Curcumin Reduces Trabecular and Cortical Bone in Naïve and Lewis Lung Carcinoma-bearing Mice

LIN YAN[1], JOHN A. YEE[2] and JAY CAO[1]

[1]*U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND, U.S.A.;*
[2]*Department of Biomedical Sciences, School of Medicine, Creighton University, Omaha, NE, U.S.A.*

Abstract. *The present study investigated the effects of curcumin on bone microstructure in non-tumor-bearing and Lewis lung carcinoma-(LLC)-bearing female C57BL/6 mice. Morphometric analysis showed that dietary supplementation with curcumin (2% or 4%) significantly reduced the bone volume to total volume ratio, connectivity density and trabecular number, and significantly increased the structure model index (an indicator of the plate- and rod-like geometry of trabecular structure) and trabecular separation in vertebral bodies compared to controls in both non-tumor-bearing and LLC-bearing mice. Similar changes in trabecular bone were observed in the femoral bone in curcumin-fed mice. Curcumin significantly reduced the cortical bone area to total area ratio and cortical thickness in femoral mid-shaft, but not in vertebral bodies, in both non-tumor-bearing and LLC-bearing mice. Curcumin feeding reduced plasma concentrations of osteocalcin and increased tartrate-resistant acid phosphate 5b in mice regardless of the presence of LLC, indicating that curcumin disrupts the balance of bone remodeling. Our results demonstrated that curcumin reduced the trabecular bone volume and cortical bone density. The skeleton is a favored site of metastasis for many types of cancers, and curcumin has been investigated in clinical trials in patients with cancer for its chemopreventive effects. Our results suggest the possibility of a combined effect of cancer-induced osteolysis and curcumin-stimulated bone loss in patients using curcumin. The assessment of bone structural changes should be considered for those who participate in curcumin clinical trials to determine its effects on skeleton health, particularly for those with advanced malignancies.*

Correspondence to: Lin Yan, USDA, ARS, Grand Forks Human Nutrition Research Center, 2420 2nd Avenue North, Grand Forks, ND 58202, U.S.A. Tel: +1 7017958499, Fax: +1 7017958220, e-mail: lin.yan@ars.usda.gov

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Curcumin [(1*E*,6*E*)-1,7-*bis* (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], commonly known as turmeric, is a phenolic compound derived from the *Curcuma longa* plant. Curcumin has been traditionally used in Ayurvedic medicine for its antiinflammatory (1), antioxidant (2) and antimicrobial (3) therapeutic properties. In recent years, curcumin has been reported to have anticancer activities (4, 5). In a previous study, we found that dietary supplementation with curcumin does not reduce spontaneous metastasis of Lewis lung carcinoma (LLC) to lungs of mice, but it significantly enhances the growth of pulmonary metastases (6). These observations raise a concern about the use of curcumin as a chemopreventive agent for treatment of patients with advanced cancer (7-11).

Other concerns about the clinical use of curcumin are its effects on normal organ functions. Available studies have suggested that curcumin may have bone-protective effects. *In vitro* studies showed that curcumin promotes apoptosis and inhibits bone resorption by rabbit osteoclasts (12) and inhibits osteoclastogenesis in murine cells induced by receptor activator of nuclear factor kB ligand (RANKL) (13). Animal studies showed that curcumin feeding can increase trabecular bone mass and improve bone mineral density in adult APP/PS1 transgenic mice (14) and reduce ovariectomy-induced bone loss in rats (15, 16). This suggests that curcumin might be useful in prevention and treatment of osteopenia and osteoporosis. However, from a preliminary analysis of randomly selected bones in our previous study (6), we found that trabecular bone volume in distal femurs was reduced in mice on curcumin-supplemented diets (unpublished data). This raises a concern about the safety of curcumin treatment in patients with advanced cancer who are often at the risk of bone metastasis, *e.g.* those who participate in curcumin cancer prevention clinical trials (7-11) and about the possible detrimental interaction of malignancy with curcumin on skeleton deterioration. The hypothesis tested in the present study was that dietary supplementation with curcumin reduces bone microstructure in mice. To test this hypothesis, we conducted a morphometric analysis of

vertebral bodies and femurs collected from non-tumor-bearing mice fed curcumin-supplemented diets. We also examined the microstructure of bones from LLC-bearing mice collected from our previous study (6).

Materials and Methods

This study was approved by the Animal Care and Use Committee of the Department of Agriculture, Agricultural Research Service (USDA, ARS), Grand Forks Human Nutrition Research Center (approval number: YAN015). The procedures followed the National Institutes of Health guidelines for the care and use of laboratory animals (17).

