BONE PROTECTIVE EFFECTS OF DRIED PLUM

AND ITS POLYPHENOLS UNDER INFLAMMATORY

AND OXIDATIVE STRESS CONDITIONS

By

SO YOUNG BU

Bachelor of Science in Food and Nutrition
Sookmyung Women's University
Seoul, Korea
1998

Master of Science in Food and Nutrition
Sookmyung Women's University
Seoul, Korea
2000

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2007

BONE PROTECTIVE EFFECTS OF DRIED PLUM

AND ITS POLYPHENOLS UNDER INFLAMMATORY

AND OXIDATIVE STRESS CONDITIONS

Dissertation Approved:

Dr. Brenda J. Smith
_____
Dissertation Adviser

Dr. Barbara J. Stoecker
_____
Chair

Dr. Cheng-I Wei
_____
Member

Dr. James E. Breazile
_____
Member

Dr. A. Gordon Emslie
_____
Dean of the Graduate College

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDIX B

LIST OF ABBREVIATION

AIDS: acquired immune deficiency syndrome
ALP: alkaline phosphatase
AP-1: activator protein-1
AR-S: Alizarin Red S
BMA: bone mineral area
BMC: bone mineral content
BMD: bone mineral density
BMP-2: bone morphogenic protein-2
BV/TV: bone volume per total volume
CAPE: caffeic acid phenethyl ester
COL-I: type I collagen
COX-2: cyclooxygenase-2
μCT: microcomputed tomography
DMEM: Dulbecco's modified eagle's medium
DP: dried plum
Dpd: deoxypyridinoline crosslinks
DXA: dual energy x-ray absorptiometry
EGCG: epigallocatechin-gallate
ERK: extracellular signal regulated kinase
ESR: evaluation of scavenging activity
FBS: fetal bovine serum
FE: finite element analysis
GI: gastrointestinal
GST: glutathione S transferase
H^+-ATPase: H^+-adenosine triphosphate
H_2O_2: hydrogen peroxide
HAART: highly active antiretroviral therapy
HIV: human immunodeficiency virus
IFN: interferon
IGF-I: insulin-like growth factor-I
IL-1β: interleukin-1β
IL-6: interleukin-6
iNOS: inducible nitric oxide synthase
IBD: inflammatory bowel disease
IP: intraperitoneal
JNK: c-Jun N terminal kinase
LIF: leukemia inhibitory factor
LOX: lysyl oxidase

LPS: lipopolysaccharide
MAPK: mitogen activated protein kinase p38
M-CSF: macrophage-colony stimulating factor
α-MEM: alpha Minimum Essential Medium
MS: multiple sclerosis
NF-κB: nuclear factor-kappaB
NFATc1: nuclear factor of activated T cells-c1
NO: nitric oxide
OCN: osteocalcin
OPG: osteoprotegerin
ORAC: oxygen radical absorbance capacity
ORX: orchidectomized
Osx: Osterix
OVX: ovariectomized
PGE_2: prostaglandin E_2
PTH: parathyroid hormone
RA: rheumatoid arthritis
RANK: receptor activator of nuclear factor-kappa B
RANKL: receptor activator of nuclear factor-kappa B ligand
ROS: reactive oxygen species
SD: Sprague Dawley
SDS-PAGE: sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SLE: systemic lupus erythematosus
SMI: structure model index
SOD: superoxide dismutase
TbN: trabecular number
TbSp: trabecular separation
TbTh: trabecular thickness
TGF-β: tumor growth factor-β
TNFR: tumor necrosis factor receptor
TNF-α: tumor necrosis factor-α
TRAF: tumor necrosis factor receptor associated factor proteins
TRAP: tartrate-resistant acidic phosphatase
TRT: testosterone replacement therapy
VOI: volume of interest

CHAPTER I

Background Information

Osteoporosis, or porous bone, is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and increased susceptibility to fractures (Benhamou et al., 2001; World Health Organization, 1994). This disease represents a major public health issue for 44 million Americans, 68% of whom are women and 32% of whom are men (Black and Cooper, 2000). Although the overall prevalence of fracture is higher in women, men generally have higher rate of fracture-related mortality (Center et al., 1999). In the United States, 10 million individuals have been diagnosed with osteoporosis and an additional 34 million have low bone mass and are at increased risk of osteoporosis (National Osteoporosis Foundation, 2007). Osteoporosis is responsible for more than 1.5 million fractures annually in the U.S., including 300,000 hip fractures, 700,000 vertebral fractures, 250,000 wrist fractures, and more than 300,000 fractures at other sites (NIH, 2000). Estimated national direct expenditures for osteoporosis-related fractures including hospitals and nursing homes, are $14 billion each year (Black and Cooper, 2000; NIH, 2000). Osteoporosis is most often associated with gonadal hormone deficiency (Gabet et al., 2005; Pacifici, 1996), unfavorable nutritional status (Holick, 2004), skeletal unloading (Smith et al., 2002), ethanol consumption (Kabat et al., 1994), corticosteroid use (Adachi et al., 2001; Hofbauer et al., 1999) or inflammatory conditions.

Recently, the relationship between altered immune function and bone loss has become a major topic of interest in effort to better understand the pathophysiology of osteoporosis. Conditions associated with chronic elevation of pro-inflammatory mediators have been implicated in the alterations in bone metabolism that negatively impact bone mass and microarchitecture (Pacifici, 1996; Smith et al., 2006). Normally, inflammation is a physiological response that protects against invading microorganisms and infectious agents, and precedes tissue repair; however, dysregulation of the immune response can lead to extensive tissue damage, disability, and death (Mehta et al., 1998). Many human chronic inflammatory diseases are associated with sustained release of inflammatory mediators. A significant body of literature has documented the relationship between osteopenia or osteoporosis and inflammatory conditions such as human immunodeficiency virus (HIV) (Mondy and Tebas, 2003; Paton et al., 1997), chronic periodontitis (Van Dyke and Serhan, 2003), inflammatory bowel disease (Bernstein et al., 2003), chronic pancreatitis (Haaber et al., 2000; Mann et al., 2003), rheumatoid arthritis and lupus erythematosus (Romas et al., 2002; Uaratanawong et al., 2003). Furthermore, evidence of estrogen's anti-inflammatory properties has resulted in postmenopausal osteoporosis being viewed as an age-related condition associated with alterations in immune function (Pacifici, 1996).

Chronic elevation of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and prostaglandin E_2 (PGE$_2$) can disrupt normal bone remodeling and ultimately lead to bone loss (Ishimi et al., 1990; Miyaura et al., 2003; Romas et al., 2002; Sakuma et al., 2000; Tatakis, 1993). In general, these pro-inflammatory mediators act both directly and indirectly to increase osteoclastogenesis,

prevent osteoclast apoptosis (Johnson et al., 1989; Lorenzo et al., 1987), and/or inhibit osteoblast activity (Wallach et al., 1993). The result of these alterations in bone metabolism is an increase in the bone resorption by osteoclasts relative to the activity of the bone forming osteoblasts.

Stimulation of osteoclast differentiation and activity by these inflammatory mediators appears to occur, at least in part, through enhanced expression of receptor activator of nuclear factor-kappa B ligand (RANKL) by osteoblasts in the presence of macrophage-colony stimulating factor (M-CSF). Upon initiation of the inflammatory response, the osteoblast secretes RANKL which in turn binds to receptor activator of nuclear factor-kappa B (RANK) on osteoclast precursor cells and initiates their differentiation into osteoclasts. Both osteoclast differentiation and activity increase in response to RANKL-RANK interaction (Khosla, 2001). The action of RANKL can be inhibited by its decoy receptor, osteoprotegerin (OPG). OPG, a member of the TNF-receptor family, is also expressed by osteoblasts and acts to inhibit bone resorption by binding with strong affinity to RANKL, thereby preventing the RANKL-RANK interaction (Hofbauer and Heufelder, 2001; Saidenberg-Kermanac'h et al., 2004). OPG may also be pivotal in modulating inflammation mediated bone diseases (Bernstein et al., 2005; Redlich et al., 2004; Saidenberg-Kermanac'h et al., 2004). Administration of OPG attenuates bone loss and inhibits rheumatoid arthritis in TNF-α transgenic mice (Redlich et al., 2004). Therefore, regulation of this RANKL/OPG/RANK system may be one of the key determinants of balance between bone formation and bone resorption under inflammatory conditions. The interrelationship between bone metabolism and the innate immune response likely explains why medical conditions typified by chronic elevation of

pro-inflammatory mediators are associated with bone loss characterized by decreased

bone mineral density (BMD) and deterioration of trabecular bone microarchitecture

(Mondy and Tebas, 2003; Romas et al., 2002; Serrano et al., 1995).

While several FDA-approved pharmacological treatments for osteoporosis are

available, side-effects of these drugs remain an important consideration. For example, the

anti-resorptive agent, alendronate, reduces fracture of the spine and hip by approximately

50% in clinical trials (Liberman et al., 1996; Liedholm and Linne, 1996; McGrath, 1996);

however, bisphosphonates such as alendronate are poorly absorbed and the large doses

required which result in gastrointestinal (GI) complications for many individuals. In

addition to the GI complications, long-term use of bisphosphonates has recently been

linked to osteonecrosis of the jaw (Rodan and Martin, 2000). Recommending long term

use of other anti-resorptive agents, such as hormone replacement therapy (HRT) and

selective androgen receptor modulators (SARM) (Hanada et al., 2003; Rosen J, 2002) is

controversial due to the uncertainty of their efficacy and potential to increase the risk of

certain cancers (Cappon et al., 2004; Delmas et al., 1997; Rodan and Martin, 2000; Terry

et al., 2004). Intermittent parathyroid hormone (PTH) treatment is currently the only

FDA-approved anabolic agent that can restore bone mass for individuals with established

osteoporosis (Kroll, 2000; Rattanakul et al., 2003). Although this treatment provides the

first therapeutic option for people with significant bone loss, it is not a reasonable

treatment for many osteoporosis patients due to the cost of the drug, the inconvenience of

daily injections, the uncertainty of long-term treatment, and the risk of side-effects

(Hodsman et al., 2005). Ideally, it would be beneficial to offer natural therapies that could

be easily incorporated into the diet, which would reduce the risk of osteoporosis or could

even be combined with pharmacological therapies to treat established osteoporosis.

Previous epidemiological studies have shown that consumption of legumes, fruits, and vegetables (Muhlbauer et al., 2003; New et al., 2000), rich in phenolic compounds and their derivatives (Hegarty et al., 2000; Johnell O, 2005), reduce the incidence of osteoporosis. Diets high in fruits and vegetables have been linked to an increase in bone accrual in boys aged 8-20 y (Vatanparast et al., 2005a) as well as a reduction in the rate of bone loss in premenopausal women (Macdonald et al., 2005) and aging men (Tucker et al., 2002). Consumption of beverages containing polyphenols such as green tea has been associated with increased BMD (Wu et al., 2002). In addition, polyphenolic compounds and their derivatives, which have antioxidant and anti-inflammatory properties, have been shown to inhibit osteoclast differentiation and activity (Muhlbauer et al., 2003; Woo et al., 2004), directly stimulate osteoblasts, and favorably alter bone formation markers (Horcajada-Molteni et al., 2000; Mizutani et al., 1998).

Dried plums (*Prunus domestica L.*) are a rich source of polyphenols (1100-2600 mg/kg) and a number of micronutrients (e.g. vitamin K, potassium and boron) involved in bone metabolism (Fang et al., 2002; Hollman and Katan, 1999; Nakatani et al., 2000). The polyphenol content of this fruit contributes to its high oxygen radical absorbance capacity rating (ORAC rating), resulting in the highest ORAC score among the most commonly consumed fruits and vegetables (Kayano et al., 2004). 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 may enhance bone formation as indicated by increased serum bone-specific alkaline phosphatase (ALP) and insulin-like growth factor (IGF)-I (Arjmandi et al., 2002). Studies utilizing female animal models of

osteopenia indicated that diets supplemented with dried plum could not only prevent (Arjmandi et al., 2001), but also reverse (Deyhim et al., 2005) bone loss due to ovarian hormone deficiency. Recent studies evaluating bone recovery following skeletal unloading demonstrated that dried plum had comparable effects to intermittent PTH therapy and that the polyphenols in dried plum may account for most of these effects (unpublished data).

Muhlbauer and colleagues (Muhlbauer et al., 2003) were the first to demonstrate that in male rats dried plum strongly inhibited bone resorption compared to other fruits and vegetables. Our recent report (Franklin et al., 2006) showed that the bone protective effects of dried plum on BMD and trabecular bone microarchitecture in gonadal hormone deficient male animals were even greater than those observed in females. Dietary supplementation with dried plum prevented the orchidectomy-induced decrease in bone mass and mechanical strength which was accompanied by improved bone microarchitecture. Circulating biochemical markers of bone metabolism and local gene expression data suggested the improved bone quality resulted from increased bone formation and decreased bone resorption, which was mediated by the IGF-I and RANK pathways, respectively.

In gonadal hormone deficiency, TNF-α is considered one of the predominant pro-inflammatory mediators of bone loss (Cenci et al., 2000). TNF-α inhibits osteoblast activity and bone mineralization by down-regulating IGF-I (Gilbert et al., 2000) and ALP (Pischon et al., 2004), and suppresses the expression of transcription factors, Osterix (Lu et al., 2006) and Runx2 (Gilbert et al., 2002). 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 (Pacifici, 1996). Therefore, dried plum's polyphenols may mediate its bone protective effects by down-regulating the production of inflammatory mediators involved in osteoclastogenesis and osteoclast activity as well as by altering osteoblast signaling and function.

Research Objectives and Hypotheses

The purpose of this research project is to further our understanding of the molecular and cellular mechanisms by which dried plum and its polyphenols improve bone mass and microarchitecture. To accomplish this purpose, *in vivo* and *in vitro* studies have been designed to address the following objectives.

Study 1

Nine-month-old orchidectomized (ORX) osteopenic rats will be randomly assigned to either control or a dried plum supplemented diet (i.e. 25% w/w) to investigate the ability of dried plum to reverse gonadal hormone deficiency-induced deterioration in bone mass and microarchitecture after bone loss has occurred in ORX males. The anabolic effects of dried plum will be compared to those of PTH.

Objective 1: To determine if dietary supplementation with dried plum can restore bone mass (BMD) and bone mineral content (BMC) in gonadal hormone deficient osteopenic male rats and to compare the degree of restoration to that of PTH.

Objective 2: To investigate whether dietary supplementation with dried plum can reverse

the deterioration in microarchitecture of trabecular and cortical bone in osteopenic male rats and to compare its effects to PTH.

Hypothesis 1: Dried plum supplementation will restore bone density and trabecular bone microarchitecture in osteopenic gonadal hormone-deficient male rats and these effects will be similar to the anabolic agent, PTH.

Objective 3: To evaluate the extent to which dried plum alters biochemical markers of bone formation and resorption by assessing serum ALP and urinary deoxypyridinoline (Dpd) and calcium excretion in osteopenic male rats. The alterations in biochemical markers will be compared to the positive control, PTH.

Hypothesis 2: Osteopenic orchidectomized male rats consuming the dried plum supplemented diet will experience an increase in serum ALP, but a decrease in urinary Dpd and calcium excretion. The alterations in ALP will be similar to the effects of PTH, but the decrease in bone resorption indicators will not mimic PTH.

Study 2

RAW 264.7 bone marrow macrophages will be used to evaluate the ability of dried plum polyphenols to down-regulate inflammatory mediators and inhibit osteoclast differentiation and activity under inflammatory and oxidative stress conditions. Inflammation will be induced by stimulating cells with lipopolysaccharide (LPS) while oxidative stress will be induced by stimulation with hydrogen peroxide (H_2O_2). The effects of the polyphenol extracts will be assessed on macrophages (i.e. no RANKL added to the culture) and during osteoclastogenesis (i.e. RANKL added over 5 days).

Objective 4: To determine whether the dried plum polyphenols decrease the inflammation- and oxidative stress-induced osteoclast differentiation and activity by examining the TRAP-positive, multinucleated osteoclasts and resorption pit formation.

Hypothesis 3: Dried plum polyphenols will decrease osteoclast differentiation and activity under inflammatory and oxidative stress conditions.

Objective 5: To evaluate the ability of dried plum polyphenols to down-regulate the production of inflammatory mediators (i.e. nitric oxide and TNF-α) and key enzymes (cyclooxygenase-2, COX-2 and inducible nitric oxide synthase, iNOS) regulating the production of inflammatory mediators that promote osteoclastogenesis under inflammatory and oxidative stress conditions.

Hypothesis 4: Dried plum polyphenols will decrease the production of inflammatory mediators by murine macrophages and osteoclasts induced by inflammation and oxidation.

Study 3

MC3T3-E1 pre-osteoblast cells will be used to evaluate the effects of dried plum polyphenols on osteoblast activity and function under normal and inflammatory conditions. TNF-α will be used to induce the inflammatory response in osteoblasts. Alterations in osteoblast gene expression of transcription and growth factors and cytokines will also be assessed.

Objective 6: To determine whether dried plum polyphenols increase osteoblast activity and function under normal and TNF-α-induced inflammatory conditions by investigating the intra- and extra-cellular ALP activity and mineralized nodule formation.

Hypothesis 5: Dried plum polyphenols will up-regulate ALP activity and mineralized nodule formation in osteoblast cells under both normal and inflammatory conditions.

Objective 7: To determine whether the dried plum polyphenols will alter osteoblast gene expression of key transcription factors (i.e. Runx2 and Osterix) and growth factors (i.e. IGF-I) in the presence or absence of TNF-α.

Hypothesis 6: Dried plum polyphenols will increase the gene expression of Runx2, Osterix and IGF-I under normal and inflammatory conditions.

Objective 8: To determine whether the dried plum polyphenols will alter the gene expression of RANKL and OPG in the presence or absence of TNF-α.

Hypothesis 7: Dried plum polyphenols will decrease the RANKL expression without altering the expression of OPG under normal and inflammatory conditions.

Limitations

The dose of dried plum (25%, w/w) incorporated into the animal diet in Study 1 may be considered quite high if attempts are made to extrapolate this dose to the human diet. The intent of this study was not to be a dose-seeking experiment, but rather to compare dried plum's effects on the restoration of bone mass, microarchitecture and metabolism with those of the potent anabolic agent, PTH. Previous studies have shown

that 25% is the most efficacious dose of dried plum (Franklin et al., 2006) and is well-tolerated by rodents without any GI side-effect, such as diarrhea and vomiting (unpublished observation). Hence in order to optimize the effects of dried plum in comparison to PTH to better understand its mechanism of action, this high dose of dried plum was utilized.

Another potential limitation to the *in vivo* study is related to the use of the ORX rat model of osteoporosis. Although gonadal hormone deficiency-induced bone loss occurs relatively abruptly in females during the early postmenopausal years, age-related changes in gonadal hormone deficiency in males tend to be more gradual and extend over the course of decades in the human lifespan. Therefore, some have suggested that the ORX rat model may not represent bone loss associated with aging in males. However, one may argue that treatments that are efficacious in the ORX male model, which represents an exaggerated response, are likely to be even more effective when applied to osteoporosis in men.

In the *in vitro* studies one of the possible limitations is that the polyphenols that were tested were extracted from dried plum. As is the case of any extract, this does not represent a purified mix of the polyphenols found in plums. A significant amount of sugar and other nutrient and non-nutrient components may remain. Even though these components may potentially confound treatment effects, the data in this study show similar effects in terms of down-regulating inflammatory mediators, decreasing osteoclast differentiation and increasing osteoblast activity as other phenolic compounds reported in the literature (Chen et al., 2001; Mizutani et al., 1998; Wattel et al., 2003). Attempts to further purify the polyphenols to remove other compounds, especially the remaining

carbohydrates, has resulted in conformational changes that alter the polyphenol's structure (unpublished observation). Studies using the available synthetic version of the phenolic compound in dried plum or proportionate formulations of those found in the fruit are warranted. Such studies would provide the opportunity to clarify the effects of the polyphenols on osteoclasts and osteoblasts, and to address whether the effects reported here are the results of individual compounds or the action of the polyphenols as a whole. However, the problem with this approach is that neochlorogenic acid, the predominant polyphenol in dried plums is very difficult to synthesize and synthetic versions are not currently available.

In addition to issues regarding composition of polyphenols, relatively little is known about the alterations in these compounds' chemical structure during their absorption and metabolism. Further *in vivo* studies are needed to identify the phenolic compounds that appear in the serum and in tissues such as bone as well as their impact on antioxidant systems and immune function.

Format of Dissertation

The three experiments included in the dissertation are organized as individual manuscripts and written using the guide for instruction for authors from *Osteoporosis International*, *Bone* and *Calcified Tissue International*, respectively. Additional data and/or information that were not included in the manuscripts have been included in the appendix.

CHAPTER II

REVIEW OF LITERATURE

Osteoporosis and Prevalence

Osteoporosis is a disease characterized by low bone mass and structural

deterioration of bone tissue, leading to bone fragility and increased susceptibility to

fractures (Kopera, 1989). Osteoporosis is a major public health threat for 44 million

Americans, 68% are women and 32% men (Black and Cooper, 2000). Although the

overall prevalence of fracture is higher in women, men generally have higher rates of

fracture-related mortality (Center et al., 1999). In the United States, 10 million

individuals have been diagnosed with osteoporosis and more than 34 millions have low

bone mass or osteopenia and are at increased risk for this disease. The number of older

adults (i.e. over 65 years) has increased dramatically during the last few decades and is

expected to continue to increase over the next 50 years (Black and Cooper, 2000). Today,

it is estimated that worldwide more than 495 million individuals are 65 years of age and

older, and this number is expected to increase to about 1,574 million by the year 2050

(U.S. Census Bureau, 2007). These demographic changes are likely to result in increased

incidence of hip fractures from 1.66 million in 1990 to 6.26 million in 2050. It is

estimated that approximately one-half of all hip fractures occurring in elderly people in

1990 occurred in Europe and North America; however, in view of the growth of the aging

Asian population it is expected that by 2050 greater than 50% of all hip fracture will

occur in Asia (Christodoulou and Cooper, 2003).

In the U.S., osteoporosis is responsible for more than 1.5 million fractures

annually, including 300,000 hip fractures, 700,000 vertebral fractures, 250,000 wrist

fractures, and more than 300,000 fractures at other sites (Black and Cooper, 2000; NIH,

2000a). In those who recover from the fracture, more than half of the patients have

permanently impaired mobility (Cooper et al., 1993). Estimated direct expenditures in the

U.S. (i.e. hospitals and nursing home) for osteoporosis and related fracture are $14 billion

each year (Cummings, 1998), and the financial and health-related costs of osteoporosis

are expected to rise in future generations (Ben et al., 2001; Reeve et al., 2003).

Risk Factors for Osteoporosis

Osteoporosis is most often associated with low BMD occurring in response to

gonadal hormone deficiency (Gabet et al., 2005; Pacifici, 1996), skeletal unloading

(Smith et al., 2002), unfavorable nutritional status (Holick, 2004), ethanol consumption

(Kabat et al., 1994), corticosteroid use (Adachi et al., 2001; Hofbauer et al., 1999a), or

inflammatory disease.

Aging as well as adverse environmental factors may lead to decreased serum

estrogen and hypogonadism which is associated with low BMD in both females and

males (Bilezikian et al., 1998; Dopp et al., 1999; Genant et al., 1989). Premature

menopause, especially before the age of 45, is a strong determinant of bone loss and

increased risk of fracture among women (Christodoulou and Cooper, 2003). Recent

studies suggested that estrogen has anti-inflammatory properties and contributes to the inhibition of osteoclast function as shown by increased bone resorption activity observed with diminished estrogen levels in both males and females (Cenci et al., 2000).

Physical loading and mechanical stress are known to increase BMD and certain forms of weight-bearing exercise may retard bone loss (Bikle et al., 2003; Smith et al., 2002). Reduced physical activity or immobilizations have negative effects on the skeleton as evidenced by the significant reduction in bone mass observed with prolonged bed rest (Palle et al., 1992). Similar degrees of bone loss have also been reported in the microgravity environment of space (Carmeliet et al., 2001; Vico et al., 2000). Moreover, epidemiological studies have shown that a relationship exists between physical inactivity in the elderly and the risk of hip and vertebral fracture (Ammann and Rizzoli, 2003; Arden et al., 1999; Argani et al., 2001). Although some of this effect might be due to the increased risk of falling, the importance of physical activity on skeletal health throughout the lifespan is well-documented (Karinkanta et al., 2005; Perrault, 2006).

Many nutrients have received attention regarding their role in bone health. A meta-analysis by Cummings and Nevitt (1997) showed a positive association between calcium intake and bone mass in pre-menopausal women. In middle-aged and elderly women a positive association has been reported between 25-hydroxyvitamin D concentrations and BMD (Nieves, 2003). Additionally, adequate vitamin D status in the elderly may not only influence calcium homeostasis relative to bone, but also improve muscle strength and reduce the risk and consequences of falls (Christodoulou and Cooper, 2003). Other nutrients such as potassium (Macdonald et al., 2005), vitamin K (Booth and Meyer, 1997; Vermeer et al., 1992), fluoride, copper (Rucker et al., 1975;

15

Smith et al., 2002; Strause et al., 1987; Teague and Carpenter, 1951), and vitamin C (Leveille et al., 1997; Morton et al., 2001) are also associated with osteoporotic risk. These nutrients are involved in acid-base balance (Macdonald et al., 2005), synthesis of bone matrix, and serve as cofactors for protein synthesis (Rucker et al., 1975; Smith et al., 2002; Strause et al., 1987; Teague and Carpenter, 1951), which are all required for the bone remodeling processes. However, the influence of nutritional status on osteoporotic or fracture risk is often very difficult to interpret (Buckley et al., 1996). This difficulty is due to factors such as compromised absorption and metabolic activity of nutrients associated with conditions such as gonadal hormone deficiency, disease states, and physical inactivity.

Another risk factor for osteoporosis is the consumption of large quantities of alcohol. The influence of excessive alcohol may be due to adverse effects on protein and calcium metabolism, compromised mobility, depressed gonadal function, and toxic effects on osteoblasts (Hefferan et al., 2003; Sampson, 2002). Long-term alcohol consumption can interfere with bone growth and normal bone remodeling, resulting in decreased BMD and increased risk of fracture (Williams et al., 2005). These effects may be exerted directly or indirectly through cellular activity, hormones, and growth factors that regulate bone metabolism (Callaci et al., 2004; Turner and Sibonga, 2001). Alcohol consumption during adolescence reduces peak bone mass and can result in relatively weak adult bones that are more susceptible to fracture (Ackard and Neumark-Sztainer, 2001). In adults, high levels of alcohol consumption (i.e. alcohol abuse) can disrupt the balance between bone resorption and bone formation activity (Schnitzler et al., 2005);

however, some evidence suggests that moderate consumption may decrease the risk of fracture in postmenopausal women (De Lorimier, 2000; Macdonald et al., 2004).

In addition to alcohol, an inverse relationship between smoking and BMD has also been reported (Broussard and Magnus, 2004; Gerdhem and Obrant, 2002; Hildebolt et al., 2000). Meta-analysis has demonstrated that although there was no significant difference in BMD between smokers and non-smokers at age 50; BMD in women was diminished by 2% more in smokers than in non-smokers for each 10 years of age after 50; and the difference between the two groups was 6% at age 80 years (Law and Hackshaw, 1997). These data suggest that smoking, especially in older women may increase the rate of bone loss. Moreover, an independent effect of cigarette smoking on the risk of hip fracture has been suggested by epidemiological studies (Cummings et al., 1995).

Corticosteroids are widely used therapeutic agents for the treatment of inflammatory diseases. Long-term corticosteroid use has been associated with the development of osteoporosis (Joseph, 1994). Osteoporosis has been reported to occur in up to 50% of persons who require long-term corticosteroid therapy and the risk of vertebral fractures increased by 5-fold while the risk for nonvertebral fractures increased by 2-fold (Lane and Lukert, 1998). The association between osteoporosis and corticosteroids has been identified since they were first used in humans and evidence has accumulated demonstrating that corticosteroids affect bone through multiple pathways (Kim et al., 2006). The primary action of corticosteroids on bone appears to be related to the direct inhibition of bone formation by osteoblasts and to a lesser extent, enhanced bone resorption by osteoclasts. Impaired calcium absorption and increased urinary

calcium loss leading to hyperparathyroidism have also been implicated as having a role in these detrimental corticosteroid-elicited effects on bone biology (Canalis, 1996).

Other risk factors for osteoporosis include a variety of endocrine disorders such as hyperparathyroidism, Cushing's syndrome (Ohmori et al., 2003), malignant disease (e.g. myeloma, lymphoma) (Atoyebi et al., 2002), and chronic renal failure (Cunningham et al., 2004). Additionally, there is evidence that osteopenia and osteoporosis are closely related to chronic inflammatory diseases (Hassoun et al., 1997; Romas et al., 2002; Uaratanawong et al., 2003; Van Dyke and Serhan, 2003). Importantly, immune response mediators known to be involved in chronic inflammatory diseases have been shown to be intimately involved in bone metabolism and induce bone loss by increasing bone resorption and decreasing bone formation (Pischon et al., 2004). Although therapeutic agents commonly used in the treatment of these conditions such as glucocorticoids (Adachi, 2001; McLaughlin et al., 2002) or highly active antiretroviral therapy (HAART) (McDermott et al., 2001; Pan et al., 2004) can accelerate the rate of bone loss, osteopenia also develops independent of treatment and may be mediated via host responses to the underlying medical conditions (Van Dyke and Serhan, 2003).

Bone Loss in Inflammatory Conditions

Inflammation is a normal physiological response that protects the host from invading microorganism and infectious agents, and precedes tissue repair (Cotran, 1999). Dysregulation of the immune response, however, can lead to extensive tissue damage, disability and death. Bone loss is a common clinical complication that presents in a variety of diseases associated with chronic elevation of pro-inflammatory mediators. For

example, periodontal disease, characterized by gingival inflammation and alveolar bone resorption resulting from primarily gram-negative bacteria, causes destruction of bone and the collagen that support the teeth (Van Dyke and Serhan, 2003; WactawskiWende et al., 1996). Osteopenia has been reported in 67-71% of patients infected with human immunodeficiency virus (HIV) and another 13-21% of HIV-infected patient are osteoporotic (Amiel et al., 2004; Landonio et al., 2004; Moore et al., 2001; Paton et al., 1997). Although the etiology of HIV-associated osteopenia is unclear, elevated proinflammatory cytokines and 1,25-dihydroxyvitamin D insufficiency are likely contributors (Bruera et al., 2003; Carr et al., 2001; Huang et al., 2001; Jain and Lenhard, 2002; Mondy and Tebas, 2003). Perhaps the most common immune-related diseases associated with bone loss are the autoimmune diseases. Rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis (MS) and inflammatory bowel disease (IBD) present with a number of factors that may have detrimental effects on bone including, but not limited to, chronic inflammatory arthritis, reduced physical activity, cytokine-promoted bone resorption, renal impairment, vitamin D insufficiency and glucocorticoid use (Di Munno et al., 2004). Furthermore, there is increasing evidence of estrogen's role in immune function which has resulted in postmenopausal osteoporosis being viewed as an age-related condition associated with alterations in immune function (Pacifici, 1996).

Bone Remodeling Unit and Primary Regulators

Bone is a mineralized tissue that has both multiple mechanical and metabolic functions and is continually remodeled by the action of osteoblasts and osteoclasts

(Manolagas, 2000). Bone remodeling is a complex process involving a number of cellular functions directed at coordinated resorption and formation of new bone (Manolagas, 2000). Bone mass is preserved by a tight balance between these two processes with remodeling occurring at discrete locations known as basic multicellular units. Osteoblasts are bone forming cells derived from mesenchymal stem cells that differentiate into a pre-osteoblastic stromal cells and eventually into osteoblasts (Kartsogiannis and Ng, 2004). Several molecules are associated with the osteoblast's function which includes the deposition and maintenance of mineralized skeletal structure. For instance, once matrix synthesis begins in osteoblast cultures, the cells differentiate as genes encoding for ALP, type I collagen (COL-I), and osteocalcin (OCN) are activated. Osteoblasts eventually become embedded in the extracellular matrix consisting mainly of collagen fibrils and matrix mineralization begins as mineral deposits extend along and within the collagen fibrils (Kartsogiannis and Ng, 2004; Sudo et al., 1983).

Osteoclasts are large multinucleated cells derived from the hematopoietic progenitors of the monocyte-macrophage lineage. These cells have the capacity to resorb mineralized tissue due to their polarized morphology and ability to adjoin the calcified matrix to form a ruffled border in resorption (Roodman, 1999). Bone resorption is initiated by the attachment of osteoclasts to the target matrix and demineralization involves acidification of the extracellular microenvironment, a process mediated by a vacuolar H^+-adenosine triphosphate (H^{+-}ATPase) (Mattsson et al., 1994; Silver et al., 1988). Acidic conditions mobilize the bone mineral and the demineralized organic component is degraded by a lysosomal protease, cathepsin K (Gowen et al., 1999; Li et

al., 1999). The products of bone degradation are endocytosed by osteoclasts, transported to and released at the cell's anti-resorptive surface (Nesbitt and Horton, 1997).

At any given time the skeleton has approximately a million basic multicellular units in various stages of the remodeling sequence: 1) initiation and organization of the basic multicelluar units, 2) activation of osteoclasts, 3) resorption of old bone, 4) recruitment of osteoblasts, 5) formation of new bone or osteoid, and 5) mineralization (Mundy GR, 1999). Bone remodeling is regulated by systemic hormones and local factors, which influence proliferation, differentiation, recruitment and activation of osteoclasts and osteoblasts (Abbas et al., 2003; Kikuchi et al., 2001b; Zhao et al., 2002). These local factors, synthesized by osteoblasts and osteoclasts, include growth factors, cytokines and prostaglandins. Abnormalities in any of these factors in favor of up-regulating osteoclasts activity may lead to pathological bone resorption as seen in inflammatory conditions and/or gonadal hormone deficiency (Jones et al., 2002; Rifas et al., 2003; Roodman, 1999).

Gonadal Hormone Deficiency and Bone Loss

Estrogen deficiency in males and females is one of the most frequent cause of osteoporosis and insufficient skeletal development (Ensrud et al., 1995; Jennings et al., 1998). In both men and women, there is a steady decline in unbound estrogen levels with aging, exacerbated in women at menopause by a marked decrease in estrogen levels (Drake et al., 2003; Nelson et al., 2002). Although in men there is no abrupt cessation of gonadal function, they do experience an age-related decrease in unbound sex steroids (i.e. testosterone) resulting from progressive increases in circulating sex-hormone-binding

globulin (Riggs et al., 2002). Decreased serum testosterone results in the reduction of the aromatization of testosterone to estradiol (Howell and Shalet, 2001) or a direct effect on osteoblast and osteoclast activity (Francis, 1999) which ultimately leads to bone loss (Falahati-Nini et al., 2000; Isaia et al., 1992).

The term postmenopausal osteoporosis was first defined by Albright and colleagues in 1941 (Albright and Reifenstein, 1941) and much of the initial research focused on the role of estrogen deficiency in calcium balance (Heaney, 2001; Rosen et al., 1997). Because estrogen is involved in the regulation of serum calcium, estrogen deficiency leads to a slight elevation of plasma calcium, hypercalciuria and an overall negative calcium balance (Gallagher et al., 1980). Recently, attention has shifted toward the role of estrogen in the activation of the estrogen receptor (Grundberg et al., 2003) and/or inflammatory cytokines (Roden et al., 2004; Safadi et al., 2000). Osteoblasts and osteoclasts express functional estrogen receptors which bind to transcription factor such as nuclear factor kappa-B (NF-κB) (Orwoll et al., 1991). When estrogen receptors are activated, they can prevent the binding of NF-κB to promoter sites of cytokines such as IL-6 and IL-1, and block the synthesis of these cytokines (Stein and Yang, 1995). Studies (Cenci et al., 2000; Ryan and Nicklas, 2004) have shown that estrogen is involved in the anti-inflammatory processes due to the up-regulation of pro-inflammatory cytokines such as TNF-α, IL-I and IL-6 in the absence of gonadal hormones. Estrogen deficiency increases inflammatory mediators and leads to the accelerated bone turn over, which is characterized by an increase in bone resorption relative to bone formation, resulting in low bone mass and compromised bone microarchitecture (Audran et al., 2001).

Inflammatory Mediators and Gonadal Hormone Deficiency-induced Bone Loss

Up-regulation of inflammatory mediators such as TNF-α, IL-1β and IL-6 is associated with the pathophysiology of bone loss which occurs in conditions such as gonadal hormone deficiency, skeletal unloading as well as chronic inflammatory diseases (Grano et al., 2002; Pacifici, 1996; Rivera et al., 2003; Speyer et al., 2005). Under normal conditions, growth factors, cytokines, hormones and adhesion molecules act as the local messengers to control essentially all biological processes that include cell growth, development, repair, fibrosis and normal bone remodeling (Manolagas, 2000). However, the persistent elevation of the pro-inflammatory mediators can disrupt normal bone remodeling and consequently lead to bone loss (Ishimi et al., 1990; Miyaura et al., 2003; Romas et al., 2002; Sakuma et al., 2000; Tatakis, 1993). These pro-inflammatory cytokines, products of stromal cells and monocytes, increase osteoclast differentiation and bone resorption directly or by up-regulating RANKL and OPG production by osteoblasts (Theoleyre et al., 2004).

