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Dried plum polyphenols attenuate the detrimental effects of TNF-α on osteoblast function coincident with up-regulation of Runx2, Osterix and IGF-I☆

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Abstract

Previous studies have demonstrated that dried plums which contain high amounts of polyphenols can restore bone mass and structure, and significantly increase indices of bone formation. The purpose of this study was to determine how dried plum polyphenols influence osteoblast activity and mineralized nodule formation under normal and inflammatory conditions. MC3T3-E1 cells were plated and pretreated with dried plum polyphenols (0, 2.5, 5, 10 and 20 μg/ml) and 24 h later stimulated with TNF-α (0 or 1.0 ng/ml). The 5, 10 and 20 μg/ml doses of polyphenols significantly increased intracellular ALP activity under normal conditions at 7 and 14 days, and restored the TNF-α-induced suppression of intracellular ALP activity by 14 days ($P<.001$). Polyphenols also increased mineralized nodule formation under normal and inflammatory conditions. In the absence of TNF-α, 5 μg/ml of polyphenols significantly up-regulated the growth factor, IGF-I, compared to controls, and the 5 and 10 μg/ml doses increased the expression of lysyl oxidase involved in collagen crosslinking. TNF-α decreased the expression of Runx2, Osterix and IGF-I, and polyphenols restored their mRNA levels to that of the controls. Although TNF-α failed to alter lysyl oxidase at 18 h, the polyphenols up-regulated its expression ($P<.05$) in the presence of TNF-α. As expected, TNF-α up-regulated RANKL mRNA and polyphenols suppressed RANKL expression without altering OPG. Based on these findings, we conclude that dried plum polyphenols enhance osteoblast activity and function by up-regulating Runx2, Osterix and IGF-I and increasing lysyl oxidase expression, and at the same time attenuate osteoclastogenesis signaling.

Keywords: Bone; Antioxidants; Osteoporosis

1. Introduction

Normal bone homeostasis is maintained by a balance between bone formation and bone resorption [1]. Conditions in which bone formation by osteoblasts is decreased relative to the activity of bone resorpting osteoclasts result in a net loss of bone mass [2,3]. Osteoblasts, fibroblast-like cells derived from a mesenchymal lineage, synthesize enzymes and matrix proteins involved in the formation of mineralized bone [4] and serve as a source for osteoclast differentiation factors including receptor activator of NF-κB ligand (RANKL) [5]. Inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 decrease osteoblast activity and stimulate osteoblasts to produce inflammatory cytokines such as RANKL, prostaglandin E_2 (PGE_2) and IL-1 that can enhance osteoclast differentiation and activity [6,7].

In gonadal hormone deficiency, TNF-α has been identified as one of the predominant pro-inflammatory mediators of bone loss [8]. TNF-α inhibits osteoblast activity and bone mineralization by down-regulating growth factors such as

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insulin-like growth factor (IGF)-I [9] and alkaline phosphatase (ALP) [10] involved in the formation of hydroxy apatite, and decreasing the expression of lysyl oxidase, the enzyme responsible for collagen crosslinking [11]. Suppression of osteoblast activity by TNF-α is associated with down-regulation of transcription factors, Osterix and Runx2, which regulate the expression of ALP and IGF-I as well as several bone matrix proteins including osteopontin and bone sialoprotein [12,13]. TNF-α also alters osteoblast signaling by increasing RANKL production which promotes osteoclast differentiation and activity [6]. Under conditions of gonadal hormone deficiency, decreased osteoblast activity and promotion of osteoclast differentiation by inflammatory cytokines such as TNF-α are associated with a defective antioxidant system [14]. Supplementation with antioxidants attenuates ovariectomy-induced bone loss by suppressing TNF-α and enhancing bone formation [15].

Some polyphenolic compounds and their derivatives, which reside in fruits and vegetables, have antioxidant and anti-inflammatory properties that have been shown to influence both osteoclasts and osteoblasts. For example, polyphenols such as caffeic acid, resveratrol and rutin inhibit osteoclast differentiation and activity [16,17], directly stimulate osteoblasts, and favorably alter bone formation markers [18,19]. Caffeic acid, one of the polyphenols in dried plums (*Prunus domestica* L.), has been reported to reverse the oxidative stress (H_2O_2)-induced decrease in ALP and type I collagen expression by osteoblasts as well as the phosphorylation of Runx2 [20]. Resveratrol, the major phenolic compound in grapes, stimulates the proliferation and differentiation of osteoblasts and increases intracellular ALP activity and bone morphogenic protein (BMP-2) expression [19]. Rutin is another polyphenol found in plums [21] and is reported to increase serum osteocalcin and bone mineral density (BMD) in estrogen-deficient osteopenic rats [18]. Findings from these studies suggest that a variety of individual phenolic compounds modulate osteoblast activity and signaling, and that an optimal combination of these compounds may have anabolic effects on bone.

Dried plum, a rich source of polyphenols [21], has been shown to positively influence bone mass, bone microarchitecture and serum markers of bone metabolism [16,22,23]. A short-term study of postmenopausal women consuming approximately 100 g of dried plum per day (i.e., 10–12 dried plums) demonstrated that dried plum increased serum bone-specific ALP and IGF-I [23]. Data from animal studies indicate that dried plum enhances circulating IGF-I in gonadal hormone deficiency models of osteoporosis [22,24] and effectively restored bone in osteopenic ovariectomized female rats [25]. Recently, dried plum's ability to restore bone mass and microarchitecture in osteopenic gonadal hormone-deficient male rats was compared to the anabolic agent, parathyroid hormone (PTH) [26]. Dried plum completely reversed the decrease in bone mass compared to sham-operated control animals and had similar effects to PTH on vertebral trabecular bone architecture and biomechanical properties. Although other plant-based foods with relatively high phenolic compound content such as soy favorably modulate bone metabolism, their ability to restore bone in osteopenic animal models appears to be somewhat limited [27].

