreed with the data from studies in rodents which reported that a low-carbohydrate/HFD may result in significantly more visceral and bone marrow fat, induce a lower BMD and reduce bone formation (25,15). The present study indicated that PPAR γ and Wnt/ β -catenin were expressed in opposite directions in the bone from obese animals. The causal

mechanism leading to the increased expression of PPAR γ but decreased expression of β -catenin remains unclear. Further molecular mechanisms responsible for the downregulation of β -catenin but upregulation of PPAR γ in the bone from obese subjects remain to be elucidated.

In summary, the present study demonstrated that an *ad libitum* HFD resulted in obesity in mice and was correlated with a lower bone quality compared with the control diet group. The impaired bone growth in the HFD-induced obese mice may have been due to an altered stromal cell differentiation potential towards either the adipocytes or osteoblasts. In addition, an *ex vivo* bone marrow cell culture showed that the number of CFU-OBs per bone was significantly lower in the samples from the HFD mice compared with the controls. The results suggested that HFD-induced obesity in growing mice may affect the total available osteoblastic cell differentiation progenitors in the bone, while increasing adipogenesis. This may result in negative consequences for the bone later on in adult life.

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