TNF-α is considered the main pro-inflammatory cytokine that stimulates osteoclast activation and inflammatory cytokines produced by macrophages during estrogen hormone deficiency (Roggia et al., 2001). Decreases in circulating estrogen stimulate interferon (IFN)-γ expression by T cells and enhance class II transactivator (CIITA); a transcriptional co-activator that up-regulates antigen presentation (e.g. class II MHC) (Cenci et al., 2003) and increases TNF-α. Additionally, under gonadal hormone deficiency TNF-α inhibits osteoblast activity, maturation (Abbas et al., 2003; Kikuchi et al., 2001) and bone mineralization by down-regulating IGF-I (Gilbert et al., 2000) and ALP (Pischon et al., 2004), and suppressing the expression of transcription factors,

Osterix (Lu et al., 2006) and Runx2 (Gilbert et al., 2002). TNF-α promotes osteoblast and marrow stromal cell expression of RANKL, activates RANK signaling in macrophages, induces osteoclast differentiation and ultimately increases bone resorption activity (Kanematsu et al., 2000). During the early stages of osteoclastogenesis, TNF-α also increases the osteoclast precursor pool size (Erwig et al., 1998). TNF-α inhibitors have been shown to decrease the CD11b^{+} osteoclast precursor cells in arthritis patients (O'Gradaigh et al., 2004; Ritchlin et al., 2003). Furthermore, TNF-α under estrogen deficiency is associated with a defective intracellular antioxidant system (Jagger et al., 2005) and supplementation with ascorbic acid inhibits TNF-α-induced osteoclast precursor cell proliferation and differentiation (Iqbal et al., 2006).

IL-1, which is a pleiotropic cytokine, induces the expression of a large variety of pro-inflammatory and hematopoietic cytokines such as leukemia inhibitory factor (LIF) and IL-6 (Dinarello, 1994). Like TNF-α, IL-1 initiates a signaling cascade through the activation of NF-κB and c-Jun N terminal kinase (JNK) which promotes osteoclast survival (Lee et al., 2006). The importance of IL-1 signaling is demonstrated by the fact that transgenic mice deficient in the IL-1 receptor are resistant to ovariectomy-induced bone loss (Lorenzo, 1991; Lorenzo et al., 1999) and treatment with IL-1 receptor antagonist attenuates bone resorption in rheumatoid arthritis patients (Bresnihan et al., 1998) and inhibits osteoclast activity in the bone marrow of multiple myeloma patients (Jimi et al., 2004).

Another pro-inflammatory cytokine involved in bone metabolism is IL-6. The main effect of IL-6 on bone is stimulation of osteoclastogenesis and bone resorption (Dai et al., 2000; Ota et al., 2001). IL-6 effects are interconnected with those of other pro-

inflammatory cytokines. For example, IL-6 stimulates osteoclast formation in human

marrow cultures by inducing IL-1 release and the addition of anti-IL-1 inhibits osteoclast

formation induced by IL-6 (Palmqvist et al., 2002). Inversely, IL-6 mediates the

stimulatory effects of TNF-α and enhances PTHrP-mediated hypercalcemia and bone

resorption by increasing the pool of osteoclastic progenitors and their differentiation into

mature osteoclasts (Devlin et al., 1998). IL-6, like other resorptive agents, stimulates

osteoclast activity and bone resorption by an indirect mechanism, increasing interaction

between RANKL and RANK (Nakchbandi et al., 2001).

Other inflammatory mediators that play a role in bone metabolism include the

prostaglandins (PG) (Feyen and Raisz, 1987). Prostaglandin production, especially PGE_2,

is regulated primarily by cylcooxygenase (COX)-2 which is highly responsive to

systemic and local cytokines (Miyaura et al., 2003; Raisz, 2001). In bone, prostaglandins

produced by osteoblasts act as potent stimulators of bone resorption by increasing

osteoclast differentiation (Okada et al., 2000) and at the same time are required for

normal bone formation (Li et al., 1995). PGE_2 stimulates RANKL production, decreases

OPG secretion by osteoblasts, and up-regulates RANK expression in osteoclasts (Liu et

al., 2005). PGE_2 also induces periosteal and endocortical bone formation, principally by

stimulating local osteoprogenitor cells on the adjacent endocortical surface (Ramirez-

Yanez et al., 2004). In PGE_2 receptor knock-out mice, osteoclast differentiation and

activity under inflammatory conditions (IL-1 or TNF-α) was decreased, providing

support for the role of PGE_2 in osteoclast function (Sakuma et al., 2000). Further

evidence of the involvement of PGE_2 in osteoclastogenesis was demonstrated by the

observation that osteoclast differentiation and activity were suppressed by treatment with

COX-2 inhibitors (Suda et al., 2004). Studies have shown that the response of bone cells to PGE_2 is related to the dose and route of COX-2 inhibitor administration (Desimone et al., 1993; Yao et al., 1999). These agents have been shown to attenuate bone turnover in postmenopausal women (Raisz, 2001), but long-term effects of potent COX-2 inhibitors causing too great of a reduction in PGE_2 may impair normal osteoblast function (Einhorn, 2003).

Another important inflammatory mediator, nitric oxide (NO) has been recognized for its role in the pathogenesis of osteoporosis due to its ability to regulate bone turnover (Pacifici, 1996). Increasing evidence supports a role for NO as an autocrine/paracrine mediator involved in the regulation of osteoclast (Zheng et al., 2006) and osteoblast function (Lin et al., 2003). iNOS, the enzyme that catalyzes the conversion of L-arginine to NO (Cuzzocrea et al., 2003), is involved in mediating the bone loss that occurs in conditions such as periodontitis (Alayan et al., 2006) and ovarian hormone deficiency (Cuzzocrea et al., 2003). The inducible forms of NOS are stimulated by inflammatory mediators such as IFN-γ, TNF-α and IL-1 (Damoulis and Hauschka, 1994; Deng et al., 1993; Lee et al., 2004a; Palmer et al., 1993). NO is required for bone resorption induced by IL-1, stimulates osteoclastogenesis through the activation of NF-κB, and contributes to osteoclast survival (Lee et al., 2004; van't Hof et al., 2000). NO also acts in conjunction with pro-inflammatory cytokines to promote osteoblast cell death (Damoulis and Hauschka, 1997). Like prostaglandins, NO has dual effects on bone metabolism. Pharmacological doses of NO donors were reported to increase bone mass in animal models (Wimalawansa, 2000). These higher concentrations (>100 μM) have been shown to increase osteoblast activity and nodule formation *in vitro* (Koyama et al., 2000). In

contrast, NO plays a role in osteoclastic bone resorption (Jung et al., 2004), and even though low levels appear essential for osteoclastic activity, higher NO concentrations inhibit resorption (Chae et al., 1997; Ralston et al., 1995).

Stimulation of osteoclast differentiation and activity by the afformentioned inflammatory mediators appears to occur, at least in part, through enhanced expression of RANKL by osteoblasts in the presence of macrophage-colony stimulating factor (M-CSF). Osteoclasts which differentiate from hematopoietic precursor cells, require factors that promote their differentiation and survival (Roodman, 1999). Such factors include M-CSF and RANKL which are produced by osteoblasts as well as activated T lymphocytes. RANKL acts by binding to its receptor, RANK, on the surface of osteoclast precursor cells, stimulating their differentiation into mature osteoclasts (Jones et al., 2002; Khosla, 2001). The action of RANKL is prevented by OPG, a soluble decoy receptor expressed by osteoblasts that competes with RANK for binding to RANKL (Khosla, 2001; Saidenberg et al., 2002; Schoppet et al., 2002).

RANKL is a protein of 317 amino acids which belongs to the TNF super family and whose mRNA is largely expressed in bone, bone marrow and lymphoid tissues (Khosla, 2001). The predominant role of this cytokine in bone is the stimulation of osteoclast differentiation and activation and the inhibition of osteoclast apoptosis (Fuller et al., 1998). Together with M-CSF, RANKL is required *in vivo* for the complete differentiation of osteoclastic precursors into mature osteoclasts (Jones et al., 2002; Khosla, 2001). For example, RANKL knock-out mice present with severe osteopetrosis and loss of osteoclasts (Kong, 1999). RANKL exists both in a soluble and a membranous forms (Ikeda et al., 2001). The soluble form, which correspond to the c-terminal end of

membranous RANKL, may be produced either directly by the cell through an alternative splicing followed by an excretion in the extra-cellular medium, or by a proteolytic cleavage of membranous RANKL (Verrier et al., 2004) and then interact with its specific receptor RANK (Ito , 2005).

RANK is a transmembrane protein of 616 amino acids which belongs to the TNF receptor (TNFR) super family. This natural receptor of RANKL is expressed primarily on the cells of the monocyte/macrophage lineage including osteoclastic precursors, B and T cells, dendritic cells and fibroblasts (Khosla, 2001). RANK is also present at the surface of mature osteoclasts. RANK, like other TNFR-related proteins, is known to activate a cascade of intracellular signaling events, including recruitment of TNFR associated factor proteins (TRAF), activation of transcription factors (i.e. NF-κB p50 and p52, activator protein (AP)-1 and nuclear factor of activated T cells-2), activation of mitogen activated protein kinase cascades (extracellular regulated kinase, c Jun N terminal kinase) and induction of Src- and phosphatidylinositol 3 kinase-dependent Akt activation (Lee and Kim, 2003). The binding of RANKL to RANK stimulates differentiation of osteoclastic precursors as well as the activation of mature osteoclasts. The RANK activation by RANKL is followed by its interaction with various components of the signaling pathway (Hsu et al., 1999a; Lomaga et al., 1999).

OPG which is synthesized as a protein of 401 amino acids is cleaved to give a 380 amino acid mature protein (Khosla, 2001). OPG loses its transmembrane and cytoplasmic domains and is released as a soluble form by stromal cells and osteoblast cells. Thus OPG differs from the other members of the TNFR superfamily which remain mainly membrane associated (Tan et al., 1997). In contrast to RANKL, OPG knockout mice

become osteoporotic (Mizuno et al., 2002) due to OPG's ability to bind with RANKL and down-regulate the RANKL signaling through RANK. OPG represents an antagonist endogenous receptor that neutralizes the biological effects of all forms of RANKL and thus acts as an inhibitor of bone resorption (Khosla, 2001; Saidenberg et al., 2002; Schoppet et al., 2002). The biological effects of OPG on bone cells include inhibition of terminal stages of osteoclast differentiation, suppression of mature osteoclast activation and induction of apoptosis (Khosla, 2001; Saidenberg et al., 2002; Schoppet et al., 2002). It should be noted that OPG can also directly inhibit osteoclastic activity, independently of RANKL through interaction with a receptor yet to be characterized on osteoclasts (Hakeda et al., 1998).

A number of factors have been shown to influence RANKL and OPG, and lead to alterations in the RANKL/OPG ratio. Hormones, vitamins, and other molecules are known to influence the production of RANKL and/or OPG by stromal osteoblasts and hence the RANKL/OPG ratio. For example, PTH and glucocorticoid enhance RANKL and inhibit OPG production (Huang et al., 2004). $1\alpha,25$-dihydroxyvitamin D_3 increases RANKL production with no effect on OPG (Kondo et al., 2004). Vitamin K has been shown to stimulate osteoblastogenesis and inhibit osteoclastogenesis in bone marrow cells through the expression of RANKL (Koshihara et al., 2003). Estrogen and tumor growth factor β (TGF-β) enhance OPG production while IFN-γ inhibits RANKL induced osteoclastogenesis (von Stechow et al., 2004). More recently IL-7 has been shown to induce bone loss via RANKL production by T cells (Lubberts et al., 2003).

Gonadal Hormone Deficiency and Oxidative Status

Recently, it has been suggested that reactive oxygen species (ROS) play an important role in postmenopausal bone loss (Basu et al., 2001; Maggio et al., 2003). Decreasing intracellular antioxidants such as glutathione and thioredoxin has been shown to increase osteoclast activity via up-regulation of TNF-α in ovarian hormone deficient animal models of osteopenia (Jagger et al., 2005; Muthusami et al., 2005). Administration of agents that increase the intracellular concentrations of the antioxidant (e.g. glutathione) in osteoclasts prevents bone loss during estrogen deficiency (Lean et al., 2003). Inflammatory mediators may deplete physiological antioxidants such as glutathione or decrease the activity of the enzyme such as glutathione S transferase (GST) (Miller et al., 2003), superoxide dismutase (SOD) (Mccord and Edeas, 2005) and heme oxygenase (Chauveau et al., 2005). In response to inflammatory mediators, reactive oxygen species such as O_2^-, H_2O_2, NOO^- may be produced that directly or indirectly enhance inflammation through the activation and phosphorylation of stress kinases (c-Jun N terminal kinase, extracellular signal regulated kinase, mitogen activated protein kinase p38) (Haddad, 2002) and redox-sensitive transcription factors such as NF-κB and AP-1 (Rahman et al., 2004). Activation of the redox sensitive transcription factor, NF-κB, has been shown to up-regulate inflammatory cytokines (Rahman et al., 2004) and enhance osteoclastogenesis (Jagger et al., 2005). Thus, compounds that scavenge free radicals have the potential to down-regulate the inflammatory response (Lean et al., 2003). In OVX animals, administration of antioxidants (e.g. *N*-acetyl cysteine or ascorbic acid) increased bone levels of thiol antioxidants (i.e. glutathione and thioredoxin) and decreased TNF-α production (Lean et al., 2003). Pegylated catalase, a compound that

30

breaks down H_2O_2, decreased bone resorptive activity following ovariectomy (Lean et al., 2005). In addition to synthetic antioxidants, natural polyphenols which are abundant in fruits and vegetables, have shown positive effect on bone metabolism under gonadal hormone deficiency (Fonseca and Ward, 2004; Horcajada-Molteni et al., 2000; Soung et al., 2006).

Fruit and Vegetable Consumption and Bone Health

Previous epidemiological and experimental studies have shown that consumption of legumes (Hallund et al., 2006; Engelen et al., 2007), fruits and vegetables (Muhlbauer et al., 2003; New et al., 2000) rich in phenolic compounds and their derivatives (Hegarty et al., 2000; Johnell O, 2005), reduce the incidence of chronic inflammatory disease such as cardiovascular disease, various types of cancers and osteoporosis. Bazzano and colleagues (Bazzano et al., 2002) reported that adults (25-74 years) consuming fruits and vegetables 3 times per day experienced a 27% lower cardiovascular disease mortality (p=0.008) compared to those who consumed fruits and vegetables <1 time per day. Jansen et al (2004) showed that daily consumption of 200 g of fruit by elderly men (65-84 years) lowered the risk of lung, colon and prostate cancer. Diets high in fruits and vegetables have been linked to an increase in bone accrual in boys aged 8-20 y (Vatanparast et al., 2005) as well as a reduction in the rate of bone loss in pre-menopausal women (Macdonald et al., 2005) and aging men (Tucker et al., 2002). A retrospective study with healthy adult women showed that femoral neck BMD was higher in women who consumed greater amounts of fruits in their childhood (New et al., 2000). These studies provide strong evidence supporting the importance of fruit and vegetable

consumption on bone health.

Dried Plums and Bone Health

In terms of fruits with particular bone-modulating properties, dried plum, *Prunus domestica L.*, has been shown to have potent effects on bone health. 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 may enhance bone formation as indicated by increased serum bone-specific ALP and IGF-I (Arjmandi et al., 2002). Muhlbauer and colleagues (Muhlbauer et al., 2003) investigated the influence of various plant-based foods on bone resorption in male rats. Of the fruit and vegetables considered in this study, dried plum (1 g/d) proved to be one of the most potent inhibitors of bone resorption. Studies utilizing female animal models of osteopenia, indicated that diets supplemented with dried plum not only prevent (Arjmandi et al., 2001), but also reverse (Deyhim et al., 2005) bone loss due to ovarian hormone deficiency. Other recent studies evaluating bone recovery following skeletal unloading demonstrated that dried plum had comparable effects to intermittent PTH therapy on vertebral bone mineral content (BMC) and trabecular bone volume (BV/TV) of the distal femur (unpublished data). Our recent report (Franklin et al., 2006) showed that the bone-protective effects of dried plum on BMD and trabecular bone microarchitecture in gonadal hormone deficient male animals were even greater than those observed in females. Dietary supplementation with dried plum prevented the orchidectomy-induced decrease in bone mass and mechanical strength which was accompanied by improved bone microarchitecture. Serum biochemical markers of bone metabolism and local gene and protein expression data

suggested the improved bone quality resulted from increased bone formation and decreased bone resorption, which was mediated by the IGF-I and RANK pathways, respectively. These studies provide strong evidence that dried plum may provide a natural alternative in terms of osteoporosis treatment.

Bone Protective Component in Dried Plum

Despite the very promising findings related to dried plum and bone health in both male and female animal models, the component of dried plum responsible for the bone-protective effects remains unclear. Dried plums contain relatively high amounts of boron, copper, potassium, vitamin K, and phenolic compounds (Stacewicz-Sapuntzakis et al., 2001). Any one of these components alone or in combination may provide the bioactive component in dried plum responsible for the bone protective effects.

Dried plum is high in the trace element boron (2-3 mg/100g)(Stacewicz-Sapuntzakis et al., 2001). The amount of boron in a 100 g serving of dried plums is equal to the average daily intake for adult males (2.23 ± 1.3 mg/ day). In human and animal studies, boron deficiency has been shown to increase urinary calcium excretion while supplementation decreased the excretion of calcium (Nielsen, 2004; Sheng et al., 2001a). Several animal studies (Armstrong and Spears, 2001; Hunt et al., 1994; Sheng et al., 2001b) have demonstrated that boron also plays a role in the metabolism of magnesium and phosphorus ,which are two of the micronutrients required for mineralization of bone matrix. Additionally, boron supplementation has been shown to increase steroid hormone concentrations in animals and humans (Hunt et al., 1997). Naghii and colleagues (Naghii and Samman, 1997) showed that rats consuming 2 mg boric acid/day for 4 weeks

experienced a significant increase in plasma testosterone. However, other studies have not observed an effect of boron supplementation on steroid hormone metabolism (Beattie and Peace, 1993; Gallardo-Williams et al., 2003), so the role of the boron provided by dried plum on bone in gonadal hormone deficiency remains in question.

In addition to boron, a 100 gram serving of dried plum also contains 20% of the daily requirement for copper (Stacewicz-Sapuntzakis et al., 2001). Copper plays a key role in skeletal health as a component of lysyl oxidase, an enzyme involved in collagen-crosslink formation (Rucker et al., 1998). Collagen crosslinks provide tensile strength to bone (Riggins et al., 1979) and several animal studies report compromised skeletal integrity as a result of copper deficiency (Rucker et al., 1975; Smith et al., 2002; Strause et al., 1987; Teague and Carpenter, 1951). Copper supplementation (15mg/kg diet) has been shown to prevent the OVX-induced decrease of BMD (Rico et al., 2000). In humans, a relationship between copper deficiency and osteoporosis has been demonstrated. Conlan and colleagues (Conlan et al., 1990) reported reduced serum copper in elderly patients with fractures of the femoral neck compared with age- and gender-matched controls. Furthermore, copper status was reported to be a more important predictor of bone health in individuals who were immobilized than calcium status (Lappalainen et al., 1982). However, in the absence of copper deficiency, copper supplementation did not improve bone markers (Cashman et al., 2001) and may have interfered with the anabolic action of other nutrients (e.g. zinc)(Lai and Yamaguchi, 2005). Although adequate copper status is important to bone health, supplementing copper at the levels found in dried plum is not likely to have anabolic effects capable of reversing bone loss in gonadal hormone deficient osteopenic animal models.

Dried plums also have relatively high potassium content (745mg/100g) which has anti-resorptive effects on bone (Macdonald et al., 2005) and decreased urinary calcium excretion (Rafferty et al., 2005). Potassium has been recognized for its buffering ability in reducing blood acidity (Marangella et al., 2004). Metabolic acidosis induces bone loss by stimulating bone resorption and inhibiting bone formation (Krieger et al., 1992). Clinical studies with postmenopausal women showed that both potassium intake and fruit and vegetable consumption decreased dietary acid overload that may have detrimental effects on skeletal health (Frassetto et al., 2005; Sellmeyer et al., 2002). Higher potassium intake especially from fruits and vegetables is associated with higher BMD in the elderly (69-97 year old) (Tucker et al., 2001). The need to encourage adequate potassium intake from fruit and vegetables is one of the primary reasons for the "5 to 10 a day" recommendation (Demigne et al., 2004). Due to its strong anti-resorptive properties, potassium may contribute to dried plum's potent effects on bone, but the anabolic action of dried plum previously demonstrated (Deyhim et al., 2005) make the anti-resorptive action of potassium alone unlikely responsible for the overall effects.

One other micronutrient in dried plum that may account for some of the effects of dried plum on bone formation is vitamin K (59.5 µg phylloquinone per 100 g DP). Vitamin K is a fat-soluble vitamin that functions as a cofactor for enzymes involved in the synthesis of blood coagulation factors and is required to facilitate carboxylation of proteins such as osteocalcin in bone and reduces urinary calcium excretion (Booth and Meyer, 1997; Vermeer et al., 1992). *In vitro*, vitamin K directly stimulates osteoblast differentiation and inhibits osteoclastogenesis (Koshihara et al., 2003). Observational studies indicate that vitamin K intake and serum levels are positively related to BMD and

35

elderly osteoporotic patients who sustain fractures are often reported to have lower serum

vitamin K (Hart et al., 1985; Szulc et al., 1993; Tamatani et al., 1998). Epidemiologic

studies have also shown that higher vitamin K intake is related to lower fracture

incidence (Booth et al., 2000; Feskanich et al., 1999; Hodges et al., 1993; Stone et al.,

1999). Furthermore, a high percentage of undecarboxylated serum osteocalcin, as seen

with low serum vitamin K, may be a predictor of fracture risk (Kohlmeier et al., 1997b;

Luukinen et al., 2000; Vergnaud et al., 1997). Several vitamin K supplementation studies

(Kohlmeier et al., 1997a; Luukinen et al., 2000; Vergnaud et al., 1997) have found

reductions in calcium excretion and bone resorption, and increased serum levels of

undecarboxylated osteocalcin. Vitamin K supplementation was reported to decrease

serum osteocalcin and lumbar bone mass in children receiving long-term glucocorticoid

treatment (Inoue et al., 2001), and increase serum osteocalcin and ALP during space

flight (Vermeer et al., 1998). A compound derived from vitamin K (i.e. menatetrenone)

had a positive effect on BMD when given to women in large doses with osteoporosis

(Orimo et al., 1998) and strokes (Sato et al., 1998). Pharmacological doses of vitamin K_2

(45mg) have also been related to a lower rate of bone loss and a lower incidence of

fracture (Braam et al., 2003). Recently vitamin K has been reported to have anti-

inflammatory properties (Ohsaki et al., 2006). Therefore, vitamin K may possibly

contribute to the effects of dried plum on bone metabolism and further studies are

warranted to determine its contribution.

Polyphenols and Anti-inflammatory Properties

Polyphenols, recognized for their free radical scavenging properties, are perhaps

one of the most intriguing groups of potential bone protective components in dried plum.

The high polyphenol content in dried plum (1600-3700mg/kg) (Fang et al., 2002;

Nakatani et al., 2000b) results in this dried fruit having one of the highest ratings of

oxygen radical absorbance capacity (ORAC), among the commonly consumed fruits and

vegetables (Dikeman et al., 2004; Fang et al., 2002; Ronald, 1999). The predominant

polyphenols in dried plum are neochlorogenic acid, chlorogenic acid and

cryptochlorogenic acid (Fang et al., 2002). Four more minor phenolic components in

plums include caffeic acid, rutin, cinnamic acid and coumarins (Fang et al., 2002).

Table 1. Composition of phenolic compounds in dried plum (per 100g fruit)
(Stacewicz-Sapuntzakis et al., 2001)

Phenolic Compounds	Amount per 100 g of Dried Plum
Neochlorogenic acid	131 mg
Chlorogenic acid	44 mg
Rutin	3.3 mg
p-Coumaric acid	1.0 mg
Caffeic acid	0.9 mg
Total Polyphenols	**184 mg**

In addition to antioxidant properties, polyphenols have suppressed the synthesis

of inflammatory mediators and/or redox sensitive transcription factors (Carluccio et al.,

2003; Maggi-Capeyron et al., 2001; Pendurthi et al., 2002). For instance, caffeic acid

inhibits the production of TNF-α and NO, and the activation of NF-κB in hippocampal

cultures (Montpied et al., 2003). Resveratrol, a polyphenol present in most red wines,

decreased the expression of COX-2 and synthesis of prostaglandins by murine

macrophages (Martinez and Moreno, 2000).

These antioxidative and anti-inflammatory properties of phenolic compound are

likely to influence the activity of osteoclasts and osteoblasts. For example, quercetins have been shown to inhibit osteoclast differentiation and activity (Muhlbauer et al., 2003; Woo et al., 2004), directly stimulate osteoblasts and favorably alter bone formation markers (Horcajada-Molteni et al., 2000; Mizutani et al., 1998). Flavonoids such as kaempferol and quercetin, known to suppress iNOS and COX-2 (Chen et al., 2001), inhibited RANKL-induced osteoclast differentiation and bone resorption activity (Rassi et al., 2005; Wattel et al., 2003; Wattel et al., 2004; Woo et al., 2004). EGCG, a major phenolic compound in green tea, suppressed osteoclast formation by inhibiting the generation of free radicals (Nakagawa et al., 2002). Two of the phenolic compounds in dried plum, chlorogenic acid and caffeic acid, have been reported to inhibit RANKL-induced osteoclast differentiation and activity (Kayano et al., 2004; Tang et al., 2006) while another polyphenol in dried plum, rutin, has been shown to inhibit ovariectomy-induced osteopenia in rats by decreasing bone resorption and increasing serum osteocalcin (Horcajada-Molteni et al., 2000). Resveratrol, the major phenolic compound in grapes, stimulated the proliferation and differentiation of osteoblast cells and increased intracellular ALP activity and bone morphogenic protein (BMP-2) production (Chang et al., 2006; Mizutani et al., 1998). Another polyphenol in dried plum, caffeic acid, reversed oxidative stress (H_2O_2)-induced decrease of gene expression involving osteoblast activity such as ALP, type I collagen and phosphorylation of Runx2 (Bai et al., 2004). The results of these studies indicate that many of the polyphenolic compounds that block oxygen derived free radicals, down-regulate inflammatory molecules and consequently inhibit osteoclastogenesis and osteoclast activity (Lean et al., 2003), and stimulate osteoblast activity. Therefore dried plum which contains high amounts of polyphenols, could

effectively down-regulate the production of inflammatory mediators that impact

osteoclast and osteoblast activity, signaling and function. Hence the properties of

polyphenols in dried plum which scavenge free radicals and inhibit inflammatory

mediators involved in bone metabolism may account for the beneficial effects of dried

plum on bone health, especially in terms of mediating the effects under inflammatory and

oxidative stress conditions.

The following chapter has been published (accepted for publication) in the *Osteoporosis International* and appears in this thesis with the journal's permission

CHAPTER III

Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats

So Young Bu[1], Edralin A. Lucas[1], Melissa Franklin[1], Denver Marlow[2], Daniel J. Brackett[3,4], Emily A. Boldrin[1], Latha Devareddy[5], Bahram H. Arjmandi[5], Brenda J. Smith[3,4]

[1]Department of Nutritional Sciences, College of Human Environmental Science, Oklahoma State University, Stillwater, OK 74078;
[2]College of Veterinary Medicine, Oklahoma State University, Stillwater, OK 74078;
[3]Department of Surgery, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190;
[4]Veterans Affairs Medical Center, Oklahoma City, OK 73190;
[5]Department of Nutrition, Food & Exercise Sciences, Florida State University, Tallahassee, FL 32306

Corresponding author: Brenda J. Smith, O'Donoghue Research Building, P.O. Box 26901 WP 2140, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190; (405) 271-2601; Brenda-Smith@ouhsc.edu

Abstract

This study was designed to investigate the extent to which dried plum (DP) reverses bone loss in osteopenic orchidectomized (ORX) rats and to compare its effects to parathyroid hormone (PTH). Fifty, 6-month-old male Sprague Dawley rats were sham-operated or ORX and bone loss was confirmed after 90 days. The ORX groups were assigned to control (AIN-93M) diet, 25% DP diet, or PTH (80μg/kg) for 90 days. DP induced an 11% increase in vertebral and femoral BMD compared to ORX-controls. BMD in the PTH-treated group was increased by 20.7% (vertebra) and 17.9% (femur). Vertebral trabecular bone volume (BV/TV) and number were increased by DP and trabecular separation was decreased compared to controls, which were similar to PTH. Alterations in trabecular bone of the femur were similar to those in the vertebra, but DP did not restore BV/TV to the same extent. Cortical thickness was improved by DP and further enhanced by PTH. DP tended to decrease urinary deoxypyridinoline and calcium, but did not alter alkaline phosphatase or osteocalcin. We conclude that though the degree of improvement was not equivalent to PTH with regard to all parameters, DP reverses bone loss due to ORX and the mechanisms should be further investigated.

Introduction

While most treatment options for osteoporosis have targeted postmenopausal bone loss, over the past decade the clinical efficacy of these therapeutic strategies for osteopenic or osteoporotic males has been evaluated on a more limited basis [1,2]. Clinical trials have shown that in males with osteoporosis, testosterone replacement therapy (TRT) produces moderate increases in bone mineral density (BMD) [2], but anti-resorptive therapy (i.e. alendronate) alone [3] or in combination with TRT [4] has a more pronounced effect. The anabolic agent, parathyroid hormone (PTH), has also been shown to enhance bone mass of the spine and hip in men with idiopathic disease, and increase bone turnover [1]. Evidence from studies in male gonadal hormone deficient animals, has shown that synthetic androgen treatment (i.e. 7-alpha-methyl-19-nortestosterone) suppresses the accelerated rate of bone turnover and bone loss [5]. Additionally, bisphosphonates, such as etidronate and alendronate, prohibited bone loss in this model [6,7] and intermittent PTH markedly increased cortical and trabecular thickness [8]. The results of these clinical and animal studies indicate the response of gonadal hormone deficient males to pharmacological therapies is similar to that observed in females.

In addition to pharmacological agents, nutrient supplements and functional foods [9-11] have also been considered as therapeutic options for men with osteopenia and osteoporosis, providing a cost effective and convenient alternative with minimal side-effects [12]. For instance, calcium and vitamin D supplementation (500 mg Ca with 700 IU cholecalciferol) significantly increased femoral neck, lumbar spine, and total body BMD in healthy men aged 65 and older [11]. However, neither calcium nor vitamin D supplements alone or in combination have been able to reverse the bone loss [10,13,14].

Functional foods and their components such as soy proteins [9], isoflavones [15], and dried plum [16-18] have been evaluated for their ability to attenuate bone loss. Soy protein, perhaps the most extensively studied natural product in the prevention and/or treatment of osteoporosis, has been shown to increase serum insulin-like growth factor (IGF)-I which is associated with bone formation in healthy men [9]. Isoflavones in the context of both casein and soy protein sources also provided modest protection against bone loss in orchidectomized male rats [15] by enhancing bone alkaline phosphatase (ALP), osteocalcin and type I collagen [19]. More recently dried plum (*Prunus domestica L.*) has provided some of the most promising evidence of a natural product's effects on bone mass and microarchitecture in terms of both prevention and reversal of bone loss [16-18,20].

Studies utilizing female animal models of osteopenia indicated that diets supplemented with dried plum could prevent [21] and reverse [16] bone loss due to ovarian hormone deficiency. Additionally, we have observed that dried plum enhanced bone recovery in female rats following hindlimb unloading and restored trabecular bone in a comparable manner to PTH (unpublished data). Muhlbauer and colleagues [17] were the first to demonstrate that in male rats dried plum strongly inhibited bone resorption compared to other fruits and vegetables. Our recent report [18] showed that the bone protective effects of dried plum on BMD and trabecular bone structure in gonadal hormone deficient male animals was even greater than those previously reported in females [21]. Dietary supplementation with dried plum prevented the orchidectomy-induced decrease in bone mass and mechanical strength which was accompanied by improved bone microarchitecture [18]. The improved bone quality was associated with

alterations in both bone formation and resorption, which were mediated by the IGF-1 and receptor activator of nuclear factor-kappa B (RANK) pathways.

Based on our previous findings in orchidectomized males, we hypothesized that dried plum may have anabolic properties enabling it to reverse bone loss in osteopenic male animals. Thus, the purpose of this study was to evaluate whether dried plum, a rich source of polyphenols and micronutrients (e.g. vitamin K, potassium and boron) involved in bone metabolism [22], could restore the bone mass and structure in gonadal hormone deficiency-induced osteopenic male rats. Furthermore, the efficacy of dried plum in reversing bone loss was compared to that of the anabolic agent, PTH.

Materials and Methods

Animals Care, Diet and Necropsy

Fifty, 6-month-old male Sprague Dawley (SD) rats (Harlan, Indianapolis, IN) were housed in an environmentally controlled animal care laboratory upon arrival. Animals were acclimated to standard temperature, light:dark (12:12) conditions and AIN-93 control diet [23] for 5 days. The animals were then anesthetized with ketamine (70mg/kg body weight) and xylazine (3mg/kg body weight) and either sham-operated (Sham: one group) or orchidectomized (ORX: four groups) to induce bone loss over a 90 day period. Thereafter, one Sham and one ORX group animals was DXA scanned, sacrificed, and excised bones analyzed using microcomputed tomography to verify that bone loss had occurred. The remaining three ORX groups were randomly assigned to either standard AIN-93M diet (control), control diet with 25% (w/w) dried plum, or

control diet plus PTH injections (80µg PTH/kg body weight 3 times per week) for an additional 90-day treatment period. The amount of dried plum incorporated into the diet was based on the most effective dose from our previous study [18]. The diets were designed to be isocaloric, isonitrogenous and had similar calcium and phosphorous content [18]. The dose of PTH utilized in this study was selected based on previous reports demonstrating significant anabolic effects on bone mass and strength [24] and no differences in a 3-day compared to a 5-day dosing regimen [25]. The animals were match fed to the group that consumed the least amount each week, had free access to deionized water, and were weighed on a weekly basis. At the end of the 90-day treatment period, rats were placed in metabolic cages and fasted 24 hours prior to necropsy. Urine was collected in acid-washed tubes over a 12 hour period. Total urine volume was measured, centrifuged for 20 minutes at 4,000 rpm and aliquotted for calcium, deoxypyridinoline (Dpd) and creatinine analyses. For necropsy, animals were anesthetized with a mixture of ketamine/xylazine (70 and 3 mg/kg body weight, respectively) and scanned with dual energy x-ray absorptiometry (DXA) to measure whole body BMD. Animals were then bled from the abdominal aorta and serum was separated from whole blood by centrifugation (4,000 rpm) for 20 minutes and stored at -20˚C. Bones (i.e. left femur and 4th lumbar vertebrae) were collected, cleaned and stored at 4˚C for later analyses. The coagulating gland, which atrophies in the absence of testosterone [26], was collected and weighed to confirm orchidectomy. All procedures were performed in strict accordance with the protocol approved by the Animal Care and Use Committee at Oklahoma State University.

Bone Mineral Area, Content and Density

Whole body DXA scans (DXA, Model QDR-4500A Elite, Hologic Waltham, MA) were performed at the beginning of the study (i.e. baseline), 90 days after surgery and 90 days post treatment (i.e. final) to assess whole body bone mineral area (BMA), bone mineral content (BMC), BMD and body composition (i.e. fat and lean mass). Excised femurs and vertebrae were also scanned to measure the local BMA, BMC and BMD. All analyses were performed using the Regional High Resolution software package designed for studying small animals (Hologic Waltham, MA).

Bone Microarchitecture

For determining the effect of dried plum diet on trabecular and cortical bone architecture, the femur and vertebra were evaluated using μCT (MicroCT40, SCANCO Medical, Switzerland). The distal end of the femur was scanned and 150 (~2.4 mm) of the 350 acquired images were analyzed within the volume of interest (VOI). The VOI was identified by semi-automatically placed contours that included only secondary spongiosa within the metaphyseal region beginning 25 slices (400μm) from the growth plate. Cortical bone of the femur mid-diaphysis was evaluated by acquiring 34 slices at the midpoint, and evaluating 30 (480 μm) of these slices. Trabecular bone of the vertebral body was scanned in a cranial-caudal direction so that 530 images were acquired. The VOI was comprised of the secondary spongiosa in 300 cross-sectional slices of the vertebral body, excluding the growth plate regions. All scans were performed utilizing a 1024 x 1024 matrix resulting in an isotropic voxel resolution of 22 μm^3. An integration time of 70 milliseconds per projection was used with a rotational step of 0.36 degrees. All

specimens were analyzed at a threshold of 240. The VOI was assessed for structural

parameters including trabecular bone volume per unit of total volume (BV/TV),

trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp),

structure model index (SMI), linear x-ray attenuation coefficient (Linear Attenuation) and

connectivity density (Connectivity). Cortical parameters included cortical thickness, area,

and porosity and medullary area.

Biomechanical Testing of Vertebra with Finite Element Analysis

The image acquired with μCT allowed further evaluation of the biomechanical

parameters on trabecular bone structures using finite element analysis (FE) software

(Scanco Medical). Previous biomechanical studies have shown that computed

tomography(CT)-based finite element (FE) models are highly correlated with actual

compressive strength of bone specimens [27]. In this study, a micromechanical finite

element (FE) model was constructed by converting bone voxels from the VOI into 8-

node brick elements [28]. The elements in this FE model have linear, elastic and isotropic

material properties described by a Poisson's ratio of 0.3 and a Young's modulus of

10GPa [28]. Compression testing was simulated on the reconstructed 3-D images of each

vertebral body based on an average of 970,103 total elements and 1,294,292 nodes. Total

force, which is consistent with ultimate force, physiological force, stiffness, size-

independent stiffness, and average von Misses stresses were determined [29].

Biochemical Markers of Bone Metabolism

Serum ALP activity, an indicator of bone formation, was measured using an alkaline phosphatase substrate kit (Bio-Rad Laboratories, Hercules, CA). Total serum protein was measured by the BCA method [30] and the values of ALP activity were expressed in nanomol of *p*-nitrophenol per μg of protein following a 30 minute incubation period. The intra- and inter- assay coefficients of this process were 4.4 % and 5.7 %, respectively. For the determination of treatment effects on bone resorption, urinary excretion of Dpd was assessed using the Pyrilinks-D ELISA kit (Metra Biosystems, Mountain View, CA) and microplate reader (ELx808 Ultra Microplate Reader). The sensitivity of Dpd measurement is 1.1 mmol/L, and the intra- and inter- assay variability were 4.8% and 8.4%, respectively. Dpd concentrations were also standardized to creatinine determined by an ACE clinical analyzer (Alfa Wassermann, West Caldwell, NJ) based on colorimetric change. The intra- and inter- assay coefficients of variation in creatinine measurement were 1.0% and 2.6%, respectively.

