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Authors

Shahnazari, Mohammad
Turner, Russell T
Iwaniec, Urszula T
et al.

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Dietary dried plum increases bone mass, suppresses proinflammatory cytokines and promotes attainment of peak bone mass in male mice

Mohammad Shahnazari[a,*], **Russell T. Turner**[b], **Urszula T. Iwaniec**[b], **Thomas J. Wronski**[c], **Min Li**[d], **Mario G. Ferruzzi**[d], **Robert A. Nissenson**[a], **Bernard P. Halloran**[a]

[a]Veterans Affairs Medical Center, and Department of Medicine, University of California, San Francisco, CA

[b]Skeletal Biology Laboratory, College of Public Health and Human Science, Oregon State University, Corvallis, OR

[c]Department of Physiological Sciences, University of Florida, Gainesville, FL

[d]Departments of Food Science and Nutrition Science, Purdue University, West Lafayette, IN

Abstract

Nutrition is an important determinant of bone health and attainment of peak bone mass.Diets containing dried plum (DP)have been shown to increase bone volume and strength. These effects may be linked to the immune system and DP-specific polyphenols. To better understand these relationships, we studied DP in skeletally mature (6-month-old) and growing (1- and 2-month-old) C57Bl/6 male mice. In adult mice, DP rapidly (<2 weeks) increased bone volume (+32%) and trabecular thickness (+24%). These changes were associated with decreased osteoclast surface (Oc.S/BS) and decreased serum CTX, a marker of bone resorption. The reduction in Oc.S/BS was associated with a reduction in the osteoclast precursor pool. Osteoblast surface (Ob.S/BS) and bone formation rate were also decreased suggesting that the gain in bone in adult mice is a consequence of diminished bone resorption and formation, but resorption is reduced more than formation. The effects of DP on bone were accompanied by a decline in interleukins, TNF and MCP-1, suggesting that DP is acting in part through the immune system to suppress inflammatory activity and reduce the size of the osteoclast precursor pool. Feeding DP was accompanied by an increase in plasma phenolics, some of which have been shown to stimulate bone accrual. In growing and young adult miceDP at levelsas low as 5% of diet (w/w) increased bone volume.At higher levels(DP 25%),bone volume was increased by as much as 94%. These data demonstrate that DP feeding dramatically increases peak bone mass during growth.

Keywords

Plum; Bone; Osteoclast; Osteoblast; Nutrition; Peak bone mass

[*]Corresponding author. shahmod@gmail.com (M. Shahnazari).

1. Introduction

Nutrition plays an important role in bone metabolism and, during growth, attainment of peak bone mass. Diets containing dried plum (DP) (*Prunus domestica L.*) have been shown to have dramatic effects on bone [1–5]. DP can increase cancellous bone volume in adult (6-month-old) and aged (18-month-old) mice by 65% and 33%, respectively [1]. Although the response of bone to DP diminishes with aging, the bone-accruing effects occur at all ages. DP is reported to be as effective as intermittent PTH injection in restoring bone volume in orchidectomized rats and prevents bone loss and restores bone already lost due to gonadal hormone deficiency in osteopenic animals [2–5]. Along with the improvement in bone volume, bone strength increases with DP feeding [2]. DP has also been shown to increase bone mineral density (BMD) in humans suggesting that DP or DP extracts may prove to have therapeutic effects in patients with low BMD [6].Whether DP can increase bone accrual during growth has not been studied.

The increase in bone volume induced by DP is associated with decreases in bone surface lined by osteoclasts(Oc.S/BS) and osteoblasts (Ob.S/BS) [7]. Bone formation rate also decreases [7].It appears that in the adult mouse the gain in bone induced by DP reflects both decreased bone resorption and formation but that the decrease in resorption must exceed the decrease in formation. The mechanisms responsible for these changes are not known but could reflect a reduction in the progenitor populations for these cells. Consistent with this idea DP has been reported to decrease the bone marrow lymphoblast populations thus linking the effects of DP to the immune system [4,5].

In vitro, crude dried plum extracts containing plum-specific phenolics have been shown to inhibit osteoclastogenesis by down-regulating *nfatc1* and inflammatory mediators [8]. Using an osteoblastic cell line, it has been shown that the expression of runx2, osterix and IGF-I, genes coding for proteins associated with osteoblast differentiation, is up-regulated by the same phenolic-rich extract of dried plum used in the osteoclast studies [9]. These studies suggest that the phenolic compounds found in DP may play a role in its effects on bone.

In the studies reported here, we sought to determine whether the effects of DP on bone were associated with changes in the osteoclast/ osteoblast precursor populations and serum concentrations of immune-related cytokines. We also determined whether DP can increase bone volume during growth and thus promote attainment of peak bone mass. Whether dietary DP alters the plasma concentration of specific phenolic compounds was also assessed.

2. Material and methods

2.1. Animal protocol

Growing and skeletally mature C57BL/6 male mice were obtained from Jackson Laboratory (Sacramento, CA) and housed individually in an environmentally controlled laboratory animal research facility and acclimated for 2 weeks before starting experiments. We selected male mice to allow comparison with the previous studies of the skeletal effects of plum in male mouse model [1].

2.1.1. Experiment 1: Effects of DP on bone in skeletally mature mice—

Skeletally mature mice (6 months old) were assigned to either AIN-93M (control diet) or DP 25% (AIN-93M control diet containing 25% dried plum, w/w, dried plum <4% water) fed for 1, 2 or 4 weeks (n=15/group). The DP 25% dose was selected based on its effectiveness to alter bone turnover as observed in our previous studies [1]. Control and experimental diets were formulated in a pellet form and contained an equal amount of energy, protein, fat, carbohydrate, calcium, phosphorus and other nutrients. Details on the description of the diets are published elsewhere [1]. Food intake and body weight were recorded weekly. The animal protocol for the study was in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at the Veterans Affairs Medical Center, San Francisco. After 4 weeks of treatment, mice were evaluated using an NMR-MRI Body Composition Analyzer for body fat mass, lean mass, free water and total body water (EchoMRI, Houston, TX, USA). Animals were scanned without anesthesia or sedation and restrained (3 min) using manufacturer-provided holders to restrict their movement. Mice also were injected subcutaneously with calcein (10 mg/kg) and demeclocycline (10 mg/kg) (both from Sigma–Aldrich, St Louis, MO, USA) 7 and 2 days before euthanasia, respectively, to label bone mineralizing surfaces and measure bone formation rate. At the time of euthanasia, blood was collected for biomarker assays of bone turnover and immune cytokines, and polyphenol assays, tibias and femurs for bone marrow cell culture, μCT analysis, dynamic and static histomorphometry and quantification of gene expression in mineralized tissue.

2.1.2. Experiment 2: Effects of DP on bone in growing mice—

Growing mice (1 and 2 months old) were assigned to either AIN-93G (control diet) or DP 5, 15 or 25% (n=8/group) and fed for 4 weeks. Attainment of peak bone mass and changes in osteoblasts and osteoclasts were primary endpoints in this experiment.