Based on the findings from these animal and clinical studies [22–26], we anticipate that components of dried plum such as its polyphenolic compounds mediate these anabolic effects on bone by altering osteoblast signaling, maturation and/or activity. Hence, the purpose of this study was to investigate how polyphenols extracted from dried plum stimulate osteoblast activity and mineralized nodule formation under normal and inflammatory conditions.

2. Methods and materials

2.1. Materials and Reagents

MC3T3-E1 (RIKEN No. RCB1126), mouse calvarial pre-osteoblastic cells were obtained from Riken BioResource Center (Ibaraki, Japan). Fetal bovine serum (FBS) and penicillin G-streptomycin were purchased from GIBCO-BRL (Grand Island, NY, USA). Minimum essential medium (α-MEM), ascorbic acid, β-glycerophosphate, alizarin red-S and mouse TNF-α were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dried plum powder was generously provided by the California Dried Plum Board. For ALP activity measurements, an alkaline phosphatase substrate kit from Bio-Rad (Hercules, CA, USA) was used. Unless otherwise listed, all other chemicals were reagent grade and obtained from Fisher Scientific.

2.2. Experiment 1

The objective of Experiment 1 was to evaluate the effects of polyphenols extracted from dried plum on osteoblast activity and function under normal and inflammatory conditions. MC3T3-E1 cells were plated at a density of 1×10^5 cells/ml in six-well plates (n=3) and cultured in α-MEM containing 10% FBS, 2 mM L-glutamine and 100 U/L penicillin G and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 48 h. After cells reached confluence, the medium was replaced with α-MEM containing 10 mM β-glycerophosphate and 25 μg/ml ascorbic acid to facilitate in vitro mineralization. Cells were treated with 0, 2.5, 5, 10 or 20 μg/ml of dried plum polyphenols for 24 h and then stimulated with 0 or 1 ng/ml of TNF-α, which was the minimum dose needed to significantly inhibit ALP activity and mineralization as determined in preliminary studies. Culture medium, which included TNF-α and the dried plum polyphenol doses described above, was replaced every 3 days. For ALP activity measurements, culture media and cell monolayers were harvested at 7 and 14 days after confluence. For analysis of mineralized nodule formation, cells were fixed at 28 days and stained with alizarin red-S as described below.

Table 1
Primer sequences for real-time PCR

Transcript	Sequence (5′–3′)
RANKL	F: CTG ATG AAA GGA GGG AGC AC
	R: GAA GGG TTG GAC ACC TGA ATG C
OPG	F: TCC TGGCAC CTA CCT AAA ACA GCA
	R: ACA CTG GGC TGC AAT ACA CA
Runx2	F: TGC TTC ATT CGC CTC ACA AA
	R: TTG CAG TCT TCC TGG AGA AAG TT
Osterix	F: CCT CTC GAC CCG ACT GCA GAT C
	R: AGC TGC AAG CTC TCT GTA ACC ATG AC
IGF-I	F: CTT CAC ATC CTC TCT ACC T
	R: ATT CTG TAG GTC TTG TTT CC
Lysyl oxidase	F: GGC GCC AGA CAA TCC AAT GGG AG
	R: GCC TGG ATG TAG TAG GGA TCG GG
GAPDH	F: CCG GTG CTG AGT ATG TCG
	R: CCC TGT TGC TGT AGC CGT A

2.3. Extraction of polyphenols from dried plum

Polyphenols were extracted from whole dried plum powder using a modified version of the method described by Kim et al. [28]. Ethanol extraction was repeated twice using 80% ethanol while sonicating with pulsated nitrogen gas. The volume of extract was reduced using roto-evaporation and then freeze-dried. The total polyphenol content of the extract was quantified based on the Folin-Calteau assay [29] and the effects of polyphenols on cell viability were assessed using resazurin assay [30] which relies on the ability of viable cells to reduce the resazurin dye to the fluorescent resorufin.

2.4. Intracellular and extracellular ALP activity

After 7 and 14 days of treatment with polyphenols, media was collected and the cell monolayer was gently washed twice with ice-cold phosphate buffered saline (PBS). Cells were lysed with 0.2% Triton x-100, the lysate was centrifuged at 14,000×*g* for 5 min and the clear supernatant was used for the measurement of ALP activity and protein concentration. ALP was determined based on the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol by spectroscopy at 405 nm according to the manufacturer's instruction (Bio-Rad). Total protein was assessed using the BCA method [31] and ALP activity was expressed in nanomole of *p*-nitrophenol produced per minute per microgram of protein.

2.5. Nodule formation

The extent of mineralized nodule formation based on staining density and number of nodules was determined by alizarin red-S (AR-S) staining at 28 days [32]. Briefly, cells were washed twice with PBS and then fixed in ice-cold 70% ethanol for 1 h at room temperature. Following another wash with PBS, cells were stained with 40 mM alizarin red-S (pH 4.2) for 10 min at room temperature. Digital images of the stained matrix were acquired using a digital camera (Canon, Japan) and the number of mineralized nodules per well was counted. For the quantification of staining density, alizarin red-S staining was released from the cell matrix by incubation with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 15 min. The alizarin red-S concentration was determined by measuring the absorbance at 562 nm.

