

# ***N*-Acetylcysteine Supplementation Decreases Osteoclast Differentiation and Increases Bone Mass in Mice Fed a High-Fat Diet<sup>1–3</sup>**

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## **Abstract**

Obesity induced by high-fat (HF) diets increases bone resorption, decreases trabecular bone mass, and reduces bone strength in various animal models. This study investigated whether *N*-acetylcysteine (NAC), an antioxidant and a glutathione precursor, alters glutathione status and mitigates bone microstructure deterioration in mice fed an HF diet. Forty-eight 6-wk-old male C57BL/6 mice were randomly assigned to 4 treatment groups ( $n = 12$  per group) and fed either a normal-fat (NF (10% energy as fat)) or an HF (45% energy as fat) diet ad libitum with or without NAC supplementation at 1 g/kg diet for 17 wk. Compared with the NF groups, mice in the HF groups had higher body weight, greater serum leptin concentrations and osteoclast differentiation, and lower trabecular bone volume, trabecular number, and connectivity density ( $P < 0.05$ ). NAC supplementation increased the serum-reduced glutathione concentration and bone volume and decreased osteoclast differentiation in HF-fed mice ( $P < 0.05$ ). We further demonstrated that osteoclast differentiation was directly regulated by glutathione status. NAC treatment of murine macrophage RAW 264.7 cells in vitro increased glutathione status and decreased osteoclast formation. These results show that NAC supplementation increases the bone mass of obese mice induced by an HF diet through elevating glutathione status and decreasing bone resorption. J. Nutr. 144: 289–296, 2014.

## **Introduction**

Obesity, a major public health problem affecting >300 million people globally, is a risk factor for many chronic health disorders, including osteoporosis, mainly because of the increase in inflammatory production and oxidative stress that comes with obesity (1–3).

Recent animal and epidemiologic findings have demonstrated that obesity is inversely associated with bone mass in a variety of conditions, such as aging (4), postmenopausal estrogen deficiency (5), high-fat (HF)<sup>4</sup> diet-induced obesity in an animal model (6,7), chronic use of glucocorticoids (5,8), thiazolidinedione treatment of diabetes (9), or physical activity (10). Excessive fat mass is also found to be associated with bone fragility (11–13). We and others

have demonstrated that obesity induced by an HF diet either increases bone resorption or decreases bone formation, leading to reduced bone mass and strength (6,7,14–16) in various animal models, despite an increase in body weight.

Increased oxidative stress and elevated production of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, are possible causal factors affecting bone metabolism in obesity among several mechanisms that have been proposed (5,17). Proinflammatory cytokines and oxidative stress are capable of stimulating osteoclast differentiation and activation (18–22), and reactive oxygen species (ROS) directly stimulate osteoclast formation and activity, leading to bone resorption (20,21). Restoring antioxidant defenses by administration of *N*-acetylcysteine (NAC), an antioxidant and glutathione precursor, or ascorbate inhibited the receptor activator of NF- $\kappa$ B ligand (RANKL)-induced osteoclastogenesis (23) and prevented estrogen deficiency-induced bone loss in mice (24). In contrast, increasing oxidative stress by depleting glutathione with a specific glutathione synthesis inhibitor, L-buthionine-(S,R)-sulphoximine (BSO), increased osteoclast differentiation and caused substantial bone loss (24). Furthermore, NAC supplementation in diet has been shown to mitigate bone loss and the downregulation of Wnt signaling, as well as the decrease in serum bone-formation markers in the ethanol-induced oxidative stress mouse model (25).

To investigate the effects of NAC supplementation on bone metabolism in obesity, we evaluated bone microstructure of the distal femur by microcomputed tomography (micro-CT) and cultured bone marrow cells for osteoclast differentiation

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<sup>3</sup> Supplemental Table 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

<sup>4</sup> Abbreviations used: BSO, L-buthionine-(S,R)-sulphoximine; BV, bone volume; Conn.Dn, connectivity density; GSH, reduced glutathione; GSSG, oxidized glutathione disulfide; HF, high-fat (45% energy as fat); Itgb3, integrin  $\beta$ 3; micro-CT, microcomputed tomography; NAC, *N*-acetylcysteine; NF, normal-fat (10% energy as fat); RANK, receptor activator NF- $\kappa$ B; RANKL, receptor activator of NF- $\kappa$ B ligand; ROS, reactive oxygen species; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TRAP, tartrate-resistant acid phosphatase; TV, tissue volume.

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from mice fed a normal-fat (NF) or HF diet supplemented with or without NAC (1 g/kg diet) for 17 wk. In addition, we measured glutathione status and osteoclast formation in cultured osteoclast-like RAW 264.7 cells treated with BSO and NAC. We hypothesized that NAC supplementation would elevate glutathione status and improve bone microstructure in HF diet-induced obese mice.