Statistical Analysis

Statistical analysis was performed using SAS Version 9.0 (SAS Institute, NC). The significance of treatment effects was analyzed by the one-way ANOVA model followed by post hoc analysis using the Fisher's least squares means separation test when F values were significant. Data are presented as means $\pm$ standard error (SE). In all statistical comparisons, differences with $p < 0.05$ were considered significant.

Results

Body Weight, Tissue Weights and DXA

Throughout the treatment period, all animals continued to gain weight and no differences in body weight were observed among groups **(Table 1)**. Results of body composition analyses by DXA demonstrated that the consistent body fat percentage and lean body mass were maintained by all groups (*data are not shown*). At 90 days post orchidectomy, coagulating gland weight in ORX-animals were significantly decreased compared to Sham-control and confirmed the success of orchidectomy (Table1). As may have been anticipated, whole body BMD was reduced (*p*<0.005) at 90 days post orchidectomy, but no significant changes in BMC and BMA were observed **(Table 2)**.

By the end of the treatment period, the dried plum diet restored whole body BMD to that of the 9-month old sham controls (Table 2). Femoral BMD and BMC (Table 2) were increased in the ORX-DP group by 6% and 11%, respectively, compared to ORX-controls. Dried plum diet significantly increased vertebral BMD and tended (*p*=0.0566) to increase BMC (Table 2). No change was observed in either femoral or vertebral BMA with the dried plum diet. A more pronounced increase in BMD of the femur and vertebra occurred with PTH in conjunction with enhanced BMC. These treatment effects on BMD by dried plum and PTH were primarily associated with an increase in the amount of bone mineral rather than a change of bone size.

Microarchitectural Properties of the Femur and Vertebra

Representative reconstructed 3-D images generated from μCT scans of the femur metaphysis **(Figure 1 a-c)** and vertebral body **(Figure 1 d-f)** showed that dried plum

improved trabecular bone microstructure. Compared to the ORX animals consuming the

control diet, the trabecular network was more evenly distributed, and the struts more

numerous and connected in the ORX-DP group. Additionally, the degree of structural

restoration conferred by dried plum appeared to be similar to PTH-treated group with the

exception of the thickness of the trabecular struts (Figure 1).

These observations from the 3-D images were confirmed by quantifiable changes

in the morphometric parameters. In the distal femur metaphysis the dried plum diet

significantly increased BV/TV (35.7 %) and TbN (23.9 %), and decreased TbSp (33 %)

compared to ORX controls **(Figure 2 a,b & d)**. Dried plum also improved the ORX-

induced alterations in the vertebra by enhancing BV/TV (34.5 %) and TbN (24.4 %), and

depressing TbSp (42.8 %) (Figure 2 a,b & d). The only trabecular parameter that was not

impacted by dried plum in the femur metaphysis and vertebra was TbTh (Figure 2c).

Compared to ORX-controls, the dried plum diet decreased SMI, indicative of a more

plate-like arrangement of the trabeculae in the distal femur (p=0.0358) and tended to

decrease vertebral SMI (p=0.0804) **(Table 3)**. Trabecular bone connectivity in the distal

femur was increased by dried plum diet (p<0.05) and comparable to that of PTH.

However, increases in connectivity density in the vertebra did not reach the level of

statistical significance (p=0.0664) with either treatment. Linear attenuation, a parameter

associated with trabecular density, was increased in the dried plum group at the distal

femur (p<0.0001) and vertebra (p<0.005) compared to ORX-controls. This increase in

linear attenuation was comparable to the effects observed with PTH in the vertebra, but

not in the femur. PTH enhanced BV/TV to a greater extent in the femur metaphysis

(140.5%) than the vertebra (47.4%) and increased TbTh at both sites (i.e. femur=34.3%

and vertebra=23.3%). Although dried plum did not have as great of an impact on BV/TV of the femur, its effects on vertebral BV/TV and parameters related to distribution of the trabeculae (i.e. TbSp and TbN) at both sites, as well as connectivity density in the femur and linear attenuation of the vertebra were similar to that of PTH.

Analysis of cortical bone at the femoral mid-diaphysis demonstrated that dried plum significantly increased cortical thickness (20%) and area (15.1%) compared to controls. PTH decreased the medullary area ($p<0.001$) in conjunction with increasing cortical thickness ($p<0.0001$) and area ($p<0.0001$). Neither the dried plum diet nor PTH altered cortical porosity over the 90-day treatment period. Dried plum enhanced cortical thickness and area, but the extent to which cortical bone was improved was not as great as that of PTH.

Biomechanical Assessment by FE Analysis

The influence of the dried plum on bone biomechanical properties of the vertebrae was evaluated by the simulated compression testing with µCT generated data **(Table 4)**. Compared to the ORX controls, the ORX-DP group experienced an approximately 6-fold increase in the total and physiological force ($p<0.05$). There were no statistical differences between the total and physiological force of the dried plum- or the PTH-treated groups. However, it should be noted that the PTH induced an 11-fold increase in the total force and physiological force, respectively, and the variability in the response to PTH may account for the lack of a statistical difference between the ORX-DP and ORX-PTH groups. Trabecular bone stiffness was increased ($p<0.005$) 6-fold with dried plum which was statistically similar to the PTH response, but size independent stiffness was

not altered by either treatment. Compared to the ORX-control, dried plum decreased the average von mises stresses by 66.9% ($p<0.005$) and the degree of decrease was similar to that of PTH (71.9%). These results indicate that dried plum significantly improved the trabecular microarchitecture of the vertebra to the extent that biomechanical parameters were enhanced.

Bone Biomarkers

As indicators of the treatment effects on bone metabolism, serum ALP and osteocalcin, and urinary Dpd excretion were assessed. No alterations were observed in the bone formation markers (i.e. serum ALP & osteocalcin) in response to ORX (*data not shown)* or treatments at the end of 90 days (**Table 5**). Total urinary excretion of Dpd, a marker of bone resorption, was increased by ORX (*data are not shown*) at 90 days post orchidectomy compared to Sham controls. Dried plum suppressed urinary Dpd expressed as 12 hr total Dpd (Table 5) and Dpd per unit of creatinine by approximately 60%, but only the 12 hr total Dpd tended to approach statistical significance (p=0.0925). Additionally, dried plum diet tended to decrease (*p*=0.0546) urinary calcium excretion by 42.6%, which may be related to net renal acid excretion as well as bone resorption [31]. In contrast, PTH did not alter urinary Dpd or calcium excretion at the end of the 90 day treatment period in spite of the restoration of bone mass and structure.

Discussion

In this study, dried plum was evaluated as an alternative treatment option for established osteopenia in response to androgen hormone deficiency. The findings of this study suggest that dried plum markedly improved bone mass, trabecular and cortical bone microarchitecture, and strength which were negatively impacted by ORX. Vertebral and femoral BMD were increased by approximately 11% with dried plum compared to animals on the control diet. The effects of dried plum on vertebral bone density were approximately 50% of those of the PTH, but dried plum had proportionately a greater effect (i.e. 60%) on bone biomechanical properties. These finding suggest that dried plum may positively effect protein matrix synthesis, regulation of osteoblast and osteoclast activity, and bone turn over rate which are factors that determine overall bone strength [32]. In contrast to other natural compounds and nutrient supplements such as soy isoflavones, calcium and vitamin D which demonstrate a limited ability to restore bone mass and structure [9,15], dried plum effectively increased the bone density and enhanced both trabecular and cortical microarchitecture.

Previously, Wang et al. [33] reported that after 9 months of age most indices of bone mass and structure decline in SD males rats. Based on these observations, age-related bone loss would be expected in the rats used in the current study (i.e. 9 months of age at initiation of treatment). Even though a Sham control group was not included during the treatment phase of this study, the increase in whole body BMD in dried plum- and PTH-treated animals were similar to 9-month-old Sham animals that were used to confirm bone loss. This suggests that BMD in dried plum- and PTH-treated animals was recovered during 90 day-treatment period. Dietary supplementation with dried plum

55

restored whole body, femoral and vertebral BMD, which was decreased in response to gonadal hormone deficiency. This improvement in BMD coincided with increased BMC and no alteration in BMA suggesting that the restoration of BMD was associated with the greater bone mineral accrual. There are concerns regarding the abrupt withdrawal of gonadal hormones in the ORX animal model which does not typically occur in men with the exception of severe hypogonadism due to castration [34]. A gradual reduction in serum testosterone and estrogen has been strongly associated with age-related bone loss in males [35] and therefore, the treatment effects observed with dried plum and PTH in the ORX animal model in this study likely represent overcoming an exaggerated skeletal response.

Even though DXA provides a valuable tool for evaluating bone density, areal BMD measurements are somewhat limited [32]. 3-D assessment of trabecular and cortical bone microarchitecture provides additional insight into the skeletal response to treatments. Deterioration of trabecular bone microarchitecture is a common feature of bone loss due to orchidectomy [35]. Reconstructed 3-D images from μCT showed marked enhancement of trabecular bone structure in animals consuming the dried plum supplemented diet which was supported by quantitative data showing in increase in BV/TV and TbN, and decreased TbSp parameters. Because there were no changes in trabecular thickness, we hypothesize that the decrease of TbSp may be due to the formation of new trabaculae rather than thickening of existing trabeculae, but this would need to be tested in future studies. Previous reports have shown that supplementation with dried plum restored trabecular BV/TV and TbN in osteopenic ovariectomized and skeletal unloaded female rat models [16]. The restoration of bone density as well as

trabecular bone structure in osteopenic male animals reported here appear to be unique to dried plum and have not been observed with other natural alternative treatments [36,37]. For example, soy isoflavone prevented the decrease in bone density and trabecular structures in gonadal hormone deficiency induced osteopenia [15], but could not reverse the decrease of vertebral BMD and structure after bone loss had occurred [37]. Nonetheless, dried plum was able to restore ORX-induced decrease in bone mass and structural properties indicating that dried plum may be a potent and effective treatment option for male osteopenia.

In addition to the deterioration in trabecular bone, cortical bone was also compromised in response to orchidectomy. Previous animal studies demonstrated that significant cortical thinning occurs after orchidectomy due to the decrease of cortical thickness and cortical area [18,8]. Treatment with dried plum increased cortical thickness and area at the femur mid-diaphysis without affecting medullary area. By comparison, PTH increased the cortical thickness and area, and at the same time decreased medullary area. Increased cortical thickness is associated with an increase in periosteal bone formation on the external surface of the cortical bone, enhanced endocortical bone formation and/or a decrease in resorption on the endocortical surface [32]. Our results indicate that the increase of cortical thickness by dried plum may be due to periosteal expansion rather than an increase in endocortical bone formation as observed with PTH [8].

Ultimately, prevention of fracture is the goal of any osteoporosis treatment, therefore the impact of dietary supplementation with dried plum on bone strength should be considered [35]. Simulated compression testing was utilized with FE analysis

employing an isotropic model that has been shown to predict the mechanical behavior of trabecular bone even though it has anisotropic material properties [27]. The dried plum diet restored trabecular bone strength in this study as demonstrated by the 6-fold increase in total force, physiological force and stiffness compared to controls. Although the degree of enhancement of these biomechanical properties was not as great as PTH (i.e. ~11-fold increase), no statistically significant differences were detected between the two treatment groups. Simulated compression tests showed that total force was increased with dried plum to the level of PTH. Dried plum decreased the von mises stress indicating enhanced resistance to relative deformation. Although the dried plum diet increased stiffness, the lack of a change when size (cross sectional area of trabaculae within vertebra) is considered (i.e. size independent stiffness) indicates the increase of stiffness was attributable to an increase in trabeculae accumulation and distribution rather than an increase of actual rigidity of bone. Because the mechanical properties of bone are associated with the quality of bone tissue and its spatial distribution [38], the increased BMD and the improved trabecular bone microarchitecture in conjunction with the enhanced bone strength confirm improved bone quality with the dried plum supplementation.

Similar to our findings with dried plum, drugs and other alternative treatment which have anti-resorptive or anabolic properties have been shown to attenuate the process of the bone loss in male hormone deficiency induced osteopenia [8,39]. The bisphosphonate, etidronate (10mg/kg injection) increased trabecular bone volume and mineral apposition rate in male rats after orchidectomy [39]. However, in this study [39], treatments were initiated two weeks post orchidectomy and the short time period to

induce osteopenia makes it difficult to assess the efficacy of this drug in reversing bone loss in this animal model. Intermittent PTH has also been used to manage osteopenic/osteoporotic conditions due to its anabolic properties [25,8]. In this study we used PTH as a positive control, which enabled us to interpret the degree of anabolic properties of dried plum. The dose of PTH utilized in this study has been previously reported to enhance bone density and biomechanical properties [24]. Consistent with previous findings [40,41], PTH restored the ORX-induced decrease of bone mass, enhanced cortical and trabecular bone structure as well as bone compressive strength. Although the effects of dried plum in this study cannot be considered equivalent to PTH, the marked improvement in bone structural and biomechanical properties suggests that dried plum has similar anabolic effects.

In lieu of these findings, the question remains as to how dried plum influences bone metabolism. In this study, there was some indication that dried plum depressed bone resorption in male osteopenic orchidectomized rats. Total urinary Dpd excretion in animals fed the dried plum diet was 60.8% lower than ORX control animals and urinary calcium excretion tended to be reduced compared to controls. Although these results only represent a single snapshot of the alterations in bone metabolism occurring after 90 days of treatment, they suggest that changes in bone density and structure induced by dried plum may be mediated to some extent through suppression of bone resorption. These changes are similar to other anti-resorptive therapies [7,39] and consistent with previous observations that dried plum suppressed bone resorption [17,18]. However, anti-resorptive action alone is not enough for the restoration of bone because suppressing bone resorption limits the potential increase of bone mass due to the reduction of

remodeling space [42]. Based on the limitations of anti-resorptive therapies to restore

bone mass and structure, our findings suggest that dried plum may have anabolic

properties. The previous clinical trial and animal studies provide strong evidence of the

bone forming activity of dried plum. For instance, postmenopausal women consuming

approximately 100 g of dried plum per day (i.e. 10-12 dried plums) experienced an

increase in serum bone-specific ALP and IGF-I [20]. Data from animal studies indicate

that dried plum also enhanced circulating IGF-I in orchidectomized male [18] and

ovariectomized female [21] rats. However, as previously mentioned, caution should be

used in the interpretation of biochemical marker data at a single time point to determine

the influence of dried plum on bone metabolism. For example, the effects of treatments

such as PTH on bone formation markers have been reported to occur at early time points

[43] which may explain the lack a detectable change in bone formation markers in the

current study. Further studies examining the influence of dried plum on bone metabolism

over time and at the cellular level are likely to provide more insight in the influence of

dried plum on bone metabolism.

In spite of the very promising findings related to dried plum and bone health in

both male and female animal models and postmenopausal women, the component(s) of

dried plum responsible for such marked effects on bone are still under investigation.

Compared to other fruit and vegetables, dried plums contain relatively high amounts of

polyphenols (184 mg/100g), potassium (732mg/100g) and vitamin K (59.5ug/100g) [22].

Polyphenolic compounds and their derivatives, which constitute many of the pigments in

fruits and vegetables, have been shown to inhibit bone resorption due to their antioxidant

and inflammatory properties [17,44]. In addition to their effects on bone resorption,

polyphenols also directly stimulate osteoblasts and favorably alter bone formation markers which would suggest potential anabolic properties [45,46]. Resveratrol, the major phenolic compound in grapes that has been extensively investigated for its health benefits, stimulated the proliferation and differentiation of osteoblast cells and increased intracellular ALP activity and bone morphogenic protein (BMP-2) production [46]. Rutin, which is one of the polyphenols found in dried plums [22] increased serum osteocalcin and BMD in estrogen deficient osteopenic rats [45], but the effects on bone mass were not as remarkable as those of dried plum. Apart from the polyphenols, another potential component of dried plum that may be involved in the bone modulating effects of dried plum is potassium. Studies have shown that potassium is associated with enhancing bone health by decreasing bone resorption via its pH buffering capacity [47,48]. Potassium supplementation also increased bone formation markers such as serum IGF-I in postmenopausal women [48]. A third component of dried plum known to play a role in bone metabolism is vitamin K. Vitamin K facilitates the carboxylation of proteins such as osteocalcin and consequently is involved in bone matrix synthesis [49]. Vitamin K supplementation was reported to increase serum osteocalcin and lumbar bone mass in children receiving long-term glucocorticoid treatment [50] and increase osteocalcin and alkaline phosphatase during space flight [51]. In the current study, no alterations in osteocalcin or alkaline phosphatase were noted after 90 days of treatment. *In vitro*, vitamin K directly stimulates osteoblast differentiation and inhibits osteoclastogenesis [49]. Although supplemental potassium, vitamin K and individual polyphenols such as rutin positively influence bone metabolism, no studies to date have reported effects on bone as pronounced as those observed with the whole dried fruit.

Although non-pharmacological therapies for osteoporosis may be appealing treatment options for many individuals, most of these natural alternatives have limited ability to restore bone density and structure, once bone loss has occurred. In this study we have shown that as a natural alternative, dried plum has potent effects on bone in terms of bone mass, microarchitecture and strength in osteopenic male rats. These changes may be mediated in part through the suppression of bone resorption, but the fact that the restoration in some of the bone structural and biomechanical parameter shares some similarities with PTH should not be overlooked. Further investigation is needed on a mechanistic level to clarify the influence of dried plum on bone metabolism as well as clinical studies to determine the therapeutic efficacy for men with osteopenia and/or osteoporosis.

Acknowledgements

References

1. Kurland ES, Cosman F, McMahon DJ et al (2000) Parathyroid hormone as a therapy for idiopathic osteoporosis in men: Effects on bone mineral density and bone markers. J Clin Endocrinol Metab 85(9):3069-3076

2. Behre HM, Kliesch S, Leifke E et al (1997) Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. J Clin Endocrinol Metab 82(8):2386-2390

3. Orwoll E, Ettinger M, Weiss S et al (2000) Alendronate for the treatment of osteoporosis in men. N Engl J Med 343(9):604-610

4. Shimon I, Eshed V, Doolman R et al (2005) Alendronate for osteoporosis in men with androgen-repleted hypogonadism. Osteoporos Int 16(12):1591-1596

5. Venken K, Boonen S, Van Herck E et al (2005) Bone and muscle protective potential of the prostate-sparing synthetic androgen 7 alpha-methyl-19-nortestosterone: Evidence from the aged orchidectomized male rat model. Bone 36(4):663-670

6. Iwamoto J, Takeda T, Katsumata T et al (2002) Effect of etidronate on bone in orchidectomized and sciatic neurectomized adult rats. Bone 30(2):360-367

7. Huuskonen J, Arnala I, Olkkonen H et al (2001) Alendronate influences bending force of femoral diaphysis after orchidectomy in rats. Ann Chir Gynaecol 90(2):109-114

8. Gabet Y, Kohavi D, Muller R et al (2005) Intermittently administered parathyroid hormone 1-34 reverses bone loss and structural impairment in orchiectomized adult rats. Osteoporos Int 16(11):1436-1443

9. Khalil DA, Lucas EA, Smith BJ et al (2002) Soy protein supplementation increase serum insulin-like growth factor-I in young and old men but does not affect markers of bone metabolism. J Nutr 132(9):2605-2608

10. Lips P, Graafmans WC, Ooms ME et al (1996) Vitamin D supplementation and fracture incidence in elderly persons - A randomized, placebo-controlled clinical trial. Ann Intern Med 124(4):400-406

11. Dawson-Hughes B, Harris SS, Krall EA et al (1997) Effect of calcium and vitamin D supplementation on bone, density in men and women 65 years of age or older. N Engl J Med 337(10):670-676

12. Dobnig H (2004) A review of teriparatide and its clinical efficacy in the treatment of osteoporosis. Expert Opin Pharmacother 5(5):1153-1162

13. Peacock M, Liu G, Carey M et al (2000) Effect of calcium or 25OH vitamin D3 dietary supplementation on bone loss at the hip in men and women over the age of 60. J Clin Endocrinol Metab 85(9):3011-3019

14. Trivedi DP, Doll R, Khaw KT (2003) Effect of four monthly oral vitamin D-3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. Brit Med J 326(7387):469-472

15. Khalil DA, Lucas EA, Smith BJ et al (2005) Soy isoflavones may protect against orchidectomy-induced bone loss in aged male rats. Calcif Tissue Int 76(1):56-62

16. Deyhim F, Stoecker BJ, Brusewitz GH et al (2005) Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause 12(6):755-762

17. Muhlbauer RC, Lozano A, Reinli A et al (2003) Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. J Nutr 133(11):3592-3597

18. Franklin M, Bu SY, Lerner MR et al (2006) Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 39(6):1331-1342

19. Soung DY, Devareddy L, Khalil DA et al (2006) Soy affects trabecular microarchitecture and favorably alters select bone-specific gene expressions in a male rat model of osteoporosis. Calcif Tissue Int 78(6):385-391

20. Arjmandi BH, Khalil DA, Lucas EA et al (2002) Dried plums improve indices of bone formation in postmenopausal women. J Women's Health Gend Based Med 11(1):61-68

21. Arjmandi BH, Lucas EA, Juma S et al (2001) Prune prevents ovariectomy-induced bone loss in rats. JANA 4(1):50-56

22. Stacewicz-Sapuntzakis M, Bowen PE, Hussain EA et al (2001) Chemical composition and potential health effects of prunes: A functional food? Crit Rev Food Sci Nutr 41(4):251-286

23. Reeves PG, Nielsen FH, Fahey GC (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing on the reformation of the AIN-76 rodent diet. J Nutr 123(11):1939-1951

24. Kostenuik PJ, Capparelli C, Morony S et al (2001) OPG and PTH-(1-34) have additive effects on bone density and mechanical strength in osteopenic ovariectomized rats. Endocrinology 142(10):4295-4304

25. Akhter MP, Kimmel DB, Recker RR (2001) Effect of parathyroid hormone (hPTH[1-84]) treatment on bone mass and strength in ovariectomized rats. J Clin Densitom 4(1):13-23

26. Sugihara A, Yamada N, Tsujimura T et al (2001) Castration induces apoptosis in the male accessory sex organs of Fas-deficient lpr and Fas ligand-deficient gld mutant mice. In Vivo 15(5):385-390

27. Kabel J, van Rietbergen B, Dalstra M et al (1999) The role of an effective isotropic tissue modulus in the elastic properties of cancellous bone. Journal of Biomechanics 32(7):673-680

28. Newitt DC, Majumdar S, Van RB et al (2002) In vivo assessment of architecture and micro-finite element analysis derived indices of mechanical properties of trabecular bone in the radius. Osteoporos Int 13(1):6-17

29. van Rietbergen B, Odgaard A, Kabel J et al (1996) Direct mechanics assessment of elastic symmetries and properties of trabecular bone architecture. J Biomech 29(12):1653-1657

30. Stoscheck CM (1990) Quantitation of Protein. Methods Enzymol 182(1):50-68

31. Lemann J (1999) Relationship between urinary calcium and net acid excretion as determined by dietary protein and potassium: A review. Nephron 81(Suppl 1):18-25

32. Ammann P, Rizzoli R (2003) Bone strength and its determinants. Osteoporos Int 14(Suppl 3):S13-S18

33. Wang L, Banu J, McMahan CA et al (2001) Male rodent model of age-related bone loss in men. Bone 29(2):141-148

34. Katznelson L, Finkelstein JS, Schoenfeld DA et al (1996) Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. J Clin Endocrinol Metab 81(12):4358-4365

35. Audran M, Chappard D, Legrand E et al (2001) Bone microarchitecture and bone fragility in men: DXA and histomorphometry in humans and in the orchidectomized rat model. Calcif Tissue Int 69(4):214-217

36. Fanti O, Monier-Faugere MC, Geng Z et al (1998) The phytoestrogen genistein reduces bone loss in short-term overiectomized rats. Osteoporos Int 8(3):274-281

37. Devareddy L, Khalil DA, Smith BJ et al (2006) Soy moderately improves microstructural properties without affecting bone mass in an ovariectomized rat model of osteoporosis. Bone 38(5):686-693

38. Legrand E, Chappard D, Pascaretti C et al (2000) Trabecular bone microarchitecture, bone mineral density, and vertebral fractures in male osteoporosis. J Bone Miner Res 15(1):13-19

39. Iwamoto J, Takeda T, Ichimura S (2004) Differential effect of short-term etidronate treatment on three cancellous bone sites in orchidectomized adult rats. Keio J Med 53(1):12-17

40. Moriyama I, Iwamoto J, Takeda T et al (2005) Comparative effects of intermittent administration of human parathyroid hormone (1-34) on cancellous and cortical bone loss in tail-suspended and sciatic neurectomized young rats. J Orthop Sci 7(3):379-385

41. Wang L, Orhii PB, Banu J et al (2001) Effects of separate and combined therapy with growth hormone and parathyroid hormone on lumbar vertebral bone in aged ovariectomized osteopenic rats. Bone 28(2):202-207

42. Hernandez CJ, Beaupre GS, Marcus R et al (2001) A theoretical analysis of the contributions of remodeling space, mineralization, and bone balance to changes in bone mineral density during alendronate treatment. Bone 29(6):511-516

43. Miyazaki T, Matsunaga T, Miyazaki S et al (2004) Changes in receptor activator of nuclear factor-kappaB, and its ligand, osteoprotegerin, bone-type alkaline phosphatase, and tartrate-resistant acid phosphatase in ovariectomized rats. J Cell Biochem 93(3):503-512

44. Woo JT, Nakagawa H, Notoya M et al (2004) Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. Biol Pharm Bull 27(4):504-509

45. Horcajada-Molteni MN, Crespy V, Coxam V et al (2000) Rutin inhibits ovariectomy-induced osteopenia in rats. J Bone Miner Res 15(11):2251-2258

46. Mizutani K, Ikeda K, Kawai Y et al (1998) Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 253(3):859-863

47. Macdonald HM, New SA, Fraser WD et al (2005) Low dietary potassium intakes and high dietary estimates of net endogenous acid production are associated with low bone mineral density in premenopausal women and increased markers of bone resorption in postmenopausal women. Am J Clin Nutr 81(4):923-933

48. Sebastian A, Harris ST, Ottaway JH et al (1994) Improved Mineral Balance and Skeletal Metabolism in Postmenopausal Women Treated with Potassium Bicarbonate. N Engl J Med 330(25):1776-1781

49. Koshihara Y, Hoshi K, Okawara R et al (2003) Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture. J Endocrinol 176(3):339-348

50. Inoue T, Sugiyama T, Matsubara T et al (2001) Inverse correlation between the changes of lumbar bone mineral density and serum undercarboxylated osteocalcin after vitamin K-2 (menatetrenone) treatment in children treated with glucocorticoid and alfacalcidol. Endocr J 48(1):11-18

51. Vermeer C, Wolf J, Craciun AM et al (1998) Bone markers during a 6-month space flight: effects of vitamin K supplementation. J Gravit Physiol 5(2):65-69

Figure legends

Figure 1. Reconstructed 3-D images of trabecular bone in the distal femur metaphysis (a-c) and vertebra (d-f) of osteopenic rats receiving control (AIN-93M) diet (a & d), dried plum diet (b & e), or PTH injection (c & f) beginning 90 days after orchidectomy.

Figure 2. Alterations in a) trabecular bone volume (BV/TV), b) trabecular number (TbN), c) trabecular thickness (TbTh) and d) trabecular separation (TbSp) at the distal femur metaphysis and vertebral body following 90 days of consumption of control diet (ORX-Control), dried plum (ORX-DP) or parathyroid hormone (ORX-PTH). Bars represent the mean ± SE for each treatment group (n=7-9 /group). Bars that share the same uppercase or lowercase are not significantly ($p<0.05$) different from each other.

Table 1. Body and Tissue Weights in Sham and Orchidectomized (ORX) Male Rats

Parameters	Sham-Control	ORX-Control	ORX-DP	ORX-PTH	*p*-Value
90 Days Post Surgery					
Body Weight (g)	469.68 ± 5.69	464.5 ± 8.0			0.9967
Coagulating Gland Weight (g)	1.90 ± 0.20[a]	0.30 ± 0.03[b]			<0.0001
Final (90 Days Post Treatment)					
Body Weight (g)		492.33 ± 12.26	472.50 ± 8.92	472.25 ± 16.61	0.4664
Coagulating Gland Weight (g)		0.19 ± 0.01	0.20 ± 0.01	0.22 ± 0.02	0.4436

Values are means ± SE, n=8-9 rats in each group.
For each parameter, values within a given row that do not share the same superscript letter are significantly different (*p*< 0.05) from each other.
ORX-Control: Orchidectomized rats on control diet based on AIN-93M rodent diet.
ORX-DP: Orchidectomized rats on control diet supplemented with dried plum (25% w/w).
ORX-PTH: Orchidectomized rats on control diet receiving subcutaneous parathyroid hormone injections (80 µg/kg body weight; 3x/wk).

Table 2. Bone Mineral Area (BMA), Bone Mineral Content (BMC) and Bone Mineral Density (BMD) in Sham-operated (Sham) and Orchidectomized (ORX) Rats

Parameters	Sham-Control	ORX-Control	ORX-DP	ORX-PTH	*p*-Value
Whole Body					
Baseline					
BMA (cm^2)	67.1871 ± 1.2046	68.3459 ± 0.9280	69.6552 ± 1.1809	70.6611 ± 1.2491	0.1732
BMC (g)	12.0483 ± 0.2168	11.8783 ± 0.3302	12.7612 ± 0.2164	12.4365 ± 0.2917	0.1395
BMD (g/cm^2)	0.1795 ± 0.0023	0.1736 ± 0.0029	0.1785 ± 0.0032	0.1806 ± 0.0006	0.2144
90 Days Post ORX:					
BMA (cm^2)	76.9824 ± 0.6125	76.7148 ± 1.0905	79.4563 ± 1.0221	77.7913 ± 1.7061	0.3189
BMC (g)	14.1579 ± 0.1701	13.2592 ± 0.2833	13.8877 ± 0.2226	13.8079 ± 0.5013	0.1741
BMD (g/cm^2)	0.1839 ± 0.0018[a]	0.1727 ± 0.0014[b]	0.1748 ± 0.0015[b]	0.1772 ± 0.0032[b]	0.0013
Final:					
BMA (cm^2)		80.2153 ± 1.5811	77.8680 ± 1.0029	79.1975 ± 1.6247	0.5179
BMC (g)		14.0036 ± 0.3507[b]	14.4503 ± 0.2612[b]	15.6332 ± 0.5243[a]	0.0209
BMD (g/cm^2)		0.1745 ± 0.0021[c]	0.1856 ± 0.0025[b]	0.1972 ± 0.0040[a]	<0.0001
Femur					
BMA (cm^2)		2.4001 ± 0.0418	2.4273 ± 0.0460	2.4631 ± 0.0429	0.5854
BMC (g)		0.5762 ± 0.0171[c]	0.6475 ± 0.0211[b]	0.7207 ± 0.0220[a]	0.0001
BMD (g/cm^2)		0.2398 ± 0.0039[c]	0.2663 ± 0.0004[b]	0.2922 ± 0.0050[a]	<0.0001
Vertebra					
BMA (cm^2)		0.6461 ± 0.0203[b]	0.6912 ± 0.0187[a,b]	0.7490 ± 0.0368[a]	0.0392
BMC (g)		0.1389 ± 0.0056[b]	0.1658 ± 0.0056[b]	0.1956 ± 0.0137[a]	0.0010
BMD (g/cm^2)		0.2147 ± 0.0034[c]	0.2398 ± 0.0040[b]	0.2592 ± 0.0062[a]	<0.0001

Values are means ± SE, n=7-9 rats in each group.

For each parameter, values within a given row that do not share the same superscript letter are significantly different (*p*< 0.05) from each other.

ORX-Control: Orchidectomized rats on control diet based on AIN-93M rodent diet.

ORX-DP: Orchidectomized rats on control diet supplemented with dried plum (25% w/w).

ORX-PTH: Orchidectomized rats on control diet receiving subcutaneous parathyroid hormone injections (80 µg/kg body weight; 3x/wk).

Table 3. Trabecular and Cortical Bone Microarchitecture in Osteopenic Orchidectomized (ORX) Rats

Parameters	ORX-Control	ORX-DP	ORX-PTH	*p*-Value
Distal Femur Metaphysis				
Connectivity (1/mm^3)	9.44 ± 1.06^b	16.71 ± 1.89^a	16.77 ± 2.24^a	0.0103
SMI	2.43 ± 0.13^a	2.02 ± 0.10^b	1.33 ± 0.14^c	<0.0001
Linear Attenuation	0.86 ± 0.02^c	0.99 ± 0.03^b	1.17 ± 0.04^a	<0.0001
Femur Mid-diaphysis				
Cortical thickness (mm)	0.59 ± 0.02^c	0.71 ± 0.02^b	0.80 ± 0.02^a	<0.0001
Cortical porosity (%)	2.72 ± 0.83	1.88 ± 0.23	1.59 ± 0.25	0.3438
Cortical area (mm^2)	5.51 ± 0.18^c	6.34 ± 0.21^b	6.96 ± 0.20^a	<0.0001
Medullary area (mm^2)	3.92 ± 0.21^a	3.61 ± 0.23^a	2.75 ± 0.22^b	0.0027
Vertebra				
Connectivity (1/mm^3)	20.41 ± 2.22	27.63 ± 0.98	23.19 ± 2.60	0.0664
SMI	1.06 ± 0.23^a	0.52 ± 0.07^{ab}	0.15 ± 0.27^b	0.0170
Linear attenuation	1.11 ± 0.07^b	1.33 ± 0.02^a	1.47 ± 0.08^a	0.0030

Values are means ± SE, n=7-9 rats in each group.

For each parameter, values within a given row that do not share the same superscript letter are significantly different ($p< 0.05$) from each other.

ORX-Control: Orchidectomized rats on control diet based on AIN-93M rodent diet.

ORX-DP: Orchidectomized rats on control diet supplemented with dried plum (25% w/w).

ORX-PTH: Orchidectomized rats on control diet receiving subcutaneous parathyroid hormone injections (80 μg/kg body weight; 3x/wk).

Connectivity: Connectivity density.

SMI: Structure model index.

Table 4. Biomechanical Properties of the Vertebral Body Using Finite Element Analyses in Osteopenic Orchidectomized (ORX) Rats

Parameters	ORX-Control	ORX-DP	ORX-PTH	p-Value
Total Force (N)	225.30 ± 101.93^a	1738.65 ± 126.21^b	2774.03 ± 861.97^b	0.0036
Stiffness (N/m x 10^3)	47.97 ± 20.98^a	359.91 ± 27.57^b	569.82 ± 176.45^b	0.036
Physiological Force (N)	0.68 ± 0.31^a	5.21 ± 0.38^b	8.32 ± 2.58^b	0.0036
Size Independent Stiffness (N/m)	184.79 ± 178.22	48.76 ± 3.00	72.38 ± 18.05	0.7151
Average Von Mises Stresses (MPa)	0.317 ± 0.04^a	0.105 ± 0.01^b	0.089 ± 0.01^b	0.0001

Values are means ± SE, n=12 in each group.
For each parameter, values within a given row that do not share the same superscript letter are significantly different ($p < 0.05$) from each other.
ORX-Control: Orchidectomized rats on control diet based on AIN-93M rodent diet.
ORX-DP: Orchidectomized rats on control diet supplemented with dried plum (25% w/w).
ORX-PTH: Orchidectomized rats on control diet receiving subcutaneous parathyroid
hormone injections (80 μg/kg body weight; 3x/wk).

