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Body Mass Influences Cortical Bone Mass Independent of Leptin Signaling

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Abstract

Obesity in humans is associated with increased bone mass. Leptin, a hormone produced by fat cells, functions as a sentinel of energy balance, and may mediate the putative positive effects of body mass on bone. We performed studies in male C57Bl/6 wild type (WT) and leptin-deficient *ob/ob* mice to determine whether body mass gain induced by high fat intake increases bone mass and, if so, whether this requires central leptin signaling. The relationship between body mass and bone mass and architecture was evaluated in 9-week-old and 24-week-old WT mice fed a regular mouse diet. Femora and lumbar vertebrae were analyzed by micro computed tomography. In subsequent studies, slowly and rapidly growing *ob/ob* mice were injected in the hypothalamus with a recombinant adeno-associated virus containing the leptin gene (rAAV-lep) or a control vector, rAAV-GFP (green fluorescent protein). The mice were maintained on a regular control diet for 5 or 7 weeks and then subdivided into groups and either continued on the control diet or fed a high fat diet (45% of kcal from fat) for 8 weeks. In the WT mice, femoral and vertebral bone mass was positively correlated with body mass (Pearson's r = 0.65–0.88 depending on endpoint). rAAV-lep therapy dramatically decreased body mass (−61%) but increased femur length. However, in the distal femur and lumbar vertebra, rAAV-lep therapy reduced cancellous bone volume/tissue volume, trabecular number and trabecular thickness, and increased trabecular spacing. The high fat diet increased body mass, irrespective of vector treatment. Total femur bone volume, length, cross-sectional volume, and cortical volume and thickness were increased in mice with increased body mass, independent of rAAV treatment. In the distal femur, increased body mass had no effect on cancellous architecture and there were no vector × body mass interactions. In WT mice, increased body mass resulted in increased (+33%) vertebral cancellous bone volume/tissue volume. Increased body mass had minimal independent effect on cancellous vertebral bone

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mass in *ob/ob* mice. Taken together, these findings suggest that increased body mass has a positive effect on femur cortical bone mass that is independent of leptin signaling.

Keywords

osteoporosis; peak bone mass; μCT; obesity; weight; body mass

1. Introduction

A low peak bone mass predisposes individuals to osteoporosis, a disease that contributes to over two million fractures annually in the United States alone [1]. The acquisition of bone mass occurs primarily during childhood and the decade following puberty, and is tightly coupled to energy metabolism. A low bone mass and increased fracture risk are associated with anorexia, whereas high bone mass and reduced fracture risk are associated with obesity [2, 3]. The increased bone mass in obese individuals has been attributed to systemic factors (e.g., elevated sex steroid and leptin levels) and increased skeletal loading due to increased body mass. Although body mass has a strong positive effect on bone mineral density in load-bearing bones of both sexes [4], lean body mass is more highly correlated with bone mass than is fat mass [5]. Furthermore, mechanical loading has direct effects on cultured osteoblasts and *ex vivo* dynamic loading stimulates bone formation [6]. In addition, disuse induced by sciatic neurotomy, hindlimb unloading, tendonotomy, casting or limb immobilization using a sling results in bone loss at the site(s) of immobilization [7]. Taken together, these observations suggest that increased weight bearing contributes, but may not be the sole factor, for determining the positive relationship between body mass and bone mass.

Leptin, the protein product of the *ob* (Lep) gene [8], is a pleiotropic hormone that acts on multiple organs, including bone. Leptin is produced predominantly by fat cells, functions as a sentinel of energy balance, and may influence bone metabolism. However, leptin has been variously reported to have bone anabolic, anti-resorptive, and anti-osteogenic effects [9–12].

The precise mechanisms of leptin action on the skeleton are not fully understood. In part, this is because the hormone has the potential to affect bone through multiple pathways; one or more indirect pathways involving central/hypothalamic relays [10, 13, 14] and a direct pathway involving the binding of leptin to its receptors on cartilage and bone cells [9, 11, 15–18]. The direct action of leptin on bone and cartilage cells is readily apparent in cell culture [15, 16, 18, 19]. Bone is highly innervated and some of the indirect effects of leptin on bone may be mediated through the sympathetic arm of the autonomic nervous system [20, 21]; the binding of leptin to its receptors in the hypothalamus stimulates the peripheral cells of the sympathetic nervous system to release noradrenalin which, in turn, could influence bone formation via adrenergic receptors expressed on osteoblasts [10, 14]. However, adrenergic receptors are widely distributed. Thus, changes in sympathetic tone could have collateral effects on bone. Additional indirect effects may be mediated through changes in systemic metabolic bone regulatory factors such as vitamin D, PTH, sex steroids, stress hormones, insulin, IL-6 and IGF-I [22–27].

The obese *ob/ob* mouse, which cannot produce leptin due to an inactivating mutation in the leptin gene, has skeletal abnormalities and exhibits a mosaic skeletal phenotype. Compared to wildtype (WT) mice, *ob/ob* mice have reduced bone length and reduced overall bone mass and strength [11, 12, 19, 28–30]. These findings suggest leptin is required for optimal peak bone growth and quality. However, ob/ob mice have increased cancellous bone in their

vertebra [10, 29], suggesting that the skeletal response to leptin is bone-specific (long bones versus vertebrae) and/or compartment-specific (cortical versus cancellous envelope).

We have recently employed leptin gene transfer technology by non-replicative, non-immunogenic and non-pathogenic virus vector (rAAV) to augment leptin availability selectively in the hypothalamus and determine if hypothalamic leptin, in addition to reversing established obesity, corrects abnormalities in bone architecture in *ob/ob* mice [30]. In brief, long-duration (15 weeks) hypothalamic rAAV-lep gene therapy in *ob/ob* mice restored all bone measurements to values that did not differ from WT mice. Specifically, leptin gene therapy increased femur length and total femur bone volume and decreased distal femur cancellous bone volume. Circulating leptin was not detected in the rAAV-lep treated mice. We interpret the results as evidence that increasing hypothalamic leptin in *ob/ob* mice to levels sufficient to prevent obesity abolishes the skeletal abnormalities that accompany the inability to produce leptin. Importantly, an additional 15 weeks of hypothalamic leptin gene therapy (total 30 weeks) had no additional effect on femur length, total femur bone mass or cancellous bone volume in distal femur or lumbar vertebra.