## Materials and Methods

**Animals, diets, and treatments.** Forty-eight male C57BL/6 mice, 6 wk old, were obtained from Charles River Laboratories (Wilmington, MA). All mice were housed in Plexiglas ventilated cages (2 mice per cage) in an environmentally controlled pathogen-free facility with a 12-h light/dark cycle. The animal protocol for the study was approved by the USDA Grand Forks Human Nutrition Research Center Animal Care and Use Committee. Mice were maintained and processed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. Mice were allowed to acclimate to our animal facility for 3 d on Purina Rat Chow #5012 (Ralston-Purina, St. Louis, MO) before being randomly assigned to 4 treatment groups ( $n = 12$  per group). During the study, mice were fed either a control purified NF diet (10% energy as fat; D12450B; Research Diets, New Brunswick, NJ) based on AIN-93G (26) or an HF diet (45% energy as fat) with extra fat from lard for 17 wk (Supplemental Table 1) or NF and HF diets supplemented with NAC (Sigma-Aldrich, St. Louis, MO) at 1 g/kg diet (27). We estimate that NAC intake was ~5–10 mg/d or 200 mg/kg, assuming that average feed intake was 5–10 g/d in mice. Mice had access ad libitum to diets and tap water throughout the study. Body weight was recorded weekly.

**Sample preparation.** At the end of the study, mice were killed with a ketamine cocktail [1.37:1 mixture of ketamine (Animal Health, St. Joseph, MO):xylazine (Phoenix Scientific, St. Joseph, MO)].

Blood samples were collected and centrifuged at  $1500 \times g$  for 20 min at  $4^{\circ}\text{C}$ , and the serum was separated and stored at  $-80^{\circ}\text{C}$  until analyzed.

The right femur and tibia of each mouse were removed and cleaned of adherent tissue for the marrow harvest. The bones were briefly immersed in 70% ethanol (3 s) and stored in  $\alpha$ -Minimum Essential Medium containing L-glutamine and nucleosides, and supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% fungizone temporarily before marrow cells were isolated and cultured as outlined below.

The left femur was collected and stored at  $-20^{\circ}\text{C}$  before being submitted to scans with micro-CT as described below.

**Bone structure evaluation with micro-CT.** The left femur from each mouse was cleaned of adherent tissue and scanned using a Scanco micro-CT scanner ( $\mu\text{CT-40}$ ; Scanco Medical, Bassersdorf, Switzerland) at  $12\text{ }\mu\text{m}$  isotropic voxel size with X-ray source power of 55 kV and 145  $\mu\text{A}$  and integration time of 300 ms. The grayscale images were processed 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 soft tissue and marrow phase. The reconstruction and 3-dimensional quantitative analyses were performed by using software provided by Scanco. The evaluation of 3-dimensional trabecular was done as described previously in detail (6). The recommended guidelines for micro-CT scanning (28) and bone histomorphometry nomenclature were followed (29).

**Biochemical measurements.** Serum concentration of leptin was measured by using a commercial ELISA kit from ALPCO Diagnostics (Windham, NH) according to the instructions of the manufacturer. Serum concentrations of TNF- $\alpha$  and IL-1 $\beta$  were measured using commercially available cytokine multiplex kits from Bio-Rad (Hercules, CA) and the Luminex200 multiplexing system (Luminex, Austin, TX).

The reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) in whole-blood samples were determined by using a Bioxytech GSH/GSSG-412 kit purchased from Oxis International (Foster City,

CA). The method is based on an enzymatic recycling method described by Griffith (30).

**Bone marrow cell harvest and osteoclast differentiation.** Bone marrow cells were harvested and cultured as described previously (14,31). For nonadherent osteoclast precursors, equal numbers of cells were cultured in either 6-well plates at  $2 \times 10^6$  cells per well for measurement of mRNA levels in osteoclasts or 96-well plates ( $2 \times 10^5$  cells per well) for measurement of osteoclast number with addition of fresh media every 2–3 d. At day 6 of culture, the cells in the 6-well plates were washed with PBS and collected for RNA isolation. For osteoclast number measurement, the cells in the 96-well plates were washed with distilled water twice and fixed with a solution (25 mL of citrate, 65 mL of acetone, and 8 mL of 37% formaldehyde) for 30 s and then stained with a commercial kit for tartrate-resistant acid phosphatase [TRAP (Sigma)] according to the instructions of the manufacturer. Multinucleated cells with  $>3$  nuclei were counted as TRAP-positive osteoclast cells under an inverted microscope.