2.6. Experiment 2

Experiment 2 was designed to investigate the dose-dependent effects of TNF-α and polyphenols alone and in combination on osteoblast gene expression. Cells were plated in six-well plates at a density of 1×10^5 cells/ml and allowed to adhere for 24 h. Culture medium was replaced with differentiation media containing 10 mM β-glycerophosphate and 25 μg/ml ascorbic acid, and the cells were treated with 0, 1 or 10 ng/ml of TNF-α to determine the alterations in gene expression associated with escalating dose. Eighteen hours was determined to be an appropriate time of TNF-α exposure to induce alterations in gene expression based on a review of the literature and a preliminary study. Therefore, cells were stimulated with TNF-α for 18 h, then harvested and total RNA isolated for the analysis of mRNA expression.

To evaluate the effects of dried plum polyphenols on gene expression, we chose the doses of polyphenols (2.5, 5 or 10 μg/ml) which effectively enhanced both ALP activity and mineralized nodule formation in Experiment 1. Cells were plated in six-well plates at a density of 1×10^5 cells/ml and allowed to adhere for 24 h. Culture medium was replaced with the differentiation media as described above. Twenty-four hours later, the cells were pretreated with 0, 2.5, 5 or 10 μg/ml of dried plum polyphenols followed by stimulation with 0 or 1 ng/ml of TNF-α. The 1 ng/ml dose of TNF-α was used to mimic the effects of a low-grade inflammatory state that significantly altered the expression of the genes of interest. After 18 h of exposure to TNF-α, the cells were collected for the analysis of mRNA levels.

2.7. Analysis of gene expression using real-time PCR

Total cellular RNA was isolated using Trizol following the manufacturer's guidelines (Invitrogen, Rockville, MD, USA). The concentration and purity of the RNA were determined by ODs measured at 260 and 280 nm. The expression of mRNA was quantified by real-time RT-PCR using a Mx3005*p* (Stratagene, La Jolla, CA, USA) with Light Cycler RNA Amplification Kit SYBR green I (Roche, Penzberg, Germany). Denatured RNA (50 ng) from cells was reverse transcribed and amplified with gene-specific primers (Table 1) under the following conditions: reverse transcription at 58°C for 10 min, inactivation of reverse transcriptase at 95°C for 30 s, and 45 cycles of 94°C for 15 s, 60°C for 20 s and 72°C for 20 s. Post-PCR melting curves confirmed the specificity of single-target amplification. The amount of mRNA for each gene was calculated using a standard curve generated from 10-fold dilutions of control RNA (Roche, Penzberg, Germany) and expression levels were normalized to GAPDH.