The objective of the present study was to evaluate the effects of global leptin deficiency, and selective central repletion of leptin on the response of long bones and vertebrae to body mass gain induced by a high fat diet. To accomplish this goal, we investigated WT and *ob/ob* mice as well as the effects of hypothalamic leptin expression following a single intracerebroventricular (icv) injection of rAAV-lep.

2. Materials and Methods

2.1. Experiment 1

A preliminary study was performed to determine if a positive relationship between body mass and bone mass exists in normal mice fed a standard diet *ad libitum*. To accomplish this, 20 male 8-week-old C57BL/6 (WT) mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Ten mice were sacrificed one week after arrival. The remaining 10 animals were maintained (2–3 mice/cage) in a temperature- and light-controlled room and fed a normal diet (LM-485, Teklad, Madison, WI; caloric density 3.4 kcal/g, 11% of kcal from fat) *ad libitum* for an additional 15 weeks and sacrificed at 24 weeks of age. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Oregon State University where the study was conducted. Two additional studies were performed to evaluate the role of leptin in mediating the skeletal response to increased body mass.

2.2. Experiment 2

Eight to ten-week-old, male, leptin-deficient *ob/ob* (weighing 40–50 g) mice (Jackson Laboratory, Bar Harbor, Maine) were used in the experiment. The animals were housed in individual cages in a temperature (21–23°C)- and light-controlled room (lights on 6am–6pm) under specific pathogen-free conditions. Food and water were available *ad libitum* to all mice. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL) where the study was conducted.

2.2a. Construction and Packaging of rAAV Vectors—The rAAV-lep and rAAV-green fluorescent protein (rAAV-GFP, control vector) vectors were constructed and packaged as described elsewhere [31]. In brief, the vector pTR-CBA-Ob EcoRI fragment of pCR-rOb containing rat leptin cDNA was subcloned into rAAV vector plasmid pAAVβGEnh after deleting the EcoRI fragment carrying the β-glucoronidase cDNA

sequence [32–36]. The control vector, rAAV-GFP, was similarly constructed to encode the GFP gene [32, 34–36].

2.2b. Vector Administration—For vector administration, the mice were anesthetized with sodium pentobarbital (60 mg/kg, ip), placed on a Kopf stereotaxic apparatus with mouse adapter for icv injection, and injected icv with either rAAV-lep (9×10^7 particles; n=17) or rAAV-GFP (9×10^7 particles; n=17). The coordinates employed for microinjector placement in the 3rd cerebroventricle were 0.3 mm posterior to bregma, 0.0 lateral to midline, and 4.2 mm below the dura [37]. Procedures to verify icv injections are detailed elsewhere [31–34, 37–43].

2.2c. Diets—At 7 weeks post-vector injection (16 weeks of age; an age when the mice are approaching skeletal maturity), rAAV-GFP and rAAV-lep groups were divided into two groups each: one group continued to consume the regular chow diet (control diet) (LM-485, Teklad, Madison, WI) and the other was switched to a high fat diet (45 % of kcal from fat, primarily from lard; Research Diets, New Brunswick, NJ; n= 8 – 9/group) fed *ad libitum* for 8 weeks. The caloric density of the control diet was 3.4 kcal/g, whereas the caloric density of the high fat diet was 4.7 kcal/g. The ingredient and nutrient composition of the two diets was not identical. However, according to the manufacturers, both diets were nutritionally complete. The mice were sacrificed at 24 weeks of age. Food intake, body mass, hormone levels (e.g., leptin and insulin), and hypothalamic mRNA expression in response to central rAAV-lep gene therapy in this experiment are detailed elsewhere [37].

2.3. Experiment 3

When placed on the high fat diet, the animals in experiment 2 were mature. Bone mass is rapidly accrued in the interval immediately following puberty. To establish the effect of growth rate, we performed an additional experiment in post-pubertal mice. The design for Experiment 3 was similar to Experiment 2 with the exception that 4-week-old, male, leptin-deficient *ob/ob* (weighing 27–31 g) and WT mice (16–18 g) (Jackson Laboratory, Bar Harbor, Maine) were used in the experiment. After a one-week acclimation, the *ob/ob* mice were anesthetized and injected with rAAV-lep or rAAV-GFP. The WT mice were left untreated. At 5 weeks post-vector injection (10 weeks of age), rAAV-GFP and rAAV-lep groups were divided into two groups each: one group continued to consume the control regular chow diet and the other was switched to the high fat diet consumed *ad libitum* for 8 weeks as in Experiment 2. At 10 weeks of age, the WT mice were also divided into two subgroups: one group continued to consume the regular control diet and the other was switched to the high fat diet. The mice were sacrificed at 18 weeks of age. The effect of central rAAV-lep gene therapy and high fat diet on body mass and hormone levels (e.g., leptin and insulin) in this experiment are detailed elsewhere [24].

2.4. Tissue Collection and Analyses

For tissue collection, the mice were anesthetized with sodium pentobarbital (60 mg/kg; ip) and killed by exsanguination. Femora and 3rd lumbar vertebrae (LV3) were excised for analysis. Micro computed tomography (microCT) was used for nondestructive three-dimensional evaluation of bone architecture. In mice from Experiments 1 and 2, right femora were scanned using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 12 × 12 × 12 μm (x-ray voltage 55 kV_P) and evaluated at a threshold of 265 (gray scale, 0–1,000) determined empirically. A similar procedure was used to scan LV3 in mice from Experiments 1, 2, and 3. Entire femora (cancellous + cortical bone) were evaluated followed by evaluation of cortical bone at the femoral midshaft and cancellous bone in the distal femoral metaphysis. For the femoral midshaft, 100 slices (1.2 mm) of bone were evaluated and total cross-sectional tissue volume (cortical and marrow

volume, mm^3), cortical volume (mm^3), marrow volume (mm^3) and cortical thickness (μm) were measured. For the femoral metaphysis, 200 slices (2.4 mm) of bone were measured and included secondary spongiosa only. Analysis of the lumbar vertebra included the total bone (Experiment 1 only) and the entire region of secondary spongiosa between the cranial and caudal growth plates. Direct cancellous bone measurements in the femur and lumbar vertebra included: cancellous bone volume (bone volume unadjusted for tissue volume, mm^3), cancellous bone volume/tissue volume (volume of total tissue occupied by cancellous bone, %), trabecular thickness (mean thickness of individual trabeculae, μm), 4) trabecular number (number of trabeculae within the samples tissue, 1/mm), and trabecular separation (the distance between trabeculae, μm) [44].

