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Original Article

Hylonome

Serum leptin levels negatively correlate with trabecular bone mineral density in high-fat diet-induced obesity mice

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Abstract

Objectives: This study evaluated the influence of diet-induced obesity on bone tissue quantity and quality in the proximal tibiae of growing mice and also examined the relationships between the serum total cholesterol, leptin, and adiponectin levels and trabecular and cortical bone mineral parameters. **Methods:** Six-week-old male C57BL/6J mice were divided into two groups; one received a control diet, and the other received a high-fat-diet. After treatment for 4, 8, or 12 weeks, the bone quantity and quality were analyzed using peripheral quantitative computed tomography (pQCT), micro-computed tomography and histomorphometry. **Results:** In the early stages, trabecular bone density decreased with an increase in the number of adipocytes and the deterioration of trabeculae. In contrast, although cortical bone formation was slower in obese mice compared with control mice, bone formation on the periosteal surface increased with age. Serum leptin levels were correlated with trabecular, but not cortical bone density, whereas neither the adiponectin nor total cholesterol level was correlated with bone mass in mice with diet-induced obesity. **Conclusions:** We conclude that bone loss at these two sites is differentially regulated in mice. Furthermore, we demonstrate that serum leptin may be a useful indicator of risk for osteoporosis associated with diet-induced obesity.

Keywords: Osteoporosis, Obesity, pQCT, Bone Quality, Leptin

Introduction

Overweight children are at higher risk for the development of type 2 diabetes, hypertension, and hyperlipidemia, which in turn increase the risk for cardiovascular disease later in life[1]. The International Obesity Task Force reported that 1 in 10 children worldwide is overweight (above the 86th percentile for body mass index); this fraction represents a total of 155 million children and a worldwide epidemic[2,3].

Although a relationship between obesity and osteoporosis has been proposed in the literature[4-6], no consensus has been reached. Fractures attributable to increased bone fragility are the primary complication of osteoporosis, a significant health problem that has increased along with obesity. Several researchers have demonstrated that increased fracture incidence has been observed in obese adolescents and children when compared with age-matched controls[4,5]. Additionally, bone fragility may occur in obese children and adolescents because of malnutrition[7]. In Japan, the National Health and Nutrition Survey showed that mean fat intake had increased from 25.8 g/day in 1961 to 53.6 g/day in 2009[8]. In animal studies, Inova-Martin et al.[9] reported that high-fat diet-induced obesity resulted in increased bone density. In contrast, Patsch et al.[10] reported that high-fat diet-induced obesity caused bone loss in mice. The reasons for these conflicting results concerning the relationship between high-fat diet-induced obesity and bone mass in animal are poorly understood. The development and significance of marrow adiposity have recently attracted attention[11,12], and several studies have elucidated the mechanisms of developing bone marrow adiposity[13,14]. However, the pathogenic mechanism underlying the relationship between high-fat diet-induced obesity and osteoporosis during childhood and adolescence has not been elucidated.

Previous investigations of the relationship between obesity

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and osteoporosis have suggested that adipose tissue may influence bone mineral density (BMD) through the production of hormones and adipokines such as leptin and adiponectin[14]. Several studies involving children have shown a direct relationship between the serum leptin concentration and bone mass[15-17], however, a conflicting study has reported that the serum leptin concentration was not related to BMD in boys and girls[18]. In adults, some studies have concluded that the serum adiponectin level is negatively correlated with BMD[19,20], whereas other investigations have demonstrated a positive correlation with BMD[21]. Thus, further research is required to determine whether serum leptin and adiponectin levels are associated with bone mass.

In the present study, we evaluated the influence of obesity induced by a high-fat diet on bone tissue quantity and quality using peripheral quantitative computed tomography (pQCT), micro-computed tomography (micro-CT), and histological analyses of the proximal tibiae from mice. We also examined the effects of a high-fat diet on bone strength using pQCT. We then evaluated the statistical relationships between the serum total cholesterol, leptin, and adiponectin levels and trabecular and cortical bone mineral parameters.

	Standard	High-fat
	w/w (%)	
Milk casein	20.0	25.6
L-cystine	0.3	3.6
Maltodextrin	0	6.0
Corn starch	39.7486	0
α-Corn starch	13.2	16.0
Sucrose	10.0	5.0
Soy bean oil	7.0	2.0
Lard	0	33.0
Cellulose powder	5.0	6.61
Mineral mix (AIN-93G)	3.5	3.5
Calcium carbonate	0	0.18
Vitamin mix (AIN-93G)	1.0	1.0
Choline bitartrate	0.25	0.25
The third butyl hydroquinone	0.0014	0
Calorie (kcal/100 g)	377.0	506.2
Ratio of fat/total calorie (%)	16.4	62.2

Table 1. Composition of experimental diet.