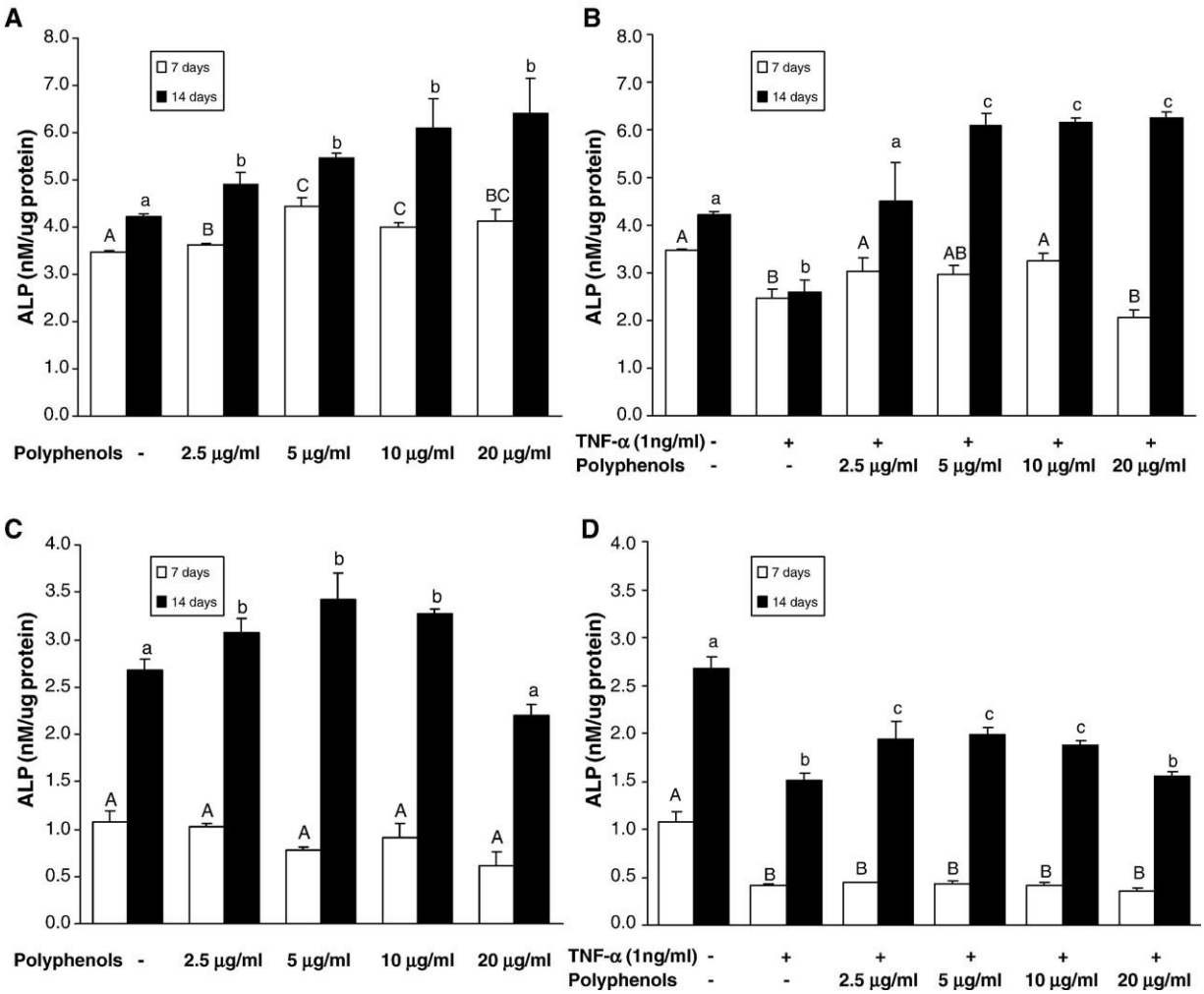

Fig. 1. Effects of dried plum polyphenol extracts on (A and B) intra- and (C and D) extracellular ALP activity in MC3T3-E1 cells. Cells were plated at 1×10^5 cells/ml, treated with 0, 2.5, 5, 10 or 20 μg/ml of dried plum polyphenol extracts (A and C) under normal conditions or (B and D) stimulated with TNF-α (1 ng/ml) for 7 and 14 days. This assay was performed in triplicate. Bars that share the same uppercase or lowercase letter are not significantly different from each other (n=3).

2.8. Statistical analysis

Statistical analysis was performed using SAS version 9.0 (SAS Institute, Cary, NC, USA). The significance of treatment effects was analyzed by one-way ANOVA followed by post hoc analysis using the Fisher's least squares means separation test. All treatments were performed in triplicate. Values were expressed as means±S.E. and difference between treatments was considered to be significant at $P<.05$. Each experiment was performed 2–3 times and the representative graphs and images are presented.

3. Results

3.1. Cell viability

No cytotoxic effects of the dried plum polyphenol extract were observed on the MC3T3-E1 cells at the doses used in this study (data not shown). Additionally, increasing dose of dried plum extract did not significantly alter cell viability as measured by the resazurin assay.

3.2. Intracellular and extracellular ALP activity

Dried plum polyphenol extract stimulated intracellular ALP activity in MC3T3-E1 cells under normal conditions at 7 and 14 days (Fig. 1A). The increase in ALP activity observed with two higher doses (10 and 20 μg/ml) was 50% greater than the controls at 14 days. TNF-α treatment significantly reduced the intracellular ALP activity by 29% and 38% at Days 7 and 14, compared to controls (Fig. 1B). In the presence of TNF-α (Fig. 1B), the 2.5 and 10 μg/ml dose of polyphenols significantly elevated intracellular ALP at 7 days, but all doses of polyphenols enhanced ALP activity by 14 days compared to cells receiving TNF-α alone. In addition to intracellular ALP, the ALP released by the cells into the media was assessed. At Day 7, no dose of dried plum polyphenols altered the extracellular ALP activity under