Table 5. Biochemical Markers of Bone Metabolism

Parameters	ORX-Control	ORX-DP	ORX-PTH	P-Value
Serum:				
ALP (nmol/g protein)	160.12 ± 19.17	146.3 ± 16.6	164.99 ± 16.6	0.7190
Osteocalcin (ng/ml)	16.3 ± 1.2	16.3 ± 1.4	12.9 ± 1.6	0.2011
Urinary:				
12h Total Dpd (nM/L)	4.77 ± 1.50	1.87 ± 0.35	5.64 ± 1.44	0.0925
Calcium (mg/dL)	67.59 ± 10.56	38.83 ± 3.88	65.46 ± 10.38	0.0546

Alkaline phosphatase (ALP); Deoxypyridinoline crosslinks (Dpd)
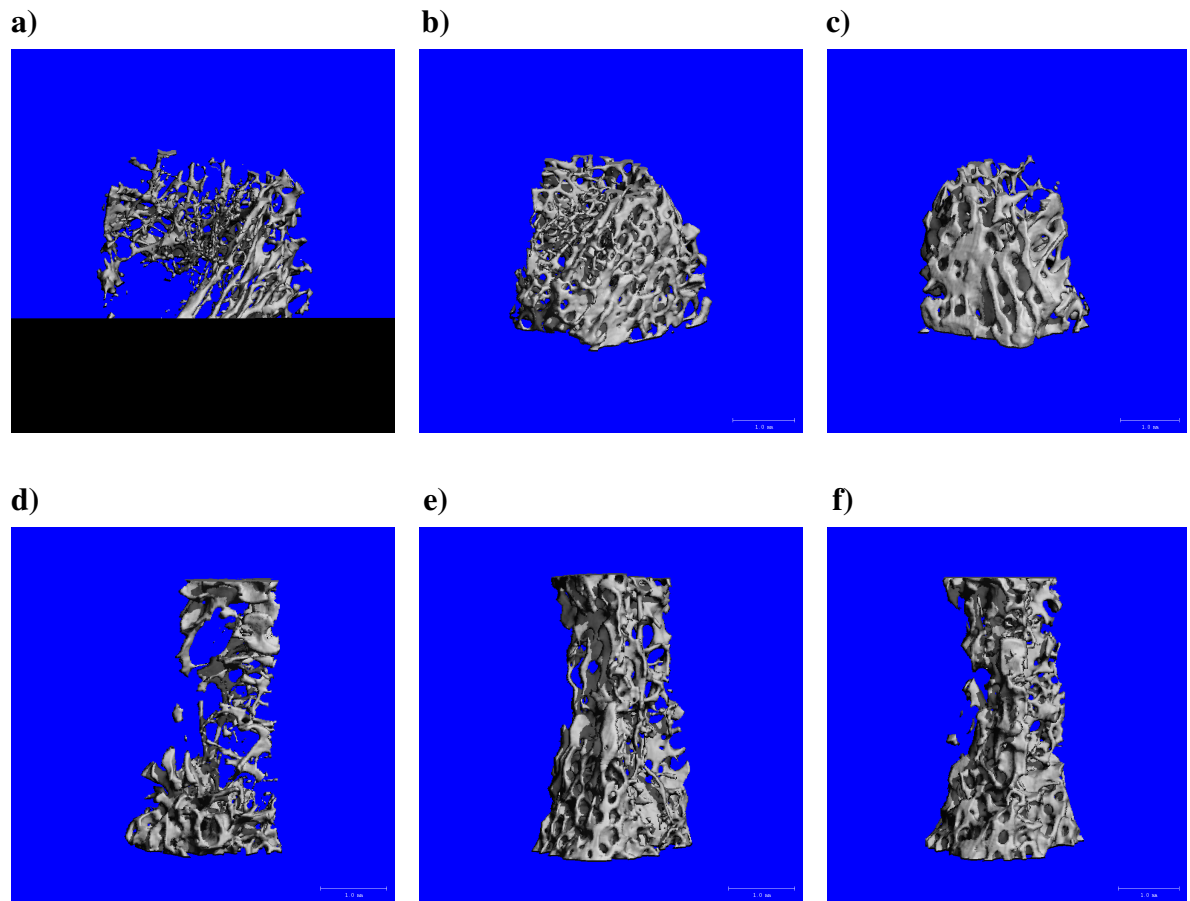Values are means ± SE, n=6-9 rats in each group.
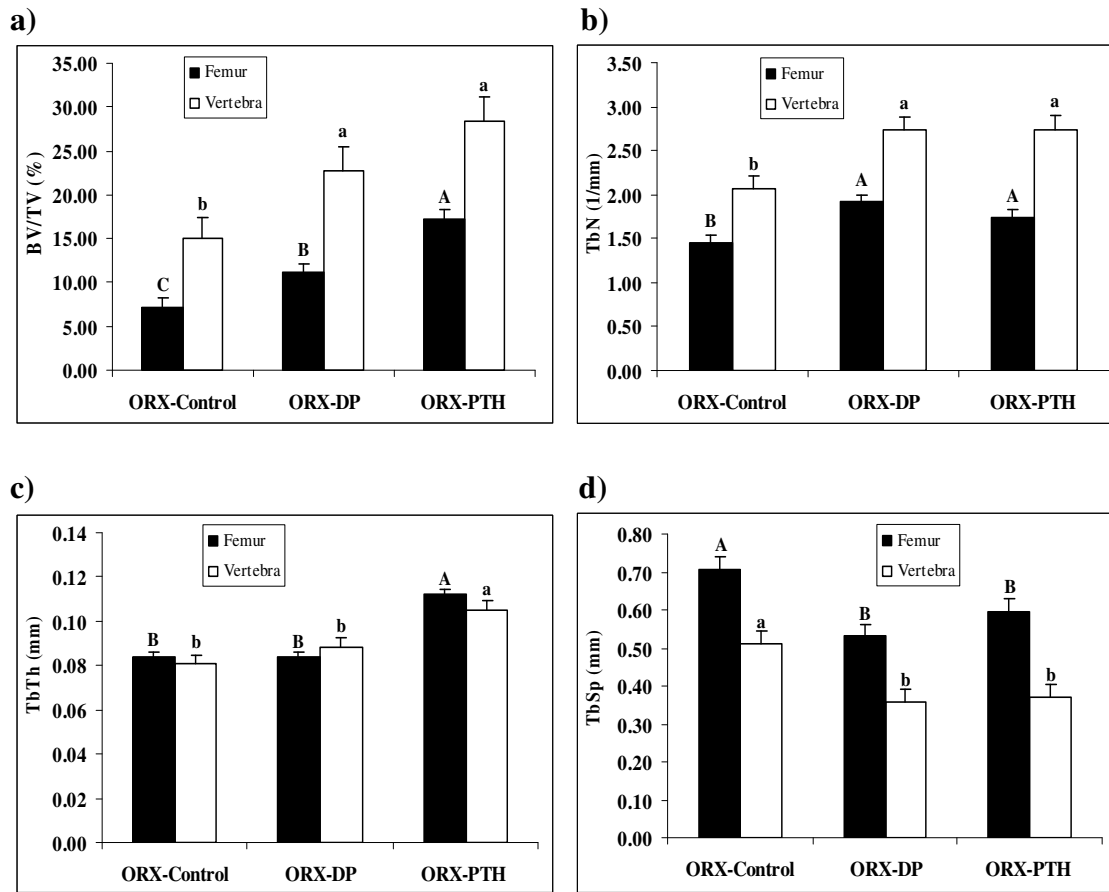ORX-Control: Orchidectomized rats on control diet based on AIN-93M rodent diet.
ORX-DP: Orchidectomized rats on control diet supplemented with dried plum (25% w/w).
ORX-PTH: Orchidectomized rats on control diet receiving subcutaneous parathyroid hormone
     injections (80 µg/kg body weight; 3x/wk).

Figure 1.

a)

b)

c)

d)

e)

f)

Figure 2

CHAPTER IV

Dried plum polyphenols inhibit osteoclastogenesis under oxidative stress and inflammatory conditions

So Young Bu[1], Megan Lerner[2,3], Barbara J. Stoecker[1], Emily Boldrin[1], Daniel J. Brackett[2,3], Edralin A. Lucas[1], Brenda J. Smith[2,3]

[1]Department of Nutritional Sciences, College of Human Environmental Sciences, Oklahoma State University, Stillwater, OK 74078;
[2]Department of Surgery, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190;
[3]Veterans Affairs Medical Center, Oklahoma City, OK 73190

Running title: dried plum polyphenols and osteoclasts

Corresponding author: Brenda J. Smith, O'Donoghue Research Building, P.O. Box 26901 WP 2140, University of Oklahoma Health Sciences Center, Oklahoma City OK 73117; (405) 271-2601; Brenda-Smith@ouhsc.edu

Abstract

Previous studies demonstrated that dietary supplementation with dried plum which contains high amounts of polyphenols, counter the detrimental influence of gonadal hormone deficiency on biochemical markers of bone resorption. This study was designed to investigate the ability of dried plum polyphenols to down-regulate inflammatory molecules known to influence osteoclast differentiation and to determine whether these alterations result in changes in osteoclastogenesis and osteoclast activity under pro-inflammatory and oxidative stress conditions. To assess the ability of polyphenols extracted from dried plum to down-regulate nitric oxide and tumor necrosis factor (TNF)-α, murine macrophages (RAW 264.7) were pretreated with the dried plum extract (0, 10, 20, or 30 μg/ml) and then stimulated with either LPS or hydrogen peroxide (H_2O_2). By 8 hr and 16 hr after treatment, dried plum polyphenols dose-dependently decreased the LPS-induced nitric oxide production by macrophages by as much as 78%. The polyphenol extract also suppressed H_2O_2-induced TNF-α secretion in macrophages over time, but exacerbated LPS-induced TNF-α secretion. LPS up-regulated cyclooxygenase (COX-2) and nitric oxide synthase (iNOS), which was attenuated by the two lower doses of dried plum polyphenols and blocked by the high dose at 16 hrs. Under normal conditions (i.e. no LPS or H_2O_2), the polyphenols down-regulated osteoclast differentiation and activity indicated by TRAP positive cell number and resorption pit assays. Dried plum polyphenol extract also suppressed ($p<0.001$) the LPS- or H_2O_2-induced osteoclast differentiation and osteoclast activity. During osteoclastogenesis, dried plum polyphenol extract decreased the LPS-induced NO and TNF-α production. In conclusion, dried plum polyphenols inhibit osteoclastogenesis and osteoclast activity, an

effect that may be mediated in part by the down-regulation of cytokines such as TNF-α, PGE_2 and NO.

Key words: bone, osteoclast, antioxidant, osteoporosis, macrophage

Introduction

Previous epidemiological and animal studies have shown that the consumption of fruits and vegetables has beneficial effects on bone health [1,2]. Natural polyphenolic compounds abundant in plant-based food products are one of the potential groups of bioactive components responsible for the enhanced bone health [3-5]. Dried plums (*Prunus domestica L.*) are a rich source of polyphenols (1.1-2.6 g/kg), neochlorogenic acid and chlorogenic acid, resulting in this fruit having one of the highest oxygen radical absorbance capacity among the commonly consumed fruits and vegetables [6,7]. Dried plum has been shown to prevent [8] and reverse [9] the decrease of bone mineral density and the deterioration of trabecular bone structure and mechanical strength due to gonadal hormone deficiency. Our recent report [8] suggested that improved bone quality by dried plum was in part due to the inhibition of bone resorption, as indicated by decreased excretion of deoxypyridinoline cross-links and the down-regulation of receptor activator of NF-κB ligand (RANKL) expression.

Normal bone remodeling requires an intricate balance between osteoblast and osteoclast activity. The pathogenesis of osteopenia or osteoporosis is characterized by accelerated osteoclast activity relative to the activity of osteoblasts that results in a net effect of bone loss. Osteoclasts, derived from hematopoietic precursor cells [10], require RANKL and macrophage-colony stimulating factors (M-CSF) to differentiate into mature osteoclasts [11]. Under conditions of gonadal hormone deficiency and chronic inflammation, RANKL-mediated osteoclastogenesis is enhanced by oxidative stress and/or pro-inflammatory molecules such as tumor necrosis factor (TNF)-α [12], interleukin(IL)-1, IL-6 [13], nitric oxide (NO) [14], and prostaglandin E_2 (PGE_2) [15]. In

the case of gonadal hormone deficiency, a reduction of thiol antioxidants (i.e. glutathione and thioredoxin) has been observed, which is indicative of increased vulnerability to oxidative stress [16]. The oxidative stress was accompanied with an increase of TNF-α production [12] resulting in increased osteoclast activity and bone loss. In PGE_2 receptor knock-out mice, osteoclast differentiation and activity under inflammatory conditions (IL-1, TNF-α, or LPS) was decreased, providing support for a role for PGE_2 and perhaps cyclooxygenase (COX-2) in osteoclast function [13]. Further evidence of the involvement of these inflammatory mediators in osteoclastogenesis was demonstrated by the observation that osteoclast differentiation and activity were attenuated with supplementation of antioxidants [16] or anti-inflammatory agents (e.g. COX-2 inhibitors) [15].

Many classes of polyphenolic compounds such as flavonoids [17,18], cathechins [19,3], and caffeic acids [20] found in fruits and vegetables are capable of scavenging reactive oxygen species [7,21] and down-regulating inflammatory mediators [22] associated with osteoclast differentiation and activity. For example, flavonoids such as kaempferol and quercetin, known to suppress iNOS and COX-2 [22], inhibited RANKL-induced osteoclast differentiation and bone resorption activity [17,18,23,4]. Epigallocatechin-3-gallate (EGCG), a major phenolic compound in green tea, suppressed osteoclast formation by inhibiting the generation of free radicals [19]. Two of the phenolic compounds in dried plum, chlorogenic acid and caffeic acid, have been reported to inhibit RANKL-induced osteoclast differentiation and activity [7,20]. Rutin, another polyphenols found in dried plum, has been shown to inhibit ovariectomy-induced osteopenia in rats, in part by decreasing bone resorption [5]. The results of these studies

81

indicate that compounds that block the oxygen derived free radicals down-regulate inflammatory molecules and lead to the inhibition of osteoclastogenesis and/or osteoclast activity [16].

Based on our previous in vivo findings related to dried plum's bone protective effects in gonadal hormone deficiency models [8,9], we hypothesized that the polyphenols extracted from dried plum would down-regulate the production of inflammatory mediators involved in osteoclastogenesis, and inhibit osteoclast differentiation and activity. The purpose of this study was to investigate the effect of polyphenols extracted from dried plum on the production of inflammatory mediators in murine bone marrow macrophages, during their differentiation into osteoclasts and on osteoclast activity under inflammatory conditions and oxidative stress.

Materials and Methods

Reagents

Murine bone marrow macrophage cells (RAW 264.7) were obtained from American Type Culture Collection (ATCC No.TIB-71, Rockville, MD). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA were purchased from GIBCO-BRL (Grand Island, NY). Penicillin G-streptomycin was purchased from Sigma-Aldrich (St. Louis, MO). Dried plum powder was provided by the California Dried Plum Board. For the detection of nitric oxide, sulfanilamide and N-ethylene diamine dihydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Mouse TNF-α ELISA kit was purchased from BD Bioscience (San Diego, CA). Anti-

mouse COX-2, anti-mouse iNOS and β-actin were purchased from Santa Cruz (San Francisco, CA). For TRAP staining, an acid phosphatase kit was purchased from Sigma-Aldrich (St. Louis, MO). Dentin disc for bone resorption assay were purchased from IDS (Fountain Hills, AZ). Unless otherwise noted, all other chemicals were reagent grade and obtained from Fisher Scientific or Sigma-Aldrich.

Extraction of Dried Plum Polyphenols

Polyphenols were extracted from whole dried plum powder using a modification of the method described by Kim and colleagues [24]. Dried plum powder (10 g) was diluted with 100 mL of 80% ethanol and sonicated for 20 minutes under pulsated nitrogen gas to reduce oxidation. Ice was added to the sonicator to ensure that the temperature did not rise above 20˚C. Following sonication, the solution was filtered through a Buchner funnel lined with filter paper (Whatman No 4) using a vacuum system. The remaining residue was then washed with 50 mL of 80% ethanol and the extraction procedure repeated. The volume of the extract was reduced to 30 ml with roto-evaporation and then freeze-dried. Total extracted polyphenols were quantified based on the Folin-Calteau assay [25].

Macrophage Culture

RAW 264.7 murine macrophage cells were maintained in DMEM containing 10% FBS, 100 U/L penicillin G and 100 mg/L streptomycin at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The media was changed every 2 days. For the experiments focused on the macrophage response, cells were plated at a density of 1×10^6

cells/ml and allowed to adhere for 6 hrs. Cells were then treated with 0, 10, 20 or 30 µg/ml of dried plum polyphenols for 2 hrs and subsequently stimulated with LPS or H_2O_2. For the analysis of NO and TNF-α production, cell media was collected at 4 hr, 8 hr and 16 hr after LPS (10ng/ml) or H_2O_2 (100uM) treatment. Protein expression analysis using western blot techniques was performed on cells harvested at 6 hr and 16 hr post LPS or H_2O_2 treatment.

Osteoclast Differentiation and Activity

Experiments were designed to evaluate the effects of the polyphenols on osteoclastogenesis and osteoclast activity under inflammatory and oxidative stress conditions. These experiments involved culturing RAW 264.7 macrophages in the presence of RANKL for 5 days to assess the effects on osteoclast differentiation or 9 days to determine osteoclast resorption pit formation. Cells were plated in 96-well plates at a density of 2×10^3 cells/well and allowed to adhere for 16 hrs. Culture medium was replaced and the cells were treated with 30 ng/ml of RANKL with media changes every 3 days. On day 4, cells were treated with 0, 10, 20 or 30 µg/ml of dried plum polyphenol extract for 2 hrs and then stimulated with LPS, H_2O_2 or vehicle. Twenty-four hours post LPS or H_2O_2 treatment, the media was collected for the analysis of NO and TNF-α, and osteoclasts were evaluated by TRAP staining as described below.

Osteoclast activity was analyzed by plating macrophages on dentine slice and the same protocol followed as described above. Cells were incubated for 9 days with 30 ng/ml RANKL and the same doses of polyphenol extract used in the osteoclast

differentiation experiment. Fifty percent of the media was replaced daily following stimulation with LPS or H_2O_2 and on day 9 the resorption pit assay was performed.

Measurement of Nitric Oxide

Nitrite levels were determined using the Griess assay [26]. Briefly, 50 μl of sample was incubated with the Griess reagent for 10 minutes and the absorbance was read at 550 nm. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0-100 μM).

Measurement of TNF-α

TNF-α production was quantified in cell culture media from macrophages and during osteoclast differentiation with a commercially available kit. Cell culture supernatants were collected and centrifuged at 3,000 g for 2 min to remove cellular debris. Samples (50 μl) were incubated with polyclonal antibodies specific for mouse TNF-α for 2 hrs and then enzyme linked substrate for 30 minute. The intensity of the color detected at 450 nm (background wavelength 570 nm) was measured after the addition of a substrate solution and TNF-α concentrations were calculated from the standard curve. The intra- and inter- assay coefficients of variation for this assay were 3.6% and 3.8%, respectively.

Western Blotting

Following the removal of culture medium, macrophages were lysed and aliquots of total protein (40 μg) were denatured with SDS buffer (50 mM Tris, pH 6.8, 2% SDS,

10 % glycerol, 1% bromophenol blue, and 470 mM β-mercaptoethanol). SDS-treated samples were then separated by SDS-PAGE using an 8% resolving gel and electroblotted to a nitrocellulose membrane. Equal transfer of proteins was confirmed by Ponceau S staining. After transfer, the membrane was blocked in blocking buffer (5% non fat dry milk in 20 mM Tris-Cl, pH 7.6) for 2 hrs. The membrane was then incubated with anti-COX2 (1:200), anti-iNOS (1:200) or anti-actin (1:500) for 2 hrs. The antigens were detected using chemiluminescent reagent following 1 hr incubation with a horseradish peroxidase-linked secondary antibody (1:1000) (UVP Bioimaging system, Upland, CA)

TRAP Staining of Multinucleated Osteoclasts

Differentiated osteoclast from RAW 264.7 macrophages were stained for TRAP expression using an acid phosphatase kit and the number of TRAP-positive multinucleated (i.e. >3 nuclei) osteoclasts per well were counted. Digital images of the TRAP staining were taken under bright field microscopy using a Bliss Imaging System (Bacus Laboratory, Lombard, IL).

Resorption Pit Assay

Following 9 days of culture, cells on dentine disc were removed with exposure to trypsin-EDTA for 15 minutes and incubated in 0.25 M ammonium hydroxide. Discs were washed with distilled water, stained with 0.5% (w/v) toluidine blue and images obtained under light microscopy (Bliss Tracer Version 0.76). Resorption pit area for each well was determined using a WebSlide Browser 3 and expressed as total resorbed area (μm^2) per each slice.

Data were analyzed using the one-way ANOVA (SAS Version 9.1, SAS Institute, NC) with Fisher's Least Squares test to determine differences among the treatments. Values were expressed as means ± standard error (SE) and P-values of <0.05 were considered to be statistically significant. Each experiment was repeated a minimum of 2-3 times and the representative data are presented.

Results

Macrophage Nitric Oxide and TNF-α Production

The effect of dried plum polyphenols on the production of inflammatory mediators by murine macrophages was investigated by measuring the accumulation of nitrite, a stable oxidized product of NO at 0, 4, 8 and 16 hr after stimulation with LPS or H_2O_2. LPS-induced NO production was detectable at 8 hrs and further increased at 16 hrs (**Figure 1A**). Similar to the controls, H_2O_2-stimulated cells did not produce NO. The increase in NO associated with LPS was down-regulated ($p<0.001$) by all three doses of polyphenols at both the 8 and 16 hr time points compared to controls (Figure 1A) and in a dose-dependent manner at 16 hrs.

Compared to the control, TNF-α production by H_2O_2-stimulated macrophages was increased 2.7-fold at 4 hr and continued to be elevated at 8 and 16 hrs (**Figure 1B**). All three doses of dried plum polyphenol extract inhibited H_2O_2-induced production of TNF-α by 4 hrs (26-28%) and continued to suppress TNF-α at 8 and 16 hrs after treatment (Figure 1B). LPS increased extracellular TNF-α up to 1000-fold over time

(**Figure 1C**); all doses of polyphenols unexpectedly exacerbated the LPS-induced macrophage TNF-α production at 8 hr and 16 hr (Figure 1C) and the increase in TNF-α by polyphenols was dose-dependent at 16 hr. Although the polyphenol extract effectively reduced TNF-α production in H_2O_2-stimulated macrophages, the polyphenols generated the opposite TNF-α response when cells were exposed to LPS.

Expression of Inflammatory-related Proteins

Due to the observation that NO was markedly increased by 8 hrs post LPS, we measured the expression of iNOS prior to and after this time. LPS up-regulated the expression of iNOS at 6 hrs after treatment and dried plum polyphenols down-regulated the LPS-induced expression in a dose-dependent manner (**Figure 2A**). Sixteen hrs after LPS, dried plum polyphenol extracts continued to suppress the iNOS expression and the highest dose of dried plum polyphenols (30 µg/ml) essentially blocked protein expression (Figure 2A) indicating that dried plum polyphenols down-regulated macrophage NO production by inhibiting iNOS protein expression.

LPS up-regulated COX-2 expression at both 6 and 16 hrs post treatment (**Figure 2B**). Except for the lowest dose (10 µg/ml), the polyphenol extract down-regulated the expression of COX-2 at 6 hrs and continued to suppress at 16 hrs. Consistent with the iNOS data, the highest dose of dried plum polyphenol extract (30 µg/ml) blocked the expression of COX-2, but only at 16 hrs (Figure 2B).

No differences in the expression of β-actin were observed with any treatments compared to control at both 6 and 16 hrs post LPS (**Figure 2C**). Neither control cells nor

cells exposed to H_2O_2 expressed iNOS (*data are not shown*), which is consistent with the lack of NO production.

Nitric Oxide and TNF-α Production During Osteoclastogenesis

Extracellular NO and TNF-α production was also assessed during osteoclastogenesis to investigate whether dried plum polyphenols altered the production of inflammatory cytokines during osteoclast differentiation. Following 4 days of exposure to RANKL, LPS increased the NO by more than 8-fold compared to controls at 24 hrs (**Figure 3A**). Treatment with polyphenols dose-dependently (*p*<0.001) decreased LPS-induced NO production. In contrast to the macrophages, dried plum polyphenols dose-dependently (*p*<0.001) inhibited LPS-induced TNF-α production by 35%, 56% and 78% in the 10, 20 and 30 µg/ml, respectively (**Figure 3B**); however, the level of inhibition did not reach the level of the control. In line with the observations in the macrophage cultures, H_2O_2 failed to stimulate cells to produce detectable levels of NO during osteoclast differentiation (*data are not shown*). None of the doses of polyphenol extract altered H_2O_2-induced TNF-α (*data are not shown*).

Osteoclast Differentiation

To determine the effect of dried plum polyphenol extract on osteoclast differentiation under normal, LPS- or H_2O_2-stimulated conditions, the number of TRAP-positive, multi-nucleated cells were counted per well (**Figure 4**). In the absence of LPS- or H_2O_2-stimulated osteoclastogenesis, 20 and 30 µg/ml of dried plum polyphenol extract inhibited differentiation of osteoclast up to 32.2% (**Figure 4A & 5A**). Both LPS and

H_2O_2 significantly increased RANKL-induced osteoclast differentiation by 127% and 30%, respectively (**Figure 4B & C**). As illustrated in **Figure 5B** and **5C**, both LPS and H_2O_2 treatment increased the size of the osteoclast, the staining density and number of nuclei in each individual osteoclast cell compared to cells treated with RANKL alone. All three doses of dried plum polyphenol extracts (10, 20 and 30 μg/ml) inhibited LPS-induced osteoclast formation (Figure 4B). The inhibition of osteoclast differentiation by the highest dose (30 μg/ml) of polyphenols was 89% higher ($p<0.01$) than the lowest dose (10 μg/ml) and comparable to the level of the control. The polyphenols also dose-dependently inhibited H_2O_2-induced osteoclast formation (Figure 4C). The two higher doses of polyphenols more effectively inhibited ($p<0.05$) osteoclast differentiation than the lowest dose. In addition to osteoclast number, a decrease in osteoclast size and TRAP staining density were observed with all three doses of dried plum polyphenol extract (**Figure 5B & C**).

Osteoclast Activity

LPS increased ($p<0.05$) the resorption pit area by 274% **(Figure 6A)** compared to the control. As illustrated in Figure 6A, LPS increased number of resorption pits as well as the resorption area. Treatment with the three doses of polyphenols significantly reduced the effects of LPS on osteoclast activity as indicated by a decrease in the resorption pit area by 63%, 87% and 90%, respectively **(Figure 6B)**. This level of suppression by all doses was comparable to the control (Figure 6B). The resorption area was significantly increased by H_2O_2 treatment and dried plum polyphenol extract decreased the H_2O_2-induced increase in resorption pit area by as much as to 94% and all

doses of polyphenols suppressed osteoclast activity below that of the controls (**Figure 6C & D**). These results suggest that the dried plum polyphenol extract significantly inhibited the activity of osteoclasts in conjunction with the suppression of their differentiation.

Discussion

Natural phenolic compounds have been proposed as one group of potential bioactive components in fruits such as dried plum responsible for the beneficial effects on bone metabolism [27,28]. In this study we investigated the influence of polyphenols extracted from dried plum on osteoclasts and found that the polyphenols suppressed osteoclast differentiation and activity under normal conditions as well as in conditions of oxidative stress and inflammation. This inhibition of osteoclastogenesis and resorpting activity occurred in conjunction with down-regulation of the inflammatory mediators, NO and TNF-α. Due to the increase in inflammatory mediators and ROS associated with gonadal hormone deficiency-induced bone loss [16], the results of this study provide further insight into the mechanisms by which dried plum preserves bone mass and microarchitecture as reported in our previous *in vivo* studies [8,29]. Although the polyphenols in dried plum are known for their potent free radical scavenging capacity [30,31], this study is the first to demonstrate that polyphenols extracted from dried plums down-regulate inflammatory mediators in osteoclast precursor cells and inhibit their differentiation into mature, bone resorpting osteoclasts.

In recent years, inflammatory mediators such as NO have been recognized for their role in the pathogenesis of osteoporosis due to their ability to regulate bone turnover [32]. iNOS, the enzyme that catalyzes the conversion of L-arginine to NO [33], is

involved in mediating the bone loss that occurs in conditions such as periodontitis [34] and ovarian hormone deficiency [35]. NO is required for bone resorption induced by IL-1, stimulates osteoclastogenesis through the activation of NF-κB, and contributes to osteoclast survival [36,37]. In the current study, dried plum polyphenols decreased the LPS-induced production of NO in pure macrophage cultures and during the differentiation of macrophages into osteoclasts. Down-regulation of NO by dried plum polyphenols was associated with decreased expression of iNOS by macrophages, similar to that observed with the synthetic antioxidant, pyrrolidine dithiocarbamate (PDTC) and the iNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) [37]. Lee and colleagues showed that both PDTC and L-NAME decreased iNOS expression and osteoclast differentiation in bone marrow cells. Previous studies have demonstrated that inflammatory stimuli (e.g. interferon-γ and LPS) up-regulate iNOS and COX-2 protein over a similar time course and that a physical interaction occurs between these two proteins [38]. Due to this interaction, iNOS inhibitors are capable of preventing PGE_2 synthesis [38,39] and therefore reduce PGE_2-mediated osteoclast differentiation [13]. COX-2 inhibitors are likewise known to suppress osteoclast differentiation and osteoclast enzymatic activity [15]. As anticipated, the down-regulation of iNOS expression with the dried plum polyphenols observed in this study occurred in conjunction with COX-2 inhibition. Other natural phenolic compounds such as kaempferol and epigallocatechin have been shown to have similar anti-inflammatory properties and to down-regulate osteoclastogenesis [19,4]. These phenolic compounds suppress RANKL-induced osteoclast differentiation and inhibit signaling kinases (e.g. c-Jun-terminal kinase, p38 MAP kinase) and transcription factors (e.g. NF-κB and AP-1) which regulate iNOS and

COX-2 expression. Thus, future studies should investigate whether the inhibition of iNOS and COX-2 associated with dried plum polyphenol treatment results from alterations in these same transcription factors and signaling kinases as observed with these other polyphenolic compounds or whether the polyphenols from dried plum have unique mechanisms of action.

Additionally, we have reported here that during osteoclastogenesis dried plum polyphenols decreased TNF-α production under both inflammatory and oxidative stress conditions. TNF-α is one of the primary pro-inflammatory cytokines responsible for the increase in bone resorption occurring with gonadal hormone deficiency [32]. During the early stages of osteoclastogenesis, TNF-α increases the osteoclast precursor pool size [40] and TNF-α inhibitors have been shown to decrease the CD11b^{+} osteoclast precursor cells in arthritis patients [41]. Erwig et al [42] demonstrated that the antioxidant, ascorbic acid, was capable of inhibiting TNF-α induced osteoclast precursor cell proliferation and differentiation [43]. Our data suggest that dried plum polyphenols may have decreased osteoclastogenesis and osteoclast activity by inhibiting the action of TNF-α when macrophages were exposed to RANKL [40] or directly blocked TNF-α receptor mediated pathways. Activation of the redox sensitive transcription factor, NF-κB, up-regulates inflammatory cytokines [44] and may synergistically enhance osteoclastogenesis [12]. Thus, compounds that scavenge free radicals such as chlorogenic acid derivatives, the primary class of polyphenols in dried plum, have the potential to down-regulate the inflammatory responses [16,31,45]. In animal models of osteoporosis, administration of antioxidants (e.g. *N*-acetyl cysteine or ascorbic acid) increased bone levels of thiol antioxidants (i.e. glutathione and thioredoxin) and decreased TNF-α production [16].

Pegylated catalase, a compound that breaks down H_2O_2, decreased bone resorptive activity following ovariectomy [46]. Thus decreased TNF-α production by dried plum polyphenols may occur as a direct effect on H_2O_2 or inhibition of ROS involved in signaling pathways that produce TNF-α.

Although dried plum polyphenol extracts significantly reduced TNF-α production during osteoclastogenesis and in H_2O_2-stimulated macrophages, the opposite response was observed when the LPS-stimulated macrophage cultures were treated with polyphenols. Previous studies [47,48] have demonstrated that antioxidant treatments do not always have uniform effects on inflammatory mediators. For instance, PDTC increased TNF-α production by LPS-stimulated macrophages, but decreased TNF-α in zymosan-stimulated (i.e. cell wall component of *Saccharomyces cerevisiae* that utilize protein kinase C activation) macrophages [47]. Ito and colleagues [49] also reported that LPS-induced production of TNF-α and IL-1 was different in pure macrophages compared to osteoclast cultures. Hence, variations in the production of inflammatory cytokines and their action on osteoclast differentiation appears to be dependent on the osteoclast's stage of maturation [15].

Aside from the inhibition of pro-inflammatory cytokines, dried plum polyphenols also suppressed osteoclast differentiation induced by exposure to RANKL (30 ng/ml) under normal conditions. Osteoclastogenesis is dependent on intracellular signaling molecules downstream of cFms and RANK including the adaptor protein, TNF receptor-associated factor (TRAF), the transcription factor, NF-κB, and the nuclear factor of activated T cells (NFAT)c1. Our findings suggest that dried plum is capable of inhibiting osteoclastogenesis independent of exogenous oxidative stress or inflammatory

conditions. This observation is comparable to other studies with polyphenolic compounds such as caffeic acid and chlorogenic acid that decrease osteoclastic function by suppressing NFATc1, the master transcriptional factor for osteoclast differentiation [50]. Caffeic acid also decreased the gene expression of cathepsin K [20], the primary cystein protease involved in bone resorption, and led to a marked decrease of osteoclastic bone resorption in rats with adjuvant-induced arthritis. The potential for dried plum polyphenols to decrease NFATc1 and proteolytic enzyme activity should be investigated to further clarify the mechanism which osteoclast activity was decreased.

Although the dried plum polyphenol extract effectively suppressed osteoclast differentiation, this extract was comprised of several types of phenolic compounds. It is unclear at this point whether the effects on osteoclasts reported here are the results of an individual phenolic compounds or the action of the polyphenols as a whole. Based on the literature [3,23,4], it would seem that a number of phenolic compounds, may have the ability to suppress osteoclast differentiation and activity, but whether there are differences in the potency of these compounds remains to be determined. In addition to the issue of the potency of polyphenolic compounds, many issues remain to be addressed related the alterations in these compounds' chemical structure that occur during the absorption and metabolism of these compounds [51,52].

In summary, we have shown that dried plum polyphenols suppressed osteoclast differentiation and activity under oxidative stress and inflammatory conditions. These effects on osteoclasts occurred in conjunction with down-regulation of NO and TNF-α production. The findings of this study suggest that the polyphenols in dried plum are at least in part responsible for the anti-resorptive effects of dried plum reported in previous

animal studies [8,9]. Additional investigations is needed to further determine the specific targets and other potential mechanisms by which dried plum's polyphenols inhibit osteoclastogenesis and depress osteoclast activity, including key transcription factors associated with osteoclast differentiation under both normal and stress conditions. It should also be noted that even though this study has focused on the anti-resorptive properties of dried plum's polyphenols, the magnitude by which dried plum restores bone mass and microarchitecture in osteopenic animal models [9,53] is not likely to be explained solely by the action of polyphenols on osteoclast. Investigation of the influence of these polyphenols on osteoblast proliferation and function should also be considered.

Acknowledgements

Reference List

[1] New SA, Robins SP, Campbell MK, Martin JC, Garton MJ, Bolton-Smith C, Grubb DA, Lee SJ, Reid DM. Dietary influences on bone mass and bone metabolism: further evidence of a positive link between fruit and vegetable consumption and bone health? Am J Clin Nutr. 2000;71:142-151.

[2] Muhlbauer RC, Lozano A, Reinli A, Wetli H. Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. J Nutr. 2003;133:3592-3597.

[3] Park EK, Kim MS, Lee SH, Kim KH, Park JY, Kim TH, Lee IS, Woo JT, Jung JC, Shin HI, Choi JY, Kim SY. Furosin, an ellagitannin, suppresses RANKL-induced osteoclast differentiation and function through inhibition of MAP kinase activation and actin ring formation. Biochem Biophys Res Comm. 2004;325:1472-1480.

[4] Wattel A, Kamel S, Mentaverri R, Lorget F, Prouillet C, Petit JP, Fardelonne P, Brazier M. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. Biochem Pharmacol. 2003;65:35-42.

[5] Horcajada-Molteni MN, Crespy V, Coxam V, Davicco MJ, Remesy C, Barlet JP. Rutin inhibits ovariectomy-induced osteopenia in rats. J Bone Miner Res. 2000;15:2251-2258.

[6] Nakatani N, Kayano S, Kikuzaki H, Sumino K, Katagiri K, Mitani T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (Prunus domestica L.). J Agri Food Chem. 2000;48:5512-5516.

[7] Kayano S, Kikuzaki H, Ikami T, Suzuki T, Mitani T, Nakatani N. A new bipyrrole and some phenolic constituents in prunes (Prunus domestica L.) and their oxygen radical absorbance capacity (ORAC). Biosci Biotech Biochem. 2004;68:942-944.

[8] Franklin M, Bu SY, Lerner MR, LE, Bellmer D, MD, LS, Arjmandi BH, BD, Lucas EA, Smith BJ. Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone. 2006;39:1331-1342.

[9] Bu SY, Lucas EA, Franklin M, Marlow D, Brackett DJ, Boldrin EA, Devareddy L, Arjmandi BH, Smith BJ. Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporosis Int. 2007;In press:

[10] Teitelbaum SL. Bone resorption by osteoclasts. Science. 2000;289:1504-1508.

[11] Hsu HL, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB, Boyle WJ. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci U S A. 1999;96:3540-3545.

[12] Jagger CJ, Lean JM, Davies JT, Chambers TJ. Tumor necrosis factor-alpha mediates osteopenia caused by depletion of antioxidants. Endocrinology. 2005;146:113-118.

[13] Sakuma Y, Tanaka K, Suda M, Yasoda A, Natsui K, Tanaka I, Ushikubi F, Narumiya S, Segi E, Sugimoto Y, Ichikawa A, Nakao K. Crucial involvement of the EP4 subtype of prostaglandin E receptor in osteoclast formation by proinflammatory cytokines and lipopolysaccharide. J Bone Miner Res. 2000;15:218-227.

[14] Jung JY, Lin AC, Ramos LM, Faddis BT, Chole RA. Nitric oxide synthase I mediates osteoclast activity in vitro and in vivo. J Cell Biochem. 2003;89:613-621.

[15] Suda K, Udagawa N, Sato N, Takami M, Itoh K, Woo JT, Takahashi N, Nagai K. Suppression of osteoprotegerin expression by prostaglandin E-2 is crucially involved in lipopolysaccharide-induced osteoclast formation. J Immunol. 2004;172:2504-2510.

[16] Lean JM, Davies JT, Fuller K, Jagger CJ, Kirstein B, Partington GA, Urry ZL, Chambers TJ. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. J Clin Invest. 2003;112:915-923.

[17] Wattel A, Kamel S, Prouillet C, Petit JP, Lorget F, Offord E, Brazier M. Flavonoid quercetin differentiation decreases osteoclastic induced by RANKL via a mechanism involving NF kappa B and AP-1. J Cell Biochem. 2004;92:285-295.

[18] Woo JT, Nakagawa H, Notoya M, Yonezawa T, Udagawa N, Lee IS, Ohnishi M, Hagiwara H, Nagai K. Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. Biol Pharm Bull. 2004;27:504-509.

[19] Nakagawa H, Wachi M, Woo JT, Kato M, Kasai S, Takahashi F, Lee IS, Nagai K. Fenton reaction is primarily involved in a mechanism of (-)-epigallocatechin-3-gallate to induce osteoclastic cell death. Biochem Biophys Res Comm. 2002;292:94-101.

[20] Tang QY, Kukita T, Ushijima Y, Kukita A, Nagata K, Sandra F, Watanabe T, Toh K, Okuma Y, Kawasaki S, Rasubala L, Teramachi J, Miyamoto I, Wu Z, Iijima T. Regulation of osteoclastogenesis by Simon extracts composed of caffeic acid and related compounds: successful suppression of bone destruction

accompanied with adjuvant-induced arthritis in rats. Histochem Cell Biol. 2006;125:215-225.