Methods

Animal care

Male C57BL/6J mice (6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed individually under a 12-h light-dark cycle, at a constant temperature of 22±1ºC and humidity of 50±5%. After acclimation for one week on a standard pelleted chow in which 16.4% of total calories were based on lipids (AIN-93G)[22], the mice were randomly divided into seven groups (n = 8 each). The mice in one group were immediately euthanized, at 7 weeks of age, with pentobarbital sodium. Three groups remained on the standard diet, and the remaining three groups were switched to a high-fat chow (62% of energy from fat). Both diets were purchased from Oriental Yeast Co. (Tokyo, Japan; Supplemental Data, Table 1). Food intake was assessed as food weight (g) per mouse per day by weighing the food in each cage dispenser, including the food that was spilled on the floor of the cage, and matched among all groups. Mice had *ad libitum* access to tap water throughout the study. Body weight was recorded weekly. After 4, 8, or 12 weeks, control and diet-induced obesity (DIO) mice were euthanized using pentobarbital sodium, and the tibiae were harvested bilaterally and cleaned of adherent tissue. Bone length was measured using a digital caliper (Mitutokyo, Kanagawa, Japan) and then analyzed by micro-CT as described below. All animal procedures were approved by the Committee for Care and Use of Laboratory Animals of Kyushu Dental College.

Peripheral quantitative computed tomography (pQCT)

Right tibiae from euthanized mice were fixed in 70% ethanol, and bone length was determined using a digital caliper. Right tibiae were then prepared for pQCT (XCT Research SA+ series; Stratec, Medizintechnik, Pforzheim, Germany) with a voxel size of 0.08×0.08×0.46 mm. The tibial metaphysis was used to measure trabecular bone density (TrBD; mg/cm^3), trabecular cross-sectional area (TrCSA; mm^2), cortical bone density (CtBD; mg/cm^3), and cortical bone cross-sectional area (CtCSA; mm^2). Measurements were taken at points representing 10% of bone length from the proximal end of the tibia, which was measured from a planar overview (scout view). The trabecular region was defined by peel mode 2 at a threshold value of 395 mg/cm^3. The cortical region was determined after setting cortical mode 1 at a threshold value of 690 mg/cm^3 [23].

Bone strength (non-invasive assessment)

Bone strength of the tibial metaphysis was estimated from the strength strain index (SSI), which was determined using pQCT as a non-invasive assessment of mechanical properties. SSI was calculated using the equation SSI= CBD · Z/NCBD, where CBD is cortical bone density (mg/cm^3), Z is the section modulus (mm^3), and NCBD is the normal physiological value of cortical bone density (1200 mg/cm^3).

CBD and Z were derived from the pQCT data[24].

Micro-computed tomography (micro-CT)

After pQCT analysis, a Scan Xmate-L090 (Comscantecno Co., Kanagawa, Japan) micro-CT machine was used to image representative proximal tibiae from control and diet-induced obesity (DIO) mice. Samples were scanned at 9-μm resolution, and tissue volume (TV, mm^3) and trabecular bone volume (BV, mm^3) were measured directly. The trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, 1/mm), and trabecular separation (Tb.Sp, μm) were calculated three-dimensionally (3D) using 3D BON software (Ratoc System Engineering, Tokyo, Japan)[25]. The measurement sites were selected within the trabecular region from the growth plate to 2 mm distally.

Parameters	7 week-old	11 week-old		15 week-old		19 week-old	
		Control	DIO	Control	DIO	Control	DIO
Body weight (g)	22.9±0.6	27.8±1.2*	32.6±1.7*†	31.8±2.3*	41.5±2.3*†	33.7±3.3*	45.3±2.5*†
Bone length (mm)	16.8±0.9	17.7±0.1*	17.5±0.3*	18.6±0.7*	18.2±0.5*	18.3±0.3*	18.2±0.5*
Total cholesterol (mg/dl)	135.7±19.2	156.7±11.3*	205.5±17.8*†	175.4±16.2*	228.4±18.9*†	182.3±23.1*	223.8±16.3*†
Triglyceride (mg/dl)	47.8±19.8	51.8±14.6	70.5±13.7	75.0±7.1*	91.8±19.9*	82.6±17.5*	116.3±6.8*†
LDL cholesterol (mg/dl)	9.8±1.7	10.8±1.5	12.3±1.7	10.8±1.0	12.0±1.0	14.0±1.6*	17.0±1.7*
HDL cholesterol (mg/dl)	71.7±2.1	81.8±2.1*	96.8±1.5*†	88.0±2.0*	91.0±2.6*	86.3±6.5*	89.7±4.5*
Insulin (ng/ml)	1.6±0.2	1.6±0.3	1.7±1.0	2.0±1.0	2.8±1.7	2.2±0.7	4.9±1.9*†
Leptin (ng/ml)	6.5±0.9	7.3±0.9	27.2±6.2*†	32.9±6.5*	71.4±18.6*†	38.2±9.2*	69.7±4.0*†
Adiponectin (μg/ml)	15.6±1.5	24.9±1.6*	26.6±3.0*	26.6±2.5*	24.9±1.9*	26.8±2.1*	24.3 ±0.5*
Calcium (mg/dl)	9.5±0.9	10.9±2.8	9.3±0.7	11.0±2.7	8.7±1.0	9.4±0.7	8.6 ±1.3
Phosphorus (mg/dl)	10.0±0.9	8.0±1.1*	9.9±1.4	8.6±1.1	14.8±6.6	8.5±2.1	17.1 ±8.2*

*Data are mean ± SD. DIO; High-fat diet-induced obesity mice. *p<.05 as compared with 7 week-old mice. †p<.05 as compared with control diet-fed mice.*

Table 2. Body compositional and serum biochemical parameters.