2.2. Osteoblast culture

Tibias and femurs were cleaned of adherent tissue, and diaphyseal bone marrow stromal cells (BMSC) were harvested and plated at 1.3×10^5 nucleated cells/cm^2 in 100-mm dishes as previously described [10]. On day 2, nonadherent cells were removed and cultured under osteoclast induction conditions as described in the next paragraph. The culture medium in the adherent cells was changed to secondary medium (αMEM supplemented with 10% fetal bovine serum, 1% P/S antibiotics, 0.1% fungizone, 50-μg/ 100-ml L-ascorbic acid and 3-mM β-glycerophosphate) to induce osteoblastogenesis. Subsequent media changes were performed every 2 days for up to 28 days. The number of colony forming units (CFU), ALP positive CFU (CFU- AP+) with diameter greater than 3 mm and mineralizing nodules was quantified.

2.3. Osteoclast culture

The nonadherent cell fraction from the BMSC cultures was removed on day 2 of culture and washed with PBS. The cells were suspended in PBS, counted using a hemocytometer and seeded into 24-well tissue culture plates at 1×10^6 cells per well. theirThe cultures were maintained for 6 days in the stromal cell culture medium supplemented with RANK-L (30 ng/ml) and M-CSF (10 ng/ml). Media was changed every 2 days, and on day 6, cells were

washed twice with PBS and stained for tartrate-resistant acid phosphatase (TRAP) using a commercial kit from Sigma–Aldrich (St Louis, MO, USA). Dark, reddish-purple multinucleated cells (>3 nuclei) were counted as TRAP+ osteoclasts.

2.4. MicroCT analysis

The left distal femur was scanned *ex vivo* with a Scanco *Viva*CT 40 (Scanco Medical, Basserdorf, Switzerland) microCT (70 kVp, 85 μA, isotropic resolution of 10.5 μm in all three spatial dimensions). The region of interest (ROI) for the distal femur included the cancellous bone compartment beginning 0.5 mm proximal to the growth plate and extending proximally 1.5 mm. A threshold was determined as 22% of the maximal gray scale to distinguish mineralized from soft tissue. Trabecular bone volume expressed as a percent of total volume (BV/TV), trabecular number (Tb.N; 1/mm), thickness (Tb.Th; μm) and structure model index (SMI; ranges from 0 to 3, with 0 = plate like and 3 = rod like) were evaluated using the software provided by manufacturer.

2.5. Bone histomorphometry

The distal end of the right femur was processed undecalcified for quantitative bone histomorphometry. Bones were fixed in 10% phosphate buffered formalin for 24 h, dehydrated in increasing concentrations of ethanol and embedded undecalcified in modified methyl methacrylate. Osteoblast (Ob.S/BS) and osteoclast (Oc.S/BS) surfaces were measured in 4 μm-thick sections stained according to the Von Kossa technique with a tetrachrome counter stain. Fluorochrome-based indices of bone formation including mineralizing surface (MS/BS), mineral apposition rate (MAR) and surface-based bone formation rate (BFR/BS, dL.S + 1/2 sL.S/BS) were measured in 8-μm thick unstained sections using an image analysis system (Osteometrics, Decatur, GA, USA). Bone histomorphometric terminology is in accordance with recommendations by the ASBMR Histomorphometry Nomenclature Committee [11]. The ROI included cancellous bone beginning 0.5 mm proximal to the growth plate and extending proximally 1.5 mm.

2.6. Measurement of bone markers in serum

Blood was collected from the heart at the time of euthanasia, and serum was separated and stored at −80°C until processed. Enzyme immunoassays were performed for measurement of N-terminal propeptide of type 1 collagen (P1NP) (Immunodiagnostic Systems Rat/Mouse P1NP EIA, LOD=7 ng/ml) and Type I collagen C-terminal telopeptide (CTX) (IDS RatLaps EIA, LOD=2 ng/ml) according to the manufacturers' protocols. All assays were performed in duplicate.

2.7. Serum cytokine assay

Using the Bio-Plex mouse cytokine immunoassay kit (Bio-Rad Laboratories, Hercules, CA, USA), serum samples (50 μL) were tested on a 96-well filtration plate for the presence of tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1a,IL-1b, IL-6,IL-9, IL-10, IL-12 (p40),IL-12 (p70), IL-13, IL-17, interferon (IFN)-c, eotaxin, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-1α, MIP-β, regulated

upon activation normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein (MCP)-1. All samples were analyzed in duplicate using the Bio-Plex suspension array system followed by data analysis using the software provided by manufacturer.

2.8. Measurement of phenolics in plasma

Blood samples were collected in lithium–heparin collection tubes and centrifuged at 6500 rpm for 10 min at 4° C to obtain plasma. Samples were acidified with 1% ascorbic acid in saline solution (w/v) in a 4:1 ratio, and purged with nitrogen gas and immediately stored in −80° C. Major phenolic components including quercetin glycosides, caffeic acid and gallic acid derivatives were profiled in the plasma of both control and DP-fed animals using liquid chromatography with mass spectrometry (LC/ TOF-MS), as described elsewhere [12].

2.9. Measurement of mRNA expression in bone

We measured gene expression levels in the freshly harvested femoral diaphyses (without marrow) and methodically flushed with PBS to minimize marrow cell contribution. We analyzed gene expression using 384-well PCR plates allowing simultaneous testing of multiple genes from control and treatment groups to minimize interplate variabilities. The following genes were analyzed: *ctsk* (cathepsin K); *nfatc1* (nuclear factor of activated T cells); *rankl* (receptor activator of nuclear factor-kappa B ligand); *opg* (osteoprotegrin), *csf1*(colony stimulating factor 1); *csf1r* (colony stimulating factor 1 receptor); *acp5* (tartrate-resistant acid phosphatase 5b); *runx2*(runt-related transcription factor 2); *osx*, (osterix); *alp1* (alkaline phosphatase); *bglap2* (bone gamma carboxyglutamate protein (osteocalcin)); *col1a1* (collagen type 1 a1); *sost* (sclerostin); and *dkk1* (dickkop-1).

RNA was extracted from the femoral diaphysis without marrow (n=10/group) using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations and reverse transcribed with Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Inventoried Taqman primers for the RNAs of interest were purchased from Applied Biosystems. Two microgram of total RNA was reverse transcribed in 100 µl of reaction mixture containing 1X PCR Buffer, 1-mM deoxynucleoside triphosphate (DNTP) mix, 5-µM random primers, 7.5-mM $MgCl_2$, 0.4-U/µl RNAase inhibitor and 2.5-U/µl MultiScribe Reverse Transcriptase. The following reverse transcription protocol was used: 25°C for 10 min, 48°C for 40 min, 95°C for 5 min. Gene expression was measured using real-time PCR in triplicate 20-°L reaction volumes containing: 10 °L Taqman Universal PCR Master Mix (Applied Biosystems), 8-µL water, 1-µL primer/probe gene assay and 1-µL cDNA template. The reactions were performed in a 7300 RT-PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 10 min, 40 cycles of 95° for 15 s and 60°C for 1 min. Analysis was carried out using the software supplied with the system, and the number of threshold cycles (Ct) required for the FAM fluorophore intensities to exceed a threshold just above background was calculated. Ct values for triplicate reactions were averaged, and ΔCt was calculated by subtracting the Ct value of *gapdh* from the Ct value of the target gene. Expression of the target genes were quantified by calculating 2^-ΔCt relative to *gapdh* as the endogenous control.

2.10. Data Analysis

All data are reported as mean ± S.D. and were analyzed using Student's *t* test comparing DP *versus* control groups or analysis of variance followed by the Tukey range test. Significance was assumed for Pb<.05.