Animals and diets. Three-week-old female C57BL/6 mice (Harlan, Madison, WI, USA) were housed 3-4 per box, in wire-topped plastic boxes, in a pathogen-free room on a 12:12-hour light-dark cycle at 22±1˚C. Three diets were compared: the AIN93G control diet (18) and that diet supplemented with 2% or 4% curcumin (w/w; purity 95%; PureBulk, Roseburg, OR, USA; Table I). All diets were presented in powdered form. Mice had free access to food and deionized water. The animals were weighed weekly.

Experimental design. All mice were maintained on the AIN93G diet for three weeks before the experimental feeding. In the experiment without LLC, mice were maintained on experimental diets for seven weeks. In the experiment with LLC (6), mice were subcutaneously injected with LLC cells after five weeks on curcumin diets. Ten days after the injection, the primary tumor was surgically removed, and the mice were maintained on their respective diets for an additional 10 days before the termination of the experiment (6). In both experiments, body composition analysis of fat and lean mass was performed on conscious, immobilized mice using quantitative magnetic resonance (Echo whole-body composition analyzer, Model 100; Echo Medical System, Houston, TX, USA) after five weeks of curcumin feeding. In the experiment without LLC, food intake and fecal excretion (n=6 per group) were recorded five days per week for three weeks from the fourth week of the experimental feeding.

Caloric intake. The gross energy of experimental diets and feces was quantified using bomb calorimetry (Model 6200, Oxygen Bomb Calorimeter; Parr Instrument, Moline, IL, USA). Daily caloric intake (kcal/d) was calculated by subtracting fecal caloric excretion (fecal caloric content × fecal excretion) from dietary caloric intake (diet caloric content × food intake).

Bone evaluation. At the end of each experiment, mice were injected intraperitoneally with a mixture of ketamine and xylazine. Vertebral columns (from 10th or 11th thoracic vertebra to sacrum) and right femurs were collected and stored in phosphate-buffered saline for microtomographic analysis of trabecular and cortical bone, and plasma was collected for measurement of osteocalcin and tartrate-resistant acid phosphatase 5b (TRAP 5b). Femurs were cleaned for physical measurements before microcomputed tomographic analysis. Femoral lengths and mid-shaft widths in both the medial-lateral and anterior-posterior axes were measured using an electronic digital caliper (Fred V. Fowler Company, Newton, MA, USA).

Lumbar vertebral bodies and right femurs were evaluated for trabecular and cortical bone structural properties using high-resolution (12-μm slice increment) microcomputed tomography (μCT-40; Scanco Medical, Basserdorf, Switzerland) with a x-ray source power of 55 keV and 145 μA and integration time of 300 ms. A fixed threshold of 275 was used to extract mineralized bone from soft tissue and bone marrow. In vertebral bodies, trabecular and cortical bone were analyzed along the entire cranial-caudal axis of the fourth lumbar vertebrae. In distal femurs, trabecular bone was evaluated in 125 slices (1.5 mm) of the metaphysis proximal to the distal growth plate, and mid-shaft cortical bone was evaluated in 100 slices (1.2 mm).

The total volume (TV, mm^3), bone volume (BV, mm^3), bone volume to total volume ratio (BV/TV, %), connectivity density (Conn.D, $1/mm^3$), structure model index (SMI, an indicator of the plate- and rod-like geometry of trabecular structure), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm) and trabecular separation (Tb.Sp, mm) were measured for trabecular bone in both vertebral bodies and distal femurs. For cortical bone, total cross-sectional area (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), cortical bone area to total area ratio (Ct.Ar/Tt.Ar, %) and cortical thickness (Ct.Th, mm) were computed from vertebral bodies and the femoral mid-shaft.

Quantification of plasma osteocalcin and TRAP 5b. Plasma concentrations of osteocalcin (Biomedical Technology, Stoughton, MA, USA) and TRAP 5b (Immunodiagnostic Systems, Scottsdale, AZ, USA) were quantified using sandwich enzyme-linked immunosorbent assay kits following the manufacturers' protocols. Samples were read within the linear range of the assay, and the accuracy of the analysis was confirmed using the controls provided in each assay kit. The lower limit of detection was 1 ng/ml and 0.1 U/l and the lower limit of quantification was 1.56 ng/ml and 0.3U/l for osteocalcin and TRAP 5b, respectively.

Statistical analyses. One-way ANOVA and Tukey contrasts were used to compare differences among the groups. All data are

Table I. *Composition of the experimental diets*[1].