Evaluation of bone marrow adiposity

Left tibiae isolated from euthanized mice were fixed in 10% neutral-buffered formalin, decalcified for 2 weeks with 10% EDTA (pH 7.4), dehydrated with increasing concentrations of ethanol, and embedded in paraffin. Paraffin block sections were cut at a thickness of 2.5 μm and stained with hematoxylin and eosin. Visible adipocytes (>30 μm in diameter) were counted within the trabecular region (i.e., from the growth plate to 2 mm distally) using a Histometry RT camera (System Supply, Nagano, Japan)[26].

Serum analyses

Blood was obtained from mice under anesthesia. Serum was isolated by centrifugation for 15 min at 3000 rpm and frozen at -80°C. Serum triglyceride, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), calcium, and phosphate levels were quantified using routine laboratory methods (Nagahama Life Science Laboratory, Shiga, Japan). Serum insulin levels were measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Ohtsuka, Ltd., Tokyo, Japan), serum leptin levels were measured using a mouse leptin ELISA kit (Morinaga Institute of Biological Science, Inc., Tokyo, Japan), and serum adiponectin levels were measured using a mouse adiponectin ELISA kit (Ohtsuka, Ltd., Tokyo, Japan) according to the manufacturer's instructions.

Statistical analysis

All data are expressed as means ± standard deviation (SD) of eight animals in each group. Differences between diet treatments (high-fat diet vs. age-matched control mice) and over time (at 11, 15, 19 weeks compared with 7 weeks) were analyzed using Student's *t*-test. The relationships between bone mineral parameters and serum total cholesterol, leptin, and adiponectin levels were evaluated using Pearson's correlation coefficient. A *p* value less than 0.05 was considered to indicate statistical significance.

Results

Effects on bone length, density, and cross-sectional area

Table 2 shows the changes in body weight and bone length observed in the control and DIO mice. At 11, 15, and 19 weeks, body weight was increased significantly in the DIO group compared with the control group. No difference in mean bone length was observed between the two groups at any time point.

TrBD was significantly lower in the DIO group compared with the age-matched controls for 15- and 19-week-old mice, and this effect was age dependent (Figure 1a). TrCSA and CtBD were also significantly lower in the DIO group compared with the control group for 19-week-old mice (Figures 1b and c, respectively). CtCSA was significantly lower in the DIO group for 15- and 19-week-old mice; however, when compared with observations at the beginning of the experiment, CtCSA increased significantly with age in both groups of mice (Figure 1d).

Representative pQCT scans of the tibial metaphysis indicated that the trabecular bone area, which is indicated in red, decreased markedly in the DIO group compared with the age-matched controls at 11, 15, and 19 weeks (Figure 2).

Effects on bone strength

The SSI values for the reference *x* (xSSI) and *y* (ySSI) axes in the tibial metaphysis are shown in Figures 3a and b. The mean xSSI decreased significantly in the DIO group compared with the age matched controls at 15 and 19 weeks. The mean ySSI was significantly lower in the DIO group compared with the age matched controls for 15 week-old mice.

Effects on bone marrow adiposity

Histomorphometry revealed an increase in the number of visible adipocytes in the bone marrow of DIO mice compared with age-matched controls (Figures 4a and b). Compared with the