3. Results

3.1. Experiment 1: Effects of DP on bone in skeletally mature mice

DP did not affect food intake (26±1.5 g/week in control vs. 26.5± 1.1 g/week in DP) or body weight (28.1±1.1 g in control vs. 28.6±0.7 g in DP) after 2 and 4 weeks of feeding in skeletally mature mice (data from 4 weeks). Body composition, including lean mass, fat mass and the hydration ratio, was not affected (data not shown).

Cancellous BV/TV increased by 32% after 2 weeks and 46% after 4 weeks of DP feeding (*P*<.0001) (Fig. 1). This was associated with increased trabecular thickness at both time points (*P*<.01), but trabecular number increased only after 4 weeks of DP (*P*<.05). The SMI which is an assessment of the trabecular structure decreased after 2 and 4 weeks of feeding, changing to more plate-like and less rod-like cancellous structure (Fig. 1). A definitive bone phenotype could not be identified after 1 week of DP feeding.

Bone histomorphometry showed a similar response to DP at both 2 and 4 weeks (Fig. 2). A sharp decline in Oc.S/BS (−43%) was observed at 2 weeks and remained suppressed at 4 weeks (−41%). Osteoblasts (Ob.S/BS) followed similar decreases at 2 weeks (−47%) and 4 weeks (−64%) (*P*<.01). Bone mineralizing surface, mineral apposition rate and surface-based bone formation rate all decreased with DP at both 2 and 4 weeks (Fig. 2).

To determine whether the changes in osteoblasts and osteoclasts on the bone surface were associated with changes in the bone marrow progenitor populations for these cells, we used bone marrow cultures to assess the numbers of multinucleated TRAP + cells and mineralized nodules (Fig. 3). Osteoclasts formed in culture of bone marrow cells from DP-fed mice were 25–30% lower at 2 and 4 weeks (*P*<.01) compared to controls, but the osteoblast precursor population in the bone marrow did not change with DP, as evidenced by formation of ALP + colonies and mineralizing nodules (Fig. 3).

The serum concentration of the bone resorption marker, CTX, was reduced (−30%) by DP (P<.01), but the marker for bone formation, P1NP, did not change compared to the control group (Fig. 4).

There was no difference in gene expression between control and DP-fed mice except for *ctsk* (encoding cathepsin k, an osteoclast protease reflecting bone resorbing activity) which was 50% lower in DP-fed mice (Fig. 5).

The serum concentrations of the immune-related cytokines known to promote osteoclastogenesis including the interleukins, TNF and MCP-1 all decreased significantly with DP (Table 1). The other cytokines measured were not affected by DP.

3.2. Experiments 2: Effects of DP on bone in growing mice

Our studies in young growing mice were designed to determine whether DP could increase cancellous bone accrual prior to peak bone mass. Cancellous bone volume fraction in mice fed DP 5%, 15% or 25% from 1 to 2 months of age increased by 12%, 36% and 64%, respectively (Fig. 6). Bone volume in mice fed DP 5%, 15% and 25% from 2 to 3 months of age increased by 25%, 49% and 94%, respectively (Fig. 6). The changes in bone volume fraction were associated with increased trabecular number at all doses and increased trabecular thickness at the highest dose. The structure of the bone, as reflected by the SMI, also changed to more plate-like and less rod-like structure. Ob.S/BS in 2-month-old mice increased by + 39%, + 57% and + 30% with DP 5%, 15% and 25%, respectively. Ob.S/BS in 3-month old mice increased with 5% DP only. The Oc.S/BS did not change with DP (Fig. 6).

Serum concentrations of several of pro- and antiinflammatory cytokines and proteins decreased with DP, as shown in Table 1.

Several individual phenolics and phenolic metabolites were observed in the plasma of mice fed 25% DP (Fig. 7 and Table 2). The plasma concentrations of these were not detectable in mice fed the control diet. These compounds included caffeic, ferulic and dihydro-caffeic and dihydroferulic acid. In addition, glucuronide conjugates of ferulic and dihydroferulic acid were detected. Further, presence of the flavonoid quercetin in methylated and sulfonated form suggests absorption and metabolism of polyphenolics from DP and presence of these compounds in circulation with access to bone compartments. Concentrations in the present study were determined to be in μM range for phenolic and nM for flavonoid metabolites, consistent with previous studies on phenolic absorption in rodents [13].

4. Discussion

Our data show that DP can rapidly increase bone volume. Significant changes can occur within 2 weeks. The rapid turnover of bone, especially in young growing mice, may provide the means for the rapid changes in bone volume. That the accumulation of bone in older mice, where turnover is more gradual, is smaller than in young mice, where turnover is rapid,is consistent with this idea. The increase in bone volume at both 2 and 4 weeks in 6-month-old mice is associated with declines in both Oc.S/BS and Ob.S/BS and bone formation rate. That bone volume increases in DP-fed mice despite the reduction in Ob.S/BS suggests that the decrease in resorption exceeds the decrease in formation. DP appears to disrupt the balance between resorption and formation with a net effect of more bone. Mineral apposition rate and mineralizing surface were both lower in DP-fed mice suggesting that not only are there fewer osteoblasts on the bone surface but that their cellular activity is reduced. These findings suggest that DP has direct effects on osteoblast function. That a diet containing only 5% dried plum can increase bone volume suggests that even modest levels of DP can have positive effects on bone. Although the amount of DP in mice diet may translate to large amounts for human intake, a recent randomized controlled trial showed that daily consumptionof 50 g of dried plum for 6 months prevented bone loss in older, osteopenic postmenopausal women [14].

The reduced Oc.S/BS was associated with a decrease in the number of TRAP + cells arising from culture of the nonadherent bone marrow stromal cells. This suggests that the reduction in Oc.S/BS may result from a decrease in the progenitor pool. It is also possible that the osteoclast precursors from DP-fed mice are less responsive to recruitment into the osteoclast lineage. This could come about as a result of intracellular changes that impair M-CSF or RANKL signaling. That the osteoblast precursor population did not change suggests that the mechanisms involved in the decline in Ob.S/BS do not include changes in the precursor population. Recruitment from the precursor pool and reduced half-life on the bone surface, however, may contribute to the decline in Ob.S/BS.

The decrease in the serum concentration of CTX is consistent with the changes in osteoclast surface. The serum concentration of P1NP was surprisingly not reduced. This seems inconsistent with the decrease in bone formation rate, but it may reflect global *versus* local activity.

Bone structure with the exception of cancellous bone volume appears normal in DP-fed mice. That all of the measured bone RNAs with the exception of *ctsk* are normal is consistent with this observation. It appears that DP has a relatively specific effect on Oc.S/BS and Ob.S/BS. It is clear that DP induces a reduction in Oc.S/BS and may also have an inhibitory effect on inherent osteoclast activity (reduction in *ctsk* expression). Interestingly when measured in bone marrow stromal cells, Smith *et al.* [7] did not find a change in *ctsk*.

Immune cells play a key role in mediating several aspects of bone biology [15,16]. Bone loss in clinical situations, such as estrogen deficiency and autoimmunity, is mediated through increased production of osteoclastogenic cytokines such as IL-1 and TNF, which act on stromal cells and osteoclast precursors [17,18]. We measured various cytokines to assess the effects of DP on immune cell activity. In general there was a decline in proinflammatory cytokines in mice fed DP (Table 1). DP may function in part by suppressing both pro- and antiinflammatory cytokines and mediators. How these changes occur is not clear.