	Control	2% Curcumin	4% Curcumin
Corn starch	395.8	395.8	395.8
Casein	200	200	200
Dyetrose	132	132	132
Sucrose	100	100	100
Corn oil	70	70	70
Cellulose	50	50	50
Mineral mix, AIN93G	35	35	35
Vitamin mix, AIN93G	10	10	10
L-Cystine	4.4	4.4	4.4
L-Methionine	0.3	0.3	0.3
Choline bitartrate	2.5	2.5	2.5
t-Butylhydroquinone	0.014	0.014	0.014
Curcumin	0	20	40
Total	1,000	1,020	1,040
Gross energy (kcal/g)			
Calculated[2]	3.82	3.88	3.94
Measured	4.33	4.42	4.41

[1]Nutrient dilution factor is 1.96% and 3.85% for 2% and 4% curcumin diets, respectively. [2]Reference (41).

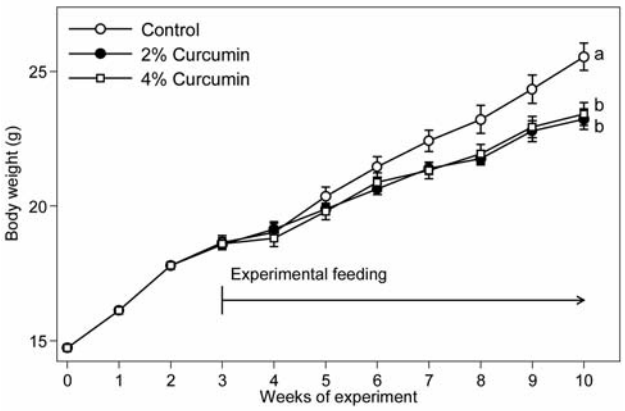

Figure 1. *Body-weight changes in non-tumor-bearing mice during the experiment. One-way ANOVA and Tukey contrasts were performed to compare differences among the control and the curcumin-fed groups. The body weight of curcumin-fed groups was significantly lower than that of the controls after seven weeks of curcumin feeding ($p \leq 0.05$). Values are means±SEM (n=15 per group).*

presented as means±SEM. Differences with a *p*-value of 0.05 or less were considered significant. All statistical analyses were performed using SAS software (version 9.3; SAS Institute, Cary, NC, USA).

Results

Dietary supplementation with 2% or 4% curcumin tended to lower mean body weight compared to the controls in non-tumor-bearing mice, and the difference was statistically significant after seven weeks of curcumin feeding ($p \leq 0.05$; Figure 1). There was no difference in lean body mass weight among the groups (average 17.32±0.14 g). The percentage fat mass was significantly lower ($p \leq 0.05$) and the percentage lean mass was significantly higher in curcumin-fed mice compared to the controls ($p \leq 0.05$; Table II). There was no difference in food intake among the groups (average=3.88±0.03 g/d). The net caloric intake was depressed by curcumin feeding ($p \leq 0.05$) which was due to increases in fecal caloric excretion by curcumin feeding ($p \leq 0.05$; Table II). There were no significant differences in mean body weight and body composition among the curcumin-fed and control groups in LLC-bearing mice (6).

There were no differences in femur length, medial-lateral axis width (WML) and anterior-posterior axis width (WAP) among the groups in either experiment. Average femur length, WML and WAP were 14.96±0.03 mm, 1.82±0.01 mm and 1.27±0.01 mm for non-tumor-bearing mice and 15.01±0.04 mm, 1.78±0.01 mm and 1.27±0.01 mm for the LLC-bearing mice, respectively.

Curcumin supplementation caused changes in the trabecular microstructure. In the vertebral body of non-tumor-bearing mice, both 2% and 4% dietary curcumin

Table II. *Body composition and caloric intake of non-tumor-bearing mice fed curcumin-supplemented diets.*

	Control	2% Curcumin	4% Curcumin
Fat body mass, %	16.69±1.29^a	11.94±0.82^b	13.05±0.59^b
Lean body mass, %	74.97±1.28^b	80.02±0.76^a	78.47±0.63^a
Net caloric intake, kcal/d	16.34±0.19^a	15.33±0.17^b	15.33±0.19^b
Caloric excretion, kcal/d	1.24±0.02^c	1.52±0.02^b	1.95±0.03^a

Values (means±SEM) in the same row with different letters are significantly different at $p \leq 0.05$. N=15 for each value for fat body mass and lean body mass, and n=6 for each value for net caloric intake and caloric excretion.

significantly reduced BV/TV by 14% ($p \leq 0.05$; Figure 2A), Conn.D. by 16-22% ($p \leq 0.05$; Figure 2C) and Tb.N. by 6-8% ($p \leq 0.05$; Figure 2G); and caused increases of 22-24% in SMI ($p \leq 0.05$; Figure 2E) and of 7-9% in Tb.Sp. ($p \leq 0.05$; Figure 2K) compared to the controls. Curcumin feeding caused similar trabecular changes in lumbar vertebrae of LLC-bearing mice (Figures 2B, 2D, 2F, 2H, 2L).