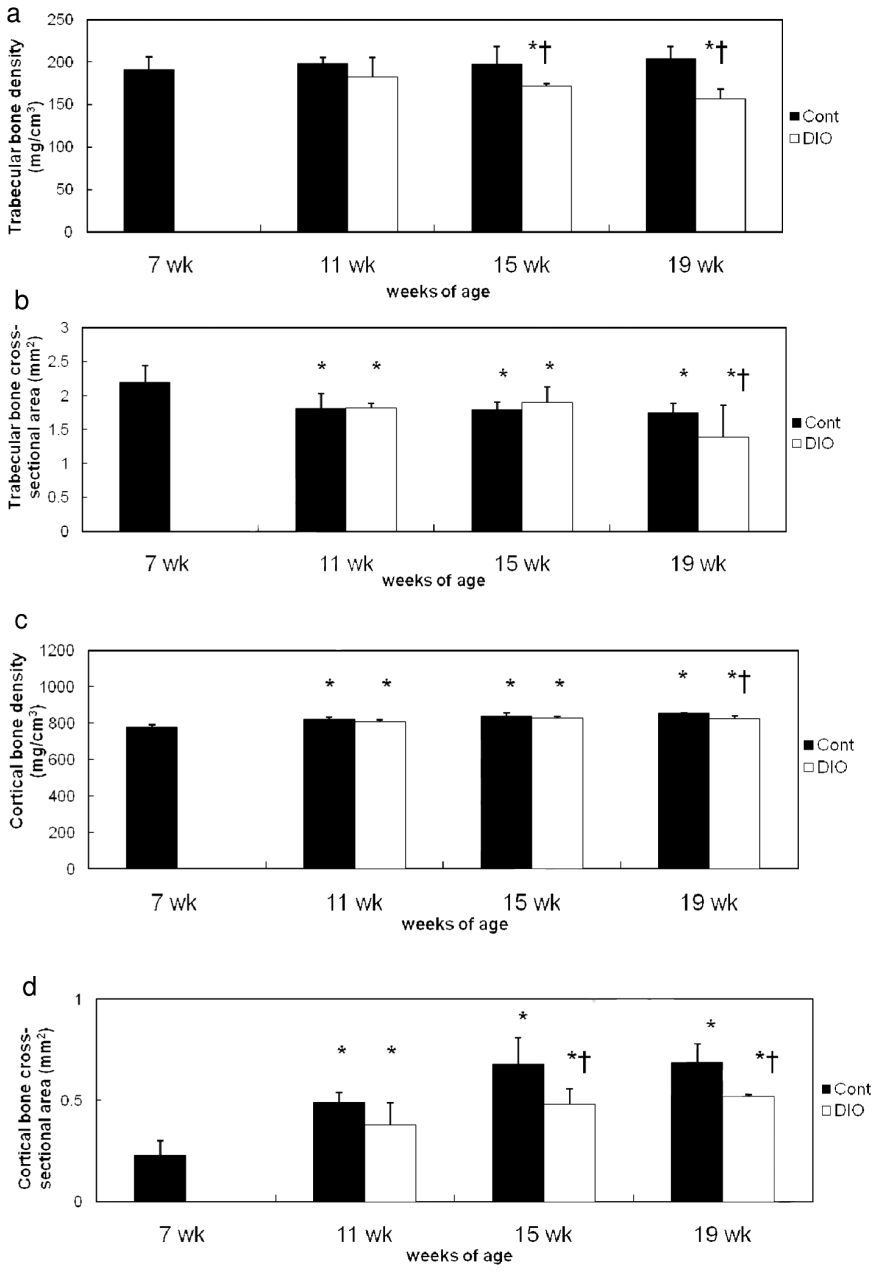

Figure 1. Bone mineral parameters: **a)** trabecular bone density, **b)** trabecular bone cross-sectional area, **c)** cortical bone density, **d)** cortical bone cross-sectional area) using pQCT of the tibial metaphysis in mice fed the standard diet (Cont) and the high fat diet (DIO). Data are mean ± SD. **P*<.05 compared with 7-week-old mice and †P<.05 compared with age-matched control mice.

adipocytes at the beginning of the experiment (7-week-old mice), the number of hypertrophic adipocytes increased significantly with age in both treatment groups. The number of hypertrophic adipocytes increased significantly in DIO mice compared with age-matched controls at 11, 15, and 19 weeks (Figure 4b).

Effects on trabecular architecture

BV/TV and Tb.N were significantly lower in the DIO group (Figures 5a and c), and Tb.Sp was significantly higher in the DIO group (Figure 5d) compared with the values in the age-matched controls for 11- and 19-week-old mice. These effects were age dependent. Compared with representative micro-CT scans of proximal tibiae from control mice, the representative micro-CT scans of proximal tibiae from DIO mice showed clear morphological abnormalities, including the deterioration of trabecular architecture. These abnormalities were observed in 11-, 15-, and 19-week-old DIO mice (Figure 6).

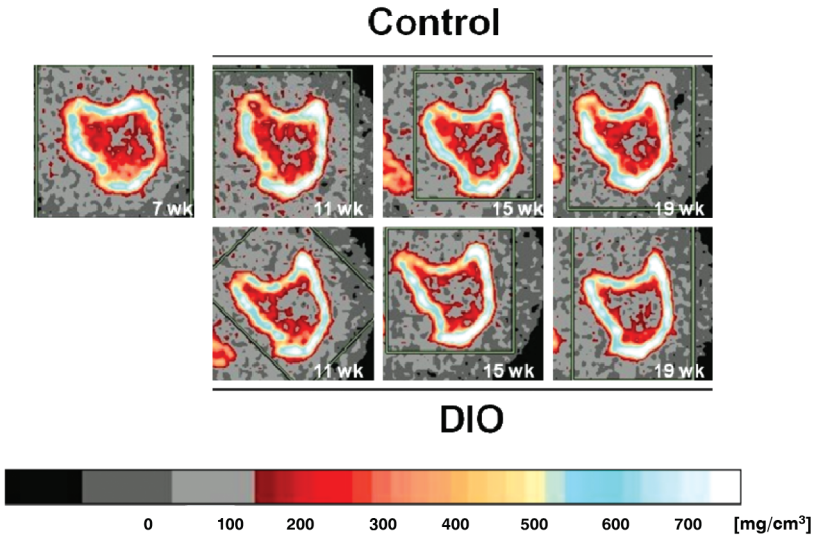

Figure 2. Representative pQCT cross-sectional transverse scans of the tibial metaphysis in mice fed the standard diet (Cont) and the high-fat diet (DIO).

