

## ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Issue: *Skeletal Biology and Medicine***Diet-induced obesity alters bone remodeling leading to decreased femoral trabecular bone mass in mice**Jay J. Cao,<sup>1</sup> Li Sun,<sup>2</sup> and Hongwei Gao<sup>3</sup>

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Obesity-derived body mass may be detrimental to bone health through not well-defined mechanisms. In this study we determined changes in bone structure and serum cytokines related to bone metabolism in diet-induced obese mice. Mice fed a high-fat diet (HFD) had higher serum tartrate-resistant acid phosphatase (TRAP) and leptin but lower osteocalcin concentrations than those fed the normal-fat diet. The HFD increased multinucleated TRAP-positive osteoclasts in bone marrow compared to the control diet. Despite being much heavier, mice fed the HFD had lower femoral bone volume, trabecular number, and connectivity density and higher trabecular separation than mice on the control diet. These findings suggest that obesity induced by a HFD increases bone resorption that may blunt any positive effects of increased body weight on bone.

**Keywords:** high-fat diet; obesity; bone; resorption; cytokine

Obesity is a major risk factor for many health disorders mainly because of its associated chronic low-grade inflammation. It shares many common environmental and genetic features with osteoporosis.<sup>1</sup> Contrary to the dogmatic view that obesity increases bone mineral density (BMD) and is a protective factor for osteoporosis in humans,<sup>2</sup> emerging data suggest that marrow adipogenesis is negatively correlated with osteoblastogenesis<sup>3,4</sup> and obesity (or excessive fat accumulation) is detrimental to bone in animals and humans.<sup>5,6</sup>

To further investigate the effects of a high-fat diet (HFD) on bones at different sites and the mechanisms through which obesity affects bone metabolism, we evaluated bone microarchitecture of distal femur by micro-computed tomography ( $\mu$ CT), measured serum cytokines related to bone metabolism, and measured osteoclast formation in bone marrow cells in an HFD-induced obese mouse model.

Our results show that obesity induced by an HFD alters the balance of bone remodeling, leading to reduced femoral bone mass in mice.

**Materials and methods****Animals and diets**

Male C57BL/6 mice purchased from Charles River Laboratories (Wilmington, MA) were housed in Plexiglas ventilated cages (two animals/cage) within a pathogen-free facility that maintained a 12:12 h light/dark cycle. Animals were maintained and processed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol for the study was approved by the United States Department of Agriculture Agricultural Research Service, Grand Forks Human Nutrition Research Center Animal Care Committee. Three days after arrival, animals were randomly assigned to two groups and fed either the normal-fat control diet

( $n = 11$ ; 10% energy as fat, D12450B, Research Diets, New Brunswick, NJ) or an HFD ( $n = 10$ ; 45% energy as fat) with extra fat from lard for 14 weeks, as described previously.<sup>5</sup> The mice had free access to tap water throughout the study.

### Serum cytokine measurements

The serum concentrations of cytokines were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits: osteocalcin (mouse osteocalcin enzyme immunoassay kit; Biomedical Technologies Inc., Stoughton, MA); leptin, osteoprotegerin (OPG), and soluble receptor activator of nuclear factor- $\kappa$ B ligand (sRANKL) (mouse ELISA kits, ALPCO Diagnostics, Windham, NH); and tartrate-resistant acid phosphatase (TRAP5b) (Immunodiagnostic System, Fountain Hill, AZ). All assays were performed according to the manufacturers' instructions.