In the distal femur of non-tumor-bearing mice, dietary supplementation with 2% and 4% curcumin reduced BV/TV by 16-22% (Figure 3A) and Conn.D. by 23-33% (Figure 3C); the differences between the 2% curcumin and the controls were at $p \leq 0.05$. Both 2% and 4% curcumin significantly reduced Tb.N. by 12-14% ($p \leq 0.05$; Figure 3G) and significantly increased Tb.Sp. by 15-17% ($p \leq 0.05$; Figure 3K) compared to the controls. In LLC-bearing mice, 4% curcumin significantly reduced BV/TV by 22% ($p \leq 0.05$; Figure 3B) and Tb.N. by 8% ($p \leq 0.05$; Figure 3H); while both 2% and 4% curcumin reduced Tb.Th. by 4-7% ($p \leq 0.05$; Figure 3J) compared to the controls.

Curcumin supplementation did not affect vertebral cortical bone neither in non-tumor-bearing nor LLC-bearing mice (Figures 4A-D). At the femoral mid-shaft, both 2% and 4% curcumin significantly lowered Ct.Ar/Tt.Ar. by 4-5% ($p \leq 0.05$; Figure 4E) and Ct.Th. by 6% ($p \leq 0.05$; Figure 4G) compared to the controls in non-tumor-bearing mice. In LLC-bearing mice, 4% curcumin significantly reduced Ct.Ar/Tt.Ar. by 4% ($p \leq 0.05$; Figure 4F) and Ct.Th. by 6% ($p \leq 0.05$; Figure 4H) compared to the controls.

Dietary supplementation with 2% and 4% curcumin reduced plasma concentrations of osteocalcin by approximately 65% compared to the controls in non-tumor-bearing mice ($p \leq 0.05$; Figure 5A) and by approximately 26% and 60%, respectively, in LLC-bearing mice ($p \leq 0.05$; Figure 5B). Curcumin supplementation resulted in dose-dependent increases of plasma TRAP 5b. Compared to the controls, the increases were 7% and 17% for 2% and 4% curcumin in non-tumor-bearing mice ($p \leq 0.05$; Figure 5C) and 14% and 35% in LLC-bearing mice ($p \leq 0.05$, Figure 5D), respectively.