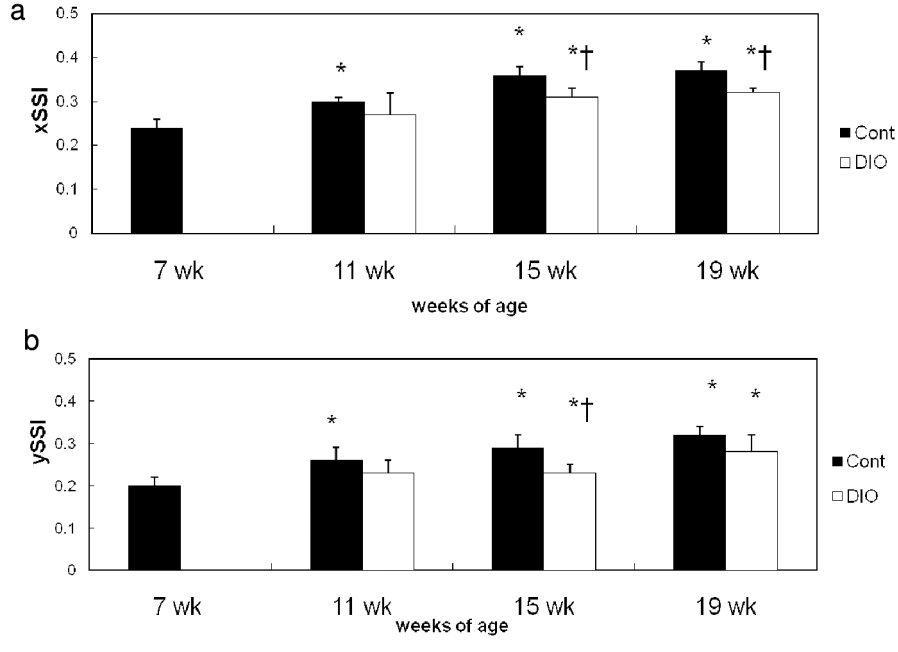

Figure 3. Non-invasive Strength Strain Index (SSI) values for the reference *x* (xSSI; a) and *y* (ySSI; b) axes of the tibial metaphysis in mice fed the standard diet (Cont) and the high fat diet (DIO). Data are mean ± SD. **P*<.05 compared with 7-week-old mice and †*P*<.05 compared with age-matched control mice.

Effects on serum parameters

Regarding changes in serum biochemical parameters, the total cholesterol and leptin levels increased significantly in the DIO group compared with the age-matched controls at 11, 15, and 19 weeks (Table 2). Serum insulin and triglyceride levels were also significantly higher in 19-week-old DIO mice compared with age-matched controls.

In all mice, the serum leptin level showed significant inverse correlations with TrBD and TrCSA, and significant positive correlations with CtBD and CtCSA. The relationships between the serum total cholesterol level and bone mineral parameters were consistent with the correlations between the leptin level and bone mineral parameters. The serum adiponectin level showed

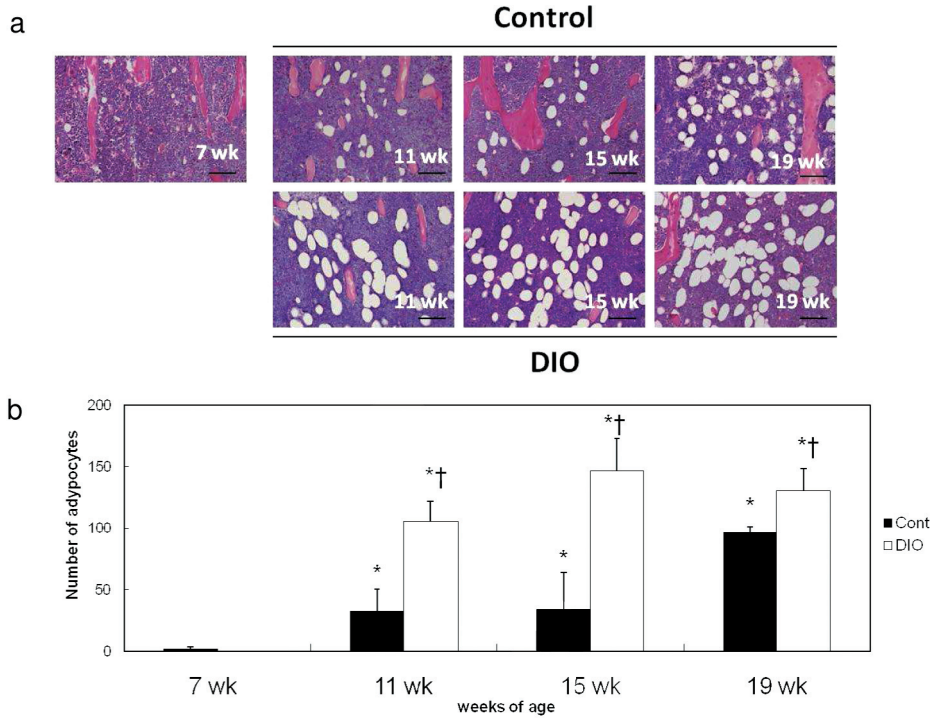

Figure 4. a) Histological images of the bone marrow obtained from standard-diet fed (Control) and high-fat diet fed (DIO) mice. Sections are stained with hematoxylin and eosin. Original magnification: 200. Bar = 100 μm. **b)** Quantization of adipocytes in standard-diet fed (Cont) and high-fat diet fed (DIO) mouse tibiae from growth plate to 2 mm beneath the growth plate. Data are mean ± SD. **P*<.05 compared with 7-week-old mice and †*P*<.05 compared with age-matched control mice.