### Harvest of bone marrow cells and osteoclast formation

Murine bone marrow cells were harvested from long bones, washed, and cultured in primary medium ( $\alpha$ -modified Eagle's medium [ $\alpha$ -MEM] with 10% fetal bovine serum and 1% penicillin/streptomycin) supplemented with 5 ng/mL macrophage colony stimulating factor (M-CSF) (Sigma-Aldrich, St. Louis, MO) overnight to deplete stromal cells. Nonadherent osteoclast precursors were collected, and equal numbers of cells were cultured in primary medium in the presence of 10 ng/mL M-CSF and 30 ng/mL RANKL (R&D Systems, Minneapolis, MN) in 96-well plates ( $2 \times 10^5$  cells/well) with addition of fresh media every 2–3 days. At day 6 of culture, the cells were washed with distilled H<sub>2</sub>O twice and fixed with a solution (25 mL citrate, 65 mL acetone, 8 mL of 37% formaldehyde) for 30 s, then stained with a commercial kit for TRAP (Sigma) according to the manufacturer's instructions. Multinucleated cells with more than three nuclei were counted as TRAP-positive osteoclast cells under an inverted microscope.

### Micro-computed tomography measurements

The trabecular and cortical microstructures of distal femurs were evaluated by using Scanco  $\mu$ CT scanner ( $\mu$ CT-40; Scanco Medical AG, Bassersdorf, Switzerland) at 6  $\mu$ m isotropic voxel size with X-ray source power of 55 kV and 145  $\mu$ A and integration time

of 300 ms. The gray-scale images were processed by using a low-pass Gaussian filter ( $\sigma = 0.8$ , support = 1) to remove noise, and a fixed threshold of 220 was used to extract the mineralized bone from the soft tissue and marrow phase. Cancellous bone was separated from the cortical regions by semiautomatically drawn contours. A total of 120 slices starting from about 0.1 mm distal to growth plate, constituting 0.70 mm length, was evaluated for trabecular and cortical bone structure by using software provided by Scanco, as described in detail previously.<sup>5</sup>

### Data analysis

Data on all parameters are expressed as group means  $\pm$  SD ( $n = 10$ –11 animals/group). Differences between the experimental groups were analyzed by using the Student's *t*-test. In all of the analyses,  $P < 0.05$  was considered to be statistically significant.

## Results and discussion

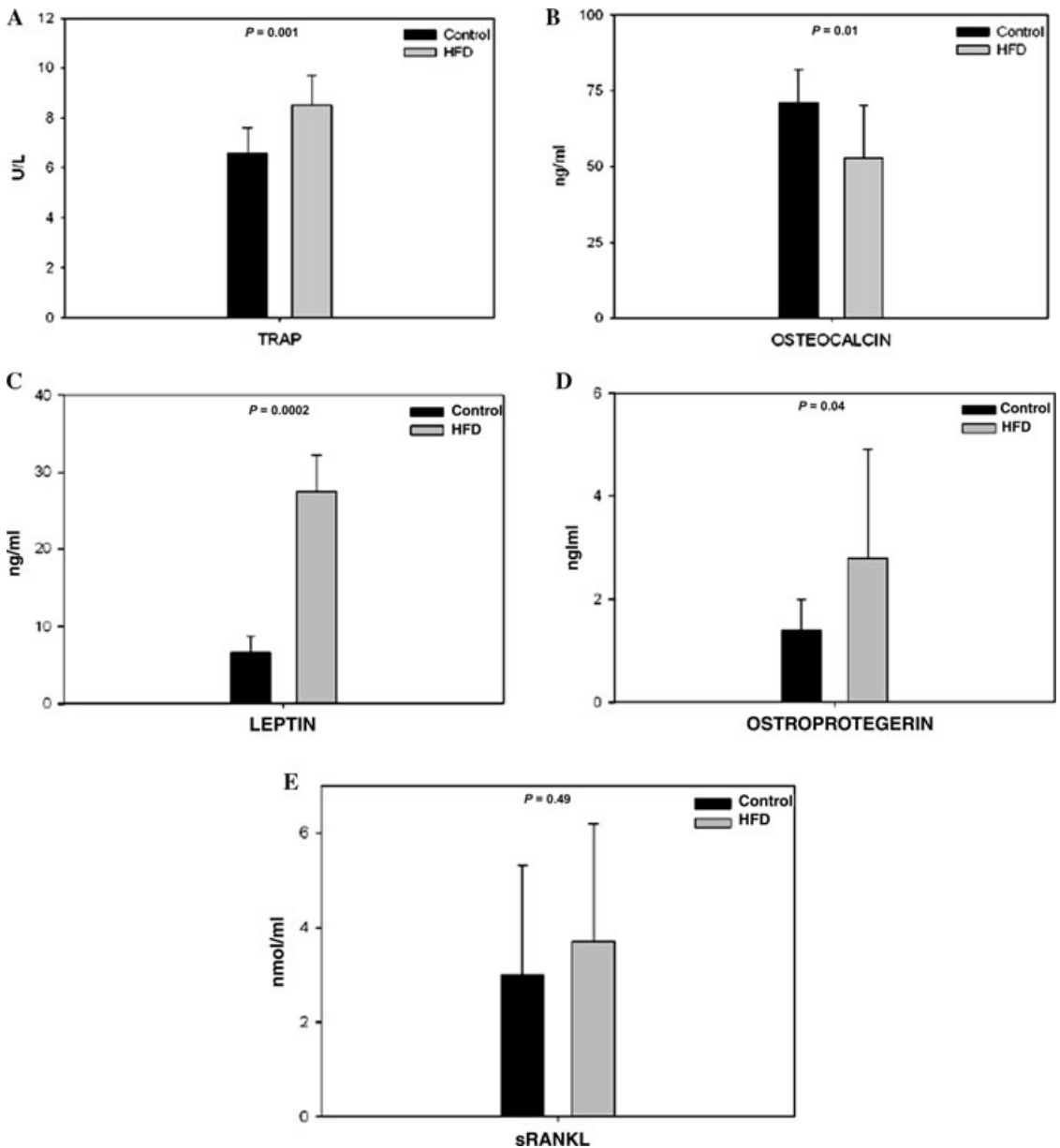
### Obesity induced by the high-fat diet increases serum tartrate-resistant acid phosphatase concentration and osteoclast formation by cultured bone marrow cells