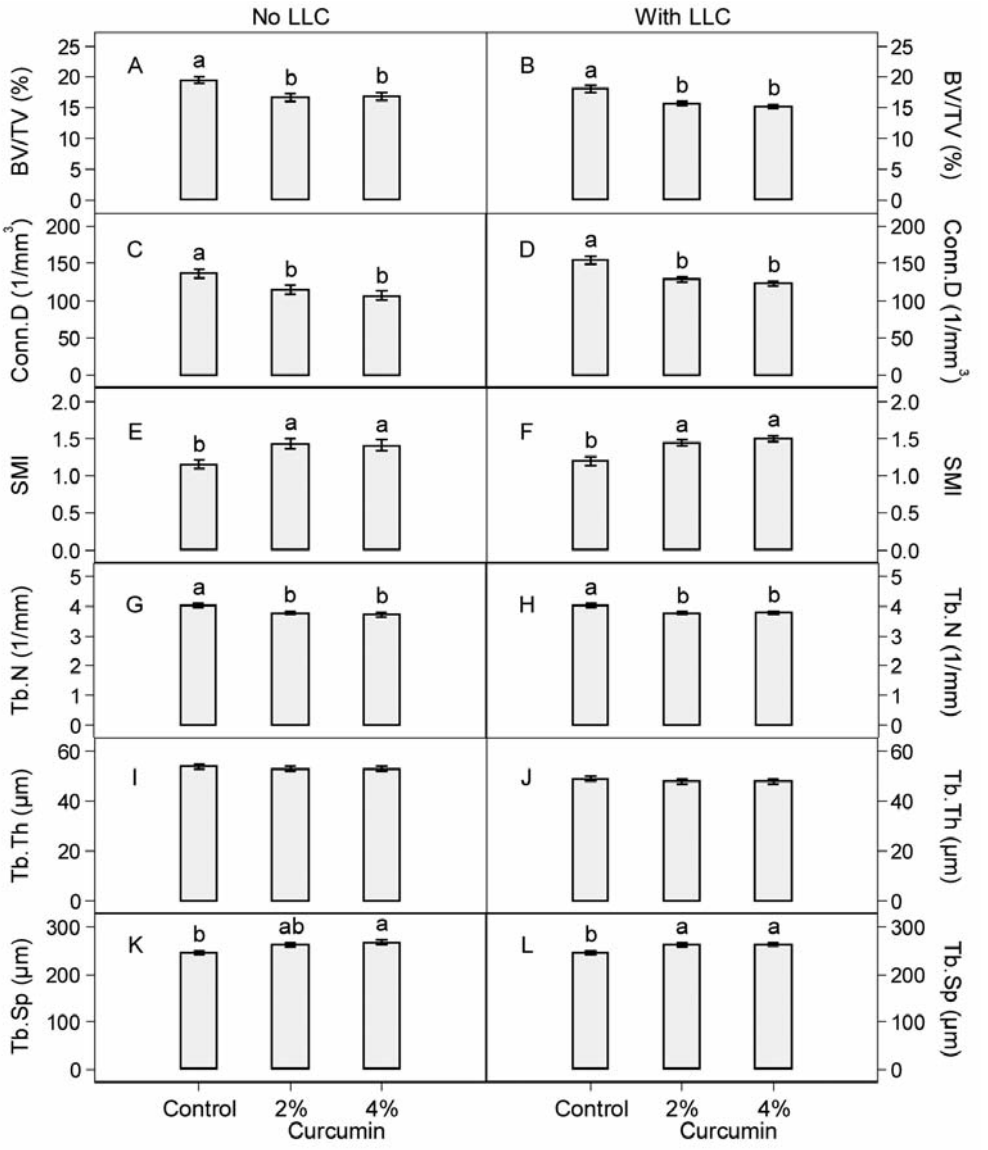

Figure 2. *Trabecular microstructural changes in the vertebral body of non-tumor-bearing (left column) and cancer-bearing mice (right column). One-way ANOVA and Tukey contrasts were used to compare differences among the control and the curcumin-fed groups. Values (means±SEM) in each panel with different letters are significantly different at p≤0.05 (n=15 for each value). LLC: Lewis lung carcinoma; BV/TV: bone volume/total volume ratio; Conn.D: connectivity density; SMI: structure model index; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation.*

Discussion

Morphometric analysis of trabecular bone demonstrated that curcumin reduced BV/TV, Conn.D. and Tb.N. and increased SMI and Tb.Sp. in both non-tumor-bearing and LLC-bearing mice. Furthermore, analysis of the cortical bone showed that curcumin reduced Ct.Ar/Tt.Ar and Ct.Th. in mice regardless of LLC. These results indicate that dietary curcumin supplementation reduces trabecular bone volume and cortical bone density in C57BL/6 mice and that this effect is not due to the presence of cancer.

The reduction of bone volume in curcumin-fed mice was associated with a decrease in plasma osteocalcin and an increase in TRAP 5b. Bone remodeling is an adaptive mechanism that controls bone mass and microachitecture throughout life. It is accomplished by the coordinated, coupled activity of bone resorption and formation. Osteocalcin is a marker of bone formation (19, 20). Changes in serum osteocalcin reflect the status of bone formation in post-menopausal osteoporosis (21, 22), and serum osteocalcin during the growth period is positively correlated to the appositional rate, the rate of longitudinal