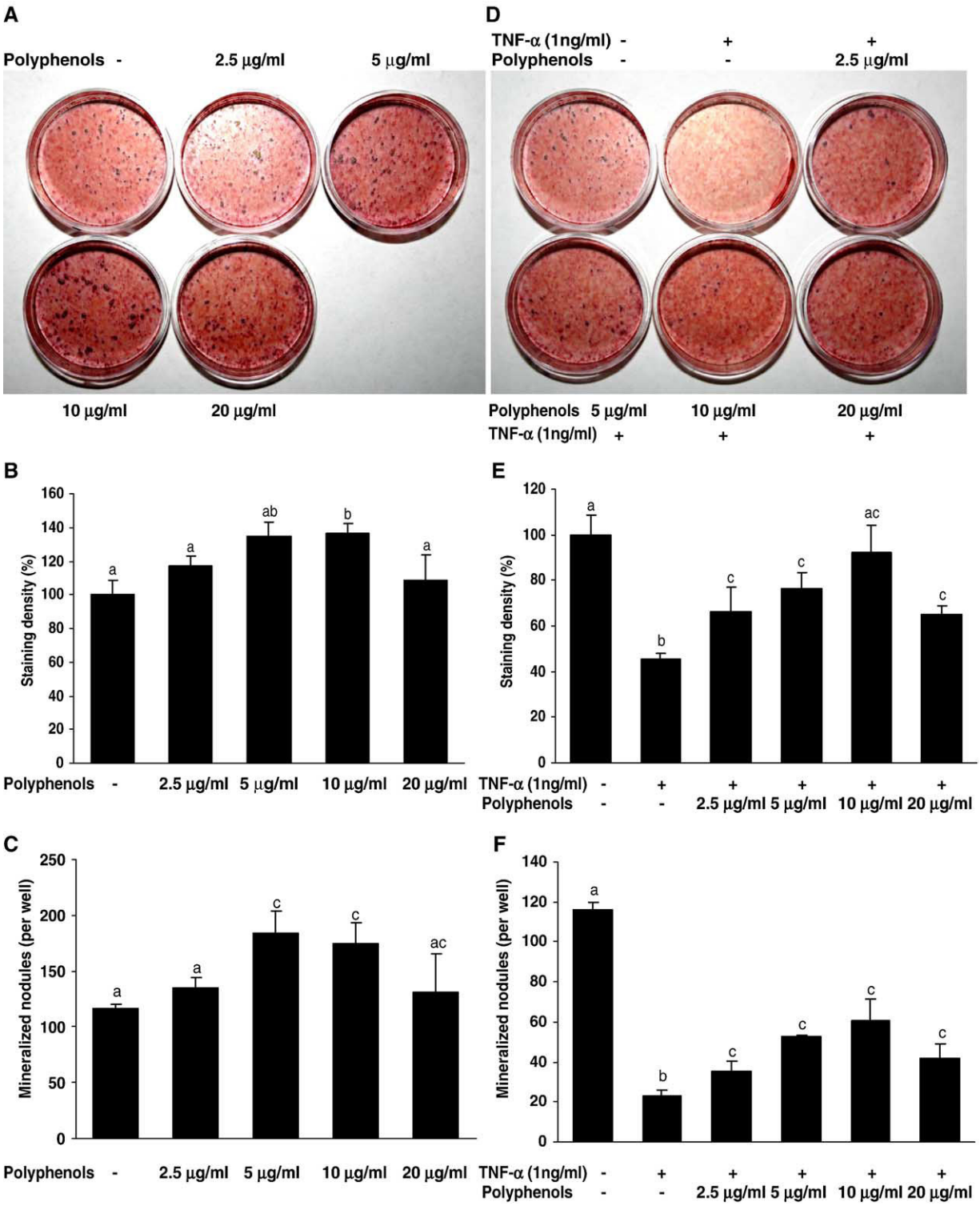

Fig. 2. Staining density and number of mineralized nodules are altered by dried plum polyphenols under (A) normal and (D) inflammatory conditions. Cells were plated at 1×10^5 cells/ml, treated with 0, 2.5, 5, 10 or 20 μg/ml of dried plum polyphenol extracts under normal conditions or stimulated with TNF-α (1 ng/ml) for 28 days. Under normal conditions, (A) Alizarin red S staining (red color) density (B) was increased in the 10 μg/ml dose and (C) nodule number was increased in both the 5 and 10 μg/ml doses. In the presence of TNF-α, (E) staining density and (F) nodule formation were increased with all doses of dried plum polyphenols. Bars that share the same letter are not significantly different from each other.

either normal or inflammatory conditions (Fig. 1C and D). After 14 days, all doses of polyphenols increased ($P<.05$) extracellular ALP activity under normal conditions (Fig. 1C) with the exception of the 20 μg/ml dose. Similar to the intracellular response, TNF-α treatment significantly reduced the extracellular ALP activity at Days 7 and 14

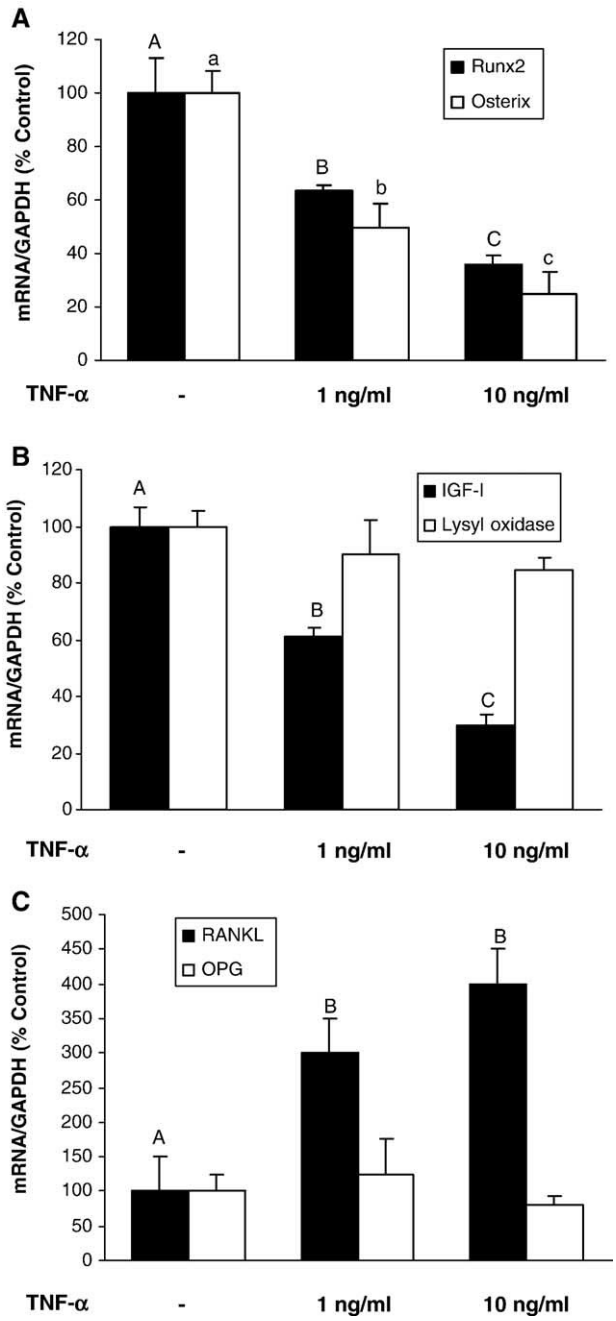

Fig. 3. Alterations in key transcription factors (Runx2 and Osterix), IGF-I, lysyl oxidase and osteoblast signaling (i.e., RANKL and OPG) in response to TNF-α. TNF-α induced dose-dependent alterations in mRNA expression of (A) Runx2 and Osterix, (B) IGF-I and (C) RANKL. Cells were plated at 1×10^5 cells/ml and stimulated with TNF-α (0, 1 or 10 ng/ml) for 18 h. Bars that share the same letter are not significantly different from each other.

compared to controls (Fig. 1D). ALP activity was increased ($P<.01$) by the 2.5, 5 and 10 μg/ml doses of polyphenols after 14 days compared to the cells receiving TNF-α.