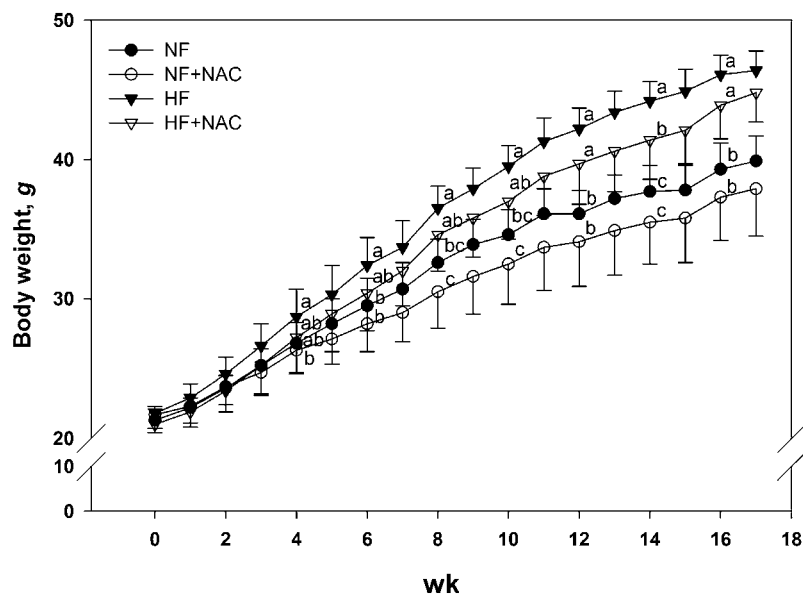
**Osteoclast-like RAW cell culture and treatment.** To determine the effects of NAC on glutathione status in osteoclasts, murine macrophage RAW cells, which can be differentiated to osteoclasts, were cultured in  $\alpha$ -Minimum Essential Medium containing L-glutamine and nucleosides, and supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% fungizone in the presence of 30  $\mu\text{g/L}$  RANKL (R&D Systems, Minneapolis, MN). Cells were cultured for 6 d in 6-well plates at  $2 \times 10^6$  cells per well for measurement of GSH and GSSG in activated osteoclasts. Cells were treated with different concentrations of BSO (Sigma-Aldrich) or NAC at the time of media change (every other day). GSH and GSSG concentrations in cells were measured as described above. For osteoclast formation, cells were seeded at  $2 \times 10^5$  cells per well and measured as described in the previous section.

**Measurement of mRNA levels in osteoclasts.** Total RNA was purified from cultured osteoclasts using TRIzol (Invitrogen, Carlsbad, CA) reagent according to the instructions of the manufacturer. Denatured total RNA from cells (2  $\mu\text{g}$ ) was reverse transcribed and amplified and quantified using a Sequence Detection System (SDS 7300) as described previously in detail (6). Relative mRNA levels were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase mRNA in the same sample. The sense and antisense primer sequences were as follows: for receptor activator NF- $\kappa\text{B}$  (*Rank*), 5'-GGT CTG CAG CTC TTC CAT GAC-3' and 5'-GAA GAG CAG AAC GAT GAG ACT-3'; for integrin  $\beta 3$  (*Itgb3*), 5'-TTC AAT GCC ACC TGC CTC AAC AAC-3' and 5'-ACG CAC CTT GGC CTC GAT ACT AAA-3'; and for glyceraldehyde 3-phosphate dehydrogenase, 5'-TGC ACC ACC AAC TGC TTA G-3' and 5'-GGA TGC AGG GAT GAT GTT C-3'. All oligonucleotide primers for PCR amplification were synthesized by Integrated DNA Technologies (Coralville, IA) with HPLC purification.

**Data analyses.** Data are expressed as group means  $\pm$  SDs. For body weight, a repeated-measures ANOVA was used to test for effects of treatment, week, and a treatment  $\times$  week interaction. All other outcomes from the animal study were analyzed using 2-factor ANOVA (JMP version 9.0.0; SAS Institute, Cary, NC). Homogeneity of variances was tested using the Brown-Forsythe test. If variances were not homogeneous, then Welch's test was used to compare means. Data from RAW cells were analyzed using 1-factor ANOVA to compare the mean values between different dosages of BSO and NAC from the controls. Tukey-Kramer multiple comparison procedure was used for post hoc comparison of treatment means. In all of the analyses,  $P < 0.05$  was considered to be statistically significant.

## Results

**Body weight.** There was no difference in initial body weight among all treatment groups (Fig. 1). Body weight increased over time regardless of diets. Starting from 4 wk after the treatment,



**FIGURE 1** Body weight changes in mice fed either a purified NF (10% energy as fat) or HF (45% energy as fat) diet supplemented with or without NAC at 1 g/kg diet for 17 wk. Values are means  $\pm$  SDs ( $n = 12$  for each group). Data were analyzed by 2-factor ANOVA, followed by Tukey-Kramer multiple comparison procedure. Labeled means for even-numbered weeks without a common letter differ, \* $P < 0.05$ , \*\* $P < 0.01$ . HF, high-fat; NAC, *N*-acetylcysteine; NF, normal-fat.

wk	0	2	4	6	8	10	12	14	16
Fat	-	-	*	**	**	**	**	**	**
NAC	-	-	-	**	**	**	**	**	**
Fat x NAC	-	-	-	-	-	-	-	-	-

body weights were higher in mice fed HF diets than those fed NF diets ( $P < 0.05$ ). Mice in the NAC groups had lower ( $P < 0.05$ ) body weights than mice without NAC treatment from 5 wk after the experiment started.

**Bone microstructure.** Neither dietary fat content nor NAC treatment had a significant effect on length or midshaft diameter of femurs of mice (Table 1). Femoral trabecular tissue volume (TV) was not significantly affected by either dietary fat level or NAC supplementation. Compared with the NF diet, the HF diet decreased bone volume (BV), BV/TV, connectivity density (Conn.Dn), and trabecular number (Tb.N), but increased trabecular separation (Tb.Sp) and structure model index (SMI) ( $P < 0.05$ ) and had no effect on trabecular thickness (Tb.Th) and bone mineral density (BMD). NAC supplementation increased BV, BV/TV,

Tb.Th, and BMD, but decreased SMI ( $P < 0.05$ ) and had no significant effects on Tb.N, Tb.Sp, and Conn.Dn.