Consistent with our findings in adult mice, the increase in bone volume in the young mice was linked to increases in both trabecular number and thickness. As a consequence of these changes connectivity density increased, and SMI decreased. The cancellous bone is becoming more plate like and less rod like. These morphometric changes are expected to improve bone quality [19].

The cellular mechanisms underlying the changes in bone in young and adult mice induced by DP appear to differ. In the adult, the primary effect appears to be decreased bone resorption (Oc.S/BS, CTX and cathepsin K are decreased), whereas in young growing mice the gain in bone is associated with an increase in Ob.S/BS. Osteoclast surface appears to be unaffected. Importantly, our data clearly show that DP can dramatically increase peak bone mass accrual during growth. This suggests that modest dietary supplementation with DP during growth and development may be an effective way to reduce osteoporotic fractures later in life.

Of the phenolics identified, caffeic acid and quercetin might be most critical in modulating bone formation and resorption. Tang *et al.* [20] reported that caffeic acid is a potent phenolic that inhibits osteoclastogenesis in rat marrow culture (IC50 5.6 uM) by down-regulating *nfatc1*. Considering that the average plasma concentration of caffeic acid found in our study was 5.27 uM, it is plausible that this phenolic was responsible in part for modulation of bone formation and resorption. In addition, quercetin has been reported to have antiinflammatory properties and inhibitory effects on osteoclastogenesis and bone resorption [21–24]. While plasma quercetin levels observed in our study were lower than that used in these *in vitro* studies, chronic exposure to DP-derived quercetin may have played role in augmenting bone volume in growing and adult animals.

In summary, we observed that dietary supplementation with DP can increase bone volume in young growing mice and thus promote attainment of peak bone mass. Dietary DP can also increase bone volume in adult and aged mice [1]. If this can be recapitulated in the human, we may have a new approach of increasing bone volume in humans of all ages.

Our findings further suggest that the gain in bone in the adult mouse reflects a decline in bone resorption where as the gain in bone in the young mouse reflects an increase in bone formation. Shrinkage of the osteoclast precursor pool may account in part for the decline in the Oc.S/BS in the adult. The broad suppression of serum proinflammatory cytokines clearly links DP to the immune system and may explain the reduction in the osteoclast precursor pool size. The appearance of phenolic compounds in the plasma known to inhibit bone resorption and stimulate osteogenesis suggests that mediationof the effects of DP on bone maybe through specific polyphenolics either found in DP or metabolized from other DP polyphenols. The gut microbiota may play an important role in this process.

The highest dietary level of DP in our study was 25% by weight. Based on a human 2000 calorie diet, a mouse diet of 25% DP is approximately equivalent to 20 DPs/day in the human. From our studies in young growing, adult and aged mice [1], we found that diets containing 5%, 15% and 25% dried plum, respectively, are needed to significantly increase bone mass. With aging bone becomes less sensitive to DP. From these findings, consumption of four DPs/day may be expected to have beneficial effects on bone in children. Human studies are needed to determine the effect of DP on bone.

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injury and exerts an anabolic effect on osteoporotic bone: the role of aryl hydrocarbon receptor as a mediator of osteogenic action. J Bone Miner Res 2011;26:2096–111. [PubMed: 21638315]

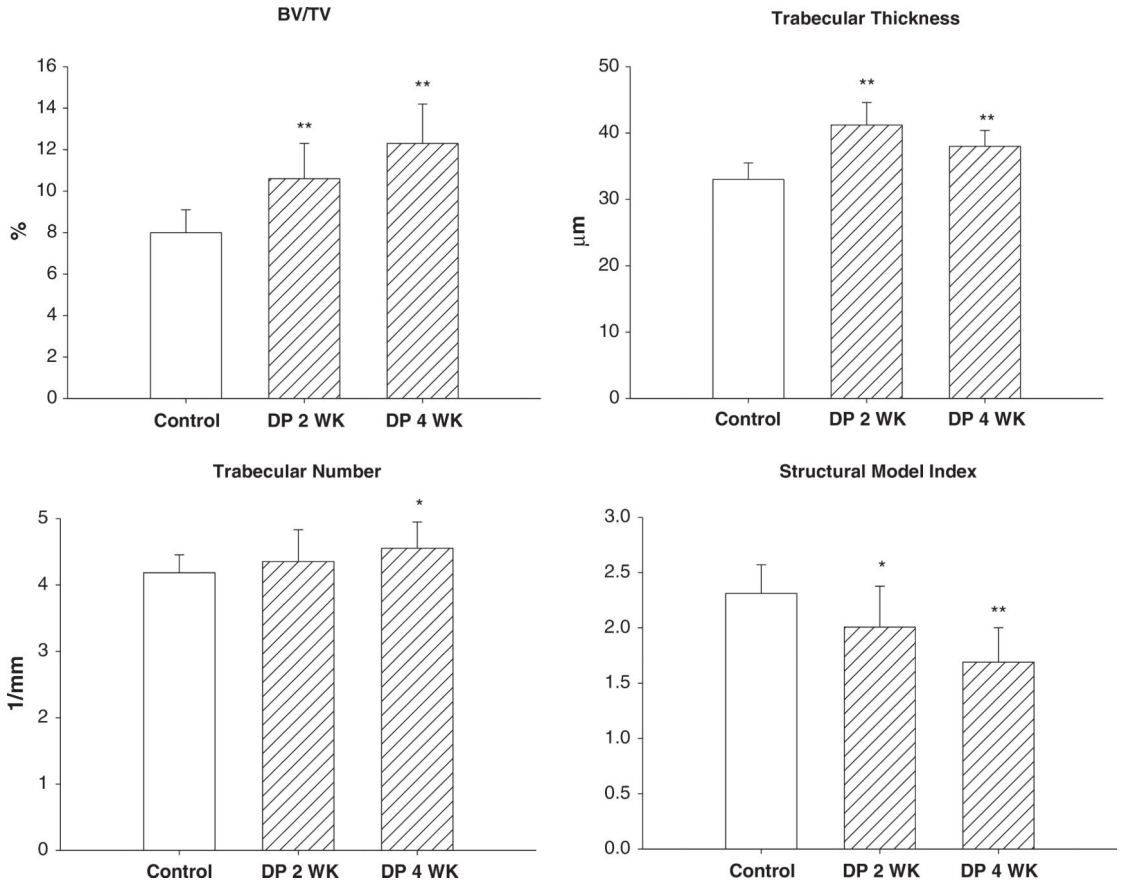


Fig. 1.

Cancellous bone volume (BV/TV)and architecture from μCT analysis in the distal femoral metaphysis in 6-month-old C57Bl/6 male micefed a control (AIN-93M) or diet containing 25% dried plum (DP) for 2 or 4 weeks (mean ± S.D., n=12–15, * P≤.05, ** P<.01 vs. control).