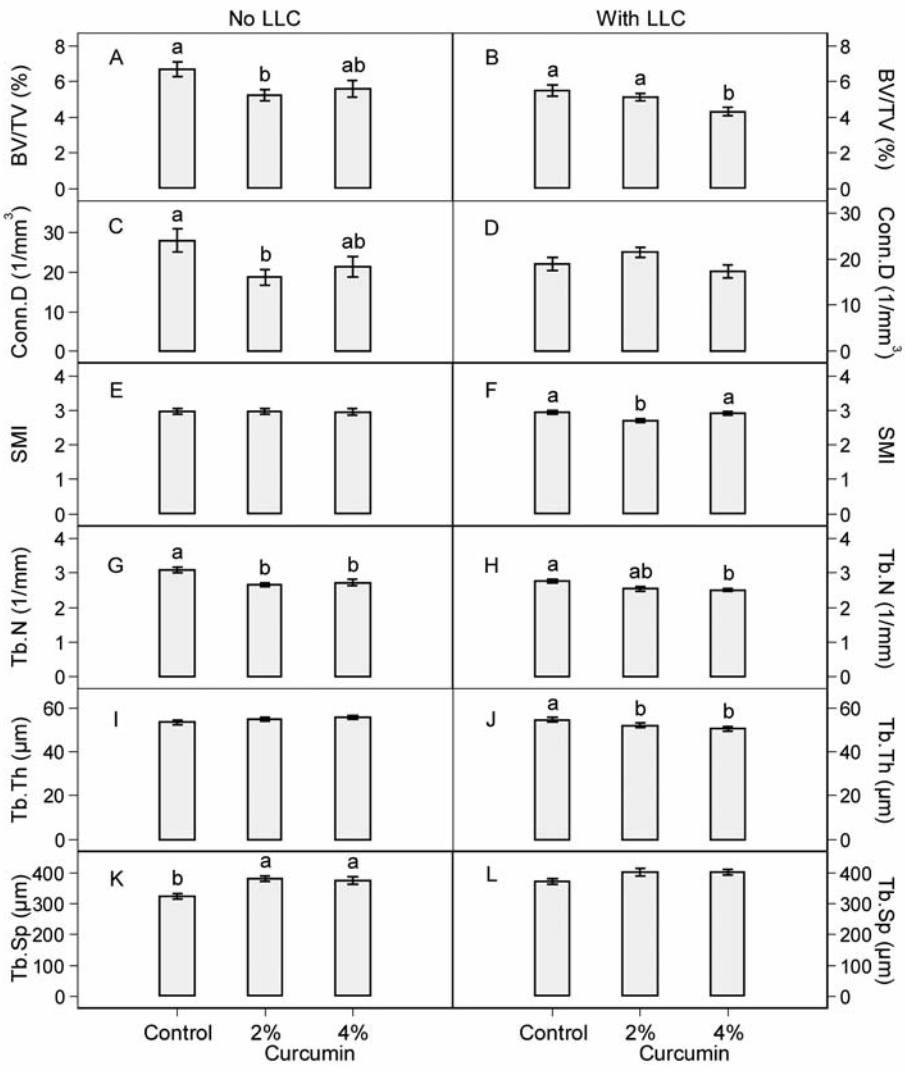

Figure 3. *Trabecular microstructural changes in the right femur of non-tumor-bearing (left column) and cancer-bearing mice (right column). One-way ANOVA and Tukey contrasts were used to compare differences among the control and the curcumin-fed groups. Values (means±SEM) in each panel with different letters are significantly different at p≤0.05 (n=15 for each value). LLC: Lewis lung carcinoma; BV/TV: bone volume/total volume ratio; Conn.D: connectivity density; SMI: structure model index; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation.*

bone growth, the rate of production of chondrocytes in growth plate and the thickness of the growth plate in animals (23). TRAP 5b is a marker of bone resorption (24, 25). The concentration of serum TRAP 5b has been shown to correlate with osteoclast surface and osteoclast number (26). Serum TRAP 5b activity is significantly elevated in patients with osteoporosis, is inversely correlated with bone mineral density (27), and is decreased after hormone replacement therapy in post-menopausal women (24). Our results suggest that curcumin-uncoupled bone resorption and formation may be responsible for the bone loss observed in curcumin-fed mice.

The present study, to our knowledge, is the first to demonstrate that curcumin can have detrimental effects on the trabecular and cortical bone. The skeleton is a major target of malignant spread and osteolytic lesions involving altered microachitecture and loss of cancellous bone are a common consequence of metastasis to bone (28, 29). Malignant cells most frequently affect those parts of the skeleton that are well-vascularized, such as the axial skeleton, the proximal ends of the long bones, vertebral column and ribs (30). Curcumin has been investigated in clinical trials in patients with cancer for its chemopreventive effects (7-11). Our results raise a concern about the use of