**Osteoclast differentiation from bone marrow cells and mRNA levels of markers of osteoclasts.** Bone marrow cells were cultured and isolated to investigate whether NAC supplementation affects osteoclast differentiation and markers (*Itgb3* and *Rank*) of osteoclasts. Compared with mice fed the NF diets, HF diet increased TRAP-positive osteoclast-like cells ( $P < 0.05$ ) (Fig. 2A, B). NAC supplementation significantly decreased osteoclast numbers regardless of dietary fat levels ( $P < 0.05$ ).

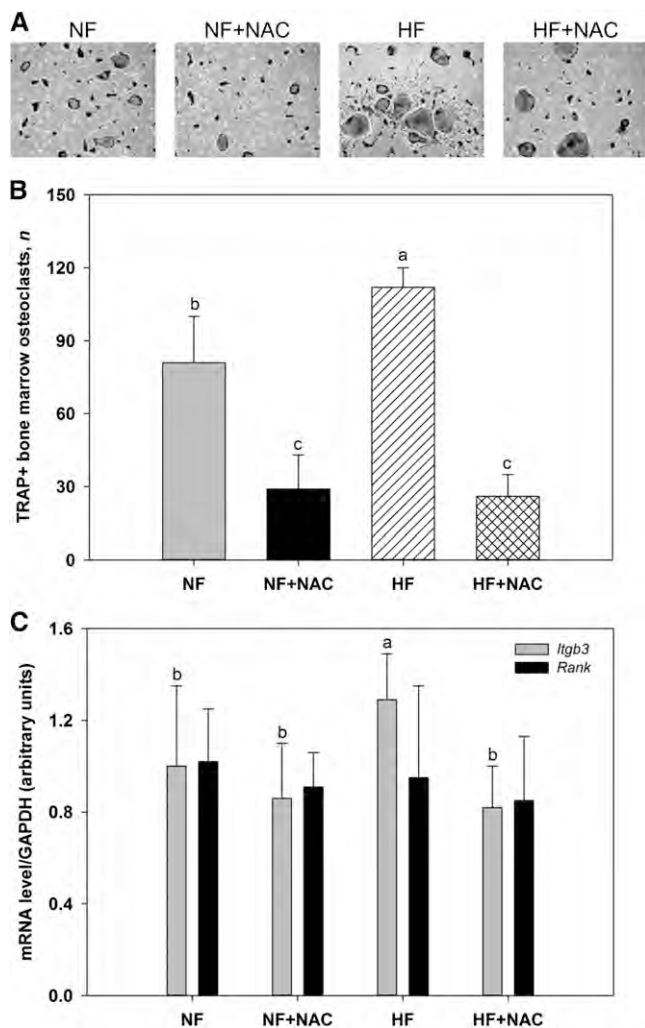
NAC supplementation but not dietary fat level decreased ( $P < 0.05$ ) the mRNA level of *Itgb3* in cultured osteoclasts (Fig. 2C). Dietary fat levels or NAC supplementation did not affect *Rank* mRNA levels in osteoclasts from bone marrow cells.

**TABLE 1** Bone microstructural parameters of distal femur in mice fed either a normal-fat purified (10% energy as fat) or a high-fat (45% energy as fat) diet supplemented with or without NAC at 1 g/kg diet for 17 wk<sup>1</sup>

Indices	Normal-fat		High-fat		ANOVA ( $P$ value)		
	-NAC	+NAC	-NAC	+NAC	Fat	NAC	Fat $\times$ NAC
Length, mm	15.6 $\pm$ 0.4	15.9 $\pm$ 0.3	15.6 $\pm$ 0.5	15.7 $\pm$ 0.4	0.437	0.090	0.445
Diameter, mm	1.30 $\pm$ 0.05	1.33 $\pm$ 0.07	1.32 $\pm$ 0.07	1.30 $\pm$ 0.07	0.716	0.847	0.155
BV, mm <sup>3</sup>	0.50 $\pm$ 0.16 <sup>a</sup>	0.59 $\pm$ 0.21 <sup>a</sup>	0.36 $\pm$ 0.11 <sup>b</sup>	0.49 $\pm$ 0.14 <sup>a</sup>	0.001	0.006	0.232
TV, mm <sup>3</sup>	3.67 $\pm$ 0.18	3.90 $\pm$ 0.43	3.69 $\pm$ 0.33	3.68 $\pm$ 0.31	0.163	0.056	0.486
BV/TV, %	13.6 $\pm$ 3.8 <sup>a</sup>	14.9 $\pm$ 3.6 <sup>a</sup>	9.8 $\pm$ 2.5 <sup>b</sup>	13.3 $\pm$ 3.3 <sup>a</sup>	0.014	0.014	0.228
Tb.N, mm <sup>-1</sup>	4.21 $\pm$ 0.28 <sup>a</sup>	4.00 $\pm$ 0.40 <sup>a,b</sup>	3.74 $\pm$ 0.44 <sup>b</sup>	3.94 $\pm$ 0.28 <sup>a,b</sup>	0.004	0.746	0.024
Tb.Th, mm	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.180	0.046	0.380
Tb.Sp, mm	0.23 $\pm$ 0.02 <sup>b</sup>	0.24 $\pm$ 0.03 <sup>a,b</sup>	0.26 $\pm$ 0.04 <sup>a</sup>	0.24 $\pm$ 0.02 <sup>a,b</sup>	0.004	0.501	0.013
Conn.Dn, mm <sup>-3</sup>	101 $\pm$ 25 <sup>a</sup>	86 $\pm$ 22 <sup>a,b</sup>	60 $\pm$ 20 <sup>c</sup>	77 $\pm$ 20 <sup>b</sup>	<0.001	0.265	0.003
SMI	2.37 $\pm$ 0.39 <sup>a,b</sup>	2.15 $\pm$ 0.40 <sup>b</sup>	2.77 $\pm$ 0.23 <sup>a</sup>	2.39 $\pm$ 0.45 <sup>a,b</sup>	<0.001	0.002	0.180
BMD, g HA/cm <sup>3</sup>	0.90 $\pm$ 0.12 <sup>a,b</sup>	0.94 $\pm$ 0.12 <sup>a</sup>	0.80 $\pm$ 0.15 <sup>b</sup>	0.92 $\pm$ 0.14 <sup>a,b</sup>	0.105	0.032	0.333