2.5. Statistical Analysis

A Pearson's correlation coefficient was used to determine the relationship between body mass and bone volume and architecture in 9- and 24-week-old WT mice fed control diet in Experiment 1. The effects of vector administration (with 2 levels, rAAV-GFP and rAAV-lep), body mass (with 2 levels: normal and high) and their interaction were investigated for each dependent variable using a two-way ANOVA. Least-squares means (LS-means) were produced when the interaction term was significant. Femur length and volume was not directly measured in several specimens (n= 9/34 distributed across treatment groups) in Experiment 2 due to minor damage, usually to the femoral head, during tissue recovery. A multiple imputation based on correlation of existing endpoints was used to estimate the missing values [45]. A t-test was used to evaluate diet effects on bone in WT mice in Experiment 3. All statistical analyses were performed using the SAS program (SAS, Cary, NC). Differences were considered significant at $p<0.05$. All data are presented as mean±SE.

3. Results

Experiment 1

The objective of Experiment 1 was to determine the relationship between body mass and bone volume in growing (9 weeks old) and adult (24 weeks old) male C57BL/6 mice fed regular control diet. Positive correlations between terminal body mass and total femur bone volume were observed at both 9 and 24 weeks of age (Figure 1a–b). Positive correlations were likewise observed between body mass and cortical bone at the femoral midshaft (cross-sectional volume, cortical volume, and cortical thickness) as well as body mass and cancellous bone (cancellous bone volume and cancellous bone volume/tissue volume) in the distal femur in mice of both age groups (Table 1). The Pearson's correlation coefficient ranged from 0.68 to 0.89, indicating that body mass accounted for 46–79% of variation in bone mass depending on site and age examined. Significant positive correlations were also noted between body mass and total vertebral bone volume (Figure 1c–d) and vertebral cancellous bone volume (Table 1) in both 9- and 24-week-old mice. The Pearson's correlation coefficient ranged from 0.65 to 0.90 depending on site and age examined. In contrast, a significant relationship between body mass and vertebral cancellous bone volume adjusted for tissue volume was not detected in either the 9- or 24-week-old mice (Table 1).

Experiment 2

The objective of Experiment 2 was to evaluate the effects of global leptin deficiency and selective central leptin repletion on the skeletal response to body mass gain induced by high fat intake. The effect of central leptin gene therapy (vector) and high fat diet on body mass is shown in Figure 2. Central rAAV-lep treatment resulted in lower body mass, whereas high fat diet resulted in higher body mass. The high fat diet increased body mass in both the rAAV-GFP-treated and rAAV-lep-treated mice without increased caloric intake [37]. There

was no interaction between vector administration and diet, indicating that high fat intake results in increased body mass, irrespective of central leptin.

The effect of central leptin gene therapy and increased body mass due to high fat intake on bone volume and architecture in the femur is shown in Figures 3–4. Femora were longer in rAAV-lep-treated mice compared to rAAV-GFP-treated mice and longer in the heavier mice fed high fat diet than those fed control diet (Figure 3a). rAAV-lep treatment had no effect on total femur bone volume whereas increased body mass resulted in greater total femur bone volume (Figure 3b). There was no interaction between vector treatment and body mass for either femur length or total femur bone volume, indicating that increased body mass results in increased femoral bone mass, irrespective of central leptin.

rAAV-lep had no significant effect on midshaft cortical bone architecture (Figure 3c–f). In contrast, increased body mass resulted in greater midshaft cross-sectional volume, cortical volume, and cortical thickness. There were no significant vector × body mass interactions for any of the cortical endpoints examined.

In the distal femur, rAAV-lep treatment resulted in lower cancellous bone volume/tissue volume (Figure 4). This was due to lower trabecular thickness in the rAAV-lep in comparison to the rAAV-GFP-treated animals. Increased body mass had no independent effect on cancellous bone volume or architecture, and no significant interactions between vector treatment and body mass were noted.

In the lumbar vertebrae, rAAV-lep resulted in lower cancellous bone volume/tissue volume, lower trabecular number and thickness, and higher trabecular spacing (Figure 5). The heavier animals fed high fat diet had higher trabecular thickness compared to controls. Although significant vector × body mass interactions were noted for cancellous bone volume/tissue volume, trabecular number, and trabecular spacing, only trabecular number was lower in leptin-deficient rAAV-GFP mice consuming high fat diet compared to rAAV-GFP mice consuming control diet.

Experiment 3

The first objective of Experiment 3 was to assess the effects of high fat diet-induced gain in body mass on bone in WT mice. In the WT mice, body mass and vertebral cancellous bone volume/tissue volume were higher in mice consuming high fat diet than in mice consuming control diet (Table 2). Significant body mass effects were not detected for trabecular number, thickness or spacing.

A second objective of Experiment 3 was to further evaluate global leptin deficiency, and selective central repletion of leptin on the skeletal response to body mass gain induced by a high fat intake. In contrast to Experiment 2, central leptin repletion and the high fat diet were initiated at an earlier age. However, the results of the two studies were nearly identical. As in Experiment 2, central rAAV-lep vector administration in *ob/ob* mice resulted in lower body mass and lower vertebral cancellous bone volume/tissue volume, lower trabecular number and trabecular thickness, and greater trabecular spacing (Table 2). Body mass had no independent effect on any of the vertebral cancellous bone endpoints evaluated. Significant vector × body mass interactions were noted for bone volume/tissue volume, trabecular number, and trabecular spacing. In the rAAV-GFP *ob/ob* mice, trabecular number was lower in animals consuming high fat diet in comparison to those consuming control diet. In the rAAV-lep *ob/ob* mice, trabecular number was higher and trabecular spacing was lower in the heavier animals consuming high fat compared to those consuming control diet.