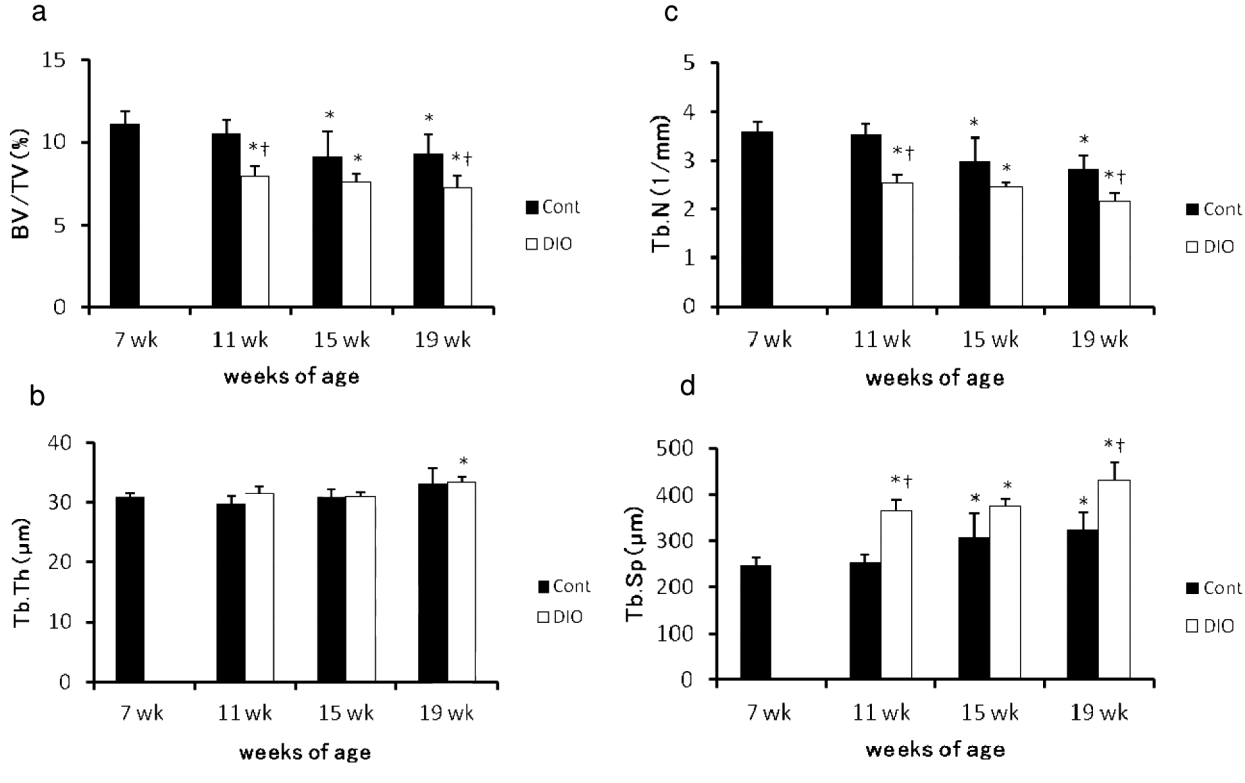

Figure 5. Bone architectural parameters of trabecular bone volume fraction (BV/TV; a), trabecular thickness (Tb.Th; b), trabecular number (Tb.N; c) and trabecular separation (Tb.Sp; d) using micro-CT of the tibial metaphysis in mice fed the standard diet (Cont) and the high fat diet (DIO). Data are mean ± SD. **P*<.05 compared with 7-week-old mice and †*P*<.05 compared with age-matched control mice.

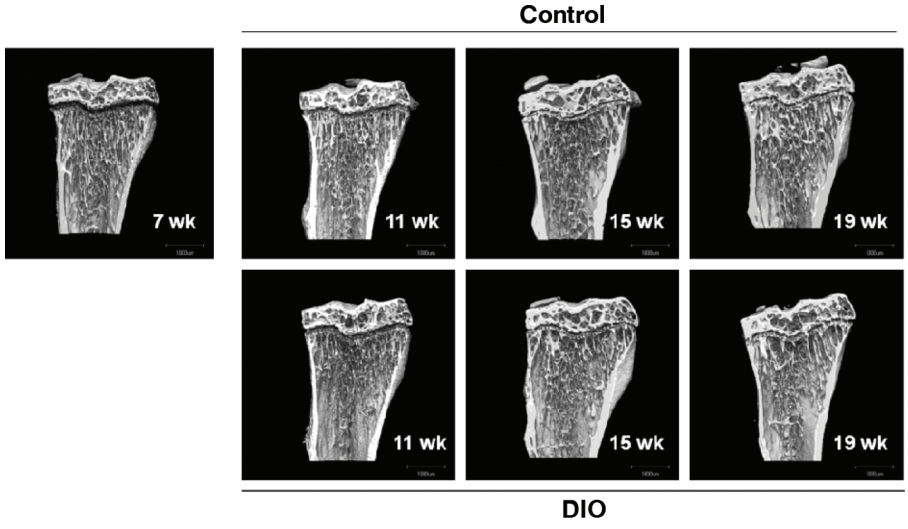

Figure 6. Representative tibial micro-CT scans in standard-diet fed (Control) and high-fat diet fed (DIO) mice.

	Trabecular bone density (mg/cm^3)	Trabecular bone cross-sectional area (mm^2)	Cortical bone density (mg/cm^3)	Cortical bone cross-sectional area (mm^2)
Total cholesterol (mg/dl)	-0.414*	-0.458*	0.568*	0.364*
Leptin (ng/ml)	-0.475*	-0.469*	0.419*	0.406*
Adiponectin (μg/ml)	0.004	-0.422*	0.626*	0.602*

*Statistically significant, *p<.05.*

Table 3. Correlations between bone mineral parameters with body compositional and serum total cholesterol, leptin and adiponectin in all mice.