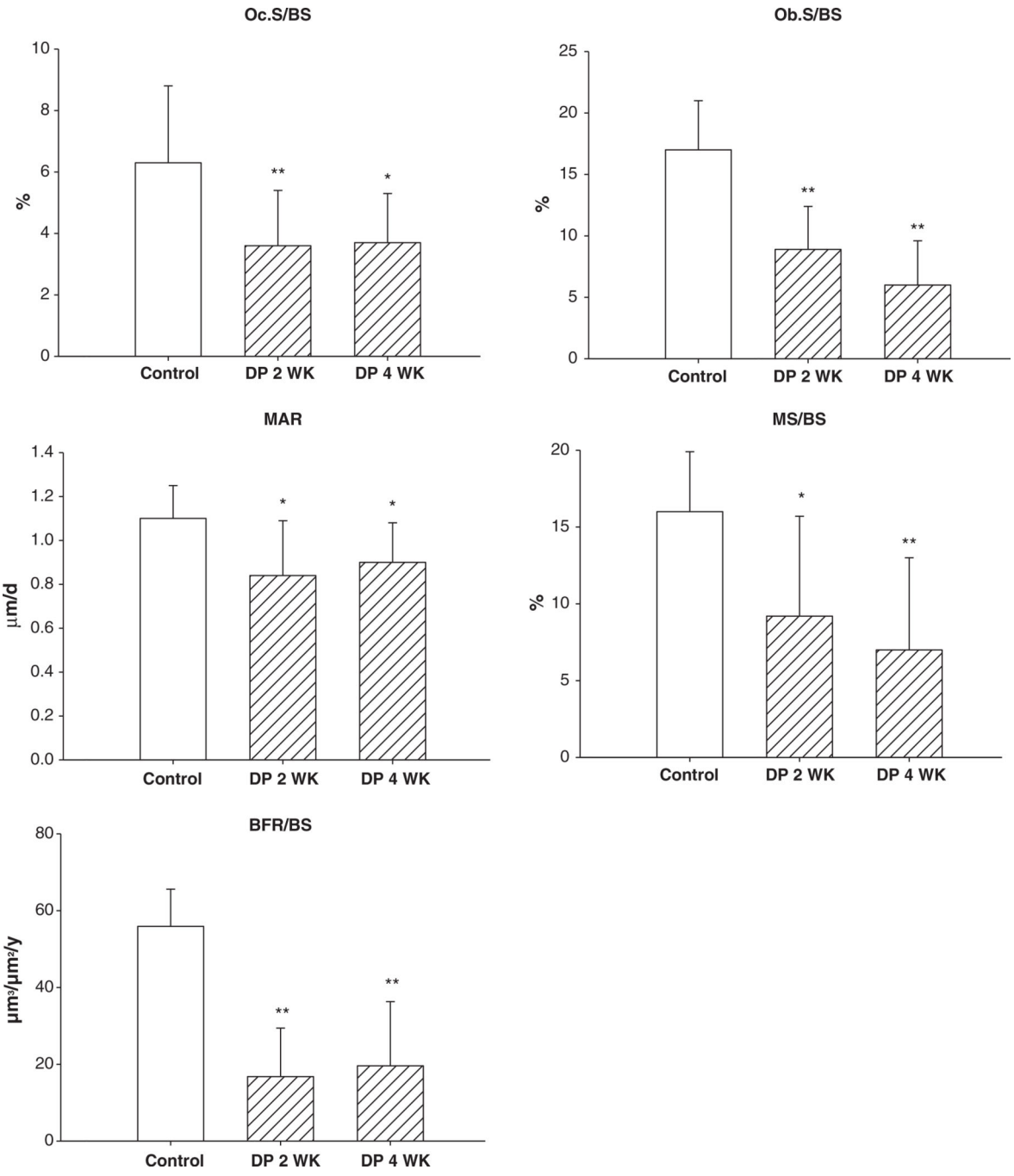


Fig. 2.

Histomorphometric measures in distal femoral metaphysis of 6-month-old C57Bl/6 male mice fed control (AIN-93M) or diet containing 25% dried plum (DP) for 2 or 4 weeks (mean ± S.D., n=12–15, * Pb<.05, ** Pb<.01 *vs.* control); Ob.S/BS, osteoblast surface; Oc.S/BS, osteoclast surface; MAR, mineral apposition rate; MS/BS, bone mineralizing surface; BFR/BS, bone formation rate.

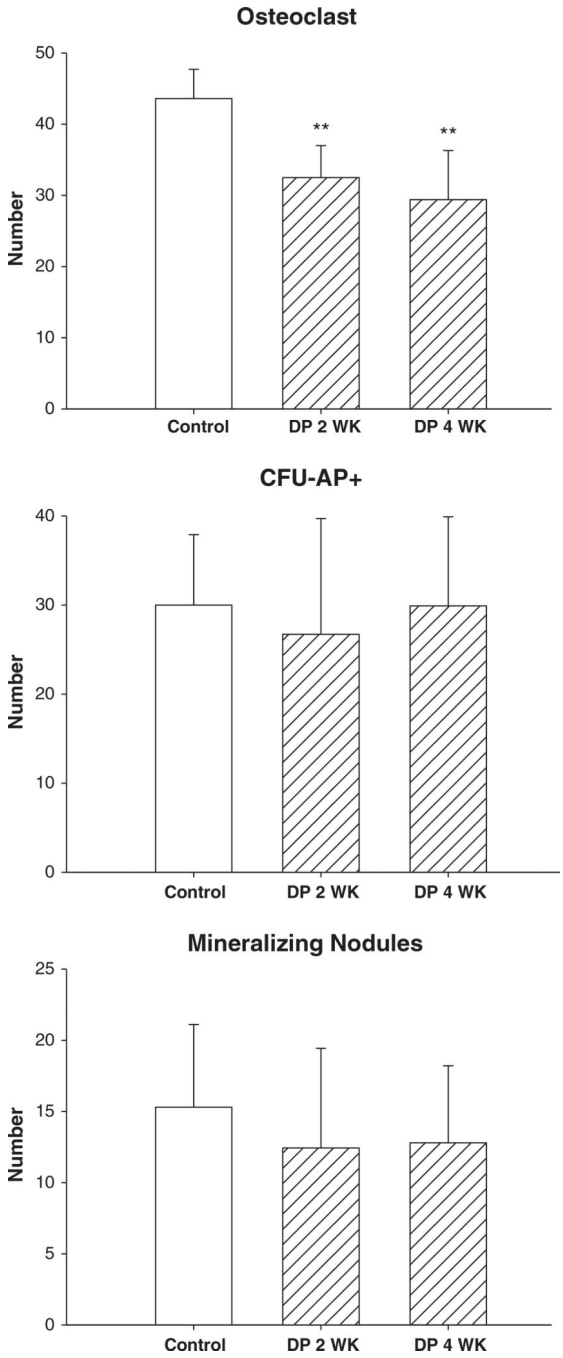


Fig. 3.

Osteoclasts (TRAP+ multinucleated cells) and osteoblasts (CFU-AP+ and mineralizing nodules) formed in culture of bone marrow cells from 6-month-old C57Bl/ 6 mice fed control (AIN-93M) or diet containing 25% dried plum (DP) for 2 or 4 weeks (mean ± S.D., n=10/group, ** P<.01 *vs.* control).

