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

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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 mice DP at levels as 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.
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Keywords: Plum; Bone; Osteoclast; Osteoblast; Nutrition; Peak bone mass

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

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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. The 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.

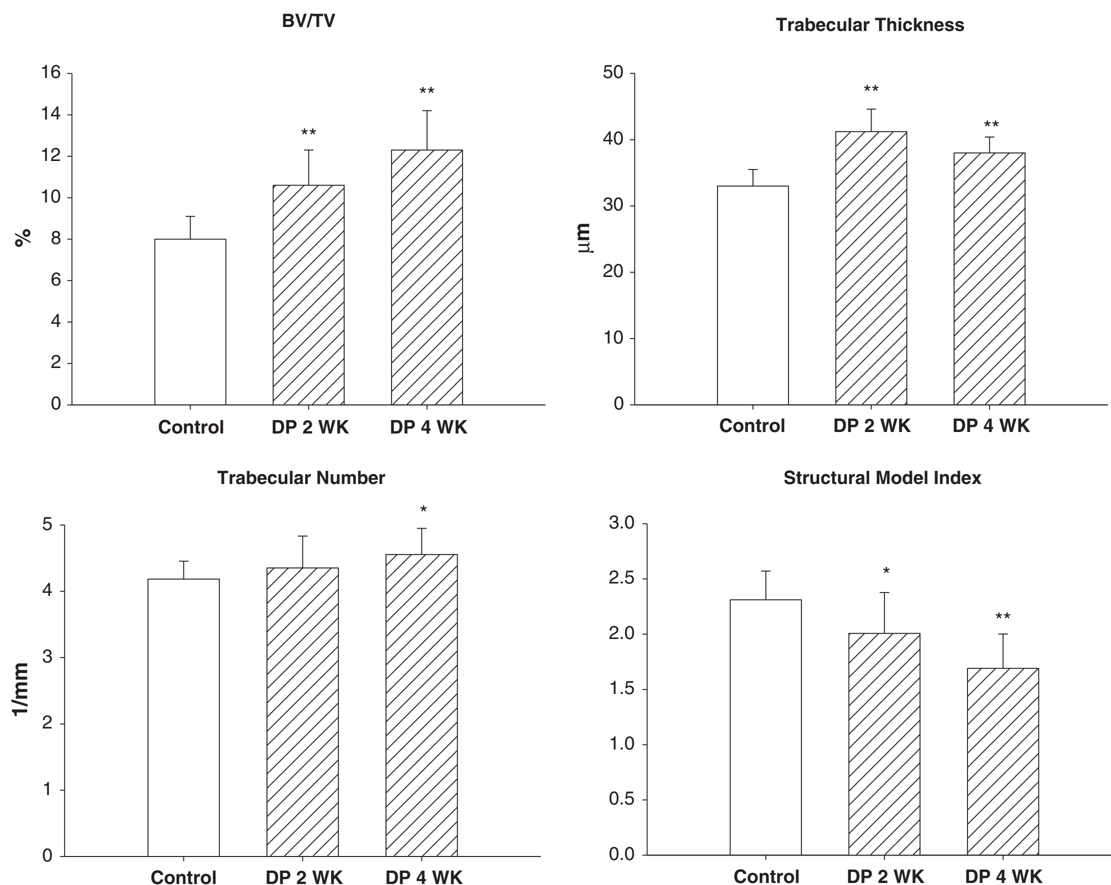

Fig. 1. Cancellous bone volume (BV/TV) and architecture from μCT analysis in the distal femoral metaphysis in 6-month-old C57Bl/6 male mice fed a control (AIN-93M) or diet containing 25% dried plum (DP) for 2 or 4 weeks (mean ± S.D., $n=12$–15, * $P\leq.05$, ** $P<.01$ *vs*. control).

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