[21] Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G. How should we assess the effects of exposure to dietary polyphenols in vitro? Am J Clin Nutr. 2004;80:15-21.

[22] Chen YC, Shen SC, Lee WR, Hou WC, Yang LL, Lee TJF. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. J Cell Biochem. 2001;82:537-548.

[23] Rassi CM, Lieberherr M, Chaumaz G, Pointillart A, Cournot G. Modulation of osteoclastogenesis in porcine bone marrow cultures by quercetin and rutin. Cell Tissue Res. 2005;319:383-393.

[24] Kim DO, Chun OK, Kim YJ, Moon HY, Lee CY. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J Agric Food Chem. 2003;51:6509-6515.

[25] Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Oxidants and Antioxidants. 1999;299:152-178.

[26] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of Nitrate, Nitrite, and [N-15]-Labeled Nitrate in Biological-Fluids. Analytical Biochemistry. 1982;126:131-138.

[27] Yang CS and Landau JM. Effects of tea consumption on nutrition and health. J Nutr. 2000;130:2409-2412.

[28] McGartland CP, Robson PJ, Murray LJ, Cran GW, Savage MJ, Watkins DC, Rooney MM, Boreham CA. Fruit and vegetable consumption and bone mineral density: the northern Ireland young hearts project. Am J Clin Nutr. 2004;80:1019-1023.

[29] Arjmandi BH, Lucas EA, Juma S, Soliman A, Stoecker BJ, Khalil DA, Smith BJ, Wang C. Prune prevents ovariectomy-induced bone loss in rats. JANA. 2001;4:50-56.

[30] Kayano S, Kikuzaki H, Fukutsuka N, Mitani T, Nakatani N. Antioxidant activity of prune (Prunus domestica L.) constituents and a new synergist. J Agric Food Chem. 2002;50:3708-3712.

[31] Nakatani N, Kayano S, Kikuzaki H, Sumino K, Katagiri K, Mitani T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (Prunus domestica L.). J Agri Food Chem. 2000;48:5512-5516.

[32] Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res. 1996;11:1043-1051.

[33] Cuzzocrea S, Mazzon E, Dugo L, Genovese T, Di Paola R, Ruggeri Z, Vegeto E, Caputi AP, Van de Loo FAJ, Puzzolo D, Maggi A. Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. Endocrinology. 2003;144:1098-1107.

[34] Alayan J, Ivanovski S, Gemmell E, Ford P, Hamlet S, Farah CS. Deficiency of iNOS contributes to *porphyromonas* gingivalis-induced tissue damage. Oral Microbiol Immunol. 2006;21:360-365.

[35] Cuzzocrea S, Mazzon E, Dugo L, Genovese T, Di Paola R, Ruggeri Z, Vegeto E, Caputi AP, Van de Loo FAJ, Puzzolo D, Maggi A. Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. Endocrinology. 2003;144:1098-1107.

[36] van't Hof RJ, Armour KJ, Smith LM, Armour KE, Wei XQ, Liew FY, Ralston SH. Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption. Proc Natl Acad Sci U S A. 2000;97:7993-7998.

[37] Lee SK, Huang H, Lee SW, Kim KH, Kim KK, Kim HM, Lee ZH, Kim HH. Involvement of iNOS-dependent NO production in the stimulation of osteoclast survival by TNF-alpha. Exp Cell Res. 2004;298:359-368.

[38] Kim SF, Huri DA, Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science. 2005;310:1966-1970.

[39] Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci U S A. 1993;90:7240-7244.

[40] O'Gradaigh D, Ireland D, Bord S, Compston JE. Joint erosion in rheumatoid arthritis: interactions between tumour necrosis factor alpha, interleukin 1, and receptor activator of nuclear factor kappa B ligand (RANKL) regulate osteoclasts. Ann Rheum Dis. 2004;63:354-359.

[41] Ritchlin CT, Haas-Smith SA, Li P, Hicks DG, Schwarz EM. Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. J Clin Invest. 2003;111:821-831.

[42] Erwig LP, Kluth DC, Walsh GM, Rees AJ. Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines. J Immunol. 1998;161:1983-1988.

[43] Iqbal J, Li S, Kumar TR, Blair HC, Zaidi M. Follicle-stimulating hormone stimulates TNF production from immune cells to enhance osteoblast and osteoclast formation. Proc Natl Acad Sci U S A. 2006;103:14925-14930.

[44] Rahman I, Marwick J, Kirkham P. Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappa B and pro-inflammatory gene expression. Biochem Pharmacol. 2004;68:1255-1267.

[45] Staniforth V, Chiu LT, Yang NS. Caffeic acid suppresses UVB radiation-induced expression of interleukin-10 and activation of mitogen-activated protein kinases in mouse. Carcinogenesis. 2006;27:1803-1811.

[46] Lean JM, Jagger CJ, Kirstein B, Fuller K, Chambers TJ. Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. Endocrinology. 2005;146:728-735.

[47] Nathens AB, Bitar R, Marshall JC, Watson RWG, Dackiw APB, Fan J, Hiscott J, Rotstein OD. Antioxidants increase lipopolysaccharide-stimulated TNF alpha release in murine macrophages: Role for altered TNF alpha mRNA stability. Shock. 2001;16:361-367.

[48] Bulger EM, Garcia I, Maier RV. Dithiocarbamates enhance tumor necrosis factor-alpha production by rabbit alveolar macrophages, despite inhibition of NF-Kappa B. Shock. 1998;9:397-405.

[49] Itoh K, Udagawa N, Kobayashi K, Suda K, Li X, Takami M, Okahashi N, Nishihara T, Takahashi N. Lipopolysaccharide promotes the survival of osteoclasts via toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. J Immunol. 2003;170:3688-3695.

[50] Komarova SV, Pereverzev A, Shum JW, Sims SM, Dixon SJ. Convergent signaling by acidosis and receptor activator of NF-kappa B ligand (RANKL) on the calcium/calcineurin/NFAT pathway in osteoclasts. Proc Natl Acad Sci U S A. 2005;102:2643-2648.

[51] Cremin P, Kasim-Karakas S, Waterhouse AL. LC/ES-MS detection of hydroxycinnamates in human plasma and urine. J Agri Food Chem. 2001;49:1747-1750.

[52] Olthof MR, Hollman PCH, Katan MB. Chlorogenic acid and caffeic acid are absorbed in humans. J Nutr. 2001;131:66-71.

[53] Deyhim F, Stoecker BJ, Brusewitz GH, Devareddy L, Arjmandi BH. Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause. 2005;12:755-762.

Legends for Figures

Figure 1. Effect of dried plum polyphenols on (A) nitric oxide (NO) and (B & C) TNF-α production in macrophages. Cells were plated at 1×10^6 cells/ml, treated with 0, 10, 20 or 30 µg/ml of dried plum polyphenol extracts and then stimulated with (A & C) LPS (10 ng/ml) or (B) H_2O_2 (100 µM) for 4, 8 or 16 hrs. At a given time point, bars that share the same letters are not significantly different ($p<0.05$) from each other. PP10: 10 µg/ml of dried plum polyphenols, PP20: 20 µg/ml of dried plum polyphenols, PP30: 30 µg/ml of dried plum polyphenols.

Figure 2. Down-regulation of (A) iNOS and (B) COX-2 protein expression by dried plum polyphenols in LPS-stimulated macrophages. β-actin (C) was used as a control. Cells were plated at 1×10^6 cells/ml and treated with 0, 10, 20 or 30 µg/ml of dried plum polyphenol extracts and then stimulated with LPS (10 ng/ml) for 6 and 16 hr.

Figure 3. LPS-induced (A) NO and (B) TNF-α production was suppressed by dried plum polyphenols during osteoclastogenesis. Cells were exposed to RANKL for 4-days and then treated with 0, 10, 20 or 30 µg/ml of dried plum polyphenols for 24 hr. Bars that share the same letters are not significantly different ($p<0.05$) from each other.
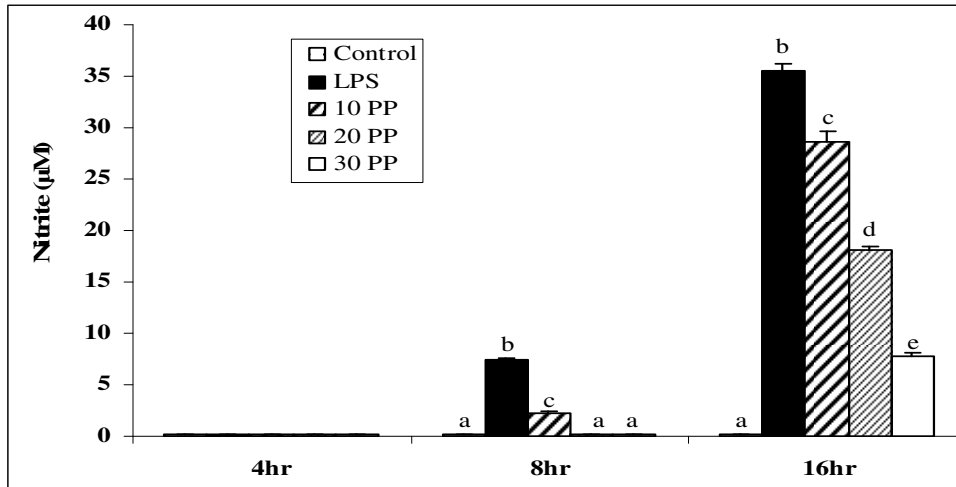
Figure 4. Dried plum polyphenols inhibit osteoclast differentiation as indicated by the number of TRAP positive cells per well under inflammatory and oxidative stress conditions. Cells were cultured with RANKL (30 ng/ml) for 4 days and treated with 0,

10, 20 or 30 μg/ml of dried plum polyphenol extracts for 2 hrs prior to stimulation with (A) Control medium, (B) LPS (10 ng/ml) or (C) H_2O_2 (100 nM). Cells were harvest 24 hr later. Bars that share the same letters are not significantly different ($p<0.05$) from each other.

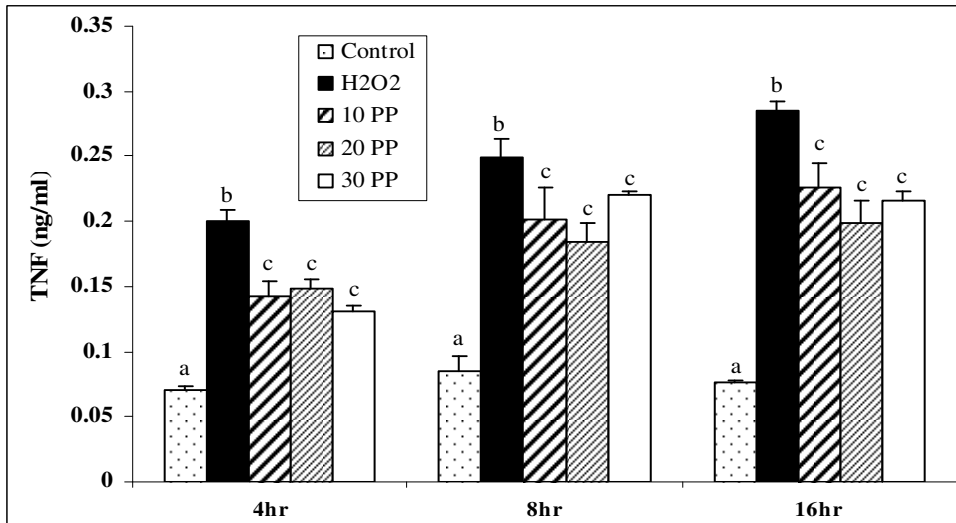Figure 5. Representative slides (5X & 20X) showing TRAP staining of LPS and H_2O_2 stimulated osteoclasts following treatment with 0, 10, 20 or 30 μg/ml of dried plum polyphenol extract. As shown, polyphenols decreased TRAP positive (purple color) cell number and size under (A) normal conditions, as well as with (B) LPS (10 ng/ml) and (C) H_2O_2 (100 nM) stimulation.

Figure 6. Effects of dried plum polyphenol extracts on (A& B) LPS (10 ng/ml) or (C&D) H_2O_2 (100 nM) induced osteoclasts activated as indicated by resorption pit area. Cells were cultured with RANKL (30 ng/ml) for 4 days and then treated with 0, 10, 20 or 30 μg/ml of dried plum polyphenol extracts for 2 hr prior to stimulation with LPS (10 ng/ml) or H_2O_2 (100 nM). Media with the respective treatments was replaced daily and discs were stained with toluidine blue on day 9. Bars that share the same letters are not significantly different from each other.
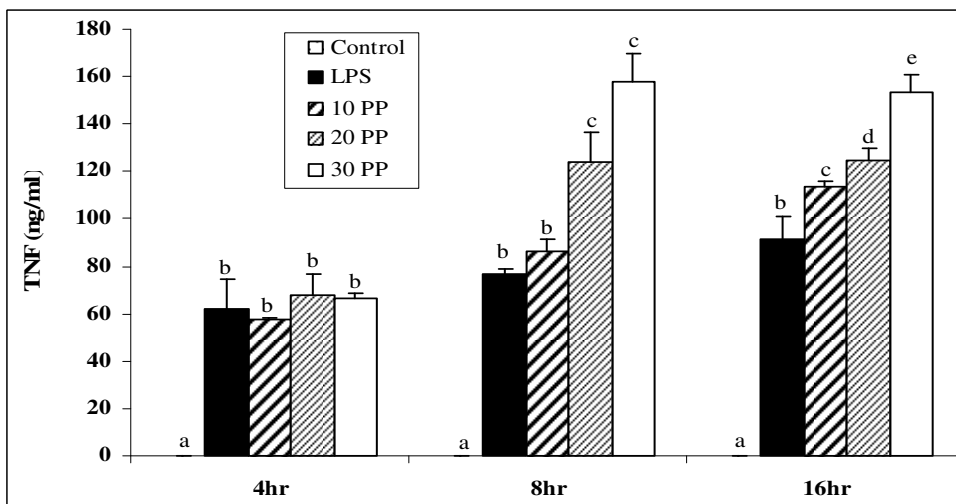
Figure 1

A) LPS

B) H₂O₂

C) LPS

105

Figure 2
A) iNOS

6hr

16hr

LPS (10ng/ml)	-	+	+	+	+
Polyphenols	-	-	10μg/ml	20μg/ml	30μg/ml

B) COX-2

6hr

16hr

LPS (10ng/ml)	-	+	+	+	+
Polyphenols	-	-	10μg/ml	20μg/ml	30μg/ml

C) β-Actin

6hr

16hr

LPS (10ng/ml)	-	+	+	+	+
Polyphenols	-	-	10μg/ml	20μg/ml	30μg/ml

Figure 3

A)

| LPS (10ng/ml) | - | + | + | + | + |
| Polyphenols | - | - | 10μg/ml | 20μg/ml | 30μg/ml |

B)

| LPS (10ng/ml) | - | + | + | + | + |
| Polyphenols | - | - | 10μg/ml | 20μg/ml | 30μg/ml |
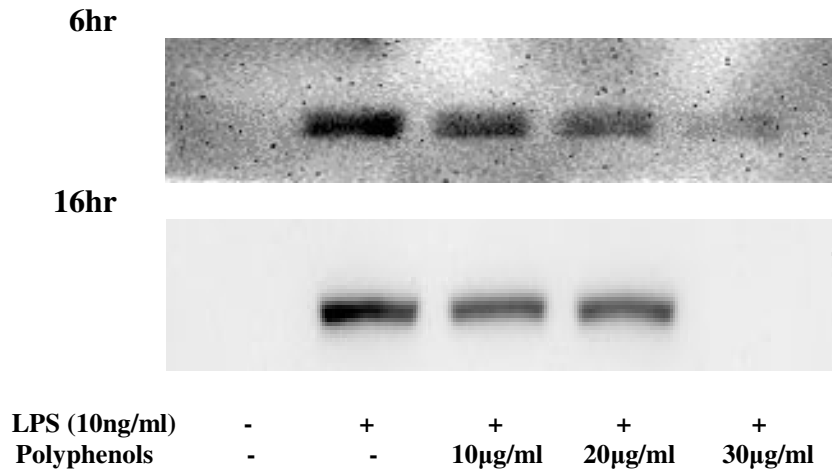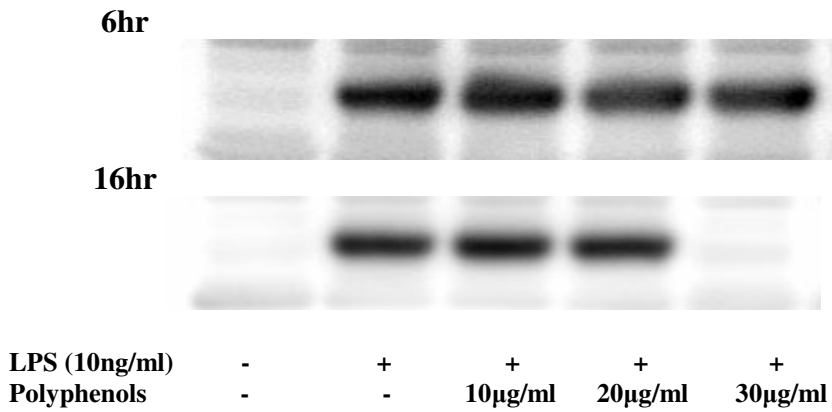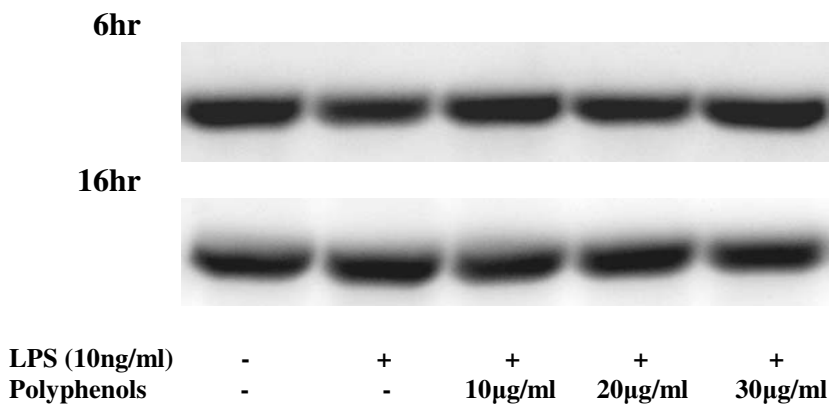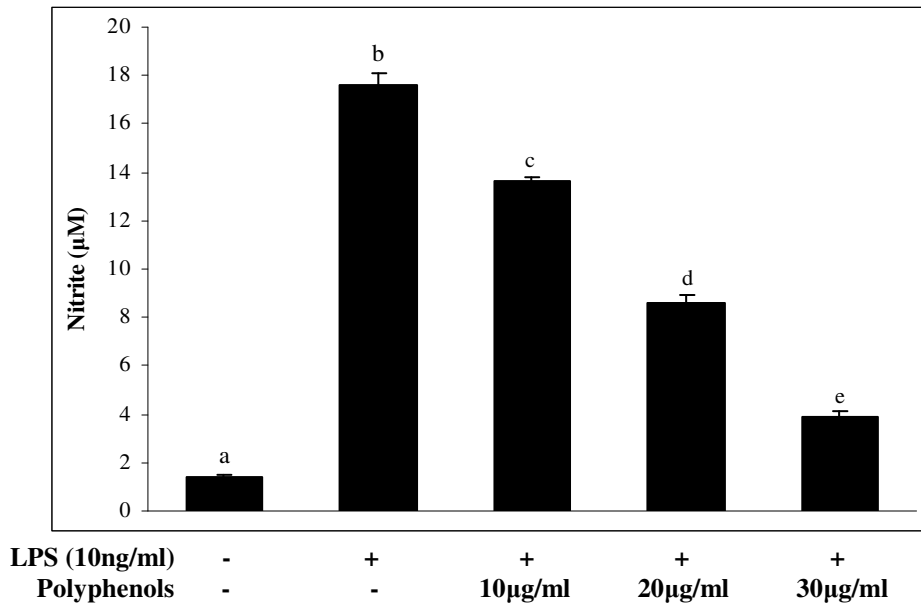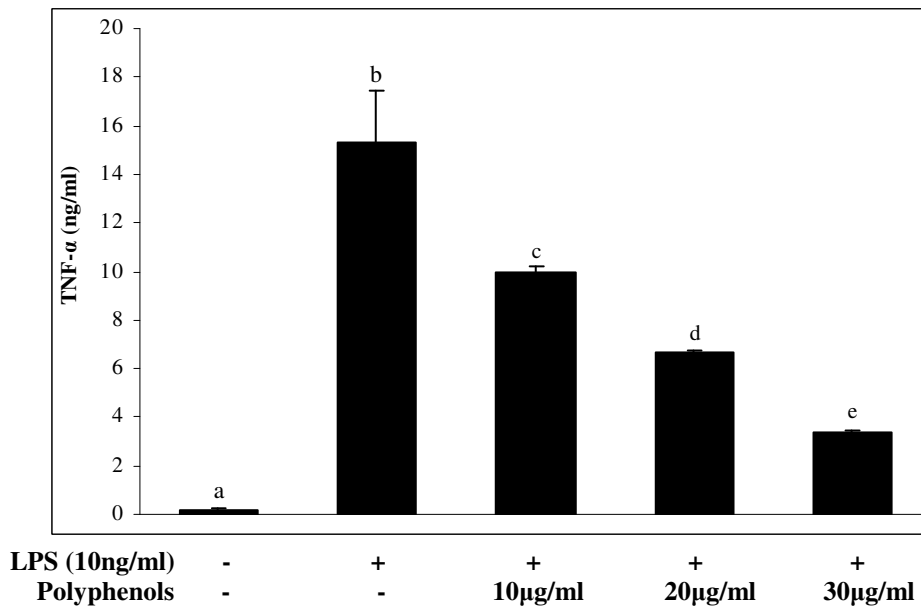
Figure 4

A)
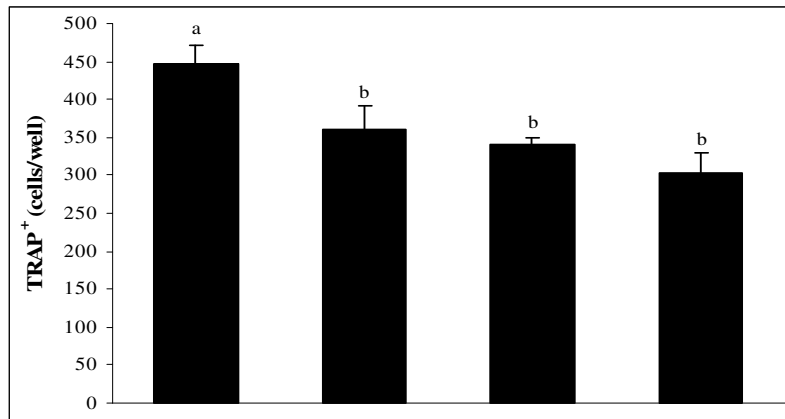
| RANKL (30ng/ml) | - | + | + | + |
| Polyphenols | - | 10μg/ml | 20μg/ml | 30μg/ml |
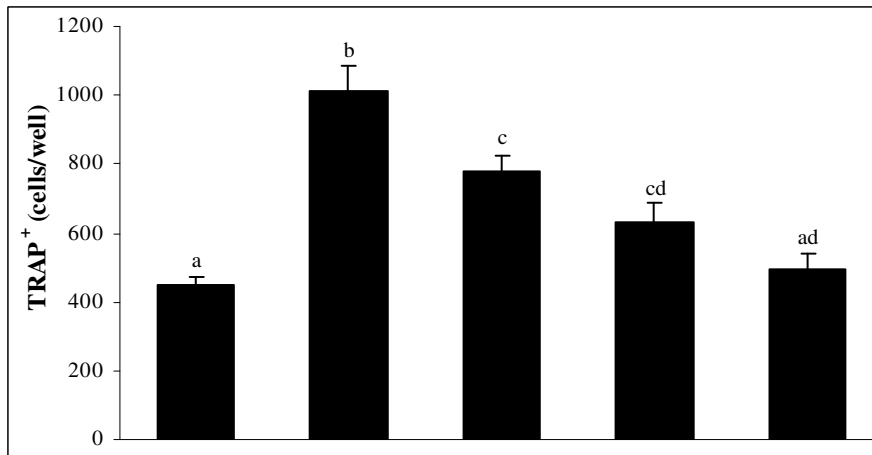
B)

| LPS (10ng/ml) | - | + | + | + | + |
| Polyphenols | - | - | 10μg/ml | 20μg/ml | 30μg/ml |

C)

| H₂O₂ (100nM) | - | + | + | + | + |
| Polyphenols | - | - | 10μg/ml | 20μg/ml | 30μg/ml |

108
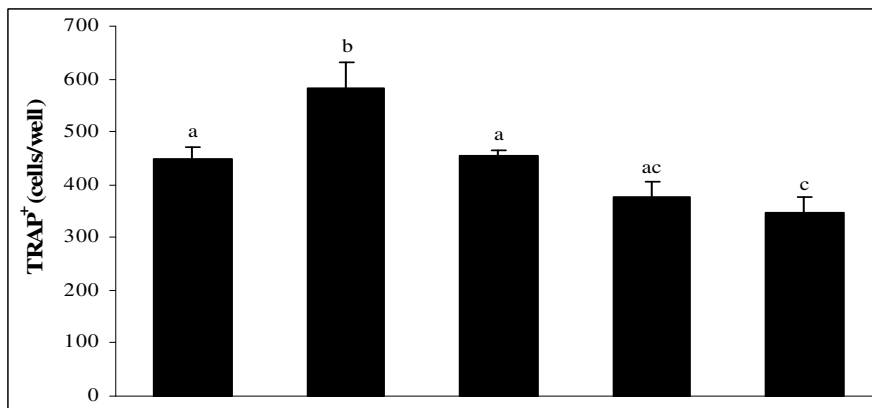
Figure 5

A)

5X
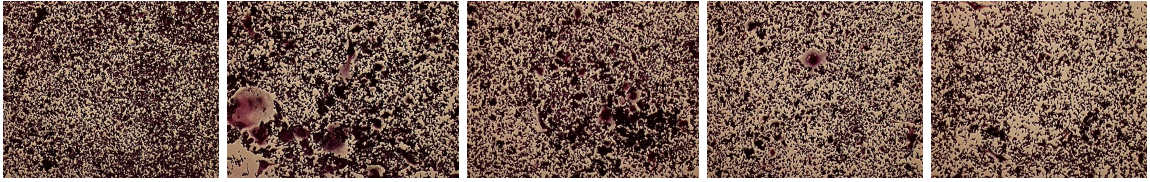
20X
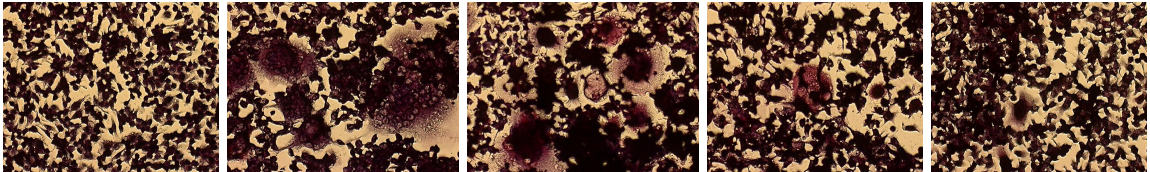
RANKL	-	+	+	+	+
Polyphenols	-	-	10μg/ml	20μg/ml	30μg/ml

B)

5X
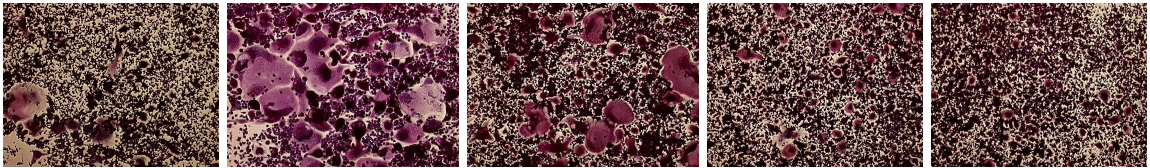
20X
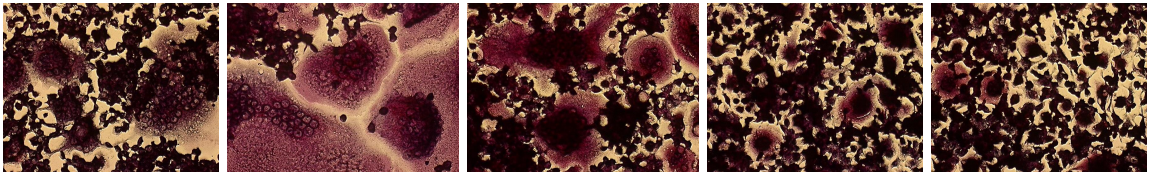
RANKL	+	+	+	+	+
LPS	-	+	+	+	+
Polyphenols	-	-	10μg/ml	20μg/ml	30μg/ml

C)

5X
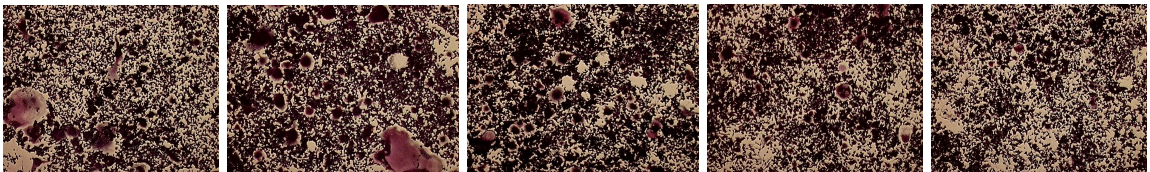
20X
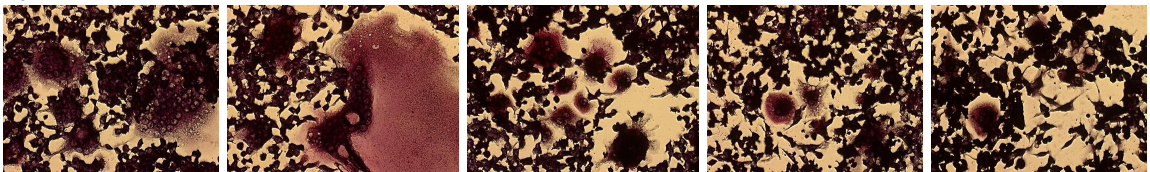
RANKL	+	+	+	+	+
H_2O_2	-	+	+	+	+
Polyphenols	-	-	10μg/ml	20μg/ml	30μg/ml

Figure 6

A)

LPS (10ng/ml) -	+	+	+	+
Polyphenols -	-	10μg/ml	20μg/ml	30μg/ml

B)

LPS (10ng/ml) -	+	+	+	+
Polyphenols -	-	10μg/ml	20μg/ml	30μg/ml

C)

H$_2$O$_2$ (100nM) -	+	+	+	+
Polyphenols -	-	10μg/ml	20μg/ml	30μg/ml

D)

H$_2$O$_2$ (100nM) -	+	+	+	+
Polyphenols -	-	10μg/ml	20μg/ml	30μg/ml
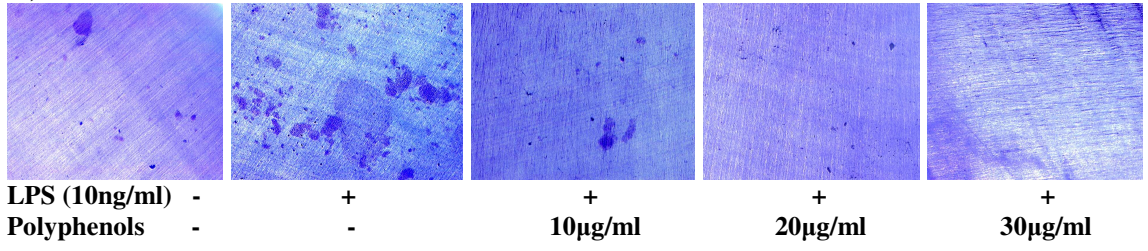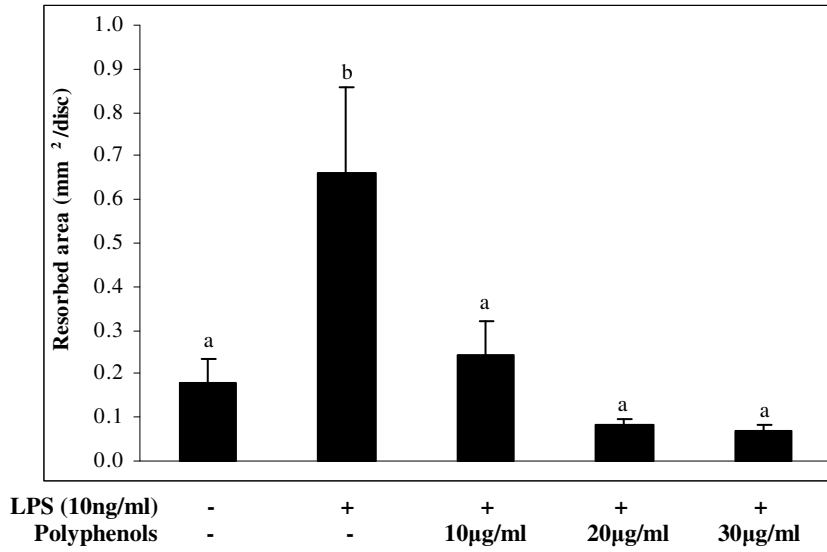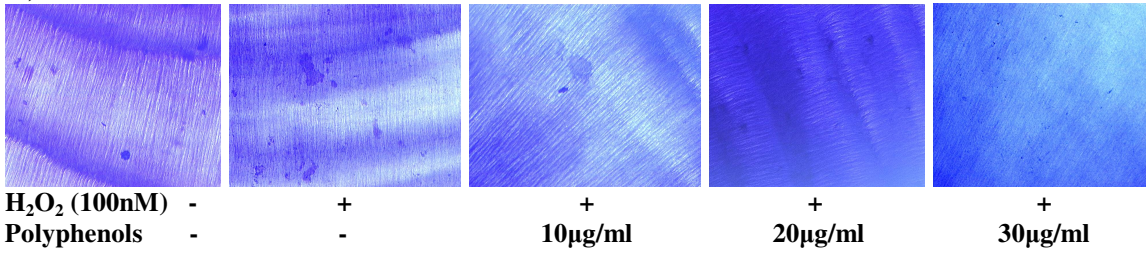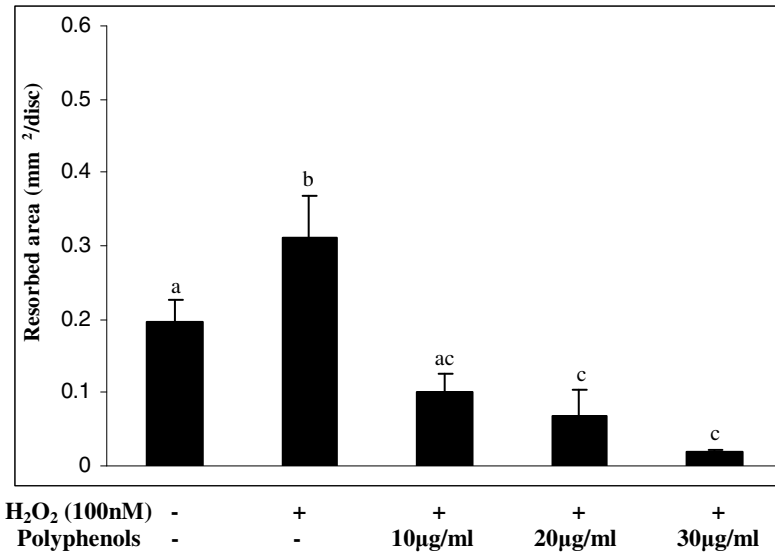
CHAPTER V

Dried plum polyphenols stimulate osteoblast activity and attenuate TNF-α-induced detrimental effects on osteoblastic function in MC3T3-E1 cells.

So Young Bu[1], Tamara S. Hunt[2], Brenda J. Smith[1,2,3]

[1]Department of Nutritional Sciences, College of Human Environmental Science,
  Oklahoma State University, Stillwater, OK 74078;
[2]Depatment of Surgery, College of Medicine, University of Oklahoma Health Sciences
  Center, Oklahoma City, OK 73190;
[3]Veterans Affairs Medical Center, Oklahoma City, OK 73190

Running title: Osteoblasts, dried plum polyphenols and TNF-α

Corresponding author: Brenda J. Smith, O'Donoghue Research Building, P.O. Box 26901 WP 2140, University of Oklahoma Health Sciences Center, Oklahoma City OK 73117; (405) 271-2601; Brenda-Smith@ouhsc.edu

Abstract

Previous studies have demonstrated that dietary supplementation with dried plum can restore bone mass and structure, and significantly increase indices of bone formation such as alkaline phosphatase (ALP) and insulin-like growth factor (IGF)-I. Potential bioactive components in dried plum responsible for these anabolic effects include its polyphenols that maintain potent antioxidant and anti-inflammatory properties. The purpose of this study was to determine how dried plum polyphenols influence osteoblast (MC3T3-E1 cells) activity and mineralized nodule formation under normal and inflammatory conditions. Cells were plated and pretreated with polyphenols (0, 2.5, 5, 10 and 20 μg/ml) extracted from dried plums and 24 hrs later stimulated with TNF-α (0 or 1.0 ng/ml). Intra- and extracellular ALP activity was measured at 7 and 14 days and mineralized nodule formation assessed at 28 days. Dried plum polyphenols at 5, 10 and 20 μg/ml significantly increased intracellular ALP activity under normal conditions at 7 and 14 days. Polyphenols also restored TNF-α -induced suppression of ALP activity at 14 days ($p<0.001$) and the restoration was comparable to the controls. Polyphenols increased mineralized nodule formation as evidenced by an increase (27.7%) in density of Alizarin red S staining and number of mineralized nodules. In the absence of TNF-α, 5 μg/ml of polyphenols significantly up-regulated IGF-I mRNA levels compared to controls. Increases in Runx2 and Osterix expression induced by polyphenols under normal conditions did not reach statistical significance. TNF-α decreased the expression of Runx2, Osterix, and IGF-I, and polyphenols restored their mRNA levels to that of the controls. In the absence of TNF-α, the lowest dose of polyphenols down-regulated the expression of RANKL. As expected, TNF-α up-regulated RANKL mRNA and the 5, 10

and 20 µg/ml doses of polyphenols decreased RANKL expression without altering OPG. We conclude that dried plum polyphenols enhance osteoblast activity and function under normal and inflammatory conditions by up-regulating growth and transcription factors as well as attenuating the inflammatory response.