3.3. Nodule formation

To determine the effect of dried plum polyphenols on osteoblast function, cells were stained with AR-S at 28 days post-confluence for the identification of mineralized nodules (Fig. 2). In the absence of TNF-α, only the 10 μg/ml dose of dried plum polyphenols significantly increased the staining density compared to controls (Fig. 2B), but both the 5 and 10 μg/ml doses increased ($P<.05$) the number of nodules (Fig. 2C). At 28 days, there was an apparent TNF-α-induced decrease ($P<.01$) in mineralized nodule formation (Fig. 2D) compared to cells receiving no TNF-α which was supported by the decrease of alizarin staining density (Fig. 2E) and number of mineralized nodules (Fig. 2F). The decrease in alizarin staining density produced by TNF-α was significantly increased by all three doses of polyphenols with the 10 μg/ml of polyphenols having the greatest effect (i.e., >1.5-fold increase in staining density) compared to the cells receiving TNF-α only. Likewise, the 10 μg/ml of polyphenols restored nodule staining density to that of the controls (Fig. 2E). These data showing that dried plum polyphenols increased the number and size of mineralized nodules under normal and inflammatory conditions suggest that dried plum polyphenols increase osteoblast activity and function.

3.4. Alterations in gene expression with TNF and polyphenols

TNF-α dose-dependently ($P<.01$) suppressed the expression of the transcription factors, Runx2 and Osterix, at 18 h (Fig. 3A). In addition to the transcription factors, TNF-α dose-dependently ($P<.01$) decreased the expression of IGF-I, with a 70% reduction observed with the higher dose of TNF-α (Fig. 3B). Neither 1 nor 10 ng/ml of TNF-α altered the expression of lysyl oxidase at 18 h (Fig. 3B). Both doses of TNF-α (1 and 10 ng/ml) increased ($P<.05$) the expression of RANKL by three- and four-fold, respectively, while no alterations in OPG expression were observed (Fig. 3C). These results suggest that TNF-α decreased ALP activity and mineralized nodule formation by down-regulating transcription and growth factor related to osteoblast maturation.

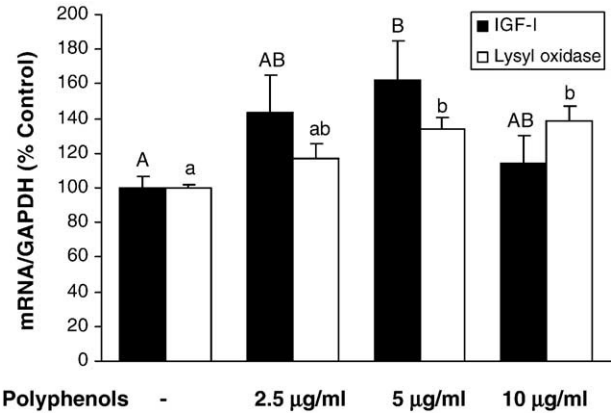

Fig. 4. Dried plum polyphenols up-regulate IGF-I and lysyl oxidase expression under normal conditions. Cells were plated at 1×10^5 cells/ml, treated with 0, 2.5, 5 or 10 μg/ml of dried plum polyphenol extracts for 42 h. Bars that share the same uppercase or lowercase letter are not significantly different from each other.