Mice fed the HFD had significantly higher concentrations of serum TRAP, a bone resorption marker (Fig. 1A) ( $P = 0.01$ ) than mice fed the control diet (group mean, control  $6.6 \pm 1.0$  vs. HFD  $8.5 \pm 1.2$  U/L). TRAP-positive osteoclast-like cells are formed when bone marrow cells are cultured in the presence of M-CSF and RANKL. Compared with mice fed the control diet, HFD-fed mice had more ( $P = 0.04$ ) TRAP-positive osteoclast-like cells at day 6 of culture (Fig. 2).

Increased serum TRAP concentrations in HFD-fed mice have been reported by other researchers.<sup>7</sup> An increased serum bone resorption marker coupled with increased osteoclast formation by bone marrow cells suggests that the HFD increases osteoclast activity and bone resorption.

### Obesity induced by the HFD decreases serum osteocalcin and increases serum leptin concentrations

The concentration of serum osteocalcin (Fig. 1B) was significantly lower in HFD mice than control mice ( $P = 0.01$ ). Serum leptin (Fig. 1C) and OPG (Fig. 1D) concentrations in mice fed the HFD were significantly higher than in mice fed the control diet

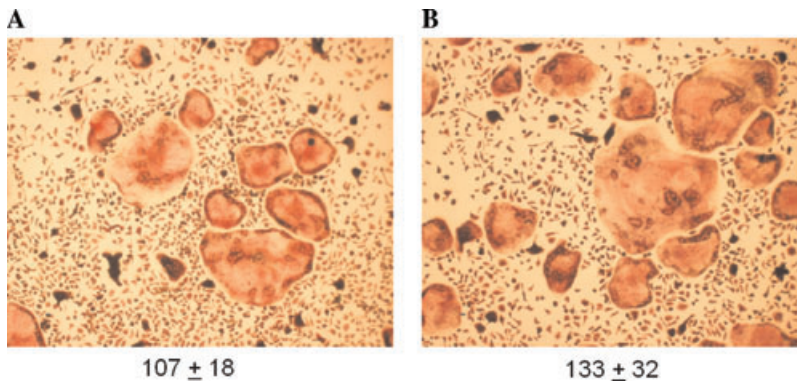


**Figure 1.** Serum concentrations of tartrate-resistant acid phosphatase (TRAP) (A), osteocalcin (B), leptin (C), osteoprotegerin (D), and soluble receptor activator of nuclear factor- $\kappa$ B ligand (sRANKL) (E) in mice fed the control and the high-fat diet (HFD). Data are mean  $\pm$  SD ( $n = 11$  for the control group and  $n = 10$  for the HFD group). Data were analyzed by using the Student's *t*-test.