Key words: bone, antioxidants, osteoporosis,

Introduction

Normal bone remodeling 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 resorbing osteoclasts may result in a net loss of bone mass (2-4). Osteoblasts, fibroblast-like cells derived from a mesenchymal lineage, synthesize enzymes and matrix proteins involved in the formation of mineralized bone (5) and serve as a source for osteoclast differentiation factors including receptor activator of NF-κB ligand (RANKL) (2;4;6). Inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 decrease osteoblast activity and at the same time stimulate osteoblasts to produce inflammatory cytokines such as RANKL, prostaglandin E_2 (PGE_2) and IL-1 that can enhance osteoclast differentiation and activity (7-9).

In gonadal hormone deficiency, TNF-α has been identified as one of the predominant pro-inflammatory mediators of bone loss (10). TNF-α inhibits osteoblast activity and bone mineralization by down-regulating growth factors such as insulin-like growth factor (IGF)-I (11) and alkaline phosphatase (ALP) (12) involved in the formation of hydroxy apatite, and decreasing the expression of lysyl oxidase, the enzyme responsible for collagen crosslinking (13). Suppression of osteoblast activity by TNF-α is associated with down-regulation of transcription factors, Osterix (14) and Runx2 (15), which regulate the expression of ALP and IGF-I as well as several bone matrix proteins including osteopontin and bone sialoprotein (16). TNF-α also alters osteoblast signaling by increasing RANKL production which promotes osteoclast differentiation and activity (17;18). Under conditions of gonadal hormone deficiency, decreased osteoblast activity and promotion of osteoclast differentiation by inflammatory cytokines such as TNF-α are

115

associated with a defective antioxidant system (19). Supplementation with antioxidants attenuates ovariectomy-induced bone loss by suppressing TNF-α and enhancing bone formation (20).

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 inhibit osteoclast differentiation and activity (21;22), directly stimulate osteoblasts, and favorably alter bone formation markers (23;24). 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 (25). 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 (24;26). Rutin is another polyphenol found in plums (27) and is reported to increase serum osteocalcin and BMD in estrogen deficient osteopenic rats (23). These data suggest that a variety of individual phenolic compounds modulate osteoblast activity and signaling, and perhaps certain combinations may have anabolic effects on bone.

Dried plum, a rich source of polyphenols (27), has been shown to positively influence bone mass, bone microarchitecture, and serum markers of bone metabolism (21;28-30). 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 (29). Data from animal studies indicate that dried plum enhances circulating IGF-I in gonadal hormone deficiency models of osteoporosis

(30;31) and was more effective than estrogen replacement in restoring bone in osteopenic ovariectomized female rats (28). 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 (32). 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). Although dried plum was not as potent as intermittent PTH therapy in restoring all bone parameters, it completely reversed the decrease in bone mass compared to sham-operated control animals and had similar effects to PTH on vertebral bone and biomechanical properties (33).

Based on the findings from these animal and clinical studies (34;35), 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.

Materials and Methods

Reagents

MC3T3-E1 (RIKEN No. RCB1126), mouse calvarial pre-osteoblastic cells were obtained from Riken BioResource Center (Ibaraki, Japan). Minimum essential medium (α-MEM), fetal bovine serum (FBS) and penicillin G-streptomycin were purchased from

GIBCO-BRL (Grand Island, NY). Ascorbic acid, β-glycerophosphate, alizarin red-S and mouse TNF-α were purchased from Sigma-Aldrich (St. Louis, MO). 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) was used. Unless otherwise listed, all other chemicals were reagent grade and obtained from Fisher Scientific.

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 6-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 hrs. 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 then treated with 0, 2.5, 5, 10 or 20 μg/ml of dried plum polyphenols for 24 hrs and then stimulated with 0 or 1 ng/ml of TNF-α. Culture medium, which included TNF-α and the dried plum polyphenol doses described above, was replaced every three days. For ALP activity measurement, 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.

Extraction of Polyphenols from Dried Plum

Polyphenols were extracted from whole dried plum powder using a modified version of the method described by Kim and colleagues as previously reported (36;37). Ethanol extraction was repeated twice using 80% ethanol while sonicating with pulsated nitrogen gas. The volume of the extract was reduced using roto-evaporation and then freeze-dried. The total extracted polyphenols was quantified based on the Folin-Calteau assay (38).

Intracellular and Extracellular ALP Activity

After 7 days 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 $14,000 \times g$ 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, Hercules, CA). Total protein was assessed using the BCA method (39) and ALP activity was expressed in nanomol of *p*-nitrophenol produced per min per µg of protein.

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. Briefly, cells were washed twice with PBS and then fixed in ice-cold 70% ethanol for 1hr 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, AR-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.

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 6-well plates at a density of 1×10^5 cells/ml and allowed to adhere for 24 hrs. 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 post TNF-α stimulation, cells were harvested and total RNA isolated for the analysis of mRNA expression.

To evaluate the effects of dried plum polyphenols on gene expression under normal and inflammatory conditions, cells were plated in 6-well plates at a density of 1×10^5 cells/ml and allowed to adhere for 24 hrs. Culture medium was replaced with the differentiation media as described above. Twenty-four hrs later the cells were pre-treated with 0, 2.5, 5, 10 or 20 μg/ml of dried plum polyphenols followed by stimulation with 0

or 1 ng/ml of TNF-α. After eighteen hours of exposure to TNF-α, the cells were collected for the analysis of mRNA levels.

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 OD's measured at 260 nm 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.

Statistical Analysis

Statistical analysis was performed using SAS Version 9.0 (SAS Institute, NC). 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. Values were expressed as means $\pm$ standard error (SE) and difference between treatments was

considered to be significant at $p<0.05$. Each experiment was performed 2-3 times and the representative graph or picture is presented.

Results

Cell Viability

No cytotoxic effects of 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.

Intracellular and Extracellular ALP Activity

Dried plum polyphenol extract stimulated ALP activity in MC3T3-E1 cells under normal conditions at 7 day and 14 days **(Figure 1A)**. The increase in ALP activity was greater than 50% with the two higher doses at day 14. TNF-α treatment significantly reduced the ALP activity by 29% and 38% at day 7 and 14, respectively **(Figure 1B)**. In the presence of TNF-α, only the 10 μg/ml dose of polyphenols significantly elevated intracellular ALP at 7 days, but all doses enhanced ALP activity by 14 days compared to cells receiving TNF-α alone (Figure 1B). 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 either normal or inflammatory conditions **(Figure 2A & 2B)**. After 14 days, all doses of polyphenols increased ($p<0.05$) extracellular ALP activity under normal conditions with the exception of the 20 μg/ml dose (Figure 2A). Similar to the intracellular response, TNF-α treatment significantly

reduced the ALP activity at day 7 and 14 (Figure 2B). ALP activity was increased ($p<$ 0.01) by the 2.5, 5, and 10 μg/ml doses of polyphenols after 14 days compared to the cells receiving TNF-α and 0 polyphenols.

Nodule Formation

To determine the effect of dried plum polyphenols on osteoblast function, cells were stained with Alizarin red S (AR-S) at 28 days post-confluence for the identification of mineralized nodules **(Figure 3 & 4)**. In the absence of TNF-α, only the 10 μg/ml dose of dried plum polyphenols significantly increased the staining density **(Figure 3 B)**, but both the 5 and 10 μg/ml doses increased ($p<0.05$) the number of nodules **(Figure 3 C)**. The increase in lowest dose (2.5 μg/ml) and highest (20 μg/ml) doses of polyphenols did not reach the level of significance suggestive of a biphasic effect. At 28 days, there was an apparent TNF-α induced decrease ($p<0.01$) in mineralized nodule formation **(Figure 4A)** which was supported by the decrease of AR-S staining density (**Figure 4B**) and number of mineralized nodule **(Figure 4C)**. The decrease in AR-S staining density resulting from TNF-α was significantly increased by the all doses of polyphenols. The 10 μg/ml of polyphenols had the greatest effect (i.e. 162% increase in staining density) compared to the cells receiving TNF-α only and restored nodule staining density to that of the controls (Figure 4B). Dried plum polyphenols prevented the TNF-α-induced reduction in the number of mineralized nodule, but the increase did not reach the level of the control cells. These data showing that dried plum polyphenols increased the number and size of mineralized nodules under normal and inflammatory conditions suggest that increased ALP by dried plum polyphenols increases mineralized nodule formation.

Alterations in Gene Expression

The effects of TNF-α on the mRNA levels of osteoblast transcription and growth factors, as well as cytokines involved in osteoclast differentiation were assessed. TNF-α dose-dependently ($p<0.01$) suppressed the expression of Runx2 and Osterix **(Figure 5A).** In addition to the transcription factors, TNF-α dose-dependently ($p<0.01$) decreased the expression of IGF-I, with a 70% reduction observed with the higher dose of TNF-α **(Figure 5B).** The 1 and 10 ng/ml of TNF-α increased ($p<0.05$) the expression of RANKL by 3-fold and 4-fold, respectively, while no alterations in OPG expression were observed **(Figure 5C).** These results suggest that TNF-α decreased ALP activity and mineralized nodule formation by down-regulating transcription and growth factor related to osteoblast maturation.

Ability of dried plum polyphenols to stimulate gene expression involving osteoblast maturation and activity

Dried plum polyphenol extract did not significantly alter the expression of Runx2 and Osterix in MC3T3-E1 cells under normal conditions **(Figure 6A)**. In the presence of TNF-α, 2.5 and 5 µg/ml doses of polyphenols completely restored (p<0.01) the TNF-α induced down-regulation of Runx2 expression to the level of control **(Figure 6B)**. Although higher doses of polyphenols did not significantly increase the Runx2 expression, increase of expression was similar to the level of control. The decrease in Osterix expression resulting from TNF-α was restored by 2.5 and 5 µg/ml of polyphenols to the level of the controls, but the difference did not reach the level of significance

compared to cells receiving TNF-α alone (Figure 6B). In addition to transcription factors, the 2.5, 5 and 10 μg/ml doses of dried plum polyphenols increased the expression of IGF-I up to 63% under normal conditions **(Figure 7A),** but only the 5 μg/ml dose reached the level of statistical significance. The highest dose (20 μg/ml) of polyphenols increased ($p<$ 0.001) the expression of IGF-I by ~2-fold in the presence of TNF-α **(Figure 7B)**. Dried plum polyphenols did not significantly increase the TNF-α-induced reduction in IGF-I expression, but the increase in expression reached the level of the control cells (Figure 7B). These data indicate that the increase in ALP activity and mineralization by dried plum polyphenols were associated with the increase in growth factor and transcription factors under inflammatory conditions. However, under normal conditions, the increase only in growth factor by dried plum polyphenols may influence the increased osteoblast activity.

Effect of dried plum polyphenols on RANKL and OPG expression

In the absence of TNF-α, only the 2.5 μg/ml dose of dried plum polyphenols significantly down-regulated the expression of RANKL but the medium doses (5 and 10 μg/ml) of polyphenols did not alter the RANKL expression suggestive of a biphasic effect. Dried plum polyphenol did not significantly alter the OPG expression compared to the control **(Figure 8A)**. In the presence of TNF-α, the 5, 10 and 20 μg/ml doses of polyphenols suppressed the TNF-α induced up-regulation of RANKL expression by 50% **(Figure 8B)**. However, dried plum polyphenols did not alter the OPG expression. These results indicate that dried plum polyphenols attenuate TNF-α induced inflammatory response in MC3T3-E1 cells.

Discussion

This study demonstrates that dried plum polyphenols stimulate osteoblast activity by increasing ALP activity and mineralized nodule formation under normal and inflammatory conditions. These changes were mediated in part through the up-regulation of growth and transcription factors which were down-regulated by TNF-α. Increased ALP and mineralization in conjunction with decreased RANKL expression in the presence of TNF-α also indicate that dried plum polyphenols may inhibit the osteoblast signaling associated with inflammatory mediators. These findings are similar to those reported in ORX animals (30), which demonstrated the dried plum's ability to suppress the ORX-induced increase in RANKL expression. These results suggests that dried plum polyphenols have anabolic potential and may provide an explanation as to how dried plum increases bone mass and improves bone microarchitecture in established osteopenic animals (28;30;40).

TNF-α is considered a central inflammatory cytokine involved in the pathophysiology of bone loss in a variety of conditions and has been shown to inhibit osteoblast activity and bone mineralization in both *in vitro* and *in vivo* studies (12;41). These effects on osteoblasts result from the down-regulation of growth factors (e.g. IGF-I) (42) and depressed enzymatic activity (12) (e.g. ALP) involved in the formation of mineralized bone matrix. In this study, TNF-α suppressed osteoblast activity, the osteoblasts' ability to form mineralized nodules and was associated with the down-regulation of IGF-I and transcription factors, Runx2 and Osterix. The binding of nuclear Runx2 (15) and Osterix (14) to osteoblast specific elements up-regulates skeletal genes and consequently stromal cells develop the osteoblast phenotype. Runx 2 has been

126

described as a master transcription factor in osteoblast differentiation from stromal

mesenchymal cells to mature osteoblasts (43) and regulates the expression of ALP and

IGF-I as well as several bone matrix proteins (16). In addition to Runx2, the transcription

factor, Osterix, regulates the expression of genes that function in osteoblast activity and

maturation (14). For example, overexpression of Osterix increases the gene expression of

bone sialoprotein, ALP, osteocalcin and osteopontin and mineralized nodule formation

(44). Previous studies have shown that suppression of osteoblast activity by TNF-α

occurs in response to decreased transcriptional activity and reduced mRNA stability

(14;15). Also, the promoter region for Runx2 contains a homologous sequence of NF-κB

and AP-1 binding sites that are capable of conferring responsiveness to TNF-α (15) and

could suppress osteoblast activity. Anti-inflammatory drugs or compounds that suppress

NF-κB or AP-1 signaling may therefore counter the negative effects of TNF-α on Runx2

expression in osteoblasts (25). Hence, our data suggest that inflammatory conditions

induced by TNF-α down-regulate Runx2 and Osterix as well as the growth factor, IGF-I,

and therapies that can counter these effects may be able to restore osteoblast activity and

function.

In addition to the alterations in osteoblast activity, TNF-α also up-regulated

osteoblast signaling for osteoclast differentiation and activity by increasing RANKL

expression. This finding is consistent with previous reports in which an increase in

RANKL mRNA levels mediated by TNF-α resulted in an increase in osteoclastogenesis

and osteoclast activity both *in vivo* and *in vitro* studies (17;18;45;46). The role of the

TNF superfamily of transmembrane proteins (i.e. RANK, RANKL and OPG) in

osteoclastogenesis via the RANK-RANKL complex has been described (47;48). When

the ligand, RANKL, binds with its receptor, RANK, osteoclast differentiation and function is enhanced (48). OPG, which is also produced by bone marrow stromal cells and osteoblasts, is a soluble decoy and therefore inhibits RANK-RANKL mediated osteoclastogenesis (48). In this study, OPG was unaltered by TNF-α, however, the changes of OPG expression by TNF-α or other inflammatory mediator reported in other literature are still inconclusive (49;50). The result of these alterations in RANKL with no change in OPG expression was a significant elevation of the RANKL/OPG ratio under the inflammatory conditions produced by TNF-α. By enhancing the signaling for osteoclastogenesis and osteoclast activity signaling and at the same time depressing the osteoblast activity, TNF-α creates potentially a worse case scenario for bone metabolism.

The intent of this study was to evaluate how dried plum polyphenols influenced osteoblasts under both normal and inflammatory conditions. Under normal conditions dried plum polyphenols increased ALP, an effect that appeared to be mediated by an increase in IGF-I, and countered the TNF-α mediated decrease in ALP activity. ALP is the enzyme involved in mineralization of the skeleton due to its ability to catalyze the hydrolysis of organic phosphate esters, thereby providing inorganic phosphates for mineralization or by removing the inorganic pyrophosphate that inhibits calcification (51;52). ALP has been widely accepted as a marker of bone formation/osteoblast activity in clinical and animal studies (53;54). For example, increased BMD in response to PTH was correlated with increased bone-specific ALP activity in postmenopausal women with osteoporosis (54). Also natural compounds beneficial to bone health such as soy isoflavones and polyphenols (e.g. rutin) are associated with an increase in ALP activity (23;53). Although dried plum polyphenols restored the TNF-α induced suppression in

ALP activity at 14 days, there were no significant changes at 7 days indicating that cells need to be exposed to polyphenols for a period of time to positively influence bone mineralization. In this study, the increase in ALP in the presence of TNF-α was associated with up-regulation of transcription factors Runx2 and Osterix. Similar to our findings with dried plum, drugs and other natural compounds which have anabolic properties have been shown to increase osteoblast activity by up-regulating transcription factors and/or growth factors. For example, the soy isoflavone, genestein increased the ALP activity and Runx2 expression in osteoblasts (55). The anabolic agent, PTH, increased mineralized nodule formation and that was mediated by increased mRNA expression of Runx2 and Osterix in osteoblast cultures (56). In addition to transcription factors, PTH restored bone mass, an effect mediated by increased IGF-I expression (57). 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 (57-59). IGF-I has been shown to influence bone mass and skeletal acquisition (60;61) although the precise mechanism remains unclear (62). In this study dried plum polyphenols increased osteoblast expression of IGF-I which is consistent with previous studies with postmenopausal women (63) and osteoporosis animal models (30). IGF-I seems to play a major role in mediating the effects of dried plum on bone formation.

In this study, RANKL was down-regulated in cells treated with the dried plum polyphenols indicating the influence of dried plum polyphenols on the osteoblast *in vitro* is consistent with the findings from our animal study (30). We had previously demonstrated that dietary supplementation with dried plum decreases the expression of

RANKL in orchidectomized rats (30). Recently we have reported that dried plum polyphenols depress RANKL-induced osteoclast differentiation and osteoclast activity (unpublished data) and have anti-inflammatory properties as indicated by the down-regulation of TNF-α and nitric oxide (37). These results related to osteoclasts combined with the findings of the current study provide strong support for the anti-resorptive action of the polyphenols in dried plum.

Mineralization of the extracellular matrix of bone is initiated by mineral crystal formation (64). Matrix vesicles containing ALP are formed by budding from the cell surface membrane. Mineral aggregation and propagation occurs along the collagen fibrils and form the mature nodule (65;66). In this study dried plum polyphenols increased mineralized nodule formation in normal and inflammatory conditions. Although promising results have been shown with all doses of dried plum polyphenols, the degree of mineralization by the highest dose (20 μg/ml) was not as effective as the lower doses. Although there are a number of possible explanations for this effect, one possibility relates to chelation of minerals. Normally, the extracellular matrix contains sufficiently high concentrations of Ca^{2+}, Pi and other trace amount of minerals (e.g. magnesium, fluoride, citrate, iron, zinc) for mineralization (67). In spite of the health benefits of polyphenols, some concern remains related to the metal/mineral ion chelating properties of polyphenols (68). As such, it is possible that the high dose of polyphenols chelated some essential minerals required for nodule formation. Further studies to evaluate the ion exchange (e.g. Ca) using isotope labeling techniques may provide information as to whether chelation is a factor.

At the present, there are a number of issues that remain related to the polyphenols in dried plum. For instance, it is unclear whether the effects on osteoblasts reported here are the results of an individual phenolic compound or the action of the polyphenols as a whole. It is also unclear whether there are differences in the potency of these polyphenolic compounds. Studies using the available synthetic versions of the phenolic compound in dried plum or proportionate formulations of those found in the fruit may provide the opportunity to clarify the polyphenols' effects on osteoblasts and to address whether the effects reported here are the results of individual compounds or the action of the polyphenols as a whole. Although several types of the polyphenolic compounds found in dried plum have been shown to favorably influence osteoclasts or osteoblasts (25;69;70), their ability to prevent bone loss or restore bone to the extent of dried plum in pre-clinical and clinical studies (28;30;40;63) appears to be somewhat limited (32). Furthermore, the alterations in gene expression presented here were the results of alterations that occurred after only 48 hours of exposure to the polyphenols and may or may not represent the long-term effects. Based on dried plum's impact on ALP activity and mineralized nodule formation, it seems that evaluation of these polyphenols on gene expression over time is warranted.

In this study dried plum polyphenols effectively enhanced the osteoblast activity and function under normal and inflammatory conditions, and these effects were mediated by an increase of growth and transcription factors, and attenuation of the inflammatory response to TNF-α. 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 (28;40). In spite of these promising results, studies conducted separately

from osteoclasts and may leave some uncertainty as to the clear effects of dried plum or

its polyphenols on interaction of osteoblasts and osteoclasts. Future studies are warranted

that include the simultaneous evaluation of these cellular activities using co-culture

systems.

Acknowledgements

Reference List

1. Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science 2000;289:1508-14.

2. Jones DH, Kong YY, Penninger JM. Role of RANKL and RANK in bone loss and arthritis. Ann Rheum Dis 2002;61:32-9.

3. Rifas L, Arackal S, Weitzmann MN. Inflammatory T cells rapidly induce differentiation of human bone marrow stromal cells into mature osteoblasts. J.Cell Biochem. 2003;88:650-9.

4. Roodman GD. Cell biology of the osteoclast. Experimental Hematology 1999;27:1229-41.

5. Rodan GA. Introduction to Bone Biology. Bone 1992;13:S3-S6.

6. Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology 2001;142:5050-5.

7. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. Journal of Clinical Investigation 2005;115:282-90.

8. Zou W, Bar-Shavit Z. Dual modulation of osteoclast differentiation by lipopolysaccharide. Journal of Bone and Mineral Research 2002;17:1211-8.

9. Suda K, Udagawa N, Sato N et al. Suppression of osteoprotegerin expression by prostaglandin E-2 is crucially involved in lipopolysaccharide-induced osteoclast formation. J Immunol 2004;172:2504-10.

10. Cenci S, Weitzmann MN, Roggia C et al. Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-alpha. Journal of Clinical Investigation 2000;106:1229-37.

11. Scharla SH, Strong DD, Mohan S, Chevalley T, Linkhart TA. Effect of Tumor-Necrosis-Factor-Alpha on the Expression of Insulin-Like Growth-Factor-I and Insulin-Like Growth-Factor Binding-Protein-4 in Mouse Osteoblasts. European Journal of Endocrinology 1994;131:293-301.

12. Gilbert L, He XF, Farmer P et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. Endocrinology 2000;141:3956-64.

13. Pischon N, Darbois LM, Palamakumbura AH, Kessler E, Trackman PC. Regulation of collagen deposition and lysyl oxidase by tumor necrosis factor-alpha in osteoblasts. Journal of Biological Chemistry 2004;279:30060-5.

14. Lu XH, Gilbert L, He XF, Rubin J, Nanes MS. Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways. Journal of Biological Chemistry 2006;281:6297-306.

15. Gilbert L, He XF, Farmer P et al. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2 alpha A) is inhibited by tumor necrosis factor-alpha. Journal of Biological Chemistry 2002;277:2695-701.

16. Harada H, Tagashira S, Fujiwara M et al. Cbfa1 isoforms exert functional differences in osteoblast differentiation. Journal of Biological Chemistry 1999;274:6972-8.

17. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1 beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 1999;25:255-9.

18. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. Journal of Clinical Investigation 2005;115:282-90.

19. Pacifici R. Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res 1996;11:1043-51.

20. Lean JM, Davies JT, Fuller K et al. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. J Clin Invest 2003;112:915-23.

21. Muhlbauer RC, Lozano A, Reinli A, Wetli H. Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. J.Nutr. 2003;133:3592-7.

22. Woo JT, Nakagawa H, Notoya M et al. Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. Biol Pharm Bull 2004;27:504-9.

23. Horcajada-Molteni MN, Crespy V, Coxam V, Davicco MJ, Remesy C, Barlet JP. Rutin inhibits ovariectomy-induced osteopenia in rats. J Bone Miner Res 2000;15:2251-8.

24. Mizutani K, Ikeda K, Kawai Y, Yamori Y. Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 1998;253:859-63.

25. Bai XC, Lu D, Bai J et al. Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappa B. Biochemical and Biophysical Research Communications 2004;314:197-207.

26. Jiunn-Kae Chang, Ya-Ling Hsu, I-Chun Teng, Po-Lin Kuo. Piceatannol stimulate osteoblast differentiation that may be mediated by increased bone morphogenetic protein-2 production. European Journal of Pharmacolgy 2006;551:1-9.

27. Stacewicz-Sapuntzakis M, Bowen PE, Hussain EA, Damayanti-Wood BI, Farnsworth NR. Chemical composition and potential health effects of prunes: A functional food? Crit Rev Food Sci Nutr. 2001;41:251-86.

28. Deyhim F, Stoecker BJ, Brusewitz GH, Devareddy L, Arjmandi BH. Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause 2005;12:755-62.

29. Arjmandi BH, Khalil DA, Lucas EA et al. Dried plums improve indices of bone formation in postmenopausal women. J.Women's Health Gend.Based Med. 2002;11:61-8.

30. Franklin M, Bu SY, Lerner MR et al. Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 2006;39:1331-42.

31. Arjmandi BH, Lucas EA, Juma S et al. Prune prevents ovariectomy-induced bone loss in rats. JANA 2001;4:50-6.

32. Picherit C, Bennetau-Pelissero C, Chanteranne B et al. Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats. Journal of Nutrition 2001;131:723-8.

33. So Young Bu, Edralin A.Lucas, MF et al. Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporosis Int 2007.

34. Smith BJ, Ethriedge A, Lucas EA, Bellmer D, Stoecker BJ, Arjmandi B.H. Dried plum promotes bone recovery comparable to parathyroid hormone in osteopenic rats following hindlimb unloading. J Bone Min.Res. 2003;18:S277 (abstr).

35. Ethriedge A, Lucas EA, Stoecker BJ, Wei CI, Arjmandi B.H., Smith BJ. Alterations in vertebral bone with hindlimb unloading and the effects of dried plum and parathyroid hormone during recovery. J Bone Min.Res. 2003;18:S278 (abstr).

36. Kim DO, Chun OK, Kim YJ, Moon HY, Lee CY. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J Agric.Food Chem. 2003;51:6509-15.

37. Bu SY, Lerner MR, Stoecker BJ et al. Dried plum polyphenols inhibit osteoclastogenesis under oxidative stress and inflammatory conditions. Bone 2007;under review.

38. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Oxidants and Antioxidants 1999;299:152-78.

39. Stoscheck CM. Quantitation of Protein. Methods Enzymol 1990;182:50-68.

40. Bu SY, Lucas EA, Franklin M et al. Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporosis Int 2007;In press.

41. Schett G, Redlich K, Hayer S et al. Osteoprotegerin protects against generalized bone loss in tumor necrosis factor-transgenic mice. Arthritis and Rheumatism 2003;48:2042-51.

42. Scharla SH, Strong DD, Mohan S, Chevalley T, Linkhart TA. Effect of tumor-necrosis-factor-alpha on the expression of insulin-like growth-factor-I and insulin-like growth-factor binding-protein-4 in mouse osteoblasts. European Journal of Endocrinology 1994;131:293-301.

43. Byers BA, Garcia AJ. Exogenous Runx2 expression enhances in vitro osteoblastic differentiation and mineralization in primary bone marrow stromal cells. Tissue Engineering 2004;10:1623-32.

44. Tu QS, Valverde P, Chen J. Osterix enhances proliferation and osteogenic potential of bone marrow stromal cells. Biochemical and Biophysical Research Communications 2006;341:1257-65.

45. Miyazaki T, Matsunaga T, Miyazaki S, Hokari S, Komoda T. Changes in receptor activator of nuclear factor-kappaB, and its ligand, osteoprotegerin, bone-type alkaline phosphatase, and tartrate-resistant acid phosphatase in ovariectomized rats. J.Cell Biochem. 2004;93:503-12.

46. Lindberg MK, Erlandsson M, Alatalo SL et al. Estrogen receptor alpha, but not estrogen receptor beta, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-kappa B ligand) ratio and serum interleukin-6 in male mice. J.Endocrinol. 2001;171:425-33.

47. Kong YY, Boyle WJ, Penninger JM. Osteoprotegerin ligand: a regulator of immune responses and bone physiology. Immunol.Today 2000;21:495-502.

48. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. Journal of Bone and Mineral Research 2000;15:2-12.

49. Iqbal J. Does TNF have anti-osteoclastogenic actions? Skeletal Development and Remodeling in Health, Disease, and Aging 2006;1068:234-9.

50. Kikuchi T, Matsuguchi T, Tsuboi N et al. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via toll-like receptors. Journal of Immunology 2001;166:3574-9.

51. Kim YJ, Lee MH, Wozney JM, Cho JY, Ryoo HM. Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2. Journal of Biological Chemistry 2004;279:50773-80.

52. Lorch IJ. Alkaline Phosphatase and the Mechanism of Ossification. Journal of Bone and Joint Surgery-British Volume 1949;31:94-9.

53. Morabito N, Crisafulli A, Vergara C et al. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study. Journal of Bone and Mineral Research 2002;17:1904-12.

54. Chen PQ, Satterwhite JH, Licata AA et al. Early changes in biochemical markers of bone formation predict BMD response to teriparatide in postmenopausal women with osteoporosis. Journal of Bone and Mineral Research 2005;20:962-70.

55. Pan W, Quarles LD, Song LH et al. Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. Journal of Cellular Biochemistry 2005;94:307-16.

56. Wang BL, Dai CL, Quan JX et al. Parathyroid hormone regulates osterix and Runx2 mRNA expression predominantly through protein kinase A signaling in osteoblast-like cells. Journal of Endocrinological Investigation 2006;29:101-8.

57. Nakajima A, Shimoji N, Shiomi K et al. Mechanisms for the enhancement of fracture healing in rats treated with intermittent low-dose human parathyroid hormone (1-34). Journal of Bone and Mineral Research 2002;17:2038-47.

58. Mohan S, Baylink DJ. Bone growth factors. Clinical Orthopaedics and Related Research 1991;30-48.

59. Zapf J, Froesch ER. Insulin-Like Growth-Factors Somatomedins - Structure, Secretion, Biological Actions and Physiological-Role. Hormone Research 1986;24:121-30.

60. Li YM, Schacher DH, Liu Q et al. Regulation of myeloid growth and differentiation by the insulin-like growth factor I receptor. Endocrinology 1997;138:362-8.

61. Patel MB, Arden NK, Masterson LM et al. Investigating the role of the growth hormone-insulin-like growth factor (GH-IGF) axis as a determinant of male bone mineral density (BMD). Bone 2005;37:833-41.

62. Ljunghall S, Johansson AG, Burman P, Kampe O, Lindh E, Karlsson FA. Low plasma levels of insulin-like growth factor 1 (IGF-1) in male patients with idiopathic osteoporosis. J Intern.Med. 1992;232:59-64.

63. Arjmandi BH, Khalil DA, Lucas EA et al. Dried plums improve indices of bone formation in postmenopausal women. Journal of Womens Health & Gender-Based Medicine 2002;11:61-8.

64. Anderson HC. Molecular biology of matrix vesicles. Clinical Orthopaedics and Related Research 1995;266-80.

65. Gorski JP, Wang AM, Lovitch D, Law D, Powell K, Midura RJ. Extracellular bone acidic glycoprotein-75 defines condensed mesenchyme regions to be mineralized and localizes with bone sialoprotein during intramembranous bone formation. Journal of Biological Chemistry 2004;279:25455-63.

66. Landis WJ, Hodgens KJ, Song MJ et al. Mineralization of collagen may occur on fibril surfaces: Evidence from conventional and high-voltage electron microscopy and three-dimensional imaging. Journal of Structural Biology 1996;117:24-35.

67. Landis WJ. Mineral characterization in calcifying tissues: Atomic, molecular and macromolecular perspectives. Connective Tissue Research 1996;35:1-8.

68. Tuntawiroon M, Sritongkul N, Brune M et al. Dose-dependent inhibitory effect of phenolic-compounds in foods on nonheme-iron absorption in men. American Journal of Clinical Nutrition 1991;53:554-7.

69. Chang H, Jin TY, Jin WF, Gu SZ, Zhou YF. Modulation of isoflavones on bone-nodule formation in rat calvaria osteoblasts in vitro. Biomedical and Environmental Sciences 2003;16:83-9.

70. Notoya M, Tsukamoto Y, Nishimura H et al. Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro. European Journal of Pharmacology 2004;485:89-96.

Legends for Figures

Figure 1. Effect of dried plum polyphenol extracts on intracellular 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 alone (A) or stimulated with TNF-α (1 ng/ml) (B) for 7 and 14 days.

Figure 2. Dried plum polyphenols increased 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 alone (A) or stimulated with TNF-α (1 ng/ml) (B) for 7 and 14 days. Bars that share the same uppercase or lowercase letter are not significantly different from each other.

Figure 3. Dried plum polyphenols increase mineralized nodule formation as indicated by Alizarin red S staining (red color) density (A & B) and number of mineralized nodule (C) under normal 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 alone for 28 days. Bars that share the same letter are not significantly different from each other.

Figure 4. Mineralized nodule formation by dried plum polyphenols under 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 and stimulated with TNF-α (1 ng/ml) for 28 days. Alizarin red S staining (red color) density (A & B) and number of mineralized nodule (C) were measured. Bars that share the same letter are not significantly different from each other.

Figure 5. TNF-α induced alterations in mRNA expression of (A) Runx2 and Osterix, (B) IGF-I and (C) RANKL and OPG. Cells were plated at 1×10^5 cells/ml and stimulated with TNF-α (1 and 10 ng/ml) for 18 hrs.

Figure 6. Effects of dried plum polyphenols on TNF-α induced alterations in mRNA expression of Runx2 and Osterix. 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 alone (A) or stimulated with TNF-α (1 ng/ml) (B) for 18 hrs. Bars that share the same uppercase or lowercase letter are not significantly different from each other.

Figure 7. Dried plum polyphenols increase mRNA expression of IGF-I. 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 alone (A) or stimulated with TNF-α (1 ng/ml) (B) for 18 hrs.

Figure 8. Effects of dried plum polyphenols on TNF-α induced alterations in mRNA expression of RANKL and OPG. 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 alone (A) or stimulated with TNF-α (1 ng/ml) (B) for 18 hrs. Bars that share the same uppercase or lowercase letter are not significantly different from each treatment.