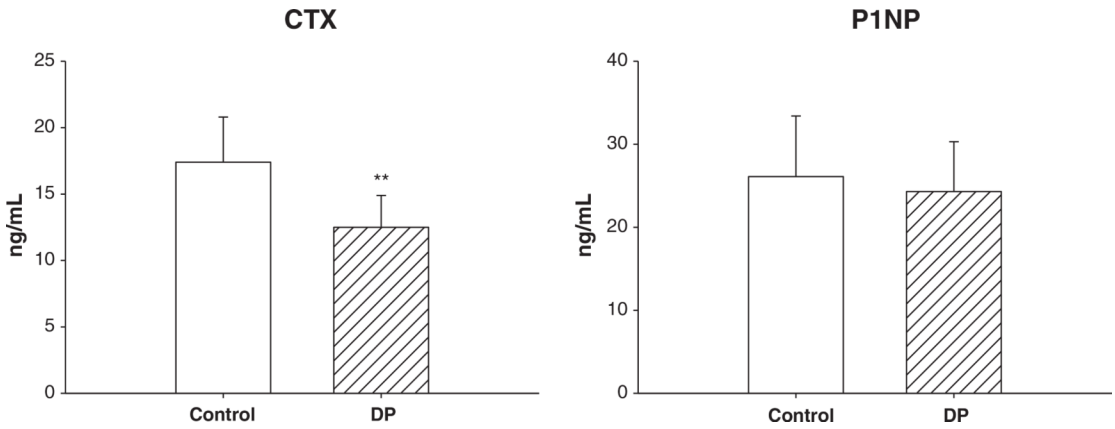


Fig. 4.

Bone resorption and formation markers in serum in 6-month-old C57Bl/6 mice fed control (AIN-93M) or diet containing 25% dried plum (DP) for 4 weeks (mean ± S.D., n=15). CTX, C-terminal telopeptide of Type 1 collagen; P1NP, N-terminal propeptide of Type 1 procollagen (mean ± S.D., n=15/group, **P≤.01 *versus* control.

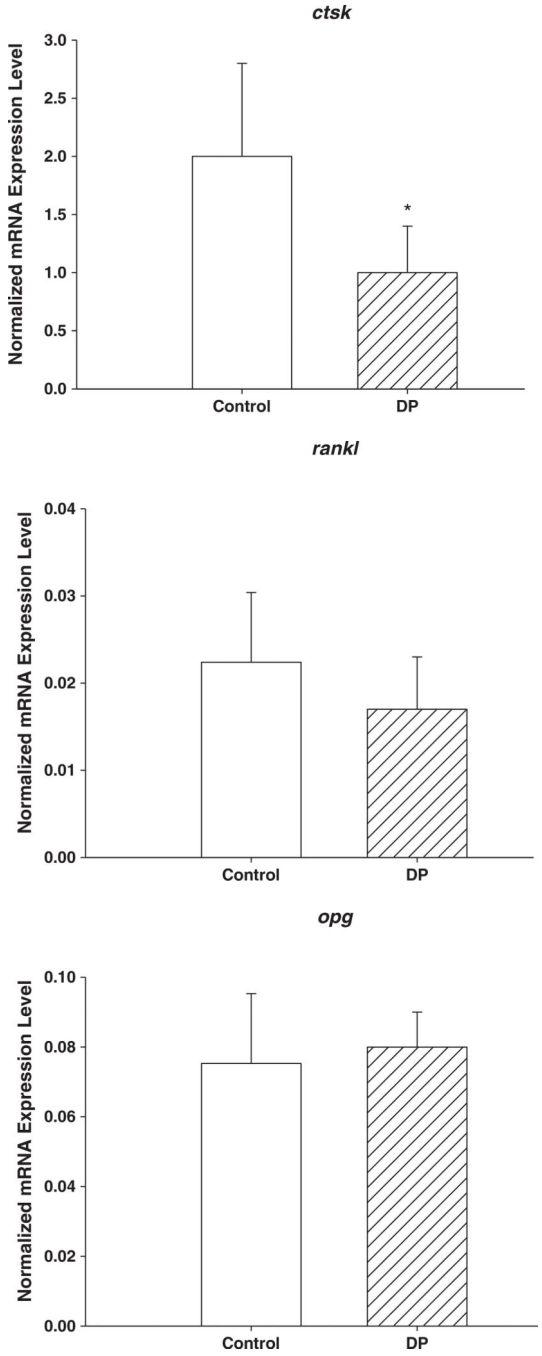
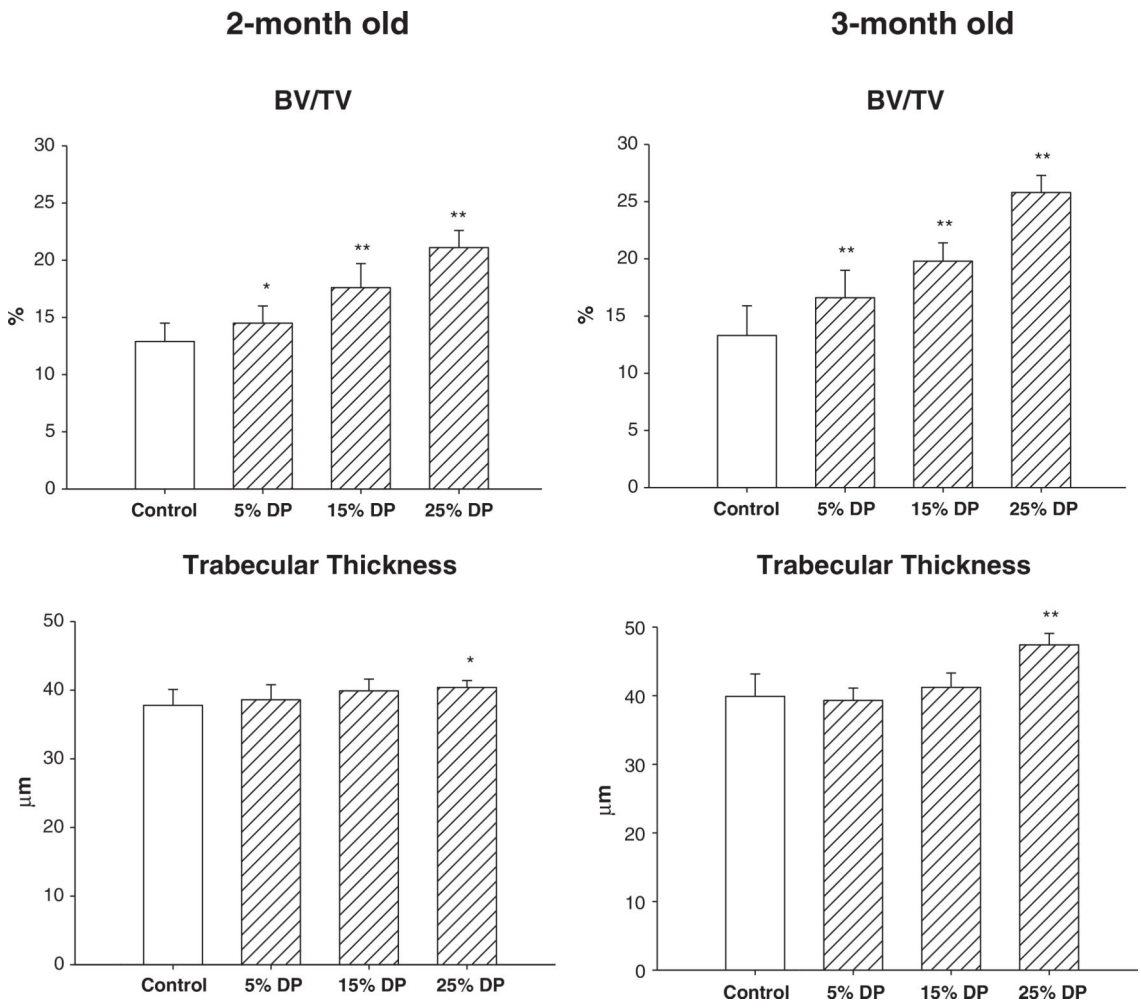
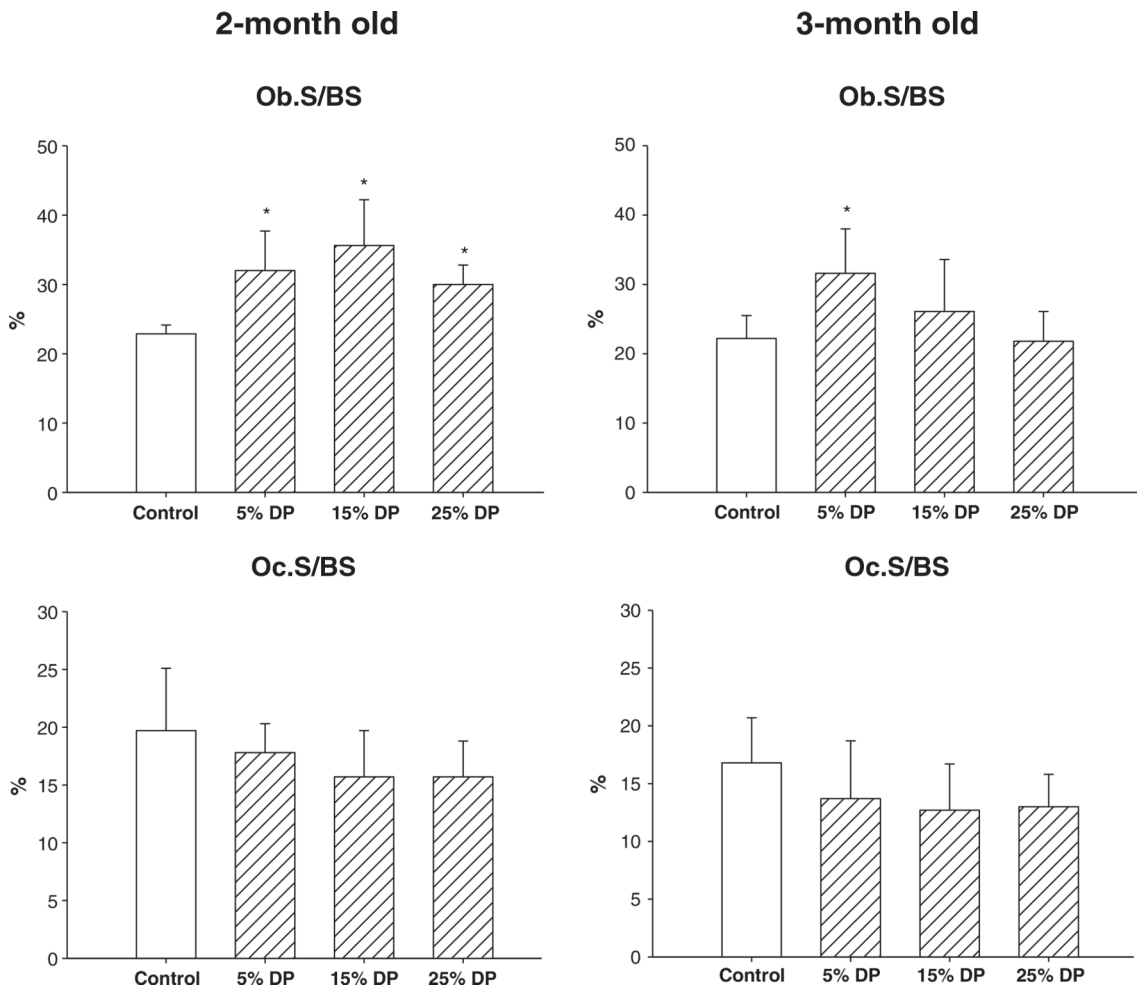