	Trabecular bone density (mg/cm^3)		Trabecular bone cross-sectional area (mm^2)		Cortical bone density (mg/cm^3)		Cortical bone cross-sectional area (mm^2)	
	Control	DIO	Control	DIO	Control	DIO	Control	DIO
Total cholesterol (mg/dl)	0.235	-0.062	-0.367	-0.179	0.806*	0.749*	0.730*	0.252
Leptin (ng/ml)	0.332	-0.615*	-0.382	-0.360	0.785*	0.564	0.747*	0.675*
Adiponectin (μg/ml)	0.249	-0.141	-0.698*	-0.077	0.742*	-0.124	0.796*	-0.368

*DIO; High-fat diet-induced obesity mice. Statistically significant, *p<.05.*

Table 4. Correlations between bone mineral parameters with body compositional and serum total cholesterol, leptin and adiponectin in Control and DIO mice.

a significant negative correlation with TrCSA, and significant positive correlations with CtBD and CtCSA (Table 3).

Considering only the control group, the leptin level showed significant positive correlations with CtBD and CtCSA.

The correlations between the serum total cholesterol level and bone mineral parameters were consistent with the correlations between the serum leptin level and bone mineral parameters. In addition, the serum adiponectin level had a significant inverse correlation with TrCSA and significant positive correlations with CtBD and CtCSA (Table 4).

In the DIO group, a significant positive correlation was observed between the serum total cholesterol level and CtBD. The serum leptin level showed a significant inverse correlation with TrBD and a significant positive correlation with CtCSA (Table 4).

Discussion

In the present results, we found significant increases in body weight and in serum total cholesterol, triglyceride, insulin, and leptin levels in mice fed a high-fat diet, compared with the values in age-matched control mice. A high-fat diet also decreased bone strength without affecting longitudinal growth in growing mice, and the decrease in bone strength was primarily attributable to retarded growth in CtCSA.

In trabecular bone, an increase bone marrow adiposity and a deterioration of trabecular structure resulted in an overall decrease in bone density in the tibiae from DIO mice. In addition, increased bone marrow adiposity and trabecular deterioration were observed by histological and micro-CT examinations of 11-week-old mice before a difference in TrBD became apparent. In contrast, Bartelt et al.[12] reported that high-fat diet-induced obesity does not significantly affect lumber vertebra bone mass in mice. They suggested that conflicting results in previous studies on the relationship between high-fat diet induced-obesity and bone mass[9,27,28] may be attributable to differences in the fatty acid profiles of the diets. A higher ratio of n-6 (linoeic acid) to n-3 (α-linolenic acid) fatty acids is associated with detrimental effects on bone health, while a lower ratio of dietary (n-6)/(n-3) fatty acids is associated with the promotion of bone formation in human and animal studies[29,30]. In the present study, the fat included in the high-fat diet was mainly from lard, which has a higher ratio of (n-6)/(n-3) fatty acids than that of the standard diet containing soy bean oil. The composition of these fatty acids may be one reason for the negative effects on bone structure in the high-fat diet group. Therefore, in order to obtain more precise data, the component ratio of dietary fatty acids should be considered when choosing standard and high-fat diets.

Other possible explanations for the conflicting findings regarding the relationship between high-fat diet-induced obesity and bone mass include the heterogeneity among skeletal sites in mice and the differences in the measurement ranges among studies. C57BL/6J mice develop age-related trabecular bone loss in the metaphyseal region of long bones compared with that in the vertebral body at a relatively young age[31]. Additionally, Bouxsein et al.[32] reported that differences in the volume of interest in measurement regions has an influence on the BV/TV value of the metaphyseal regions when using micro-CT. Therefore, we believe that compared with other skeletal regions, trabecular bone in the proximal tibia may be more susceptible to high-fat diet-induced obesity. Furthermore, no significance difference in the BV/TV was found between 15-week-old DIO mice and control mice, suggesting the occurrence of a period during bone loss that does not differ significantly between DIO and control mice.

Regardless of the changes in bone mass, several studies have shown a significant increase in the size or number of adipocytes in the bone marrow of high-fat diet-induced obesity mice[10,12,27,33]. Patsch et al.[10] suggested that high-fat diet-induced obesity causes significant bone loss in mice due mainly to resorptive changes in trabecular architecture. Obesity is associated with low-grade chronic inflammation and elevated production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6[34], which are released from adipocytes, the adipose tissue matrix, and elsewhere[35]. These cytokines have been shown to increase osteoclast differentiation via the RANKL/RANK/OPG pathway[36]. Halade et al.[37] suggested that significantly elevated levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) due to the accumulation of adipocytes in bone marrow results in an increase in bone resorption in mice fed a high-fat-diet. Furthermore, osteoclast differentiation and bone resorption are stimulated in bone marrow-derived macrophages from obese mice[38]. Therefore, we consider that the degree of bone resorption is associated with the degree of osteoclastic potential, including osteoclast formation, in the marrow. However, it is unclear what mediates enlarged and increased adipocytes to lead to such environmental changes in bone marrow, and the way in which those mediators and bone marrow adiposity affect bone metabolism. Furthermore, because adipocytes and osteoblasts are derived from a common multipotent mesenchymal stem cell, obesity may increase bone marrow adipogenesis while inhibiting osteoblastogenesis[39,40]. Therefore, we believe that a high-fat diet in mice triggers an increase in bone resorption and inhibits of bone formation, rather than directly affecting bone mass.