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
GAPDH	F: CCG GTG CTG AGT ATG TCG
	R: CCC TGT TGC TGT AGC CGT A

Figure 1. Intracellular ALP activity

A)
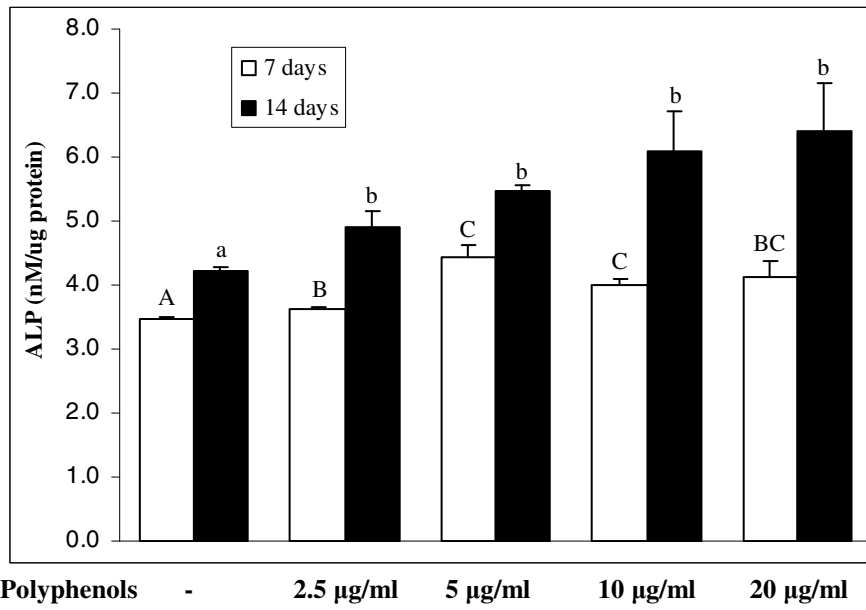
B)
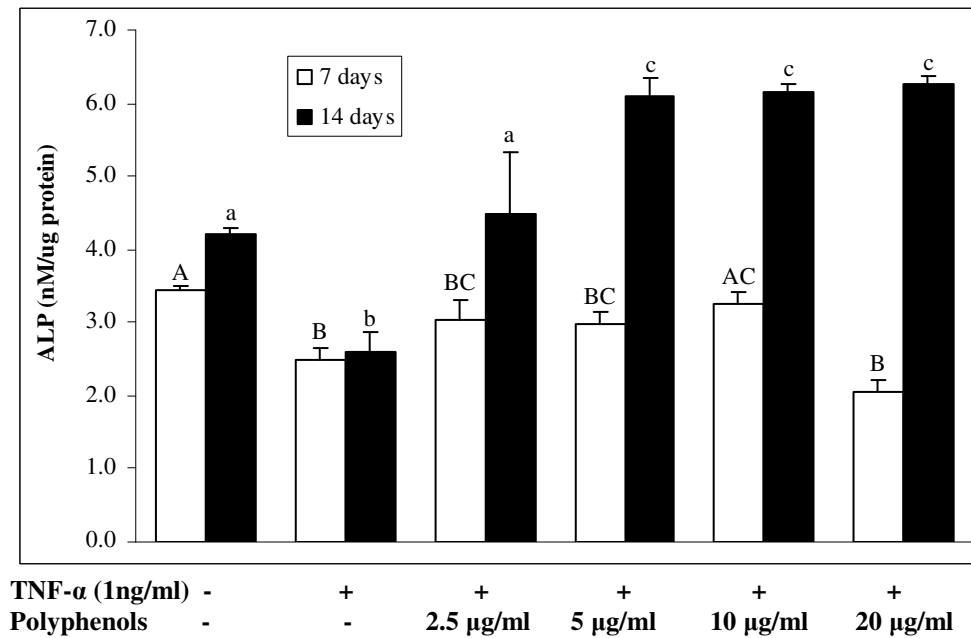
Figure 2. Extracellular ALP activity

A)
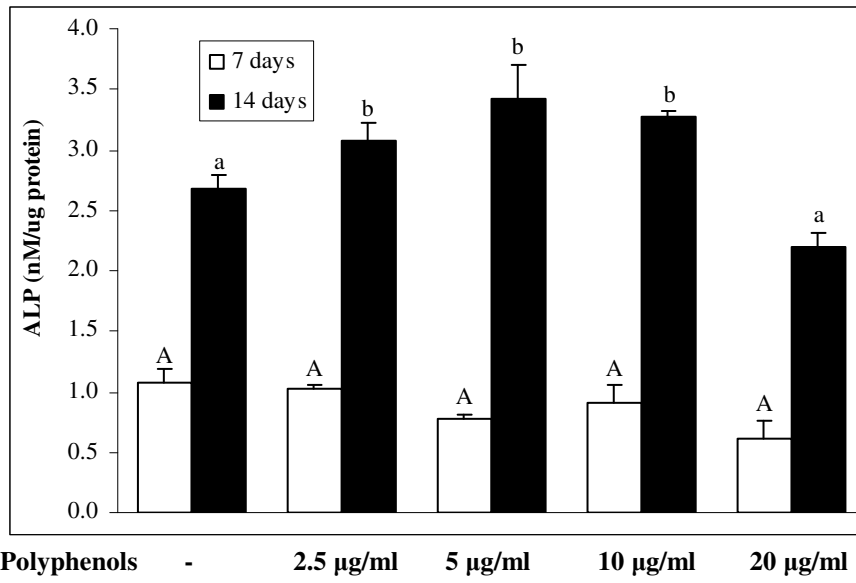
B)
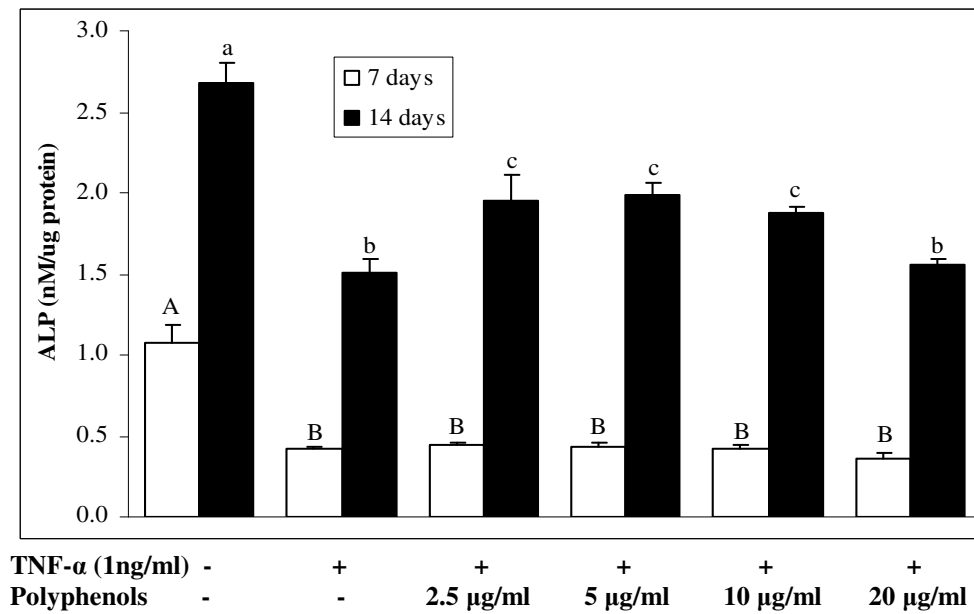
Figure 3. Nodule formation under normal conditions

A)

Polyphenols   -   2.5 µg/ml   5 µg/ml

10 µg/ml   20 µg/ml

B)
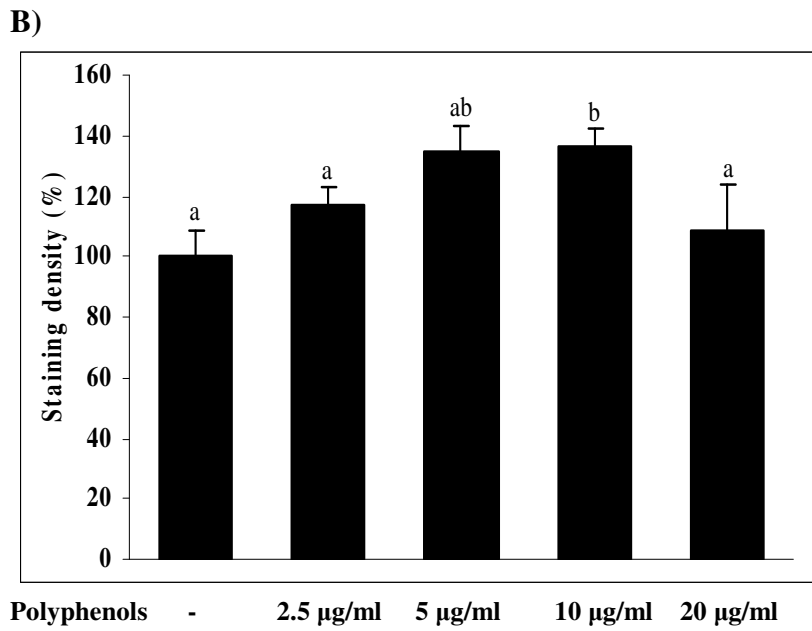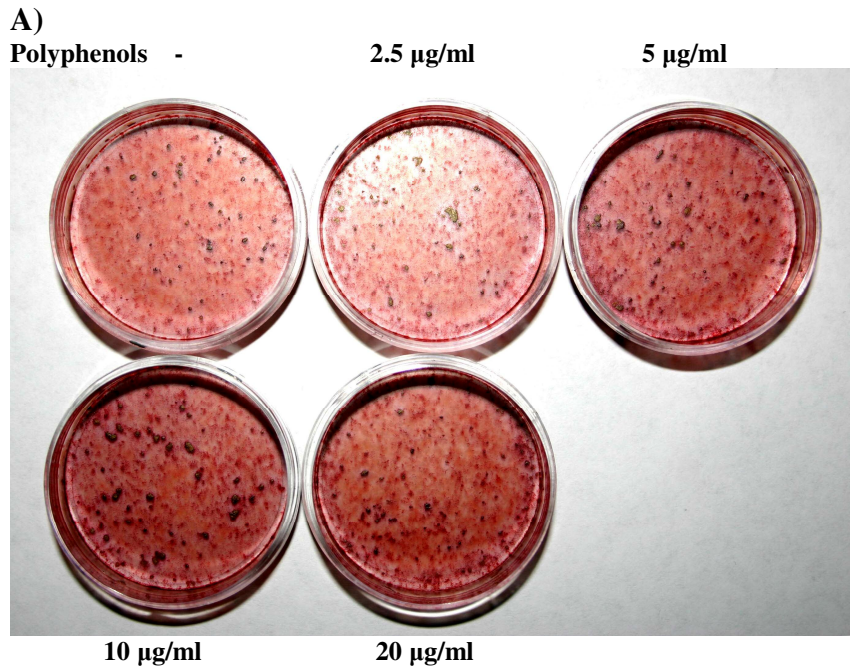
C)
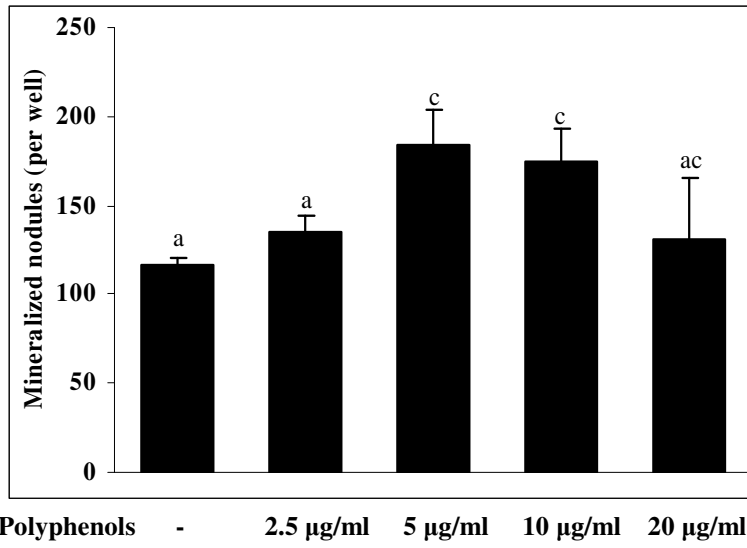
Figure 4. Nodule formation under inflammatory conditions

A)

TNF-α (1ng/ml)	-	+	+
Polyphenols	-	-	2. 5 μg/ml

Polyphenols	5 μg/ml	10 μg/ml	20 μg/ml
TNF-α (1ng/ml)	+	+	+

B)

Polyphenols	-	-	2.5 μg/ml	5 μg/ml	10 μg/ml	20 μg/ml
TNF-α (1ng/ml)	-	+	+	+	+	+

C)

| Polyphenols | - | - | 2.5 µg/ml | 5 µg/ml | 10 µg/ml | 20 µg/ml |
| TNF-α (1ng/ml) | - | + | + | + | + | + |
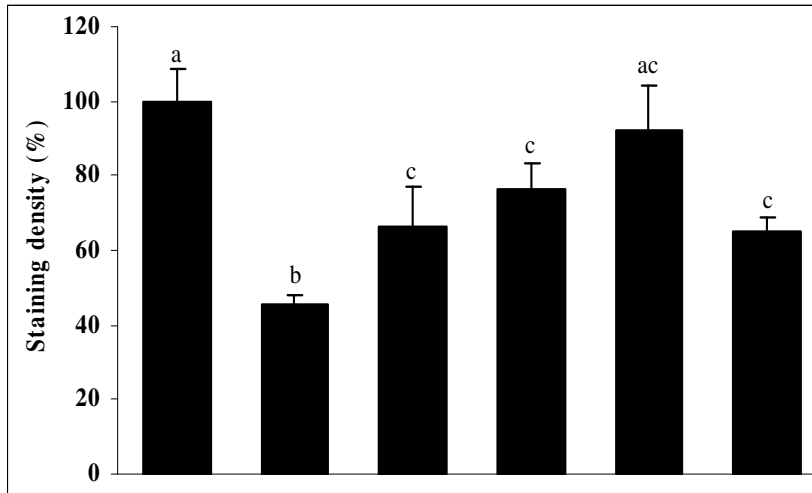
Figure 5

A)

B)

C)

Figure 6

A)

B)

149

Figure 7

A)

B)

Figure 8

A)
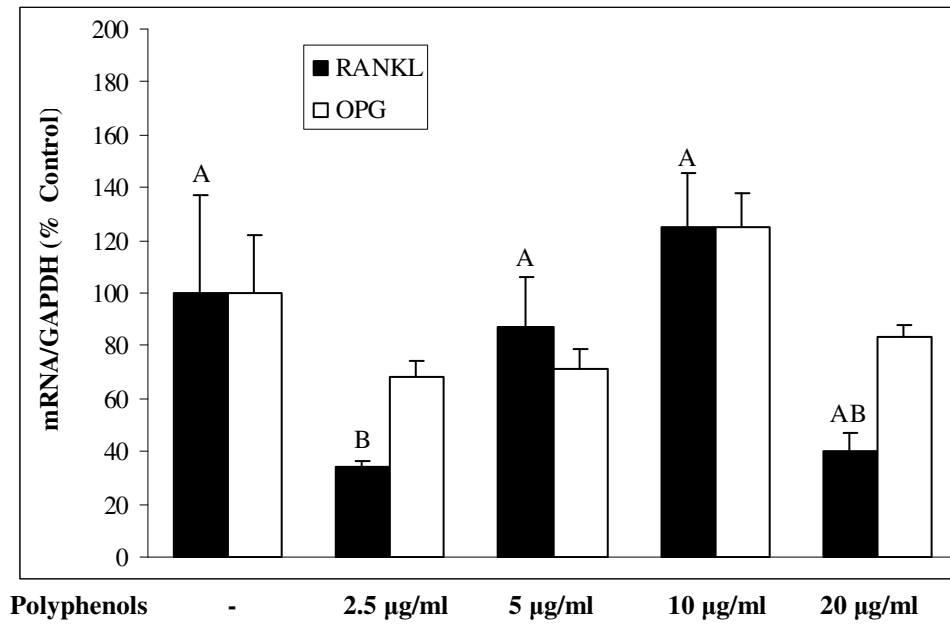
B)
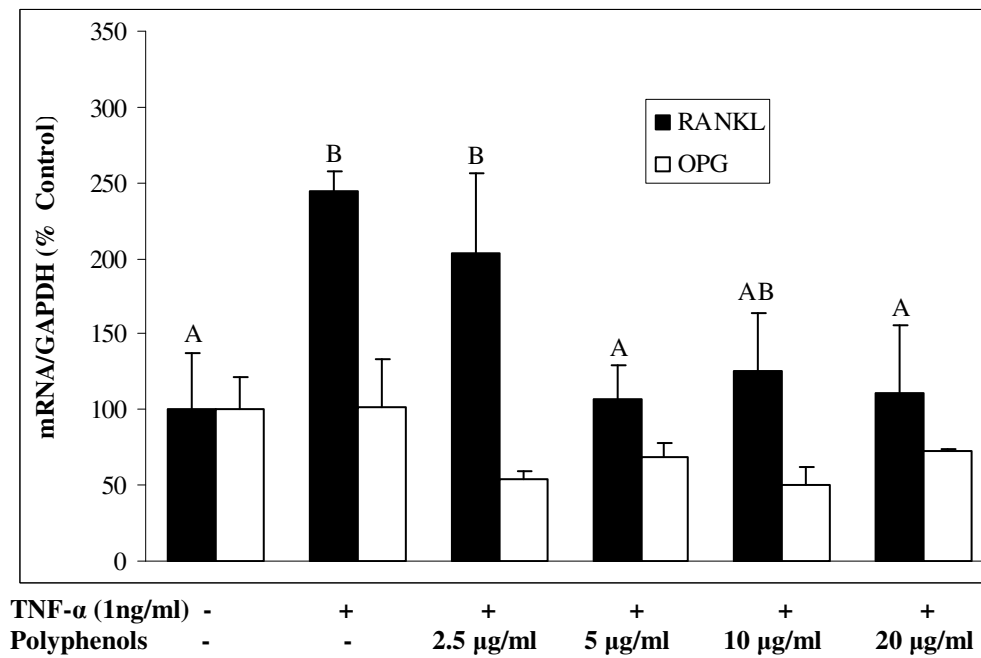
CHAPTER VI

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The purpose of this research project was to further our understanding of the molecular and cellular mechanisms by which dried plum and/or its polyphenols improve bone mass and microarchitecture. In Study 1, the dried plum diet (25%, w/w) restored bone mass (i.e. BMC and BMD) and trabecular microarchitecture in 9-month-old male osteopenic orchidectomized rats over the course of 90 days. These effects on trabecular bone, especially trabecular bone of the vertebral body, were similar to those produced by intermittent PTH therapy (80 μg/kg x3 per wk). Study 2 was designed to assess the effects of polyphenols extracted from dried plum (0, 10, 20, or 30 μg/ml), one of the groups of potential bioactive components in dried plum, on osteoclastogenesis and osteoclast activity. The polyphenols (10, 20, and 30 μg/ml) decreased the production of inflammatory mediators (i.e. NO and TNF-α) and reduced the number of TRAP-positive cells and the size of the resorption pits when the cells were stimulated with either LPS or hydrogen peroxide (H_2O_2). In Study 3, murine pre-osteoblasts (MC3T3-E1) were pre-treated with polyphenols extracted from dried plum (0, 2.5, 5, 10, or 20 μg/ml) to determine their influence on osteoblast activity, function and signaling under normal and inflammatory (i.e. stimulation with TNF-α) conditions. The polyphenols (2.5, 5, 10, and

20 µg/ml) enhanced ALP activity and mineralized nodule formation. These findings suggest that dried plum may have anti-resorptive and anabolic properties that are mediated in part by its polyphenols.

Conclusions

The purpose of this research project was to investigate the molecular and cellular mechanisms by which dried plum and its polyphenols improve bone mass and microarchitecture. To accomplish this purpose, the following 7 hypotheses were developed and tested.

Hypothesis 1: Dried plum supplementation will restore bone density and trabecular bone microarchitecture in osteopenic gonadal hormone deficient male rats and these effects will be similar to the anabolic agent, PTH.

The dried plum group experienced an increase ($p<0.05$) in vertebral (11.7%) and femoral (12.4%) BMD compared to ORX controls. Vertebral BV/TV and TbN were increased by dried plum ($p<0.05$) and TbSp was decreased. These changes in trabecular structural properties were comparable to those observed with PTH. Alterations in trabecular bone of the distal femur metaphysis were similar to those observed in the vertebra, but BV/TV was not restored to the same extent. Cortical thickness at the femur mid-diaphysis was also improved by dried plum, but the extent to which cortical bone was improved was not as great as that of PTH. Enhanced bone biomechanical properties of the vertebrae (i.e. total force and von mises stress) evaluated by the simulated compression testing indicated that dried plum significantly improved the trabecular

microarchitecture of the vertebra to the extent that biomechanical parameters were enhanced. The findings of this study suggest that dried plum markedly improved bone mass, trabecular and cortical bone microarchitecture, and strength which were negatively impacted by ORX. However, the degree of the anabolic effects on BMD and some parameters of bone microarchitecture were not as great as the effects of PTH. Based on the inability of dried plum to improve all parameters, we fail to accept Hypothesis 1. It should be noted when considering the effects of dried plum compared to a potent anabolic agent such as PTH that the ability of dried plum to restore bone is quite remarkable and shows much more promise than other natural compounds evaluated.

Hypothesis 2: Osteopenic ORX male rats consuming the dried plum supplemented diet will experience an increase in serum ALP, but a decrease in urinary Dpd and calcium excretion. The alterations in ALP will be similar to the effect of PTH, but the decrease in bone resorption indicators will not mimic PTH.

No statistically significant alterations were observed in serum ALP activity or urinary excretion of Dpd or calcium in response to 90 days of dried plum or PTH treatment. Dried plum tended to suppress urinary 12 hr total Dpd excretion (p=0.0925) by approximately 60% and calcium excretion (p=0.0546) by 42.6%; however, PTH did not affect either of these indicators of bone metabolism. It is important to note that the age of the animals and the duration of the experiment likely influenced this lack of statistically significant effects. Bone formation markers have been reported to occur at early time points in studies and only a single time point measurement may not reflect the alterations in bone metabolism that had occurred as indicated by the structural and biomechanical

analyses. The mRNA levels of indicators of osteoblast and osteoclast activity and signaling did not show any differences among treatment groups (Appendix A). Therefore, we fail to accept Hypothesis 2.

Hypothesis 3: Dried plum polyphenols will decrease osteoclast differentiation and activity under inflammatory and oxidative stress conditions.

Dried plum polyphenol extract suppressed (p<0.001) the LPS- or H_2O_2-induced osteoclast differentiation and osteoclast activity as indicated by TRAP positive cell number and resorption pit assays. Furthermore, the polyphenols also down-regulated osteoclast differentiation and activity under normal conditions (i.e. no LPS or H_2O_2). Based on the suppression of both osteoclast differentiation and activity, we accept Hypothesis 3.

Hypothesis 4: Dried plum polyphenols will decrease the production of inflammatory mediators by murine macrophages and osteoclasts induced by inflammation and oxidation.

Dried plum polyphenols dose-dependently decreased ($p<0.001$) the LPS-induced NO production by macrophages by as much as 78%. The polyphenol extract also suppressed H_2O_2-induced TNF-α production in macrophages over time, but exacerbated LPS-induced TNF-α production. LPS up-regulated COX-2 and iNOS which was attenuated by the two lower doses of dried plum polyphenols (10 and 20 μg/ml) at 6 and 16 hrs, and was blocked by the high dose at 16 hrs. During osteoclastogenesis, dried plum polyphenol extract dose-dependently decreased ($p<0.001$) the LPS-induced NO and TNF-

α production. These findings indicate that dried plum polyphenols have the ability to down-regulate inflammatory mediators which influence osteoclastogenesis. Although dried plum polyphenols effectively reduced the production of inflammatory mediators during osteoclastogenesis, they increased TNF-α production by macrophages and therefore we fail to accept Hypothesis 4.

Hypothesis 5: Dried plum polyphenols will up-regulate ALP activity and mineralized nodule formation in osteoblast cells under both normal and inflammatory conditions.

Dried plum polyphenols at doses of 5, 10, 20 μg/ml significantly increased the intracellular ALP activity in MC3T3-E1 cells under normal conditions. TNF-α produced a significant reduction in intra- and extra-cellular ALP activity over time. This decrease in ALP activity was restored by all doses of dried plum polyphenols. The TNF-α-induced decrease of ALP activity was completely prevented ($p<0.001$) by all doses of dried plum polyphenols at 14 days and the level of restoration in ALP activity was comparable to the controls. Under normal conditions, 5 μg/ml and 10 μg/ml dose of dried plum polyphenols significantly increased mineralized nodule formation. TNF-α decreased ($p< 0.01$) the mineralized nodule formation as indicated by the decrease of AR-S staining density and number of mineralized nodule. TNF-α induced a decrease in mineralized nodule formation, which was restored by the all doses of polyphenols. The restoration by the 10 μg/ml dose was comparable to the control. These results suggest that increased ALP by dried plum polyphenols led to an increase of mineralized nodule formation. Based on these findings, Hypothesis 5 is accepted.

Hypothesis 6: Dried plum polyphenols will increase the gene expression of Runx2, Osterix and IGF-I under normal and inflammatory conditions.

After 48 hrs of exposure, dried plum polyphenol extracts did not significantly alter the expression of Runx2 and Osterix under normal conditions. Under inflammatory conditions, lower doses of polyphenols (2.5 and 5 µg/ml) restored the TNF-α induced down-regulation of Runx2 expression to the level of the control. The decrease in Osterix expression resulting from TNF-α was reversed by the 2.5 and 5 µg/ml of polyphenols to the level of controls. In addition to transcription factors, the expression of IGF-I was increased up to 63% with the 2.5, 5 and 10 µg/ml doses of dried plum polyphenols under normal conditions. The highest dose (20 µg/ml) of dried plum polyphenols was the only dose that significantly enhanced IGF-I expression under inflammatory conditions. These alterations in gene expression were observed after only 48 hours of exposure to the polyphenols and may or may not represent the long-term effects. Although dried plum polyphenols were able to restore osteoblast expression of key transcription factors and growth that were down-regulated by TNF-α, in the absence of inflammation few alterations occurred. Based on these results, we fail to accept Hypothesis 6.

Hypothesis 7: Dried plum polyphenols will decrease the RANKL expression without altering the expression of OPG under normal and inflammatory conditions.

Under normal conditions, only the 2.5 µg/ml dose of dried plum polyphenols significantly down-regulated the expression of RANKL and no alterations in OPG expression were detected. In the presence of TNF-α, 5, 10 and 20 µg/ml, dried plum polyphenols significantly suppressed the TNF-α induced up-regulation of RANKL

expression by 50% (Figure 7B) with no effect on OPG. These results indicate that dried plum polyphenol attenuated TNF-α induced inflammatory signaling in MC3T3 cells. Based on these findings, Hypothesis 7 is accepted.

Recommendations

We have shown that dietary supplementation with dried plum restored BMD in osteopenic ORX rats at 12 months of age to the level of 9-month old sham-operated rats. Previously, Wang and colleagues reported that after 9 months of age most indices of bone mass and structure decline in SD males rats (Wang et al., 2001). These data suggest the dried plum increased whole body BMD to that of peak bone mass in SD rats. Additionally, in Study 1 bone formation and resorption markers and the gene expression data provided relatively little information related to the alterations in bone metabolism that occurred in response to either dried plum or PTH at 90 days. As previously indicated, caution is needed when interpreting biochemical marker data at a single time point, especially 90 days after the initiation of treatment in mature adult animals. For example, the effects of treatments such as PTH on bone formation markers have been reported to occur at early time points which may explain the lack a detectable change in bone formation markers (serum and cellular) in the current study. Future studies should be designed to compare to the influence of dried plum on bone metabolism to that of PTH over time. Such studies are likely to provide more insight in the influence of dried plum on bone metabolism.

Aside from anabolic effects of dried plum observed in Study 1, polyphenols extracted from dried plum demonstrated the ability to increase osteoblast activity and

inhibit osteoclast differentiation under normal conditions. These findings suggest that dried plum is capable of improving osteoblast activity and inhibiting osteoclast activity and differentiation independent of exogenous inflammatory conditions. Additional studies are needed to further investigate the specific mechanisms by which dried plum's polyphenols increase osteoblast activity and/or inhibit osteoclastogenesis and osteoclast activity, including key signaling molecules and other transcription factors associated with osteoblast and osteoclast differentiation as well as the interaction between osteoblasts and osteoclasts utilizing co-culture systems.

Although the dried plum polyphenol extract effectively suppressed osteoclast differentiation and enhanced osteoblast activity in the *in vitro* studies, this extract also contained carbohydrates and other nutritive or non-nutritive components, and does not represent a purified mix of the polyphenols found in plums. Having raised this issue, the polyphenol extract utilized in these studies down-regulated inflammatory mediators, decreased osteoclast differentiation and increased osteoblast activity which is similar to the effects of other phenolic compounds reported in the literatures (Chen et al., 2001; Mizutani et al., 1998; Wattel et al., 2003). Though a number of polyphenolic compounds have been shown to have positive effects on osteoclasts or osteoblasts, their ability to prevent bone loss and most importantly restore bone in physiological conditions (animal models or clinical studies), as shown by dried plum, appears to be somewhat novel. These effects observed on the cellular activity and bone histomorphometry under physiological conditions appear to be unique to dried plum.

It is unclear at this point whether the effects on osteoclasts and osteoblasts reported here are the results of an individual phenolic compound or the action of the

polyphenols as a whole. Additionally, the question remains as to whether there are differences in the potency of these compounds. Studies using the available synthetic versions of the phenolic compound in dried plum or proportionate formulations of those found in the fruit are underway and may provide the opportunity to clarify the effects of the polyphenols on osteoclasts and osteoblasts and to address whether the effects reported here are the results of individual compounds or the action of the polyphenols as a whole. However, not all of the polyphenols found in dried plum, including neochlorogenic, are available synthetically and so some limitations exist in these experiments as well.

The results of this study provide further insight into the key mechanisms by which dried plum enhances bone mass and microarchitecture. Based on the findings from these studies it appears that dried plum and its polyphenols have the ability to inhibit osteoclast differentiation and increase osteoblast activity in conjunction with attenuating inflammatory mediators. In spite of these promising results, *in vivo* and *in vitro* studies conducted separately may leave some uncertainty as to the effects of dried plum or its polyphenols on bone formation and bone resorption. Further studies are warranted that include the simultaneous evaluation of cellular activity and structural changes using dynamic bone histomorphometry under unique physiological condition. These studies will provide the opportunity to further understand dried plum's mechanism of action and whether it mimics the actions of current anti-resorptive and anabolic therapies or provides a novel mechanism for future consideration.

LITERATURE CITED

Abbas, S., Zhang, Y. H., Clohisy, J. C., Abu-Amer, Y. (2003) Tumor necrosis factor-alpha inhibits pre-osteoblast differentiation through its type-1 receptor. Cytokine 22: 33-41.

Ackard, D. M. and Neumark-Sztainer, D. (2001) Health care information sources for adolescents: age and gender differences on use, concerns, and needs. J. Adolesc.Health 29: 170-176.

Adachi, J. D. (2001) Corticosteroid-induced osteoporosis. Int. J. Fertil.Womens Med. 46: 190-205.

Adachi, J. D., Saag, K. G., Delmas, P. D., Liberman, U. A., Emkey, R. D., Seeman, E., Lane, N. E., Kaufman, J. M., Poubelle, P. E., Hawkins, F., Correa-Rotter, R., Menkes, C. J., Rodriguez-Portales, J. A., Schnitzer, T. J., Block, J. A., Wing, J., McIlwain, H. H., Westhovens, R., Brown, J., Melo-Gomes, J. A., Gruber, B. L., Yanover, M. J., Leite, M. O., Siminoski, K. G., Nevitt, M. C., Sharp, J. T., Malice, M. P., Dumortier, T., Czachur, M., Carofano, W., Daifotis, A. (2001) Two-year effects of alendronate on bone mineral density and vertebral fracture in patients receiving glucocorticoids: a randomized, double-blind, placebo-controlled extension trial. Arthritis Rheum. 44: 201-211.

Akhter, M. P., Kimmel, D. B., Recker, R. R. (2001) Effect of parathyroid hormone (hPTH[1-84]) treatment on bone mass and strength in ovariectomized rats. J. Clin. Densitom. 4: 13-23.

Alayan, J., Ivanovski, S., Gemmell, E., Ford, P., Hamlet, S., Farah, C. S. (2006) Deficiency of iNOS contributes to *porphyromonas* gingivalis-induced tissue damage. Oral Microbiol. Immunol. 21: 360-365.

Albright, F. and Reifenstein, E. (1941) Postmenopausal osteoporosis:its clinical features. JAMA 116: 2465-2474.

Amiel, C., Ostertag, A., Slama, L., Baudoin, C., N'Guyen, T., Lajeunie, E., Neit-Ngeilh, L., Rozenbaum, W., de Vernejoul, M. C. (2004) BMD is reduced in HIV-infected men irrespective of treatment. J. Bone Miner. Res. 19: 402-409.

Ammann, P. and Rizzoli, R. (2003) Bone strength and its determinants. Osteoporos. Int. 14: S13-S18.

Anderson, H. C. (1995) Molecular biology of matrix vesicles. Clin. Orthopaedics and Related Res. 1: 266-280.

Arden, N. K., Nevitt, M. C., Lane, N. E., Gore, L. R., Hochberg, M. C., Scott, J. C., Pressman, A. R., Cummings, S. R. (1999) Osteoarthritis and risk of falls, rates of bone

loss, and osteoporotic fractures. Study of Osteoporotic Fractures Research Group. Arthritis Rheum. 42: 1378-1385.

Argani, P., Iacobuzio-Donahue, C., Ryu, B., Rosty, C., Goggins, M., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Jaffee, E., Yeo, C. J., Cameron, J. L., Kern, S. E., Hruban, R. H. (2001) Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). Clin. Cancer Res. 7: 3862-3868.

Arjmandi, B. H., Khalil, D. A., Lucas, E. A., Georgis, A., Stoecker, B. J., Hardin, C., Payton, M. E., Wild, R. A. (2002) Dried plums improve indices of bone formation in postmenopausal women. J. Women's Health Gend. Based Med. 11: 61-68.

Arjmandi, B. H., Lucas, E. A., Juma, S., Soliman, A., Stoecker, B. J., Khalil, D. A., Smith, B. J., Wang, C. (2001) Prune prevents ovariectomy-induced bone loss in rats. JANA 4: 50-56.

Armstrong, T. A. and Spears, J. W. (2001) Effect of dietary boron on growth performance, calcium and phosphorus metabolism, and bone mechanical properties in growing barrows. J. Animal Sci. 79: 3120-3127.

Atoyebi, W., Brown, M., Wass, J., Littlewood, T. J., Hatton, C. (2002) Lymphoplasmacytoid lymphoma presenting as severe osteoporosis. Am. J. Hematol. 70: 77-80.

Audran, M., Chappard, D., Legrand, E., Libouban, H., Basle, M. F. (2001) Bone microarchitecture and bone fragility in men: DXA and histomorphometry in humans and in the orchidectomized rat model. Calcif. Tissue Int. 69: 214-217.

Bai, X. C., Lu, D., Bai, J., Zheng, H., Ke, Z. Y., Li, X. M., Luo, S. Q. (2004) Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappa B. Biochem. Biophysic. Res. Comm. 314: 197-207.

Basu, S., Michaelsson, K., Olofsson, H., Johansson, S., Melhus, H. (2001) Association between oxidative stress and bone mineral density. Biochem. Biophysic. Res. Comm. 288: 275-279.

Bazzano, L. A., He, J., Ogden, L. G., Loria, C. M., Vupputuri, S., Myers, L., Whelton, P. K. (2002) Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. Am. J. Clin. Nutr. 76: 93-99.

Beattie, J. H. and Peace, H. S. (1993) The Influence of A Low-Boron Diet and Boron Supplementation on Bone, Major Mineral and Sex Steroid-Metabolism in Postmenopausal Women. British J. Nutr. 69: 871-884.

Behre, H. M., Kliesch, S., Leifke, E., Link, T. M., Nieschlag, E. (1997) Long-term effect of testosterone therapy on bone mineral density in hypogonadal men. J. Clin. Endocrinol. Metab. 82: 2386-2390.

Ben Sedrine, W., Radican, L., Reginster, J. Y. (2001) On conducting burden-of-osteoporosis studies: a review of the core concepts and practical issues. A study carried out under the auspices of a WHO Collaborating Center. Rheumatol. 40: 7-14.

Benhamou, C. L., Poupon, S., Lespessailles, E., Loiseau, S., Jennane, R., Siroux, V., Ohley, W., Pothuaud, L. (2001) Fractal analysis of radiographic trabecular bone texture and bone mineral density: two complementary parameters related to osteoporotic fractures. J. Bone Miner. Res. 16: 697-704.

Bernstein, C. N., Leslie, W. D., Taback, S. P. (2003) Bone density in a population-based cohort of premenopausal adult women with early onset inflammatory bowel disease. Am. J. Gastroenterol. 98: 1094-1100.

Bernstein, C. N., Sargent, M., Leslie, W. D. (2005) Serum osteoprotegerin is increased in Crohn's disease: A population-based case control study. Inflamm. Bowel Dis. 11: 325-330.

Bikle, D. D., Sakata, T., Halloran, B. P. (2003) The impact of skeletal unloading on bone formation. Gravit. Space Biol. Bull. 16: 45-54.

Bilezikian, J. P., Morishima, A., Bell, J., Grumbach, M. M. (1998) Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency. N. Engl. J. Med. 339: 559-603.

Black, D. M. and Cooper, C. (2000) Epidemiology of fractures and assessment of fracture risk. Clin. Lab. Med. 20: 439-453.

Booth, S. L. and Meyer, J. (1997) Skeletal functions of vitamin K-dependent proteins: Not just for clotting anymore. Nutr. Rev. 55: 282-284.

Booth, S. L., Tucker, K. L., Chen, H. L., Hannan, M. T., Gagnon, D. R., Cupples, L. A., Wilson, P. W. F., Ordovas, J., Schaefer, E. J., Dawson-Hughes, B., Kiel, D. P. (2000) Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. Am. J. Clin. Nutr. 71: 1201-1208.

Braam, L. A. J. L., Knapen, M. H. J., Geusens, P., Brouns, F., Hamulyak, K., Gerichhausen, M. J. W., Vermeer, C. (2003) Vitamin K1 supplementation retards bone loss in postmenopausal women between 50 and 60 years of age. Calcif. Tiss. Int. 73: 21-26.

Bresnihan, B., Alvaro-Gracia, J. M., Cobby, M., Doherty, M., Domljan, Z., Emery, P., Nuki, G., Pavelka, K., Rau, R., Rozman, B., Watt, I., Williams, B., Aitchison, R., McCabe, D., Musikic, P. (1998) Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. Arthritis Rheum. 41: 2196-2204.

Broussard, D. L. and Magnus, J. H. (2004) Risk assessment and screening for low bone mineral density in a multi-ethnic population of women and men: does one approach fit all? Osteoporos. Int. 15: 349-360.

Bruera, D., Luna, N., David, D. O., Bergoglio, L. M., Zamudio, J. (2003) Decreased bone mineral density in HIV-infected patients is independent of antiretroviral therapy. AIDS 17: 1917-1923.

Bu SY, Lerner MR, Stoecker, B. J., Boldrin EA, Brackett DJ, Lucas EA, Smith BJ (2007a) Dried plum polyphenols inhibit osteoclastogenesis under oxidative stress and inflammatory conditions. Bone (under review)

Bu SY, Lucas EA, Franklin M, Marlow D, Brackett DJ, Boldrin EA, Devareddy L, Arjmandi BH, Smith BJ (2007b) Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. Osteoporosis. Int. (In press)

Buckley, L. M., Leib, E. S., Cartularo, K. S., Vacek, P. M., Cooper, S. M. (1996) Calcium and vitamin D3 supplementation prevents bone loss in the spine secondary to low-dose corticosteroids in patients with rheumatoid arthritis. A randomized, double-blind, placebo-controlled trial. Ann. Intern. Med. 125: 961-968.

Bulger, E. M., Garcia, I., Maier, R. V. (1998) Dithiocarbamates enhance tumor necrosis factor-alpha production by rabbit alveolar macrophages, despite inhibition of NF-Kappa B. Shock 9: 397-405.

Byers, B. A. and Garcia, A. J. (2004) Exogenous Runx2 expression enhances in vitro osteoblastic differentiation and mineralization in primary bone marrow stromal cells. Tissue Eng. 10: 1623-1632.

Callaci, J. J., Juknelis, D., Patwardhan, A., Sartori, M., Frost, N., Wezeman, F. H. (2004) The effects of binge alcohol exposure on bone resorption and biomechanical and structural properties are offset by concurrent bisphosphonate treatment. Alcohol Clin. Exp. Res. 28: 182-191.

Canalis, E. (1996) Mechanisms of glucocorticoid action in bone: Implications to glucocorticoid-induced osteoporosis. J. Clin. Endo. Met. 81: 3441-3447.