<sup>1</sup> Values are means  $\pm$  SDs ( $n = 12$  for each group). Data were analyzed by 2-factor ANOVA, followed by Tukey-Kramer multiple comparison procedure. Means in a row with superscripts without a common letter differ,  $P < 0.05$ . BMD, bone mineral density; BV, bone volume; Conn.Dn, connectivity density; HA, hydroxylapatite; NAC, *N*-acetylcysteine; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, total volume; -NAC, without *N*-acetylcysteine; +NAC, with *N*-acetylcysteine.





**FIGURE 2** Formation of TRAP-positive multinucleated osteoclasts by bone marrow cells (A, B) and expression of *Itgb3* and *Rank* (B) in cultured osteoclasts from mice fed either a purified NF (10% energy as fat) or an HF (45% energy as fat) diet supplemented with or without NAC at 1 g/kg diet for 17 wk. Values are means  $\pm$  SDs ( $n = 12$  for each group). Data were analyzed by 2-factor ANOVA, followed by Tukey-Kramer multiple comparison procedure. Labeled means without a common letter differ,  $P < 0.05$ . GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HF, high-fat; *Itgb3*, integrin  $\beta 3$ ; NAC, N-acetylcysteine; NF, normal-fat; *Rank*, receptor activator NF- $\kappa$ B; TRAP, tartrate-resistant acid phosphatase.

**Cytokine concentrations in serum and glutathione status in whole blood.** Serum leptin concentration was higher ( $P < 0.05$ ) in mice fed HF diets than those fed NF diets regardless of NAC supplementation (Fig. 3). NAC supplementation did not affect serum leptin concentrations. Serum concentrations of TNF- $\alpha$  and IL-1 $\beta$  were low, and neither NAC supplementation nor dietary fat levels affected them (TNF- $\alpha$ :  $1.2 \pm 0.6$ ,  $1.2 \pm 0.6$ ,  $1.1 \pm 0.3$ ,  $0.8 \pm 0.1$  pmol/L; IL-1 $\beta$ :  $0.45 \pm 0.10$ ,  $0.45 \pm 0.18$ ,  $0.43 \pm 0.24$ ,  $0.35 \pm 0.06$  pmol/L for NF, NF + NAC, HF, and HF + NAC groups, respectively).

Dietary fat did not affect blood GSH concentration (Fig. 4A) but increased blood GSSG concentration (Fig. 4B) and decreased the ratio of GSH to GSSG (Fig. 4C) ( $P < 0.05$ ). NAC supplementation increased blood GSH concentration ( $P < 0.05$ ) but had no effect on blood GSSG concentration or the ratio of GSH to GSSG.

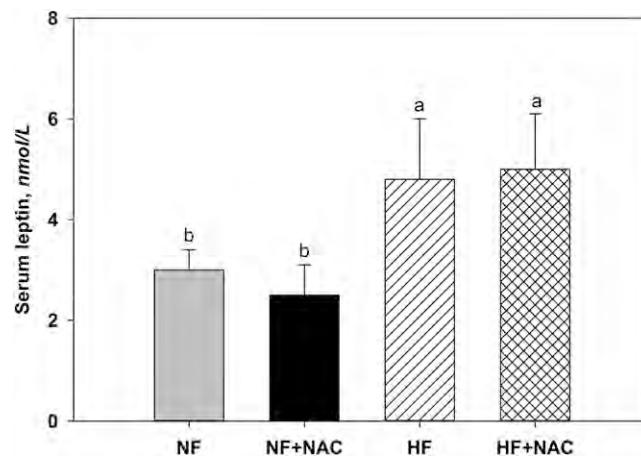
**Osteoclast formation and GSH status of RAW cells treated with BSO and NAC.** To further investigate whether NAC

affects GSH status and the role of GSH status on osteoclast differentiation in vitro, we cultured osteoclast-like RAW cells in the presence of RANKL. The  $\gamma$ -glutamylcysteine synthetase inhibitor BSO induced a biphasic effect on osteoclast differentiation, i.e., inducing differentiation at lower concentrations and inhibiting differentiation at higher concentrations (Fig. 5A), but decreased GSH and GSSG in cells (Fig. 5C, E). In contrast, the addition of up to 20 mmol/L of NAC reduced osteoclast formation (Fig. 5B) but increased GSH (Fig. 5D) content compared with cells without NAC treatment.