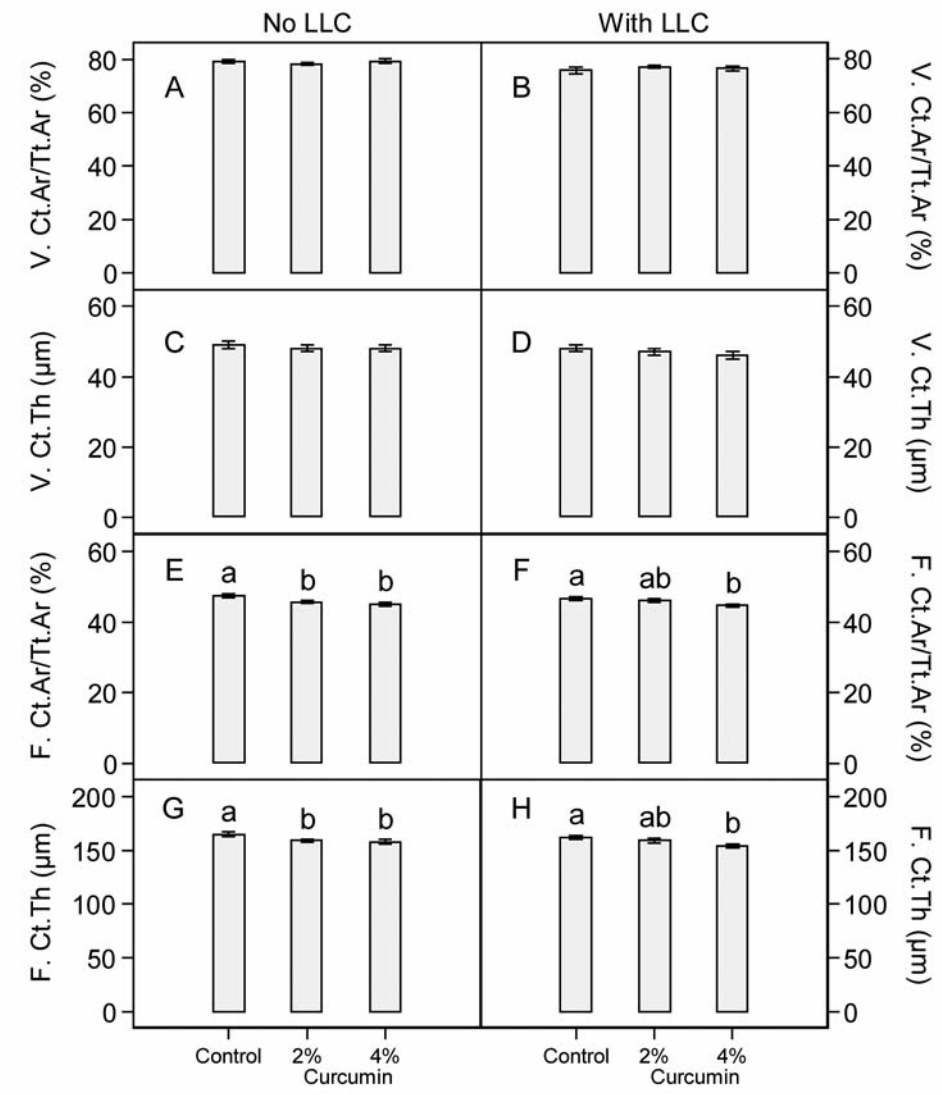

Figure 4. *Cortical bone structural changes in the vertebral body and right femur of non-tumor-bearing (left column) and cancer-bearing mice (right column). One-way ANOVA and Tukey contrasts were used to compare differences among the control and the curcumin-fed groups. Values (means±SEM) in each panel with different letters are significantly different at p≤0.05 (n=15 for each value). LLC: Lewis lung carcinoma; V.Ct.Ar/Tt.Ar: vertebral cortical area/total area ratio; V.Ct.Th: vertebral cortical thickness; F.Ct.Ar/Tt.Ar: femoral cortical area/total area ratio; F.Ct.Th: femoral cortical thickness.*

curcumin in patients with advanced cancer who are often at the risk of bone metastasis and regarding the possibility of combined bone deterioration due to cancer-induced osteolysis and curcumin-stimulated bone loss.

Metastasis-related angiogenesis and inflammation actively contribute to osteolysis. For example, breast cancer-produced vascular endothelial growth factor (VEGF) increases osteoclast formation (31) and promotes osteoclastogenesis (32). Myeloma-produced interleukin-1β (IL-1β) stimulates bone resorption, and inhibition of *IL-1β* mRNA expression in myeloma cells suppresses myeloma-mediated bone resorption (33). Significant increases in plasma concentrations of angiogenic factors (*e.g.* VEGF) and inflammatory cytokines (*e.g.* IL-1β) in LLC-bearing mice (6) suggested that bone loss in LLC-bearing mice might be caused, as least in part, by cancer-related angiogenic and inflammatory activities. However, the reduction in trabecular and cortical bone in curcumin-fed non-tumor-bearing mice demonstrates that curcumin-induced bone reduction is independent of LLC-mediated angiogenic and inflammatory activities.

There are studies showing that curcumin improves bone health in laboratory rodents. Hie *et al.* (34) reported that