( $P = 0.002$  and  $0.04$  for leptin and OPG, respectively). There was no difference in serum sRANKL (Fig. 1E) concentration between control mice and HFD mice.

Osteocalcin, noncollagenous protein secreted by osteoblasts, has often been used as a bone forma-

tion biomarker. Thus, a decrease in serum osteocalcin concentration in HFD mice suggests decreased bone formation. The finding of an inverse relationship between serum osteocalcin and fat mass is consistent with that reported in humans.<sup>8</sup> Osteocalcin has also been found to reduce fat storages



**Figure 2.** Formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts by bone marrow cells from mice fed either the control (A) or HFD (B) diet. Data are mean  $\pm$  SD ( $n = 11$  for the control group and  $n = 10$  for the HFD group). Data were analyzed using the Student's *t*-test.

and increase insulin sensitivity.<sup>9</sup> However, whether decreased serum osteocalcin levels would cause (or be caused by) fat accumulation in mice fed an HFD remains to be investigated.

Although it has been established that osteoblasts regulate the activity and recruitment of osteoclasts through the RANKL/OPG pathway, the use of serum sRANKL and OPG as bone resorption or formation biomarkers has not been widely accepted. For example, while aging is associated with significant bone loss in humans, serum OPG concentrations are reported to not change, increase, or decrease with age.<sup>10</sup>

#### Obesity induced by the HFD decreases femoral bone mass despite a significant increase in body weight

None of the cortical measurements (total area, bone area, medullary area, bone perimeter, and cortical thickness) were significantly affected by the HFD ( $P > 0.05$ ) (Table 1). The femoral trabecular bone volume of HFD mice was lower than that of control mice ( $P = 0.04$ ) (Table 1). HFD mice had 31.6% less bone volume/total volume than control mice ( $P = 0.03$ ). Compared to mice fed the control diet, mice on the HFD had lower trabecular number and connectivity density ( $P = 0.01$ ) and higher trabecular separation ( $P = 0.01$ ). There were no differences in trabecular thickness and segmented bone mineral density between mice fed the HFD and the control diet ( $P > 0.05$ ). At the end of 14 weeks, mice fed the HFD were almost 10 g heavier ( $P = 0.0001$ ) than those fed the control diet.<sup>5</sup> When adjusted to the body weight, bone volume of HFD mice was signif-

icantly lower ( $P = 0.0004$ ) than that of control mice ( $0.017 \pm 0.006$  vs.  $0.008 \pm 0.003$  mm<sup>3</sup>/g for control and HFD mice, respectively).

The increase in trabecular separation and the decrease in trabecular number and connectivity density clearly demonstrate that obesity induced by an HFD is detrimental to trabecular bone of femur in mice. These structural changes are associated with a decrease in bone formation and an increase in bone resorption in HFD-fed mice.

Our findings are in agreement with several other studies where an HFD was found to be detrimental to either bone quantity or quality<sup>11</sup> but differ with a study where body weight was found to be the main determinant of bone mass regardless of dietary fat levels.<sup>12</sup> The inconsistent findings may be the result of differences in animal species, age, gender, dietary fat levels, and time or duration of high-fat feeding.