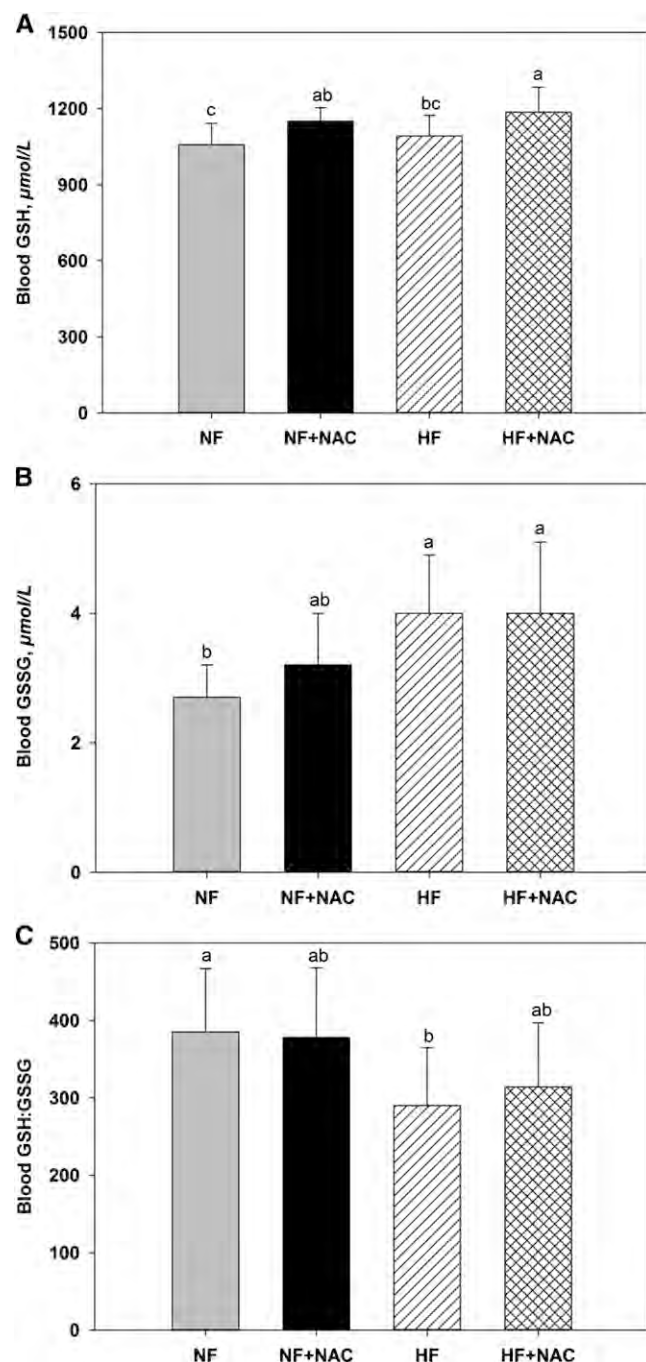
## Discussion

In the present study, we showed that NAC supplementation at 1 g/kg diet increases oxidative defense (GSH concentration in serum), decreases osteoclast differentiation from bone marrow cells, and mitigates the deterioration of bone microarchitecture in mice fed an HF (45% energy as fat) diet for 17 wk. These changes, coupled with the in vitro findings that NAC enhances glutathione status and decreases osteoclast formation, whereas BSO suppresses glutathione status and increases osteoclast formation in osteoclast-like RAW cells, indicate that NAC supplementation is beneficial to bone in HF diet-induced obese mice through increased oxidative defense and decreased bone resorption.

In general, body weight is positively correlated with BMD (32–34) because of increased mechanical loading associated with increased body weight. However, our data demonstrated that obesity induced by an HF diet is detrimental to bone structure, i.e., obese mice had lower BV, Tb.N, Conn.Dn, and BMD and higher Tb.Sp than those fed the NF diet, despite their higher body weights, a finding that is consistent with several previous studies (6,7,14–16). Several potential mechanisms have been proposed and demonstrated in various animal models regarding the detrimental effects of obesity, especially when it is induced by an HF diet, on bone metabolism (5,17). Obesity can decrease bone formation (7,16) by increasing in marrow adipogenesis at the expense of osteoblastogenesis (5) or increase bone resorption (6,7,16,35) by activation of the RANKL/RANK/osteoprotegerin pathway and/or upregulation of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (18,19,36). An



**FIGURE 3** Serum leptin concentrations in mice fed either a purified NF (10% energy as fat) or an HF (45% energy as fat) diet supplemented with or without NAC at 1 g/kg diet for 17 wk. Values are means  $\pm$  SDs ( $n = 12$  for each group). Data were analyzed by 2-factor ANOVA, followed by Tukey-Kramer multiple comparison procedure. Labeled means without a common letter differ,  $P < 0.05$ . HF, high-fat; NAC, N-acetylcysteine; NF, normal-fat.