4. Discussion

The positive relationship between body mass and bone mass in adult humans is well established [2, 3]. The results of our first study demonstrate that there is also a positive relationship between body mass and bone mass in growing and mature WT mice consuming regular mouse chow *ad libitum*. Leptin is a potential mediator of the effects of body mass on bone. In general, there is a strong positive correlation between adipose tissue mass and serum leptin. Thus, increased serum leptin levels are usually associated with weight gain. However, the relationship between fat and leptin is abolished in leptin-deficient *ob/ob* mice. We, therefore, evaluated the role of leptin in mediating the skeletal response to increased body mass by comparing the response of WT and leptin-deficient *ob/ob* mice to a high fat diet. Our results demonstrate that leptin status and body mass have independent as well as interactive effects on bone mass and architecture.

ob/ob mice are obese and compared to WT mice have a mosaic skeletal phenotype; the skeletal abnormalities include a decrease in overall bone mass, but site-specific increases in cancellous bone volume [29]. Administration of leptin to growing *ob/ob* mice restores bone architecture to near normal [30]. In concordance with our results, Ducy et al. [10] have shown that increasing hypothalamic leptin via short-term (4 weeks) icv infusion results in decreased cancellous bone volume in vertebrae of *ob/ob* mice. As shown in earlier studies [11, 12, 19, 28, 29], leptin deficiency results in decreased femur length and mass. Furthermore, administration of systemic leptin increases formation of cortical bone and corrects the growth plate abnormalities in *ob/ob* mice [11, 12, 19, 28, 29]. Increasing body mass through increased high fat intake resulted in an increase in cortical bone mass in femora of WT mice. This was independent of leptin signaling because it also occurred in leptin-deficient *ob/ob* mice. We interpret these results as evidence that increased body mass and leptin signaling have independent positive effects on cortical bone growth. In contrast, body mass had no independent effect on cancellous bone in distal femur or lumbar vertebra. However, the significant interaction between leptin status and body mass on several bone parameters suggests leptin may play a permissive role in increased body mass-induced bone changes in vertebra.

Circulating leptin was not detected in the rAAV-lep-treated *ob/ob* mice [37, 43], indicating that systemic leptin is unnecessary for the observed skeletal effects of leptin gene therapy in mice fed either control or high fat diet. Similarly, central but not systemic leptin is required for the observed effect of leptin gene therapy to reduce body mass.

Leptin deficient *ob/ob* obese mice develop multiple abnormalities in homeostatic regulatory pathways as evidenced by elevated corticosterone and decreased thyroid hormone levels [46]. Loss of leptin restraint on insulin secretion and glucose metabolism in *ob/ob* mice increases the risk for developing type 2 diabetes and gonadal insufficiency results in sterility [37, 43, 47, 48]. However, it is unlikely that the *ob/ob* phenotype is entirely due to leptin deficiency. Studies in Balb/cJ mice suggest that modifier genes alter the sterile-obese phenotype of *ob/ob* mice by reducing their obesity and stimulating their reproductive system despite the absence of leptin [49]. Nevertheless, the importance of leptin is illustrated by the observation that rAAV-lep treatment reverses the abnormalities in *ob/ob* mice [47, 48].

Increased adiposity in *ob/ob* mice is associated with increased blood levels of proinflammatory cytokines such as IL-6 and C-reactive protein [24]. Also, *ob/ob* mice have elevated serum IGF-I levels and can develop secondary hyperparathyroidism, hypercalcemia and abnormalities in vitamin D metabolism [25, 27]. A high fat diet may also impact bone regulatory factors such as insulin, IGF-I and proinflammatory cytokines [50–52]. Regarding bone, sex steroids, proinflammatory cytokines, corticosterone, thyroxine, insulin, vitamin D,

parathyroid hormone and IGF-I influence bone growth and turnover. Changes in these hormones and cytokines may play a role in the skeletal adaptation to a high fat diet, leptin deficiency, or hypothalamic rAAV-lep treatment. Thus, our data does not distinguish between the direct effects of leptin or body mass on bone from indirect effects mediated by these intermediates.

Obesity resulting from a high fat diet may be the result of decreased leptin signaling. Resistance to leptin has been postulated to occur due to reduced leptin entry across the blood brain barrier despite chronic systemic hyperleptinemia [42, 43, 53–55] or down regulation of leptin receptor signaling [56]. Alternatively, inadequate leptin signaling may be due to suboptimal hypothalamic leptin levels [57] or an opposing regulatory mechanism [58]. Central leptin therapy bypasses the blood brain barrier by providing a local source of leptin in the hypothalamus. This approach results in long duration body mass reduction in wild type and genetically obese *ob/ob* mice [26, 37, 43, 47, 48], indicating that this method does not down regulate the leptin receptor. Our replication of the effect of daily systemic and central injections [59, 60] indicates that leptin produced by increased leptin transgene expression [26, 37, 43, 47, 48] was biologically active for the duration of the 15-week experiments described here, a finding that is in agreement with even longer studies performed in rodents [26, 31, 42, 61].

It has been reported that the central effects of leptin on bone metabolism are mediated by increased sympathetic signaling [10, 14]. Neither sympathectomy nor capsaicin treatment of neonatal rats had an effect on bone growth. However, the skeletal response to a mechanical perturbation was altered [20]. Interestingly, sensory denervation increased, whereas sympathectomy decreased the normal response. Thus, leptin acting through the central nervous system may influence the bone anabolic response to increased body mass. Loading has a direct stimulatory effect on osteoblasts in culture [62] and on sympathectomized limbs [63]. It is possible, however, that the adaptive effects of leptin and body mass are more readily apparent at low levels of loading. This could explain the lack of a requirement for leptin signaling in the femur. This possibility is supported by the observation that the response of bone to systemic factors can vary between skeletal sites depending upon the magnitude of loading [64].