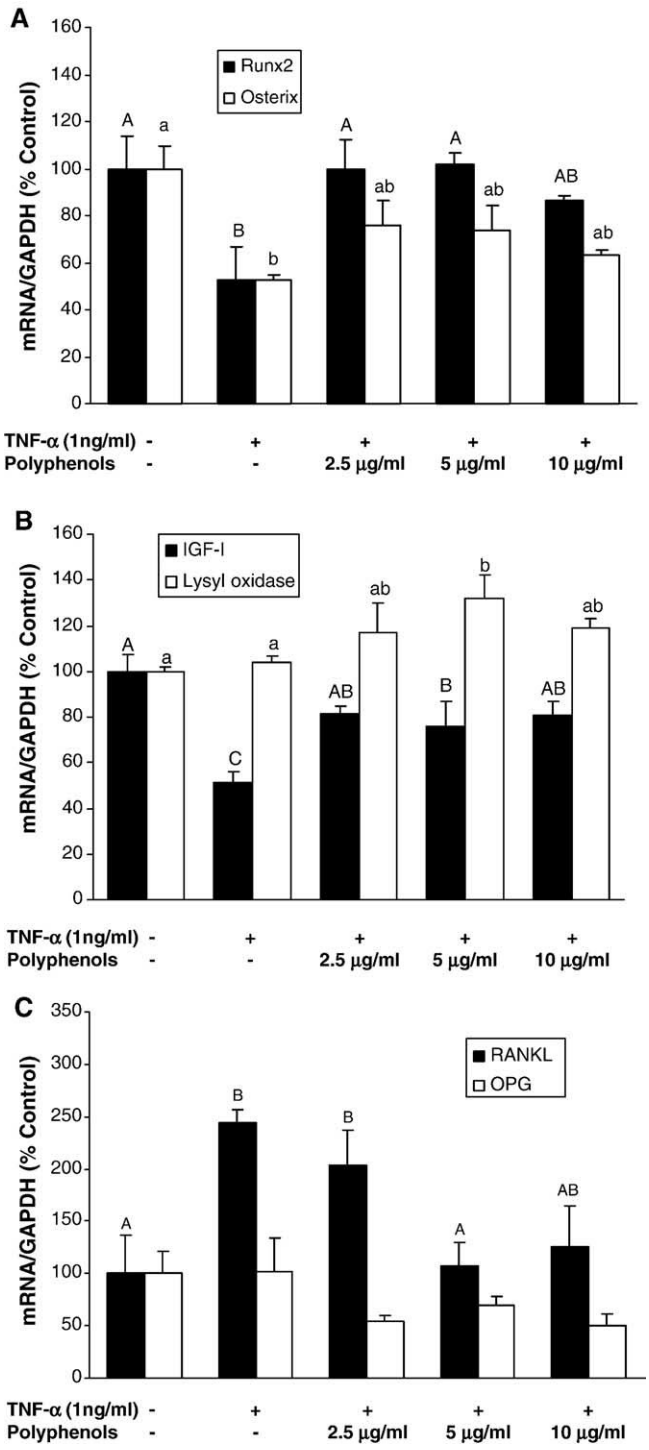

Fig. 5. Effects of dried plum polyphenols on TNF-α induced alterations in (A) Runx2 and Osterix, (B) IGF-I and lysyl oxidase, (C) RANKL and OPG expression under inflammatory conditions. Cells were plated at 1×10^5 cells/ml, treated with 0, 2.5, 5 or 10 μg/ml of dried plum polyphenol extracts for 24 h and then stimulated with TNF-α (1 ng/ml) for 18 h. Bars that share the same uppercase or lowercase letter are not significantly different from each treatment.

Under normal conditions, the 2.5, 5 and 10 μg/ml doses of dried plum polyphenols increased the expression of IGF-I up to 63% after 24 h of treatment (Fig. 4), but only the 5 μg/ml dose reached the level of statistical significance. Lysyl oxidase expression was also up-regulated ($P<.05$) in the 5 and 10 μg/ml doses of polyphenols compared to controls (Fig. 4). The dried plum polyphenol extract did not significantly alter the expression of the transcription factors, Runx2 and Osterix, by MC3T3-E1 cells in the absence of an inflammatory state (data not shown).

In the presence of TNF-α, 2.5 and 5 μg/ml doses of polyphenols completely restored ($P<.01$) Runx2 expression to the level of controls (Fig. 5A), while the 10 μg/ml dose had an intermediate effect on gene expression. Osterix expression was depressed by TNF-α, but all doses of polyphenols (2.5 , 5 and 10 μg/ml) increased the expression to a level similar to the controls. It should be noted that the polyphenols did not elevate the expression level of Osterix to the point that they were significantly different to the cells receiving TNF-α only (Fig. 5A). The suppression of IGF-I in the cells receiving TNF-α was reversed by all doses of dried plum polyphenols, and the 2.5 and 10 μg/ml doses increased IGF-I expression to a level comparable to the controls (Fig. 5B). Although TNF-α did not depress the expression of lysyl oxidase, the 5 μg/ml doses of dried plum polyphenols up-regulated ($P<.05$) its expression in the presence of TNF-α (Fig. 5B) and the other doses (2.5 and 10 μg/ml) had intermediate effects. These data suggest that the increase in osteoblast ALP activity and mineralization induced by dried plum polyphenols was associated with an ability to increase the post-translational crosslinking of collagen fibers and to counter the negative effects of TNF-α on growth and transcription factors.

3.5. Effect of dried plum polyphenols on RANKL and OPG expression

Under normal conditions, the lowest dose of dried plum polyphenols (i.e., 2.5 μg/ml) tended ($P=.08$) to down-regulate the expression of RANKL compared to control, but none of the doses of polyphenols altered RANKL or OPG expression to a level of statistical significance (data not shown). In the presence of TNF-α, the 5 and 10 μg/ml doses of polyphenols suppressed the TNF-α-induced up-regulation of RANKL expression by 50% (Fig. 5C). These alterations in RANKL occurred with no changes in OPG expression, indicating that dried plum polyphenols attenuate the TNF-α induced inflammatory response of MC3T3-E1 cells.

4. Discussion

This study demonstrates that dried plum polyphenols stimulate osteoblast activity as indicated by ALP and mineralized nodule formation under normal and inflammatory conditions. These changes were mediated in part through the up-regulation of growth and transcription factors that were down-regulated by TNF-α and an increase in lysyl oxidase, an enzyme involved in extracellular matrix synthesis.

TNF-α, recognized as a key inflammatory cytokine involved in the pathogenesis of bone loss [10,33],

suppressed osteoblast activity and the osteoblasts' ability to form mineralized nodules. These effects occurred in conjunction with the down-regulation of IGF-I and transcription factors, Runx2 [13] and Osterix [12]. Previous studies have shown that suppression of osteoblast activity by TNF-α occurs in response to decreased transcriptional activity and reduced mRNA stability [12,13]. The Runx2 promoter contains a homologous sequence of NF-κB and activated protein-1 (AP-1) binding sites that are capable of conferring responsiveness to TNF-α resulting in the suppression of osteoblast activity [13,34]. Consequently, anti-inflammatory drugs or natural compounds that suppress NF-κB or AP-1 signaling could potentially counter the negative effects of TNF-α on osteoblast Runx2 expression [20] and enhance mineralized nodule formation [35].