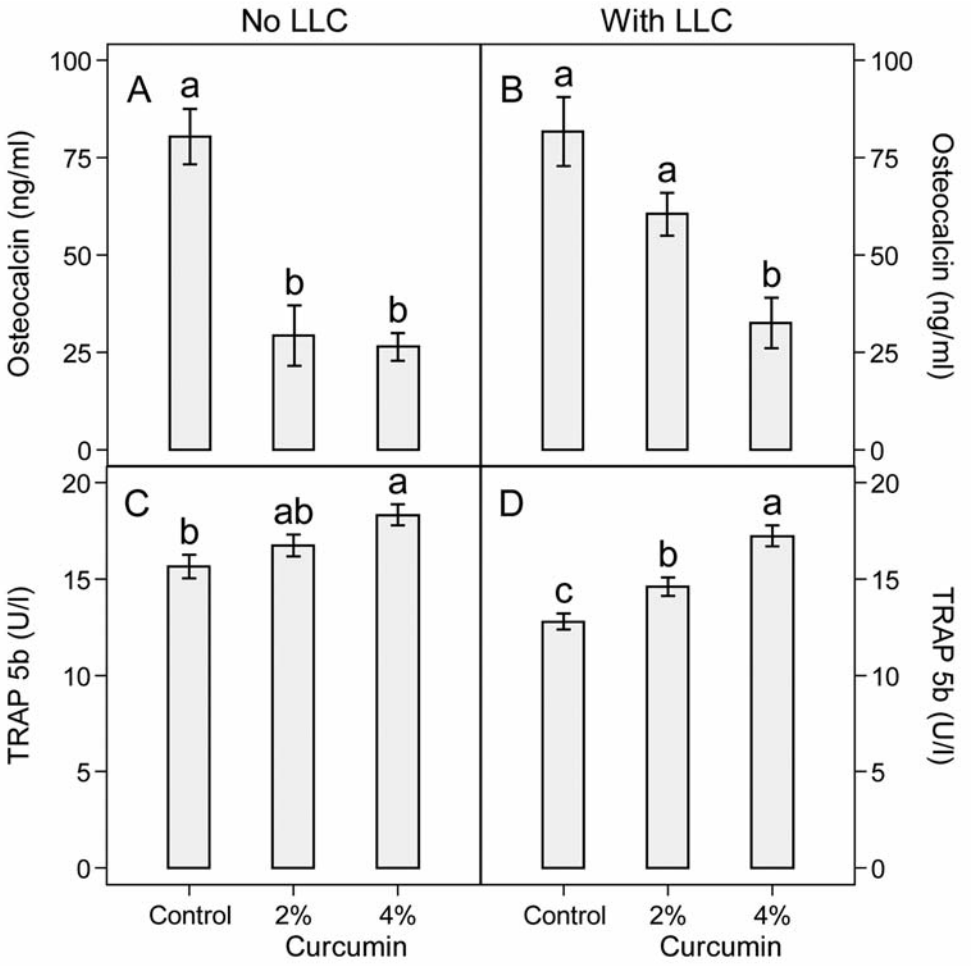

Figure 5. *Plasma concentrations of osteocalcin (A, B) and tartrate-resistant acid phosphatase 5b (TRAP 5b; C, D) in non-tumor-bearing (left column) and cancer-bearing mice (right column). One-way ANOVA and Tukey contrasts were used to compare differences among the control and the curcumin-fed groups. Values (means±SEM) in each panel with different letters are significantly different at p≤0.05 (n=8-10 for each value). LLC: Lewis lung carcinoma.*

consumption of a 0.5% curcumin diet for 14 days reduced bone resorption in rats with streptozotocin-induced diabetes. Yang *et al*. (14) found that feeding APP/PS1 transgenic mice with curcumin (0.6 g/kg) for three months improved bone microstructure and enhanced mineral density. Two studies showed that curcumin improved ovariectomy-induced bone loss in rats (15, 16); others reported that curcumin does not prevent bone loss in ovariectomized rats (35). The present study was carried out with physiologically intact mice, and curcumin feeding for a seven-week duration was initiated when mice were six-weeks old. Thus, differences in animal models, the levels of curcumin administered and the duration of experimental feeding may contribute to the different outcomes from these studies.

In the present study, curcumin was supplemented to the diet at 2% and 4% levels. These levels are within the range of 1% to 5% dietary levels that have been used in studies of curcumin and cancer prevention (5, 36, 37). The fact that curcumin feeding reduced body weight and body fat in non-tumor-bearing mice was unexpected, although increases in fecal caloric excretion explained these reductions. It does not seem likely that curcumin-induced bone loss was due to a decrease in body weight or caloric consumption because a similar bone reduction was observed in cancer-bearing mice in which curcumin feeding did not cause significant changes in body weight compared to the AIN93G-fed controls (6).

Chemoprevention studies with laboratory animals revealed that curcumin increases the incidence of intestinal carcinoma (38), increases the multiplicity of chemically-induced lung tumorigenesis (39) and enhances pulmonary metastatic growth (6). Long-term studies regarding the efficacy and safety of curcumin in laboratory rodents and particularly in humans are

very limited (40). The present study demonstrated that dietary curcumin supplementation reduced trabecular bone volume and cortical bone density in mice regardless of the presence of cancer. As the skeleton is a favored site of metastasis for many types of cancers, results from the present study suggest the possibility of a combined effect of cancer-induced osteolysis and curcumin-stimulated bone loss in patients using curcumin. The role of curcumin in skeletal health certainly warrant further investigation, and bone assessments should be performed on the participants of curcumin intervention trials, particularly on those with advanced malignancies.

Conflicts of Interest

The Authors declare no conflict of interests.

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