Severe dietary restriction, rather than a genetic defect, is the most common cause of leptin insufficiency in humans [65]. It is, therefore, possible that leptin insufficiency, as well as a low body mass, contributes to the low bone mass in adolescents with eating disorders. The composition of the normal and high fat diets in our studies was not identical. Therefore, we cannot rule out the possibility that bone active components of the diet contribute to the skeletal changes associated with a high fat diet. However, there was a significant correlation between body mass and bone mass in mice fed normal diet (Experiment 1). This result suggests that the increase in bone mass associated with feeding mice a high fat diet is associated with the increase in body mass and not the diet *per se*.

In summary, increased body mass has a positive effect on bone mass in WT mice. This was found to be the case in animals consuming normal and high fat diets. Studies in the *ob/ob* mice demonstrate that leptin is not required for increased cortical bone volume associated with increased body mass but may play a role in the positive effects of body mass gain on cancellous bone volume. These differential effects of leptin and body mass on various skeletal sites may help explain the complex skeletal phenotype in *ob/ob* mice.

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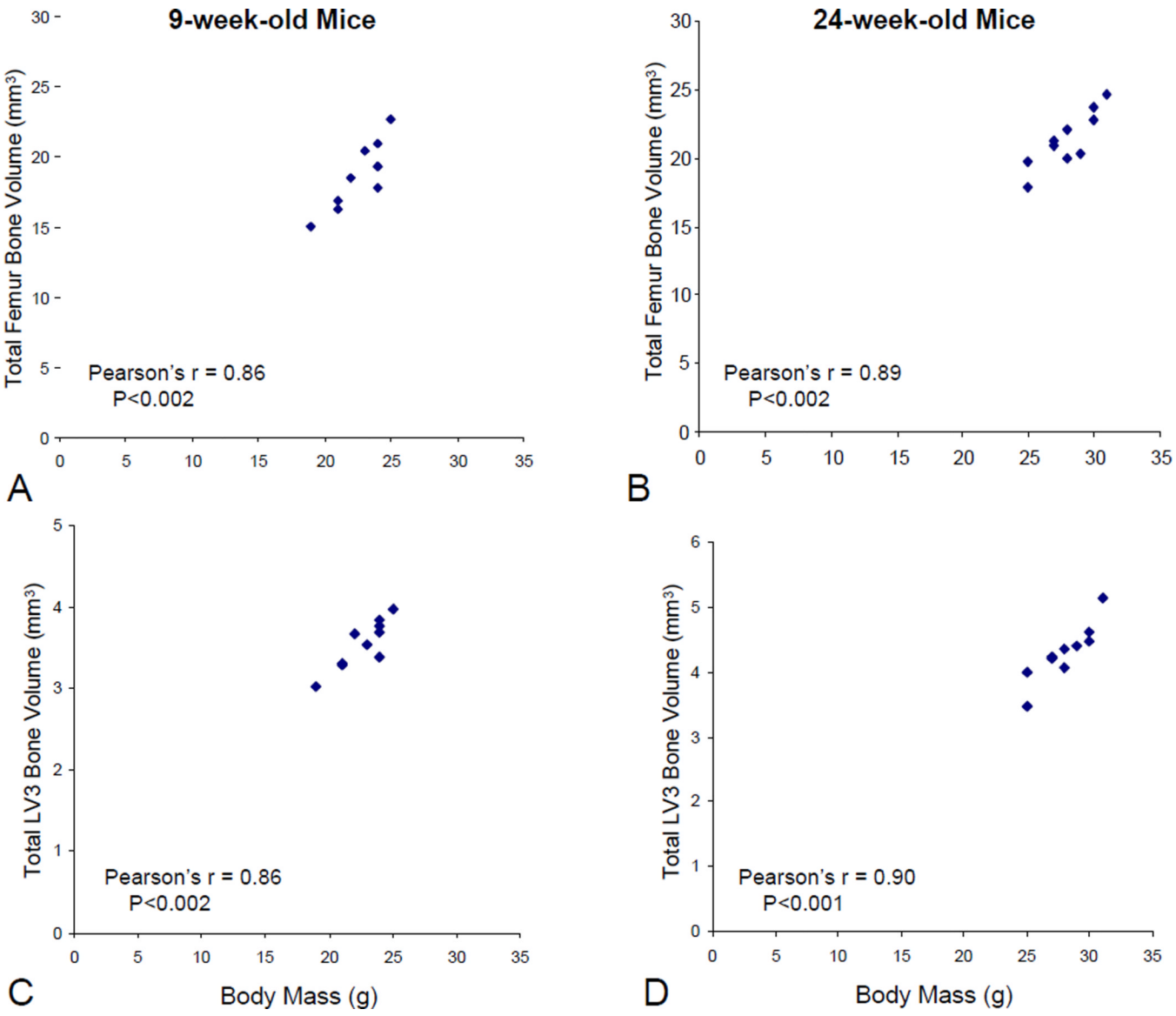

Figure 1.

Correlation between body mass and total femur bone volume (a and b) and body mass and total vertebra (LV3) bone volume (c and d) in 9 and 24-week-old male C57BL/6 mice, respectively, fed standard mouse chow (Experiment 1).