Fig. 5.

Osteoclast-related gene expression in femoral cortical bone of 6-month-old C57Bl/6 mice fed control (AIN-93M) or diet containing 25% dried plum (DP) for 4 weeks (mean ± S.D., relative to expression level of *gapdh* (mean ± S.D., n=12/group, * Pb<.05). *ctsk*, cathepsin K; *rankl*, receptor activator of nuclear factor-kappa B ligand; *opg*, osteoprotegerin.





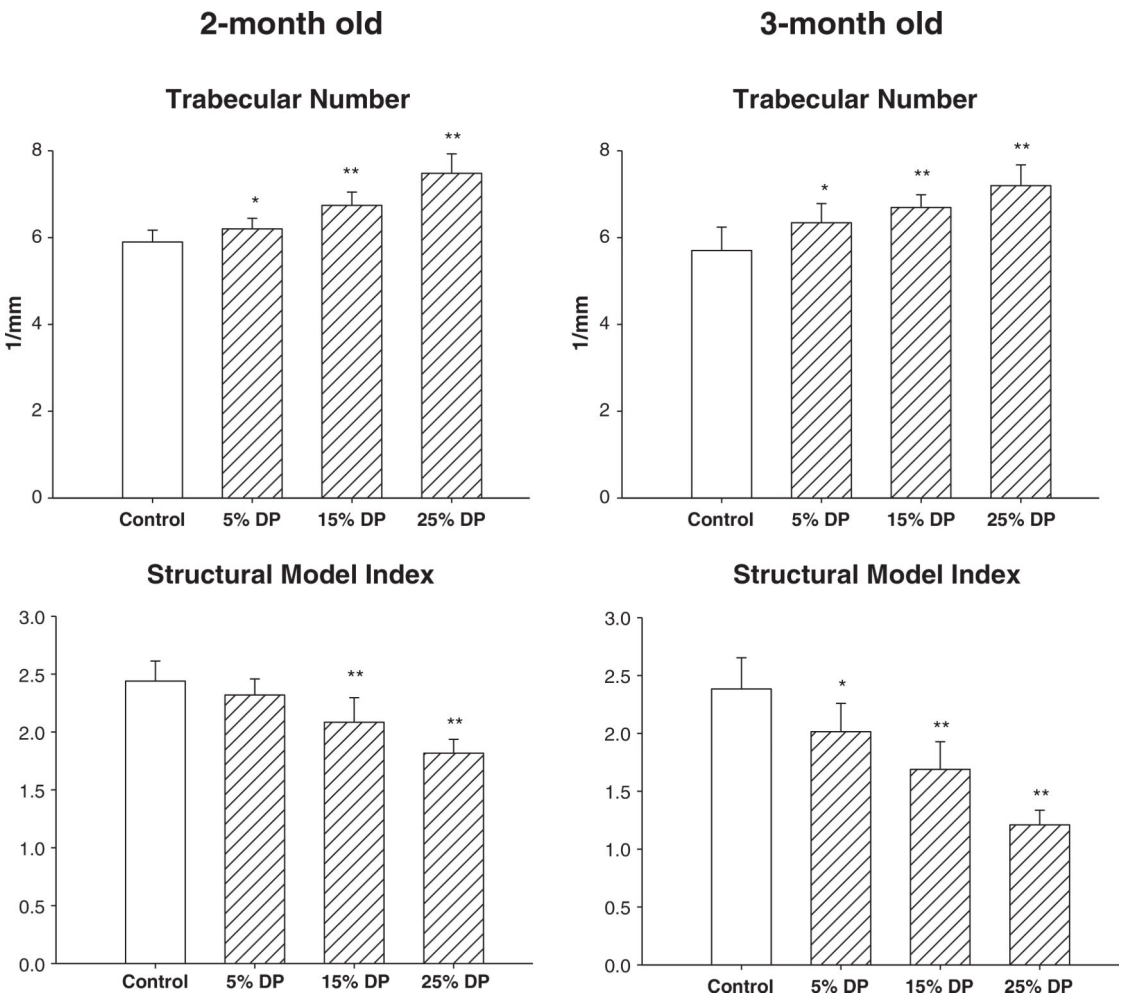


Fig. 6.