In this study, polyphenols from dried plums increased intracellular and extracellular ALP activity. ALP is known to enhance mineralization by catalyzing the hydrolysis of organic phosphate esters, thereby providing inorganic phosphates for mineralization [36]. This effect of the polyphenols on osteoblast ALP activity appeared to be mediated by an increase in IGF-I. Under inflammatory conditions we have shown that the increase in ALP was associated with up-regulation of transcription factors Runx2 and Osterix. Similar to our findings, anabolic drugs and other natural compounds such as PTH and soy isoflavones have been reported to increase osteoblast activity by up-regulating transcription factors and/or growth factors. PTH increases mineralized nodule formation, which is mediated by up-regulation of Runx2 and Osterix expression in osteoblast cultures [37], and enhances bone mass by increasing IGF-I [38]. The soy isoflavone genestein has also been shown to increase ALP activity and Runx2 expression in osteoblasts [39]. Up-regulation of IGF-I observed in the current study coincides with previous reports in which postmenopausal women [23] and animal models of osteoporosis [22] consuming diets supplemented with dried plum experienced an increase in serum IGF-I. The role of IGF-I in bone metabolism has typically been associated with the stimulation of osteoblast activity, inhibition of collagen matrix degradation, T-cell proliferation, and myeloid cell growth and differentiation [38,40]. Even though IGF-I has been shown to increase bone mass and skeletal acquisition [41,42] the precise mechanism remains under investigation.

In addition to effects on IGF-I, the polyphenolic compounds also up-regulated osteoblast expression of lysyl oxidase. Lysyl oxidase is known to be an important factor in the post-translational modification of collagen (i.e., collagen cross-linking) and thus plays an integral role in matrix mineralization and bone strength [43]. This finding is of particular interest based on our recent animal data demonstrating that dried plum exerted a greater effect on bone strength than would be expected relative to the change in bone density [26]. Increased lysyl oxidase activity in response to the polyphenols in dried plum may account for the reported improvement in bone biomechanical properties [26], which are determined by factors such as protein matrix synthesis and cellular activity [44].

Our findings suggest that the polyphenol extract not only enhanced osteoblast activity, but also inhibited osteoblast signaling. Inflammatory conditions up-regulate osteoclast differentiation and activity in part through the regulation of TNF superfamily proteins (i.e., RANK, RANKL and OPG) [45]. We have shown that TNF-α stimulated the expression of RANKL, a finding consistent with previous reports in which an increase in RANKL mRNA levels in response to TNF-α resulted in an increase in osteoclastogenesis and osteoclast activity both in vivo and in vitro [6,46]. In contrast, OPG, the soluble decoy for RANKL that inhibits RANK–RANKL binding, was unaltered by TNF-α in this study. Increased RANKL expression, with no change in OPG, results in a significant elevation of the RANKL/OPG ratio which promotes osteoclast differentiation and activity. In this study, we have shown that RANKL was down-regulated in cells treated with the dried plum polyphenols under inflammatory conditions indicating the influence of dried plum polyphenols on the osteoblast in vitro is consistent with the findings from our in vivo data [22]. We had previously reported that dietary supplementation with dried plum decreases RANKL expression in trabecular bone of orchidectomized rats [22]. More recently, we have observed that, in addition to inhibiting RANKL expression, dried plum polyphenols directly suppress osteoclast differentiation and activity in bone marrow macrophages (RAW264.7) by down-regulating TNF-α and nitric oxide production in the presence of RANKL (unpublished data). These results related to osteoclasts suggest that in addition to stimulating bone formation, dried plum polyphenols may also have anti-inflammatory and anti-resorptive effects.

Although we have demonstrated that dried plum's polyphenols stimulate osteoblast mineralized nodule formation in normal and inflammatory conditions, we should note that the degree of mineralization by the highest dose of polyphenols (20 μg/ml) was not as effective as the lower doses. One possible explanation for this observation is related to the chelation of minerals by polyphenols. Normally, the extracellular matrix contains sufficient concentrations of Ca^{2+}, Pi and other trace amount of minerals (e.g., magnesium, iron, zinc) for mineralization [47]. In spite of the health benefits of polyphenols, some concerns remain as to the metal/mineral ion chelating potential of polyphenols [48]. It is possible that, at higher doses, the polyphenols chelate some of the essential minerals required for nodule formation. In general, polyphenolic compounds are poorly absorbed and the work of Cremin et al. [49] suggests that the concentrations of polyphenols used in this study are relevant to the doses that we have found to be beneficial in vivo [22,24–26]. Further studies are needed to determine whether chelation is a factor by evaluating the dose-escalating effects of polyphenols on the free mineral concentration and

determining whether restoration of mineralization occurs with the addition of $Ca^{2+}$and Pi in the presence of high doses of polyphenols.

We have demonstrated that dried plum polyphenols effectively enhance osteoblast activity and mineralization under normal and inflammatory conditions. The findings of this study suggest that the polyphenols in dried plum are at least in part responsible for the anabolic effects of dried plum reported in previous animal studies [25,26]. Thus, it is possible that dietary consumption of dried plums could serve as a source of polyphenolic compounds that favorably modulate both bone formation and resorption, and provide a natural alternative for individuals at risk of osteoporosis. At present, it is unclear whether the effects on osteoblasts reported here are the results of individual phenolic compounds or the action of the polyphenols as a whole. Studies using proportionate formulations of the available synthetic phenolic compounds in dried plum are underway and may provide further clarification of this issue. The gene expression data presented here were the result of alterations that occurred within 42 h of treatment with the polyphenols and may or may not represent the long-term effects of these compounds. Although dried plum polyphenols restored ALP activity under inflammatory conditions at 14 days, there were no significant changes at 7 days indicating that time of exposure to polyphenols is important to positively influence bone mineralization. Based on dried plum polyphenols' impact on ALP activity and mineralized nodule formation, it seems that evaluation of these polyphenols on gene expression over time is warranted. Additionally, it should be noted that the results of this study represent the findings of an osteoblast cell line and may not reflect the effects of dried plum or its polyphenols in a more relevant physiological environment in which osteoblast and osteoclast interaction occurs. Future studies should include the simultaneous evaluation of cellular activity and signaling using co-culture systems of osteoblast and osteoclast.

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