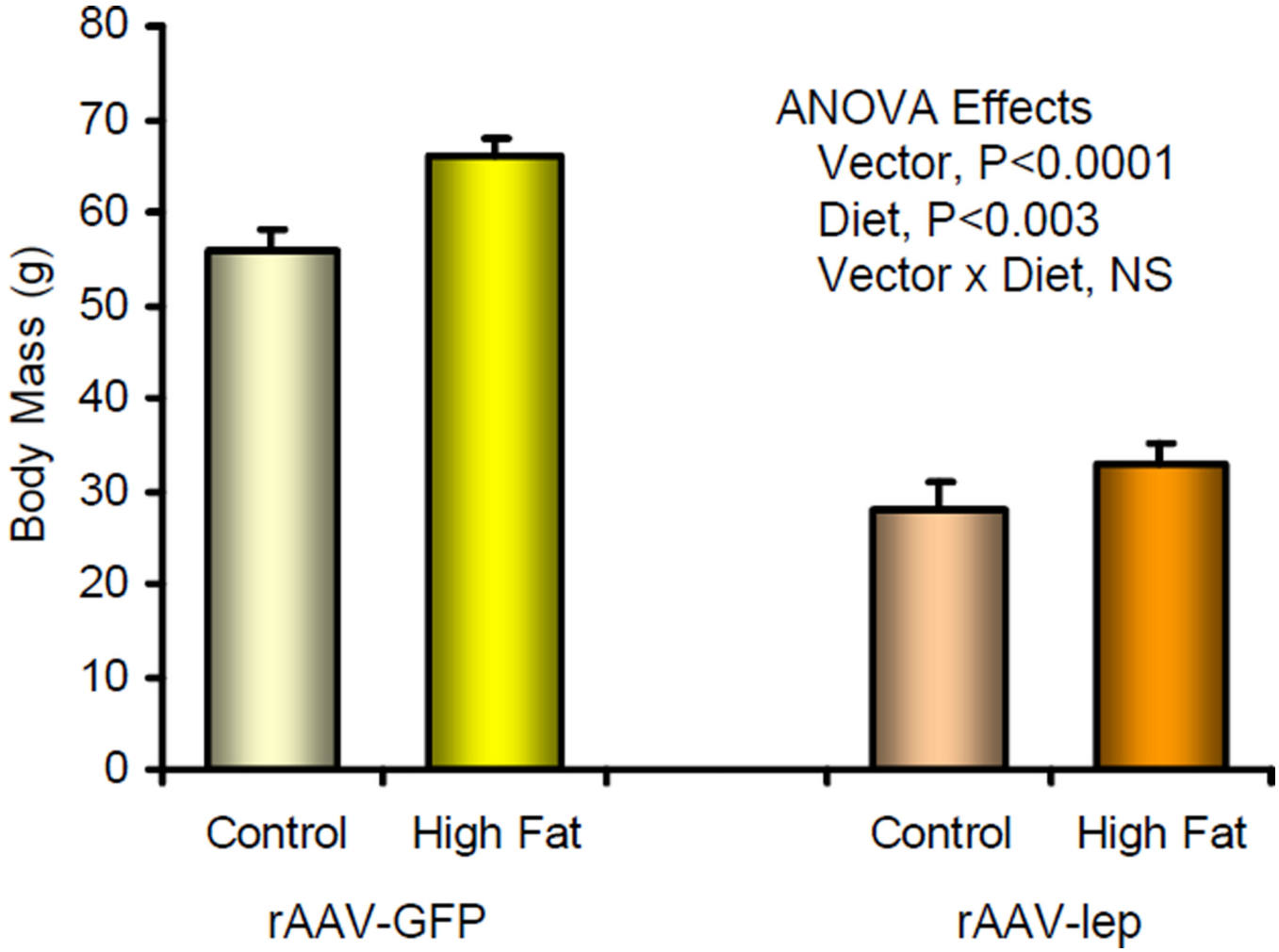

Figure 2.
Effect of central leptin gene therapy and high fat diet on body mass in *ob/ob* mice administered either rAAV-GFP (control vector) or rAAV-lep icv (Experiment 2). All data are mean ± SE. NS, not significant, P>0.05.

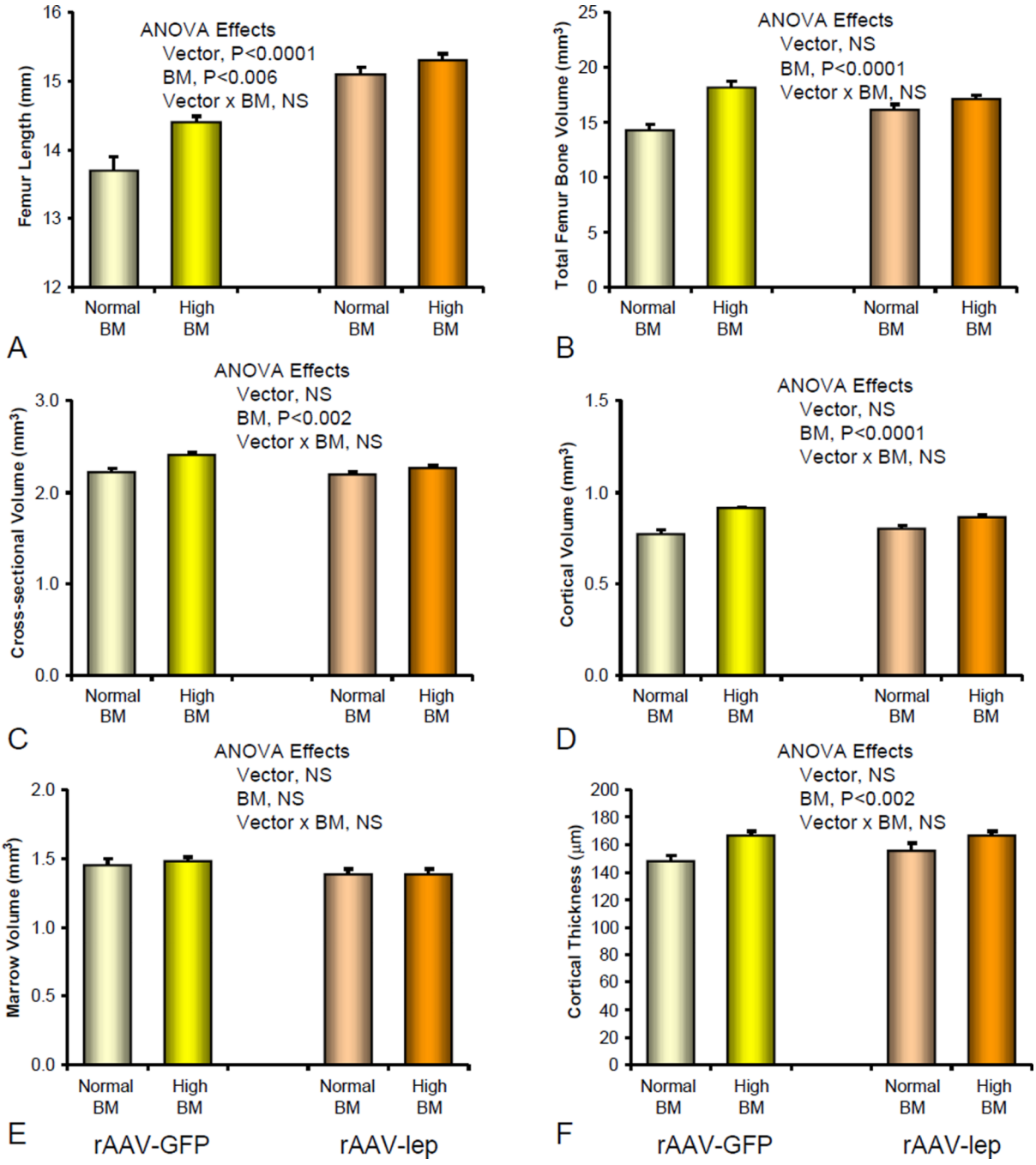

Figure 3.