Cancellous bone volume (BV/TV) and architecture (μCT) and osteoblast and osteoclast surfaces (histology) in distal femoral metaphyses in 2- and 3-month-old C57Bl/6 male mice fed control (AIN-93G) or diet containing 5, 15 or 25% dried plum (DP) for 4 weeks (mean ± S.D., n=10–12, **P*<.05, ** *P*<.01 *vs*. control).

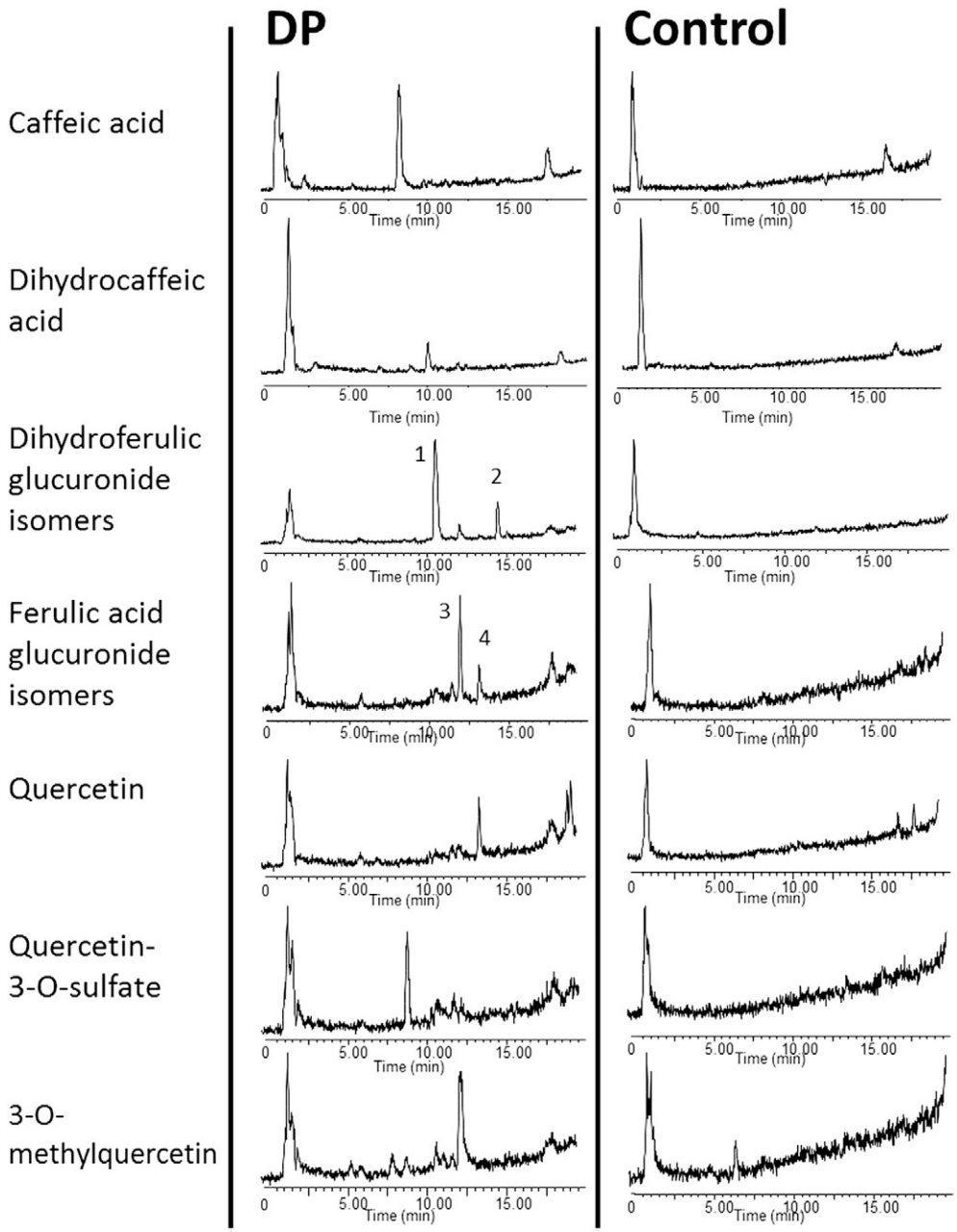


Fig. 7.

Extracted ion chromatograms of plasma from 2 months old male mice fed control (AIN-93G) or diet containing 25% dried plum (DP) for 4 weeks. Isomers of phenolic metabolites were identified as followed: 1, dihydroferulic 4-O-glucuronide; 2, dihydroferulic 3-O-glucuronide; 3, ferulic acid 4-O-glucuronide; 4, isoferulic acid 3-O-glucuronide.

Table 1

Levels of immune cytokines in the serum of 6-month-old C57B1/6 male mice fed control (AIN-93M) or 25% plum in diet (DP) for 4 weeks (mean ± S.D., $n = 8$, * $P \leq .05$, ** $P < .01$ *vs.* control)

Cytokine	Control	DP
IL 1a	15±3	9±3*
IL 1b	439±129	275±68*
IL 6	13±5	10±2
IL 10	76±26	46±26*
IL 12 (p70)	187±62	105±36**
IL 12 (p40)	151±41	117±37
IL 13	404±156	261±118*
IL 17	120±28	89±17*
TNF α	364±104	271±71*
MCP 1	237±76	174±21*
KC	27±5	35±7*
INF γ	14±6	11±4
m CSF	200±52	170±45
g CSF	55±8	75±10*
Rantes	46±11	44±14
Mip 1b	61±22	48±12
Mip 1a	17±6	13±4
Eotaxin	585±128	520±140

IL-, interleukin; TNF-α, tumor necrosis factor; MCP, monocyte chemoattractant protein; KC, keratinocyte-derived chemokine; INF γ, interferon gamma; g-CSF, granulocytecolony-stimulating factor; Mip, macrophage inflammatory proteins.

Table 2

Plasma concentrations of phenolic compounds and metabolites in 2 months old male mice fed diet containing 25% dried plum (DP) for 4 weeks

Phenolic compounds and metabolites (μmol/L)	$C_{average}$	C_{Max}	C_{Min}
Caffeic acid	5.27±1.14	10.98	1.17
Dihydrocaffeic acid	1.50±0.33	2.72	0.02
Dihydroferulic acid 4-O-glucuronide	4.99±0.44	7.13	3.27
Dihydroisoferulic acid 3-O-glucuronide	1.39±0.31	2.72	0.42
Ferulic acid 4-O-glucuronide	3.06±0.45	5.33	1.10
Isoferulic acid 3-O-glucuronide	0.80±0.21	1.83	0.02
Quercetin	0.015±0.003	0.024	0.004
Quercetin 3-O-sulfate	0.023±0.006	0.047	0.006
3-O-methylquercetin	0.032±0.004	0.046	0.013

Values were undetectable in animals fed control diet (AIN-93G).