Whether obesity is beneficial or detrimental to bone health in humans remains controversial where conclusions are mainly based on statistical correlation or modeling rather than controlled studies. The positive effects of mechanical loading conferred by body weight on bone has been well established, but the adverse effects of obesity on bone is also apparent in animal models.<sup>13</sup>

Based on our current study and those of others, obesity may affect bone metabolism through one or more of the following mechanisms. First, obesity may increase osteoclastogenesis and bone resorption. It has been established that obesity is associated with low-grade chronic inflammation and elevated production of proinflammatory cytokines, including tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$

**Table 1.** Effects of feeding high-fat diet (HFD) for 3 months on distal femoral structural parameters of mice<sup>a,b</sup>

Parameters	Control	HFD	<i>P</i> value ( <i>t</i> -test)
<b>Cortical bone</b>			
T.Ar (mm <sup>2</sup> )	1.22 ± 0.14	1.13 ± 0.09	0.10
B.Ar (mm <sup>2</sup> )	0.98 ± 0.12	0.92 ± 0.12	0.23
Me.Ar (mm <sup>2</sup> )	0.24 ± 0.04	0.21 ± 0.03	0.14
B.Pm (mm)	18.8 ± 1.7	18.0 ± 0.7	0.18
BV/TV (%)	14.5 ± 1.5	14.0 ± 2.1	0.51
Ct.Th (mm)	0.10 ± 0.01	0.10 ± 0.01	0.60
<b>Trabecular bone</b>			
BV (mm <sup>3</sup> )	0.53 ± 0.20	0.35 ± 0.17	0.04
BV/TV (%)	25.0 ± 7.5	17.1 ± 7.7	0.03
Tb.N (mm <sup>-1</sup> )	4.59 ± 0.45	3.93 ± 0.62	0.01
Tb.Th (μm)	67.2 ± 10.6	61.8 ± 9.0	0.22
Tb.Sp (μm)	192 ± 25	242 ± 49	0.01
Conn.Dn (mm <sup>-3</sup> )	182 ± 30	134 ± 34	0.01
SMI (g HA/cm <sup>3</sup> )	1.23 ± 0.57	1.71 ± 0.57	0.07
BMD (g HA/cm <sup>3</sup> )	0.93 ± 0.02	0.93 ± 0.02	0.55

<sup>a</sup>Data are mean ± SD (*n* = 11 for the control and *n* = 10 for the HFD groups).

<sup>b</sup>T.Ar, total area; B.Ar, bone area; Me.Ar, medullary area; B.Pm, bone perimeter; Ct.Th, cortical thickness; BV, bone volume; TV, total volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Conn.Dn, connectivity density; SMI, structural model index; BMD, bone mineral density.

(IL-1β), and IL-6.<sup>14</sup> These cytokines have been shown to increase osteoclast activity through regulating the RANKL/RANK/OPG pathway.<sup>15</sup> Second, because adipocytes and osteoblasts are derived from a common multipotential mesenchymal stem cell, obesity may increase bone marrow adipogenesis while inhibiting osteoblastogenesis.<sup>3,4</sup> Third, high fat may interfere with intestinal calcium absorption, as reported in some studies.<sup>16</sup> Fourth, obesity may inhibit bone formation through leptin when it binds to its receptors on ventromedial hypothalamic neurons.<sup>14</sup> Serum leptin, a small protein secreted primarily by the adipocytes, is a potent antiosteogenic cytokine.<sup>17</sup> Serum leptin was increased in mice fed the HFD by more than fourfold in our study. Elevated serum leptin may inhibit bone formation, as reported in genetic and diet-induced mouse models,<sup>17</sup> although it has also been reported to stimulate

bone formation.<sup>12</sup> It is likely that leptin levels may determine its ultimate action on bone metabolism.

In conclusion, our results show that obesity induced by an HFD decreases femoral trabecular bone mass by increasing trabecular separation and reducing trabecular number and connectivity density in young mice. Therefore, obesity induced by an HFD may alter the bone remodeling by favoring resorption that blunts any positive effects of increased body weight on bone.

### Acknowledgment

We thank Brian R. Gregoire and LuAnn Johnson for technical support.

### Conflicts of interest

The authors declare no conflicts of interest.

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