Effect of central leptin gene therapy and increased body mass (BM) on femur length (a) total femur bone volume (b), midshaft femur cross-sectional volume (c), midshaft femur cortical volume (d), midshaft femur marrow volume (e), and midshaft femur cortical thickness (f) in *ob/ob* mice administered either rAAV-GFP (control vector) or rAAV-lep icv (Experiment 2). All data are mean ± SE. NS, not significant, P>0.05.

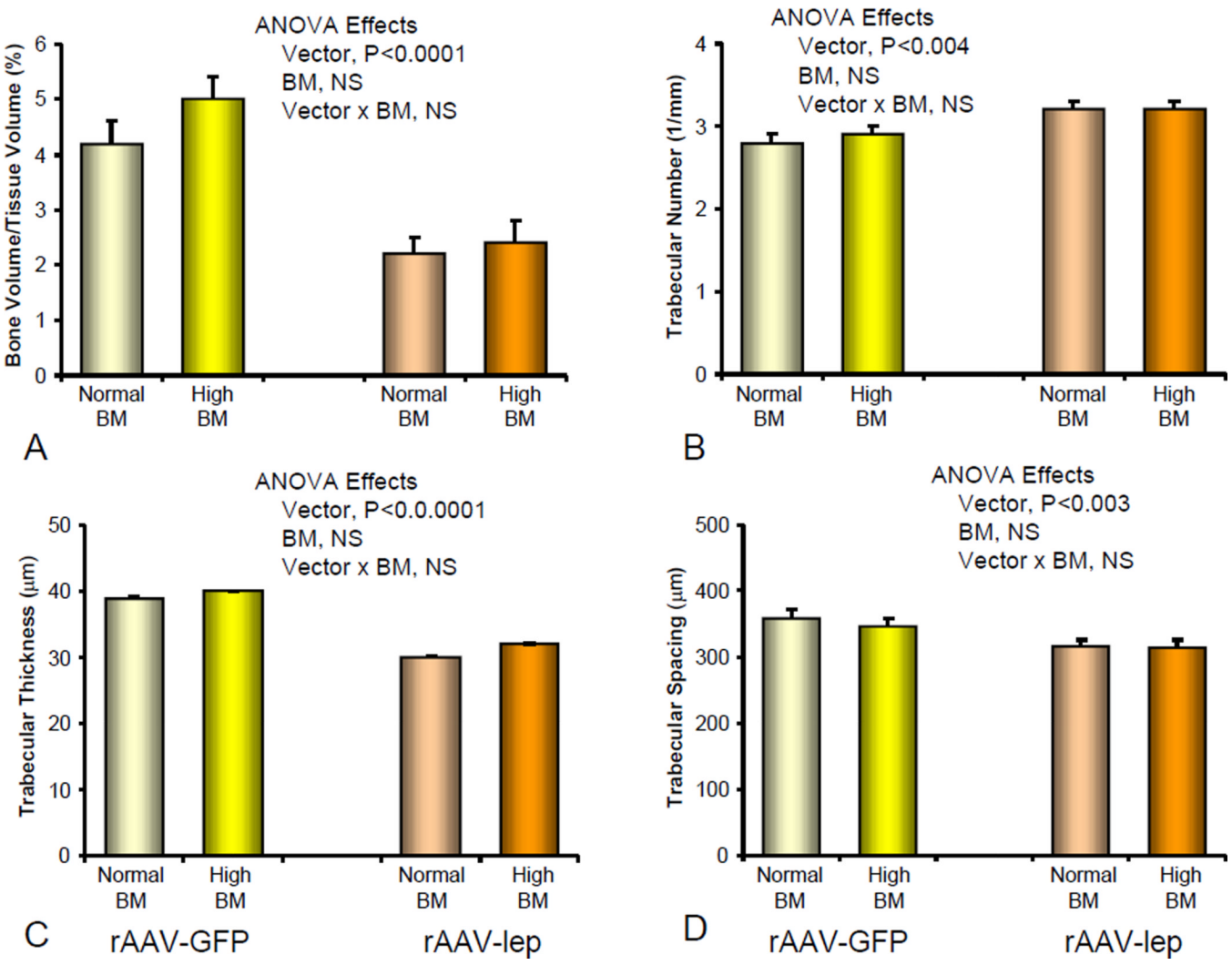

Figure 4.

Effect of central leptin gene therapy and increased body mass (BM) on distal femur bone volume/tissue volume (a), trabecular number (b), trabecular thickness (c), and trabecular spacing (d) in *ob/ob* mice administered either rAAV-GFP (control vector) or rAAV-lep icv (Experiment 2). All data are mean ± SE. NS, not significant, P>0.05.