In contrast, although cortical bone formation was slower in DIO mice compared with control mice, bone formation on the periosteal surface increased with age. These results probably reflect a high bone turnover rate in young animals. In addition, DIO mice did not show a significant decrease in CtBD compared with the control group until 19 weeks, indicating that trabecular bone architecture was more responsive than cortical bone to a high-fat diet.

These results suggest that high-fat diet-induced trabecular and cortical bone loss are differentially regulated in growing mice.

The standard tool for BMD measurement in most human studies is dual-energy X-ray absorptiometry (DXA), which provides precision and reproducibility at a relatively low radiation dose[4-6,41]. However, DXA is problematic in that it is impossible to measure volumetric bone density or to distinguish between cortical and trabecular bone. This inability to differentiate between the responses of cortical vs. trabecular bone may explain the conflicting results regarding the relationship between obesity and osteoporosis. Although pQCT and micro-CT can be used to distinguish cortical and trabecular bone, the equipment required for these techniques is less widely available[42]. In the present study, TrBD in 11-week-old mice did not differ significantly between the DIO and age-matched control mice, although BV/TV was significantly lower in 11-week-old mice than in age-matched control mice. These results suggest that the bone density obtained by pQCT is an index indicating not only bone density but also the degree of bone mineralization. Therefore, we believe that TrBD should be evaluated because it is different from BV/TV.

This study also demonstrated that the serum total cholesterol level increased significantly in DIO mice compared with age-

matched control mice. Bartelt et al.[12] demonstrated that a high cholesterol level does not have a dominant negative effect on trabecular bone mass in C57BL/6J and apolipoprotein E-deficient mice fed a standard or high-fat diet, respectively. In contrast, in the present study, only CtBD was positively related to total cholesterol in DIO mice. However, CtBD was significantly lower in 19-week-old DIO mice compared with age-matched control mice. Thus, we suggest that serum total cholesterol is not an effective indicator of the risk for osteoporosis in diet-induced-obesity. Nevertheless, in normal mice, the serum total cholesterol level and cortical bone mass are strongly correlated.

The relationship between leptin and bone is a complex one, with a diverging effect depending on whether central or peripheral mechanisms are operating[43,44]. Centrally, leptin has been shown to inhibit bone formation through a hypothalamic relay, and this effect is suppressed by β-blockers[45,46]. Peripherally, leptin has a positive effect on bone; previous studies have shown that leptin promotes increased production of the potent anti-resorptive factor, osteoprotegerin by osteoblasts[47,48]. Leptin also enhances osteoblast formation and inhibits osteoclast generation[49,50]. In the present study, serum leptin level increased significantly in mice fed a high-fat diet compared with age-matched control mice, and leptin was negatively correlated with TrBD; these results are consistent with those of Patsch et al.[10]. Leptin was also positively correlated with CtCSA in DIO mice, which is consistent with the findings of Iwaniec et al.[51]. However, the same authors demonstrated that leptin was not required for increased cortical bone volume associated with increased body mass in leptin-deficient *ob/ob* and diet-induced obesity mouse models. Indeed, CtCSA was significantly lower in 15- and 19-week-old DIO mice than in age-matched control mice, while the serum leptin level in 15- and 19-week-old DIO mice was significantly higher than that in age-matched control mice. Therefore, it appears that trabecular, rather than cortical, bone mass is correlated with the serum leptin level in DIO mice.

Plasma adiponectin levels have been reported to decrease in obese mice[52], and decreased adiponectin levels have been implicated in the development of insulin resistance in mouse models of obesity and lipoatrophy[53]. In the present study, we observed a non-significant, decreasing trend in the adiponectin level with age in DIO mice. Consistent with this, previous studies have shown that significant changes in plasma adiponectin do not appear to be involved in the early stages of obesity onset[54]. Furthermore, we did not analyze for the presence of bound, activated insulin receptor, which is an indicator of insulin resistance, in the tibial metaphysis. In a previous study, Wu et al.[55] reported that insulin receptor was expressed in the growth plate of tibiae from growing mice fed a high-fat diet (60% of energy from fat) for 6 weeks. Therefore, further examination of the tibiae from 15- and 19-week-old DIO mice would be useful to determine whether insulin resistance is occurring.

Adiponectin is thought to directly increase bone mass by suppressing osteoclastogenesis and activating osteoblastogenesis, as indicated in previous *in vitro* studies[56,57]. Williams et al.[57] reported that adiponectin-deficient (AdKO) mice, which demonstrate both insulin resistance and hyperinsulinemia, showed variably increased bone volume. The authors also demonstrated that adiponectin had a negative, indirect effect on bone mass *in vivo*. In the present study, age-dependent increase in serum insulin were not associated with a positive effect on trabecular or cortical bone mass, and no significant correlation was observed between adiponectin and bone mineral parameters in DIO mice. These results suggest that serum adiponectin is not useful as an indicator of the risk for osteoporosis in diet-induced obesity. However, in normal mice, the serum adiponectin level is strongly correlated with both trabecular and cortical bone mass.

In conclusion, this study demonstrates that the deterioration of trabecular bone architecture begins early in the development of diet-induced obesity and is followed by a decrease in cortical bone density. The difference in the response of trabecular vs. cortical bone to diet-induced obesity, suggests that bone loss at these two sites is differentially regulated in growing mice. Furthermore, our results indicate that serum leptin, in comparison with serum total cholesterol and adiponectin, may be a much more useful indicator of the risk for osteoporosis in mice subjected to diet-induced obesity.

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