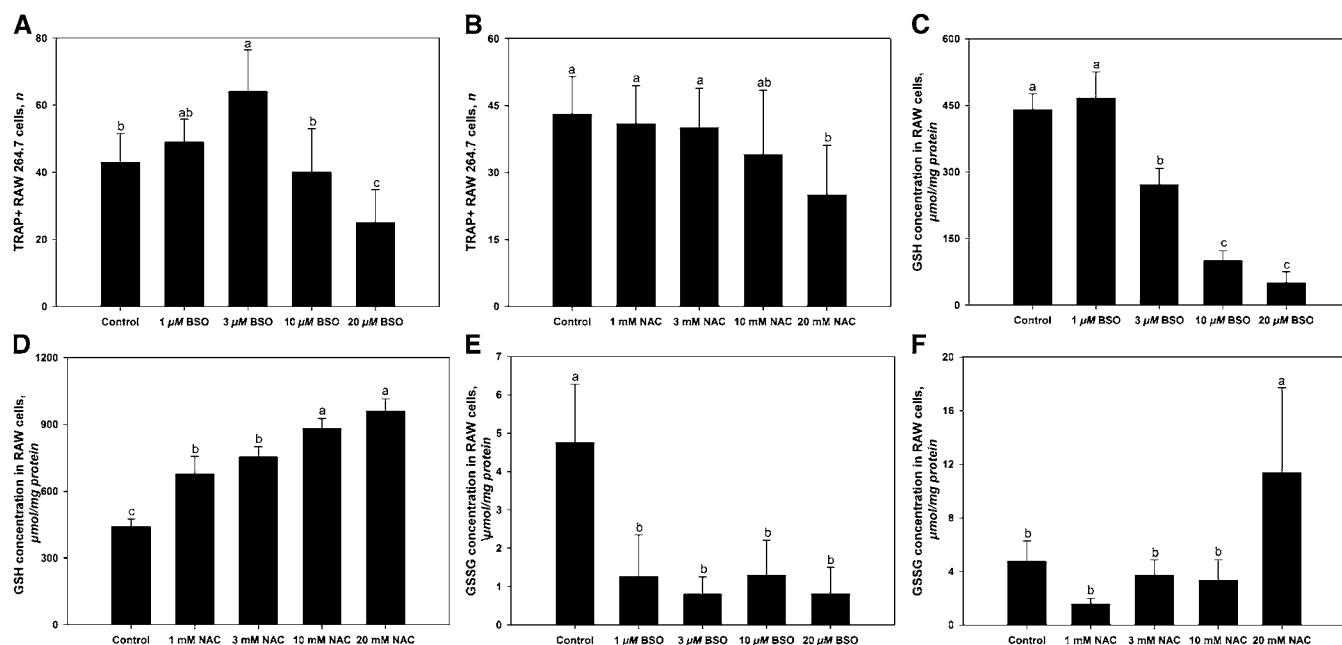
**FIGURE 4** Blood concentrations of GSH (A), GSSG (B), and the ratio of GSH to GSSG (C) mice fed either a purified NF (10% energy as fat) or an HF (45% energy as fat) diet supplemented with or without NAC at 1 g/kg diet for 17 wk. Values are means  $\pm$  SDs ( $n = 12$  for each group). Data were analyzed by 2-factor ANOVA, followed by Tukey-Kramer multiple comparison procedure. Labeled means without a common letter differ,  $P < 0.05$ . GSH, glutathione; GSSG, glutathione disulfide; HF, high-fat; NAC, N-acetylcysteine; NF, normal-fat.

HF diet may decrease calcium absorption attributable to formation of insoluble calcium soaps with FAs (37–39). Although elevated circulating concentrations of inflammatory markers with obesity have been demonstrated (1,40), we did not detect a significant difference in serum concentrations of TNF- $\alpha$  and IL-1 $\beta$  in mice fed different dietary fat levels in this study because concentrations of these cytokines were low and variations were high.

Our data showed that NAC supplementation significantly decreased body weight from 5 wk after the experiment started, a finding similar to several other studies in which administration of antioxidant NAC was found to reduce body weight gain (41–43). Although it is possible that the decreased body weight might be attributable to the reduced food intake (not measured in the current study), Kim et al. (42) reported that i.p. injection of NAC from 81 to 800  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  did not affect food intake but significantly decreased body weight in a dose-dependent manner. Souza et al. (43) also reported that food intake was not affected by administration of NAC in drinking water (2 mg/L) in high-sucrose induced obese rats. Ample evidence suggests that the reduction in body weight gain is attributed to the decrease in fat mass. NAC administration in rats resulted in a reduction of body weights and a marked reduction in visceral fat tissues (42). NAC inhibited the adipogenic transcription factors CCAAT/enhancer binding protein  $\beta$  and peroxisomal proliferator activated receptor  $\gamma$  expression in cultured adipocytes (44). Supplementing NAC in drinking water decreased the BMI in obese rats induced by a high-sucrose diet (43). Furthermore, Chang et al. (45) demonstrated that mice lacking nonselenocysteine-containing phospholipid hydroperoxide glutathione peroxidase causes accumulation of ROS and increased fat mass, whereas treatment with NAC prevents increased adiposity in these mice. However, we found that serum concentration of leptin, a small polypeptide hormone secreted primarily by the adipocytes that can be used as an indicator of adiposity, was not affected by NAC supplementation. Whether NAC affects body composition or cytokine production by adipocytes and to what extent warrants additional investigation.