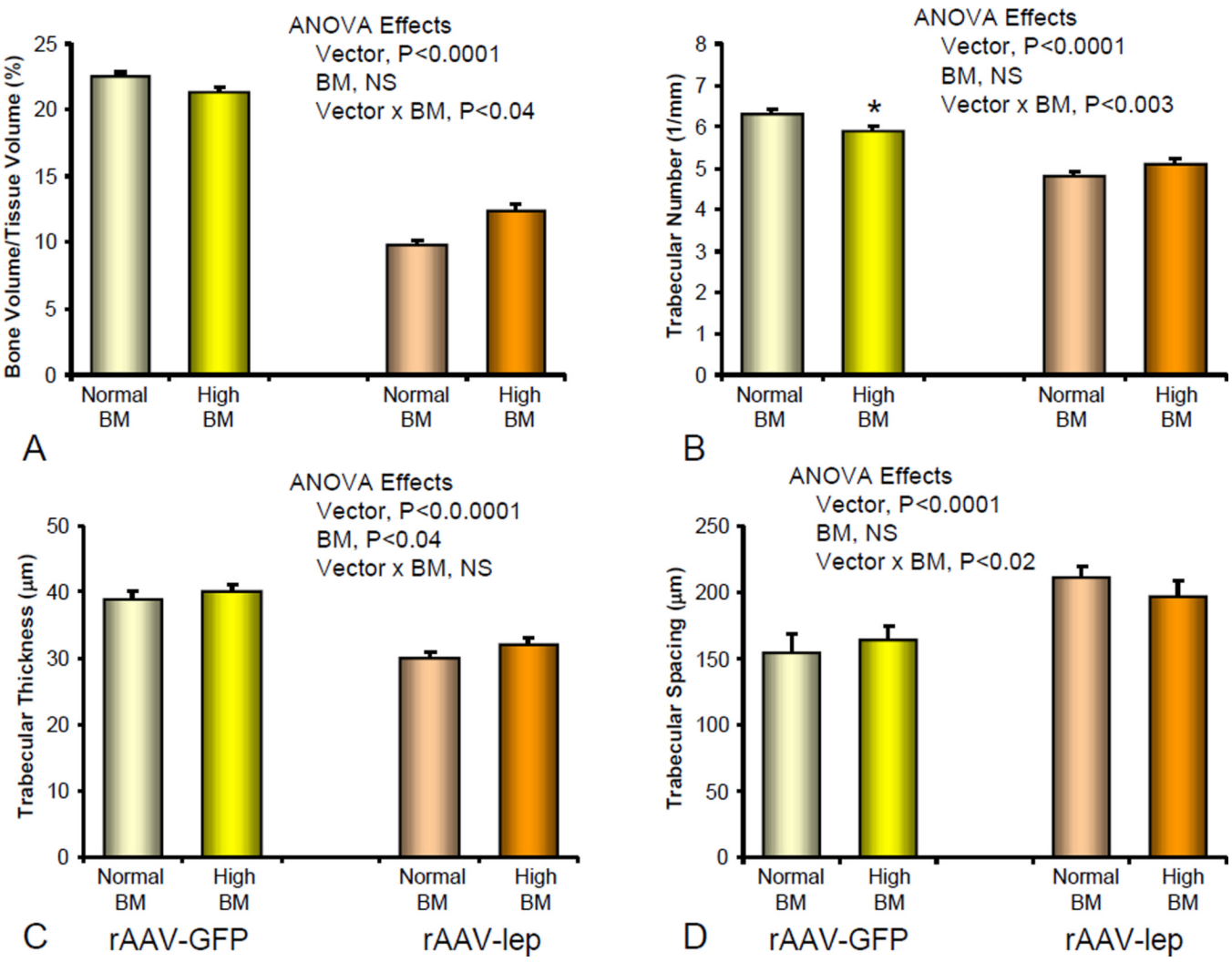

Figure 5.

Effect of central leptin gene therapy and increased body mass (BM) on vertebral (LV3) bone volume/tissue volume (a), trabecular number (b), trabecular thickness (c), and trabecular spacing (d) in *ob/ob* mice administered either rAAV-GFP (control vector) or rAAV-lep icv (Experiment 2). All data are mean ± SE. *P<0.05. NS, not significant, P>0.05.

Table 1

Correlation between body mass and cortical bone in the midshaft femur and cancellous bone in the distal femur and 3rd lumbar vertebra (Experiment 1).

	9-week-old Mice		24-week-old Mice	
	Pearson's r	**P<**	**Pearson's r**	**P<**
Midshaft Femur				
Cross-sectional volume (mm^3)	0.81	0.005	0.88	0.01
Cortical volume (mm^3)	0.88	0.001	0.86	0.02
Cortical thickness (μm)	0.80	0.006	0.75	0.05
Distal Femur				
Bone volume (mm^3)	0.69	0.03	0.80	0.02
Bone volume/Tissue volume (%)	0.68	0.04	0.68	0.05
Lumbar Vertebra				
Bone volume (mm^3)	0.65	0.04	0.82	0.004
Bone volume/Tissue volume (%)	0.59	NS	0.55	NS

NS, Not significant

Table 2

Effects of increased body mass (due to high fat intake) on vertebral (LV3) cancellous bone volume and architecture in wildtype mice and in *ob/ob* mice administered hypothalamic rAAV-GFP (control vector) or rAAV-lep (Experiment 3).

	Wild Type Mice			*ob/ob* Mice						
	Body Mass			rAAV-GFP Body Mass		rAAV-lep Body Mass		ANOVA (P<)		
Endpoint	Normal	High	ANOVA (P<)	Normal	High	Normal	High	Vector Treatment	BM	Vector × BM
Body mass (g)	24.5 ± 0.2	28.0 ± 1.0	0.005	55.2 ± 3.1	65.1 ± 1.9	21.6 ± 0.9	23.1 ± 0.8	0.0001	0.04	NS
Bone volume/Tissue volume (%)	11.4 ± 0.9	15.2 ± 1.0	0.016	24.0 ± 0.9	21.3 ± 1.7	9.0 ± 0.5	11.0 ± 0.4	<0.0001	NS	0.042
Trabecular number (1/mm)	4.6 ± 0.2	5.0 ± 1.1	NS	6.1 ± 0.1	5.7 ± 0.1 *	4.7 ± 0.1	5.0 ± 0.1 *	<0.0001	NS	0.008
Trabecular thickness (μm)	37 ± 2	39 ± 1	NS	43 ± 1	44 ± 1	33 ± 1	34 ± 1	<0.0001	NS	NS
Trabecular spacing (μm)	217 ± 8	198 ± 5	NS	159 ± 3	169 ± 5	213 ± 5	197 ± 3 *	<0.0001	NS	0.008

Data are mean ± SE

BM, Body mass

NS, Not significant

* Significantly different from Normal within same vector treatment, P<0.05