That NAC supplementation decreased osteoclast differentiation from mouse bone marrow cells and the expression of *Itgb3*, an osteoclast marker, in these differentiated osteoclasts suggests that osteoclastogenesis and bone resorption are down-regulated by NAC. Furthermore, using osteoclast-like RAW cells (in the presence of RANKL) as a model, we demonstrated that NAC increased glutathione status and decreased osteoclast formation, whereas moderate depletion of glutathione with the glutathione synthesis inhibitor BSO increased the formation of osteoclasts. These results are consistent with other studies that demonstrated that formation of TRAP-positive osteoclasts from cultured bone marrow monocytes-macrophage lineage cells was inhibited by NAC treatment (23,24). The finding that NAC decreases osteoclast activity and/or bone resorption should have significant implications on bone metabolism because, in general, all forms of acquired osteoporosis reflect increased bone resorption or activity of the osteoclast relative to bone formation or activity of the osteoblast (46). Consistent with the above results, we found that NAC supplementation at 1 g/kg diet ( $\sim 5$ – $8$  mg/d per mouse, assuming 5–8 g of food intake per day on average) increased BV, BV/TV, and Tb.Th but decreased SMI in the distal femur. Similar findings have been reported. Lean et al. (24) demonstrated that i.p. injection of NAC at 200  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  ( $\sim 6$ – $8$  mg/d for mice with body weights between 30 and 40 g) prevents estrogen deficiency-induced bone loss in mice (24). Chen et al. (25) demonstrated that supplementation of NAC in the diet at 1.4 g/kg prevents bone loss induced by elevated oxidative stress attributable to ethanol consumption in rats. Although the effects of NAC on osteoblast function and bone formation were not determined in this study, Chen et al. (25) demonstrated that NAC supplementation in rats blocks ethanol-induced downregulation of serum bone formation markers, alkaline phosphatase, and osteocalcin.

NAC can be administered through drinking water, diet, or i.p. injection in a wide range of dosages. Functional changes have been reported in mice supplemented with NAC at doses ranging



**FIGURE 5** Formation of TRAP-positive multinucleated cells (*A, B*), and concentrations of GSH (*C, D*) and GSSG (*E, F*) in cultured osteoclast-like RAW 264.7 cells treated with BSO and NAC, respectively. Values are means  $\pm$  SDs ( $n = 9$  and  $6$  for osteoclast number and glutathione status, respectively). Data were analyzed using 1-factor ANOVA to compare the mean values between different dosages of BSO and NAC to the control without treatment, and Tukey-Kramer multiple comparison procedure was used for post hoc comparison of treatment means. Labeled means without a common letter differ,  $P < 0.05$ , from the control without treatment. BSO, L-buthionine-(*S,R*)-sulphoximine; GSH, glutathione; GSSG, glutathione disulfide; NAC, N-acetylcysteine; TRAP, tartrate-resistant acid phosphatase.

from 200 mg to 30 g/kg diet depending on outcome measures (27,47,48). NAC has been demonstrated to have many biologic effects, including antioxidant (44,49), antitumor and anticancer (49), antidiabetic (50), anti-inflammation (51), and anti-aging (41) activities. NAC provides cysteine for GSH and promotes its synthesis (52). GSH is a radical scavenger that directly neutralizes superoxide anion and hydroxyl radicals, and it is also an essential cofactor in the inactivation of hydroperoxides by glutathione peroxidase; so GSH could help as a buffer against ROS, allowing cell viability and normal cell function. Our results showed that NAC treatment increases glutathione status in osteoclast-like RAW cells in vitro, and NAC supplementation in mice significantly increased serum GSH concentration in either NF or HF-fed mice compared with those mice without NAC supplementation, indicating that NAC improved antioxidant defense.

Oxygen-derived free radicals directly stimulate osteoclast activity and bone resorption (21,22). Available evidence suggests that the beneficial effect of NAC on bone metabolism is likely attributable to its ability to modulate oxidant defenses. Restoring antioxidant defenses by catalase, N-acetyl cysteine, or ascorbate inhibited RANKL-induced osteoclastogenesis (23) and prevented estrogen deficiency bone loss (20). Recently, NAC has also been shown to decrease alveolar bone loss in streptozotocin-induced diabetic rats (53). Other substances that possess antioxidant activity have been shown to inhibit osteoclast differentiation and decrease osteoclast activity and bone resorption by reducing ROS (35,54–56).

In summary, the present study examined the effects of an HF diet and NAC supplementation on bone metabolism in a mouse model and investigated the effects of NAC treatment and cellular GSH on osteoclast formation in murine macrophage RAW cells in vitro. Our data demonstrated that the HF diet is detrimental to bone microstructure, whereas NAC supplementation decreases body weight gain and mitigates adiposity-induced bone

microstructure deterioration by increasing oxidant defense in mice. Our data suggest that elevated glutathione status, and likely decreased oxidative stress, decrease osteoclast formation, and that NAC supplementation may be beneficial to bone and body composition.

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