Cappon, G. D., Horimoto, M., Hurtt, M. E. (2004) Reproductive toxicity assessment of lasofoxifene, a selective estrogen receptor modulator (SERM), in male rats. Birth Defects Research Part B-Devel. Rep. Toxicol. 71: 142-149.

Carluccio, M. A., Siculella, L., Ancora, M. A., Massaro, M., Scoditti, E., Storelli, C., Visioli, F., Distante, A., De Caterina, R. (2003) Olive oil and red wine antioxidant polyphenols inhibit endothelial activation - Antiatherogenic properties of Mediterranean diet phytochemicals. Arterioscler. Thrombo. Vascul. Biol. 23: 622-629.

Carmeliet, G., Vico, L., Bouillon, R. (2001) Space flight: a challenge for normal bone homeostasis. Crit. Rev. Eukaryot. Gene Exp. 11: 131-144.

Carr, A., Miller, J., Eisman, J. A., Coooper, D. A. (2001) Osteopenia in HIV-infected men: association with asymptomatic lactic acidemia and lower weight pre-antiretroviral therapy. AIDS 15: 703-709.

Cashman, K. D., Baker, A., Ginty, F., Flynn, A., Strain, J. J., Bonham, M. P., O'Connor, J. M., Bugel, S., Sandstrom, B. (2001) No effect of copper supplementation on biochemical markers of bone metabolism in healthy young adult females despite apparently improved copper status. Eur. J. Clin. Nutr. 55: 525-531.

Cenci, S., Toraldo, G., Weitzmann, M. N., Roggia, C., Gao, Y. H., Qian, W. P., Sierra, O., Pacifici, R. (2003) Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-gamma-induced class II transactivator. Proc. Nat'l. Acad. Sci. U.S.A. 100: 10405-10410.

Cenci, S., Weitzmann, M. N., Roggia, C., Namba, N., Novack, D., Woodring, J., Pacifici, R. (2000) Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-alpha. J. Clin. Invest. 106: 1229-1237.

Center, J. R., Nguyen, T. V., Schneider, D., Sambrook, P. N., Eisman, J. A. (1999) Mortality after all major types of osteoporotic fracture in men and women: an observational study. Lancet 353: 878-882.

Chae, H. J., Park, R. K., Chung, H. T., Kang, J. S., Kim, M. S., Choi, D. Y., Bang, B. G., Kim, H. R. (1997) Nitric oxide is a regulator of bone remodelling. J. Pharm. Pharmacol. 49: 897-902.

Chang, H., Jin, T. Y., Jin, W. F., Gu, S. Z., Zhou, Y. F. (2003) Modulation of isoflavones on bone-nodule formation in rat calvaria osteoblasts in vitro. Biomed. Environ. Sci. 16: 83-89.

Chauveau, C., Remy, S., Royer, P. J., Hill, M., Tanguy-Royer, S., Hubert, F. X., Tesson, L., Brion, R., Beriou, G., Gregoire, M., Josien, R., Cuturi, M. C., Anegon, I. (2005) Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. Blood 106: 1694-1702.

Chen, P. Q., Satterwhite, J. H., Licata, A. A., Lewiecki, E. M., Sipos, A. A., Misurski, D. M., Wagman, R. B. (2005) Early changes in biochemical markers of bone formation predict BMD response to teriparatide in postmenopausal women with osteoporosis. J. Bone Miner. Res. 20: 962-970.

Chen, Y. C., Shen, S. C., Lee, W. R., Hou, W. C., Yang, L. L., Lee, T. J. F. (2001) Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. J. Cell. Biochem. 82: 537-548.

Christodoulou, C. and Cooper, C. (2003) What is osteoporosis? Postgrad. Med. J. 79: 133-138.

Conlan, D., Korula, R., Tallentire, D. (1990) Serum copper levels in elderly patients with femoral-neck fractures. Age and Ageing 19: 212-214.

Cooper, C., Atkinson, E. J., Jacobsen, S. J., Ofallon, W. M., Melton, L. J. (1993) Population-based study of survival after osteoporotic fractures. Am. J. Epidemiol. 137: 1001-1005.

Cotran, R. S. (1999) Robbins pathologic basis of disease.

Cremin, P., Kasim-Karakas, S., Waterhouse, A. L. (2001) LC/ES-MS detection of hydroxycinnamates in human plasma and urine. J. Agri. Food Chem. 49: 1747-1750.

Cumming, R. G. and Nevitt, M. C. (1997) Calcium for prevention of osteoporotic fractures in postmenopausal women. J. Bone Miner. Res. 12: 1321-1329.

Cummings, S. R. (1998) Osteoporosis: Review of the evidence for prevention, diagnosis, and treatment and cost-effectiveness analysis - Status report. Osteoporos. Int. 8: S1-S85.

Cummings, S. R., Nevitt, M. C., Browner, W. S., Stone, K., Fox, K. M., Ensrud, K. E., Cauley, J. C., Black, D., Vogt, T. M. (1995) Risk-Factors for Hip Fracture in White Women. N. Eng. J. Med. 332: 767-773.

Cunningham, J., Sprague, S. M., Cannata-Andia, J., Coco, M., Cohen-Solal, M., Fitzpatrick, L., Goltzmann, D., Lafage-Proust, M. H., Leonard, M., Ott, S., Rodriguez, M., Stehman-Breen, C., Stern, P., Weisinger, J. (2004) Osteoporosis in chronic kidney disease. Am. J. Kid. Dis. 43: 566-571.

Cuzzocrea, S., Mazzon, E., Dugo, L., Genovese, T., Di Paola, R., Ruggeri, Z., Vegeto, E., Caputi, A. P., Van de Loo, F. A. J., Puzzolo, D., Maggi, A. (2003) Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. Endocrinology 144: 1098-1107.

Dai, J. L., Lin, D. L., Zhang, J., Habib, P., Smith, P., Murtha, J., Fu, Z., Yao, Z., Qi, Y. H., Keller, E. T. (2000) Chronic alcohol ingestion induces osteoclastogenesis and bone loss through IL-6 in mice. J. Clin. Invest. 106: 887-895.

Damoulis, P. D. and Hauschka, P. V. (1994) Cytokines induce nitric-oxide production in mouse osteoblasts. Biochem. Biophys. Res. Comm. 201: 924-931.

Damoulis, P. D. and Hauschka, P. V. (1997) Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. J. Bone Miner. Res.12: 412-422.

Dawson-Hughes, B., Harris, S. S., Krall, E. A., Dallal, G. E. (1997) Effect of calcium and vitamin D supplementation on bone, density in men and women 65 years of age or older. N. Engl. J. Med. 337: 670-676.

De Lorimier, A. A. (2000) Alcohol, wine, and health. Am. J. Surg. 180: 357-361.

Delmas, P. D., Bjarnason, N. H., Mitlak, B. H., Ravoux, A. C., Shah, A. S., Huster, W. J., Draper, M., Christiansen, C. (1997) Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N. Engl. J. Med. 337: 1641-1647.

Demigne, C., Sabboh, H., Remesy, C., Meneton, P. (2004) Protective effects of high dietary potassium: Nutritional and metabolic aspects. J. Nutr. 134: 2903-2906.

Deng, W. L., Thiel, B., Tannenbaum, C. S., Hamilton, T. A., Stuehr, D. J. (1993) Synergistic cooperation between T-cell lymphokines for induction of the nitric-oxide synthase gene in murine peritoneal-macrophages. J. Immun. 151: 322-329.

Desimone, D. P., Greene, V. S., Hannon, K. S., Turner, R. T., Bell, N. H. (1993) Prostaglandin-E(2) administered by subcutaneous pellets causes local inflammation and systemic bone loss - A model for inflammation-induced bone-disease. J. Bone Miner. Res. 8: 625-634.

Devareddy L, Khalil DA, Smith BJ, Lucas EA, Soung do Y, Marlow DD, Arjmandi BH (2006) Soy moderately improves microstructural properties without affecting bone mass in an ovariectomized rat model of osteoporosis. Bone 38: 686-693.

Devlin, R. D., Reddy, S. V., Savino, R., Ciliberto, G., Roodman, G. D. (1998) IL-6 mediates the effects of IL-1 or TNF, but not PTHrP or 1,25(OH)(2)D-3, on osteoclast-like cell formation in normal human bone marrow cultures. J. Bone Miner. Res. 13: 393-399.

Deyhim, F., Stoecker, B. J., Brusewitz, G. H., Devareddy, L., Arjmandi, B. H. (2005) Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. Menopause 12: 755-762.

Di Munno, O., Mazzantini, M., Delle Sedie, A., Mosca, M., Bombardieri, S. (2004) Risk factors for osteoporosis in female patients with systemic lupus erythematosus. Lupus 13: 724-730.

Dikeman, C. L., Bauer, L. L., Fahey, G. C. (2004) Carbohydrate composition of selected plum/prune preparations. J. Agric. Food Chem. 52: 853-859.

Dinarello, C. A. (1994) The interleukin-1 family - 10 years of discovery. FASEB J. 8: 1314-1325.

Dobnig, H. (2004) A review of teriparatide and its clinical efficacy in the treatment of osteoporosis. Expert. Opin. Pharmacother. 5: 1153-1162.

Dopp, E., Vollmer, G., Hahnel, C., Grevesmuhl, Y., Schiffmann, D. (1999) Modulation of the intracellular calcium level in mammalian cells caused by 17beta-estrodiol, different

phytoestrogens and the anti-estrogen ICI 182780. J. Steroid Biochem. Mol. Biol. 68: 57-64.

Drake, W. M., Kendler, D. L., Rosen, C. J., Orwoll, E. S. (2003) An investigation of the predictors of bone mineral density and response to therapy with alendronate in osteoporotic men. J. Clin. Endocr. Metab. 88: 5759-5765.

Einhorn, T. A. (2003) COX-2: where are we in 2003? The role of cyclooxygenase-2 in bone repair. Arthritis Res. Ther. 5: 5-7.

Engelen, M. P. K. J., Rutten, E. P. A., De Castro, C. L. N., Wouters, E. F. M., Schols, A. M. W. J., Deutz, N. E. P. (2007) Supplementation of soy protein with branched-chain amino acids alters protein metabolism in healthy elderly and even more in patients with chronic obstructive pulmonary disease. Am. J. Clin. Nutr. 85: 431-439.

Ensrud, K. E., Palermo, L., Black, D. M., Cauley, J., Jergas, M., Orwoll, E. S., Nevitt, M. C., Fox, K. M., Cummings, S. R. (1995) Hip and calcaneal bone loss increase with advancing age - longitudinal results from the study of osteoporotic fractures. J. Bone Miner. Res. 10: 1778-1787.

Erwig, L. P., Kluth, D. C., Walsh, G. M., Rees, A. J. (1998) Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines. J. Immunol. 161: 1983-1988.

Ethriedge, A., Lucas, E. A., Stoecker, B. J., Wei, C. I., Arjmandi B.H., Smith, B. J. (2003) Alterations in vertebral bone with hindlimb unloading and the effects of dried plum and parathyroid hormone during recovery. J. Bone Miner. Res. 18: S278-

Falahati-Nini, A., Riggs, B. L., Atkinson, E. J., O'Fallon, W. M., Eastell, R., Khosla, S. (2000) Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men. J. Clin. Invest. 106: 1553-1560.

Fang, N. B., Yu, S. G., Prior, R. L. (2002) LC/MS/MS characterization of phenolic constituents in dried plums. J. Agric. Food Chem. 50: 3579-3585.

Feskanich, D., Weber, P., Willett, W. C., Rockett, H., Booth, S. L., Colditz, G. A. (1999) Vitamin K intake and hip fractures in women: a prospective study. Am. J. Clin. Nutr. 69: 74-79.

Feyen, J. H. M. and Raisz, L. G. (1987) Prostaglandin Production by Calvariae from Sham Operated and Oophorectomized Rats - Effect of 17-Beta-Estradiol Invivo. Endocrinology 121: 819-821.

Fonseca, D. and Ward, W. E. (2004) Daidzein together with high calcium preserve bone mass and biomechanical strength at multiple sites in ovariectomized mice. Bone 35: 489-497.

Francis, R. M. (1999) The effects of testosterone on osteoporosis in men. Clin. Endocrin. 50: 411-414.

Franklin, M., Bu, S., Lerner, M., Lancaster, E., Bellmer, D., Marlow, D., Lightfoot, S., Arjmandi, B., Brackett, D., Lucas, E., Smith, B. (2006) Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. Bone 39: 1331-1342.

Frassetto, L., Morris, R. C., Sebastian, A. (2005) Long-term persistence of the urine calcium-lowering effect of potassium bicarbonate in postmenopausal women. J. Clin. Endocrin. Metab. 90: 831-834.

Fuller, K., Wong, B., Fox, S., Choi, Y. W., Chambers, T. J. (1998) TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J. Exp. Med. 188: 997-1001.

Gabet, Y., Kohavi, D., Muller, R., Chorev, M., Bab, I. (2005) Intermittently administered parathyroid hormone 1-34 reverses bone loss and structural impairment in orchiectomized adult rats. Osteoporos. Int. 16: 1436-1443.

Gallagher, J. C., Riggs, B. L., Deluca, H. F. (1980) Effect of estrogen on calcium absorption and serum vitamin-D metabolites in postmenopausal osteoporosis. J. Clin. Endocr. Metab. 51: 1359-1364.

Gallardo-Williams, M. T., Maronpot, R. R., Turner, C. H., Johnson, C. S., Harris, M. W., Jayo, M. J., Chapin, R. E. (2003) Effects of boric acid supplementation on bone histomorphometry, metabolism, and biomechanical properties in aged female F-344 rats. Biol. Trace Ele. Res. 93: 155-169.

Genant, H. K., Baylink, D. J., Gallagher, J. C. (1989) Estrogens in the prevention of osteoporosis in postmenopausal women. Am. J. Obstet. Gynecol. 161: 1842-1846.

Gerdhem, P. and Obrant, K. J. (2002) Effects of cigarette-smoking on bone mass as assessed by dual-energy X-ray absorptiometry and ultrasound. Osteoporos. Int. 13: 932-936.

Gilbert, L., He, X. F., Farmer, P., Boden, S., Kozlowski, M., Rubin, J., Nanes, M. S. (2000) Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. Endocrinology 141: 3956-3964.

Gilbert, L., He, X. F., Farmer, P., Rubin, J., Drissi, H., van Wijnen, A. J., Lian, J. B., Stein, G. S., Nanes, M. S. (2002) Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2 alpha A) is inhibited by tumor necrosis factor-alpha. J. Biol. Chem. 277: 2695-2701.

Gorski, J. P., Wang, A. M., Lovitch, D., Law, D., Powell, K., Midura, R. J. (2004) Extracellular bone acidic glycoprotein-75 defines condensed mesenchyme regions to be mineralized and localizes with bone sialoprotein during intramembranous bone formation. J. Biol. Chem. 279: 25455-25463.

Gowen, M., Lazner, F., Dodds, R., Kapadia, R., Feild, J., Tavaria, M., Bertoncello, I., Drake, F., Zavarselk, S., Tellis, I., Hertzog, P., Debouck, C., Kola, I. (1999) Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. J. Bone Miner. Res. 14: 1654-1663.

Grano, M., Mori, G., Minielli, V., Barou, O., Colucci, S., Giannelli, G., Alexandre, C., Zallone, A. Z., Vico, L. (2002) Rat hindlimb unloading by tail suspension reduces osteoblast differentiation, induces IL-6 secretion, and increases bone resorption in ex vivo cultures. Calcif. Tissue Int. 70: 176-185.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite, and [N-15]-labeled nitrate in biological-fluids. Anal. Biochem. 126: 131-138.

Grundberg, E., Brandstrom, H., Ribom, E. L., Johnell, O., Orwoll, E., Ljungren, O., Mallmin, H., Carling, T., Kindmark, A. (2003) Correlations of the estrogen receptor alpha coactivator RIZ1 deletion with bone mineral density in men and women. J. Bone Miner. Res. 18: S128-S128.

Haaber, A. B., Rosenfalck, A. M., Hansen, B., Hilsted, J., Larsen, S. (2000) Bone mineral metabolism, bone mineral density, and body composition in patients with chronic pancreatitis and pancreatic exocrine insufficiency. Int. J. Pancreatol. 27: 21-27.

Haddad, J. J. (2002) Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. Cellular Signalling 14: 879-897.

Hakeda, Y., Kobayashi, Y., Yamaguchi, K., Yasuda, H., Tsuda, E., Higashio, K., Miyata, T., Kumegawa, M. (1998) Osteoclastogenesis inhibitory factor (OCIF) directly inhibits bone-resorbing activity of isolated mature osteoclasts. Biochem. Biophys. Res. Comm. 251: 796-801.

Hallund, J., Bugel, S., Tholstrup, T., Ferrari, M., Talbot, D., Hall, W. L., Reimann, M., Williams, C. M., Wiinberg, N. (2006) Soya isoflavone-enriched cereal bars affect markers of endothelial function in postmenopausal women. Brit. J. Nutr. 95: 1120-1126.

Hanada, K., Furuya, K., Yamamoto, N., Nejishima, H., Ichikawa, K., Nakamura, T., Miyakawa, M., Amano, S., Sumita, Y., Oguro, N. (2003) Bone anabolic effects of S-40503, a novel nonsteroidal selective androgen receptor modulator (SARM), in rat models of osteoporosis. Biol. Pharm. Bull. 26: 1563-1569.

Harada, H., Tagashira, S., Fujiwara, M., Ogawa, S., Katsumata, T., Yamaguchi, A., Komori, T., Nakatsuka, M. (1999) Cbfa1 isoforms exert functional differences in osteoblast differentiation. J. Biol. Chem. 274: 6972-6978.

Hart, J. P., Shearer, M. J., Klenerman, L., Catterall, A., Reeve, J., Sambrook, P. N., Dodds, R. A., Bitensky, L., Chayen, J. (1985) Electrochemical detection of depressed circulating levels of vitamin-K1 in osteoporosis. J. Clin. Endocr. Met. 60: 1268-1269.

170

Hassoun, A. A., Nippoldt, T. B., Tiegs, R. D., Khosla, S. (1997) Hepatitis C-associated osteosclerosis: an unusual syndrome of acquired osteosclerosis in adults. Am. J. Med. 103: 70-73.

Heaney, R. P. (2001) Constructive interactions among nutrients and bone-active pharmacologic agents with principal emphasis on calcium, phosphorus, vitamin D and protein. J. Am. Coll. Nutr. 20: 403S-409S.

Hefferan, T. E., Kennedy, A. M., Evans, G. L., Turner, R. T. (2003) Disuse exaggerates the detrimental effects of alcohol on cortical bone. Alcoholism-Clin. Exp. Res. 27: 111-117.

Hegarty, V. M., May, H. M., Khaw, K. T. (2000) Tea drinking and bone mineral density in older women. Am. J. Clin. Nutr. 71: 1003-1007.

Hernandez CJ, Beaupre GS, Marcus R, Carter DR (2001) A theoretical analysis of the contributions of remodeling space, mineralization, and bone balance to changes in bone mineral density during alendronate treatment. Bone 29: 511-516.

Hildebolt, C. E., Pilgram, T. K., Yokoyama-Crothers, N., Vannier, M. W., Dotson, M., Muckerman, J., Hauser, J., Cohen, S., Kardaris, E. E., Hanes, P., Shrout, M. K., Civitelli, R. (2000) Alveolar bone height and postcranial bone mineral density: Negative effects of cigarette smoking and parity. J. Period. 71: 683-689.

Hodges, S. J., Akesson, K., Vergnaud, P., Obrant, K., Delmas, P. D. (1993) Circulating levels of vitamin-K1 and vitamin-K(2) decreased in elderly women with hip fracture. J. Bone Miner. Res. 8: 1241-1245.

Hodsman, A. B., Bauer, D. C., Dempster, D. W., Dian, L., Hanley, D. A., Harris, S. T., Kendler, D. L., McClung, M. R., Miller, P. D., Olszynski, W. P., Orwoll, E., Yuen, C. K. (2005) Parathyroid hormone and teriparatide for the treatment of osteoporosis: A review of the evidence and suggested guidelines for its use. Endocrine Rev. 26: 688-703.

Hofbauer, L. C., Gori, F., Riggs, B. L., Lacey, D. L., Dunstan, C. R., Spelsberg, T. C., Khosla, S. (1999a) Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology 140: 4382-4389.

Hofbauer, L. C. and Heufelder, A. E. (2001) Role of receptor activator of nuclear factor-kappa B ligand and osteoprotegerin in bone cell biology. J. Mol. Med. 79: 243-253.

Hofbauer, L. C., Khosla, S., Dunstan, C. R., Lacey, D. L., Boyle, W. J., Riggs, B. L. (2000) The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J. Bone Miner. Res. 15: 2-12.

Hofbauer, L. C., Lacey, D. L., Dunstan, C. R., Spelsberg, T. C., Riggs, B. L., Khosla, S. (1999b) Interleukin-1 beta and tumor necrosis factor-alpha, but not interleukin-6,

stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 25: 255-259.

Holick, M. F. (2004) Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am. J. Clin. Nutr. 80: 1678S-1688S.

Hollman, P. C. H. and Katan, M. B. (1999) Dietary flavonoids: Intake, health effects and bioavailability. Food Chem. Toxicol. 37: 937-942.

Horcajada-Molteni, M. N., Crespy, V., Coxam, V., Davicco, M. J., Remesy, C., Barlet, J. P. (2000) Rutin inhibits ovariectomy-induced osteopenia in rats. J. Bone Miner. Res. 15: 2251-2258.

Howell, S. and Shalet, S. (2001) Testosterone deficiency and replacement. Horm. Res. 56: 86-92.

Hsu, H. L., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Tan, H. L., Elliott, G., Kelley, M. J., Sarosi, I., Wang, L., Xia, X. Z., Elliott, R., Chiu, L., Black, T., Scully, S., Capparelli, C., Morony, S., Shimamoto, G., Bass, M. B., Boyle, W. J. (1999) Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc. Nat'l. Aca. Sci. U.S.A. 96: 3540-3545.

Huang, J. C., Sakata, T., Pfleger, L. L., Bencsik, M., Halloran, B. P., Bikle, D. D., Nissenson, R. A. (2004) PTH differentially regulates expression of RANKL and OPG. J Bone Miner. Res. 19: 235-244.

Huang, J. S., Rietschel, P., Hadigan, C. M., Rosenthal, D. I., Grinspoon, S. (2001) Increased abdominal visceral fat is associated with reduced bone density in HIV-infected men with lipodystrophy. AIDS 15: 975-982.

Hunt, C. D., Herbel, J. L., Idso, J. P. (1994) Dietary boron modifies the effects of vitamin-D-3 nutrition on indexes of energy substrate utilization and mineral metabolism in the chick. J. Bone Miner. Res. 9: 171-182.

Hunt, C. D., Herbel, J. L., Nielsen, F. H. (1997) Metabolic responses of postmenopausal women to supplemental dietary boron and aluminum during usual and low magnesium intake: Boron, calcium, and magnesium absorption and retention and blood mineral concentrations. Am. J. Clin. Nutr. 65: 803-813.

Huuskonen, J., Arnala, I., Olkkonen, H., Alhava, E. (2001) Alendronate influences bending force of femoral diaphysis after orchidectomy in rats. Ann. Chir. Gynaecol. 90: 109-114.

Ikeda, T., Kasai, M., Utsuyama, M., Hirokawa, K. (2001) Determination of three isoforms of the receptor activator of nuclear factor-kappa B ligand and their differential expression in bone and thymus. Endocrinology 142: 1419-1426.

Inoue, T., Sugiyama, T., Matsubara, T., Kawai, S., Furukawa, S. (2001) Inverse correlation between the changes of lumbar bone mineral density and serum undercarboxylated osteocalcin after vitamin K-2 (menatetrenone) treatment in children treated with glucocorticoid and alfacalcidol. Endocr. J. 48: 11-18.

Iqbal, J. (2006) Does TNF have anti-osteoclastogenic actions? Skel. Devel.Rem. Heal. Dis. Ag. 1068: 234-239.

Iqbal, J., Li, S., Kumar, T. R., Blair, H. C., Zaidi, M. (2006) Follicle-stimulating hormone stimulates TNF production from immune cells to enhance osteoblast and osteoclast formation. Proc. Nat'l. Acad. Sci. U.S.A. 103: 14925-14930.

Isaia, G., Mussetta, M., Pecchio, F., Sciolla, A., Distefano, M., Molinatti, G. M. (1992) Effect of Testosterone on Bone in Hypogonadal Males. Maturitas 15: 47-51.

Ishimi, Y., Miyaura, C., Jin, C. H., et al. (1990) IL-6 is produced by osteoblasts and induces bone resorption. J. Immunol. 145: 3297-3303.

Ito S, H. T. (2005) Crystal structure of RANK ligand involved in bone metabolism. Vitam. Horm. 2004;67:19-33.

Itoh, K., Udagawa, N., Kobayashi, K., Suda, K., Li, X., Takami, M., Okahashi, N., Nishihara, T., Takahashi, N. (2003) Lipopolysaccharide promotes the survival of osteoclasts via toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. J. Immunol. 170: 3688-3695.

Iwamoto J, Takeda T, Ichimura S (2004) Differential effect of short-term etidronate treatment on three cancellous bone sites in orchidectomized adult rats. Keio J Med. 53: 12-17.

Iwamoto, J., Takeda, T., Katsumata, T., Tanaka, T., Ichimura, S., Toyama, Y. (2002) Effect of etidronate on bone in orchidectomized and sciatic neurectomized adult rats. Bone 30: 360-367.

Jagger, C. J., Lean, J. M., Davies, J. T., Chambers, T. J. (2005) Tumor necrosis factor-alpha mediates osteopenia caused by depletion of antioxidants. Endocrinology 146: 113-118.

Jain, R. G. and Lenhard, J. M. (2002) Select HIV protease inhibitors alter bone and fat metabolism ex vivo. J. Biol. Chem. 277: 19247-19250.

Jansen, M. C. J. F., Bueno-De-Mesquita, H. B., Feskens, E. J. M., Streppel, M. T., Kok, F. J., Kromhout, D. (2004) Quantity and variety of fruit and vegetable consumption and cancer risk. Nutr. Cancer 48: 142-148.

Jennings, P. J., Janowsky, J. S., Orwoll, E. (1998) Estrogen and sequential movement. Behav. Neurosci. 112: 154-159.

Jimi, E., Aoki, K., Saito, H., D'Acquisto, F., May, M. J., Nakamura, I., Sudo, T., Kojima, T., Okamoto, F., Fukushima, H., Okabe, K., Ohya, K., Ghosh, S. (2004) Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. Nature Med. 10: 617-624.

Jiunn-Kae Chang, Ya-Ling Hsu, I-Chun Teng, Po-Lin Kuo (2006) Piceatannol stimulate osteoblast differentiation that may be mediated by increased bone morphogenetic protein-2 production. Eur. J. Pharm. 551: 1-9.

Johnell O (2005) Risk factors for hip fracture in European women: the MEDOS Study. Mediterranean Osteoporosis Study. J. Bone Miner. Res. 10: 1802-1815.

Johnson, R. A., Boyce, B. F., Mundy, G. R., Roodman, G. D. (1989) Tumors producing human tumor necrosis factor induced hypercalcemia and osteoclastic bone resorption in nude mice. Endocrinology 124: 1424-1427.

Jones, D. H., Kong, Y. Y., Penninger, J. M. (2002) Role of RANKL and RANK in bone loss and arthritis. Ann Rheum Dis 61: 32-39.

Joseph, J. C. (1994) Corticosteroid-Induced Osteoporosis. Am. J. Hospital Pharm. 51: 188-197.

Jung, J. Y., Lin, A. C., Ramos, L. M., Faddis, B. T., Chole, R. A. (2003) Nitric oxide synthase I mediates osteoclast activity in vitro and in vivo. J. Cell. Biochem. 89: 613-621.

Jung, T. Y., Pashia, M. E., Nishimoto, S. Y., Faddis, B. T., Chole, R. A. (2004) A possible role for nitric oxide in osteoclastogenesis associated with cholesteatoma. Otol. Neurotol. 25: 661-668.

Kabat, G. C., Chang, C. J., Wynder, E. L. (1994) The Role of Tobacco, Alcohol-Use, and Body-Mass Index in Oral and Pharyngeal Cancer. Int. J. Epidemiol. 23: 1137-1144.

Kanematsu, M., Sato, T., Takai, H., Watanabe, K., Ikeda, K., Yamada, Y. (2000) Prostaglandin E-2 induces expression of receptor activator of nuclear factor-kappa B ligand/osteoprotegrin ligand on pre-B cells: Implications for accelerated osteoclastogenesis in estrogen deficiency. J. Bone Miner. Res.15: 1321-1329.

Karinkanta, S., Heinonen, A., Sievanen, H., Uusi-Rasi, K., Kannus, P. (2005) Factors predicting dynamic balance and quality of life in home-dwelling elderly women. Gerontology 51: 116-121.

Kartsogiannis, V. and Ng, K. W. (2004) Cell lines and primary cell cultures in the study of bone cell biology. Mol. Cell. Endocrin. 228: 79-102.

Katznelson, L., Finkelstein, J. S., Schoenfeld, D. A., Rosenthal, D. I., Anderson, E. J., Klibanski, A. (1996) Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. J. Clin. Endocrinol. Metab. 81: 4358-4365.

Kayano, S., Kikuzaki, H., Fukutsuka, N., Mitani, T., Nakatani, N. (2002) Antioxidant activity of prune (Prunus domestica L.) constituents and a new synergist. J. Agric. Food Chem. 50: 3708-3712.

Kayano, S., Kikuzaki, H., Ikami, T., Suzuki, T., Mitani, T., Nakatani, N. (2004) A new bipyrrole and some phenolic constituents in prunes (Prunus domestica L.) and their oxygen radical absorbance capacity (ORAC). Biosci. Biotech. Biochem. 68: 942-944.

Khalil, D. A., Lucas, E. A., Smith, B. J., Payton, M. E., Arjmandi, B. H. (2002) Soy protein supplementation increase serum insulin-like growth factor-I in young and old men but does not affect markers of bone metabolism. J. Nutr. 132: 2605-2608.

Khalil, D. A., Lucas, E. A., Smith, B. J., Soung, D. Y., Devareddy, L., Juma, S., Akhter, M. P., Recker, R., Arjmandi, B. H. (2005) Soy isoflavones may protect against orchidectomy-induced bone loss in aged male rats. Calcif. Tissue Int. 76: 56-62.

Khosla, S. (2001) Minireview: The OPG/RANKL/RANK system. Endocrinology 142: 5050-5055.

Kikuchi, T., Matsuguchi, T., Tsuboi, N., Mitani, A., Tanaka, S., Matsuoka, M., Yamamoto, G., Hishikawa, T., Noguchi, T., Yoshikai, Y. (2001) Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via toll-like receptors. J. Immun. 166: 3574-3579.

Kim, D. O., Chun, O. K., Kim, Y. J., Moon, H. Y., Lee, C. Y. (2003) Quantification of polyphenolics and their antioxidant capacity in fresh plums. J. Agric. Food Chem. 51: 6509-6515.

Kim, H. J., Zhao, H. B., Kitaura, H., Bhattacharyya, S., Brewer, J. A., Muglia, L. J., Ross, F. P., Teitelbaum, S. L. (2006) Glucocorticoids suppress bone formation via the osteoclast. J. Clin. Invest. 116: 2152-2160.

Kim, S. F., Huri, D. A., Snyder, S. H. (2005) Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science 310: 1966-1970.

Kim, Y. J., Lee, M. H., Wozney, J. M., Cho, J. Y., Ryoo, H. M. (2004) Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2. J. Biol. Chem. 279: 50773-50780.

Kohlmeier, M., Saupe, J., Shearer, M. J., Schaefer, K., Asmus, G. (1997) Bone health of adult hemodialysis patients is related to vitamin K status. Kidney Int. 51: 1218-1221.

Komarova, S. V., Pereverzev, A., Shum, J. W., Sims, S. M., Dixon, S. J. (2005) Convergent signaling by acidosis and receptor activator of NF-kappa B ligand (RANKL) on the calcium/calcineurin/NFAT pathway in osteoclasts. Proc. Nat'l. Acad. Sci. U.S.A. 102: 2643-2648.

Kondo, T., Kitazawa, R., Maeda, S., Kitazawa, S. (2004) 1 alpha,25 dihydroxyvitamin D-3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. J. Bone Miner. Res. 19: 1411-1419.

Kong YY (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature. 397(6717):315-23.

Kong, Y. Y., Boyle, W. J., Penninger, J. M. (2000) Osteoprotegerin ligand: a regulator of immune responses and bone physiology. Immunol. Today 21: 495-502.

Kopera, H. (1989) Prophylaxis and Treatment of Osteoporosis. Klinische Wochenschrift 67: 557-557.

Koshihara, Y., Hoshi, K., Okawara, R., Ishibashi, H., Yamamoto, S. (2003) Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture. J. Endocrinol. 176: 339-348.

Kostenuik, P. J., Capparelli, C., Morony, S., Adamu, S., Shimamoto, G., Shen, V., Lacey, D. L., Dunstan, C. R. (2001) OPG and PTH-(1-34) have additive effects on bone density and mechanical strength in osteopenic ovariectomized rats. Endocrinology 142: 4295-4304.

Koyama, A., Otsuka, E., Inoue, A., Hirose, S., Hagiwara, H. (2000) Nitric oxide accelerates the ascorbic acid-induced osteoblastic differentiation of mouse stromal ST2 cells by stimulating the production of prostaglandin E_2. Eur. J. Pharmacol. 391: 225-231.

Krieger, N. S., Sessler, N. E., Bushinsky, D. A. (1992) Acidosis Inhibits Osteoblastic and Stimulates Osteoclastic Activity Invitro. Am. J. Physiol. 262: F442-F448.

Kroll, M. H. (2000) Parathyroid hormone temporal effects on bone formation and resorption. Bull. Math. Biol. 62: 163-188.

Kroon, P. A., Clifford, M. N., Crozier, A., Day, A. J., Donovan, J. L., Manach, C., Williamson, G. (2004) How should we assess the effects of exposure to dietary polyphenols in vitro? Am. J. Clin. Nutr. 80: 15-21.

Kurland, E. S., Cosman, F., McMahon, D. J., Rosen, C. J., Lindsay, R., Bilezikian, J. P. (2000) Parathyroid hormone as a therapy for idiopathic osteoporosis in men: Effects on bone mineral density and bone markers. J. Clin. Endocrinol. Metab. 85: 3069-3076.

Lai, Y. L. and Yamaguchi, M. (2005) Effects of copper on bone component in the femoral tissues of rats: Anabolic effect of zinc is weakened by copper. Biol. Pharm. Bull. 28: 2296-2301.

Landis, W. J. (1996) Mineral characterization in calcifying tissues: Atomic, molecular and macromolecular perspectives. Conn. Tissue Res. 35: 1-8.

Landis, W. J., Hodgens, K. J., Song, M. J., Arena, J., Kiyonaga, S., Marko, M., Owen, C., Mcewen, B. F. (1996) Mineralization of collagen may occur on fibril surfaces: Evidence from conventional and high-voltage electron microscopy and three-dimensional imaging. J. Struc. Biol. 117: 24-35.

Landonio, S., Quirino, T., Bonfanti, P., Gabris, A., Boccassini, L., Gulisano, C., Vulpio, L., Ricci, E., Carrabba, M., Vigevani, G. M. (2004) Osteopenia and osteoporosis in HIV+ patients, untreated or receiving HAART. Biomed. Pharmacother. 58: 505-508.

Lane, N. E. and Lukert, B. (1998) The science and therapy of glucocorticoid-induced bone loss. Endocrin. Metab. Clin. N. Am. 27: 465.

Lappalainen, R., Knuuttila, M., Lammi, S., Alhava, E. M., Olkkonen, H. (1982) Zn and Cu Content in Human Cancellous Bone. Acta Orthopaedica Scandinavica 53: 51-55.

Law, M. R. and Hackshaw, A. K. (1997) A meta-analysis of cigarette smoking, bone mineral density and risk of hip fracture: recognition of a major effect. Britis. Med. J. 315: 841-846.

Lean, J. M., Davies, J. T., Fuller, K., Jagger, C. J., Kirstein, B., Partington, G. A., Urry, Z. L., Chambers, T. J. (2003) A crucial role for thiol antioxidants in estrogen-deficiency bone loss. J. Clin. Invest. 112: 915-923.

Lean, J. M., Jagger, C. J., Kirstein, B., Fuller, K., Chambers, T. J. (2005) Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. Endocrinology 146: 728-735.

Lee, S. K., Gardner, A. E., Kalinowski, J. F., Jastrzebski, S. L., Lorenzo, J. A. (2006) RANKL-stimulated osteoclast-like cell formation in vitro is partially dependent on endogenous interleukin-1 production. Bone 38: 678-685.

Lee, S. K., Huang, H., Lee, S. W., Kim, K. H., Kim, K. K., Kim, H. M., Lee, Z. H., Kim, H. H. (2004) Involvement of iNOS-dependent NO production in the stimulation of osteoclast survival by TNF-alpha. Exp. Cell. Res. 298: 359-368.

Lee, Z. H. and Kim, H. H. (2003) Signal transduction by receptor activator of nuclear factor kappa B in osteoclasts. Biochem. Biophys. Res. Comm. 305: 211-214.

Legrand, E., Chappard, D., Pascaretti, C., Duquenne, M., Krebs, S., Rohmer, V., Basle, M. F., Audran, M. (2000) Trabecular bone microarchitecture, bone mineral density, and vertebral fractures in male osteoporosis. J. Bone Miner. Res. 15: 13-19.

Lemann, J. (1999) Relationship between urinary calcium and net acid excretion as determined by dietary protein and potassium: A review. Nephron 81: 18-25.

Leveille, S. G., LaCroix, A. Z., Koepsell, T. D., Beresford, S. A., VanBelle, G., Buchner, D. M. (1997) Dietary vitamin C and bone mineral density in postmenopausal women in Washington State, USA. J. Epidem.Comm. Health 51: 479-485.

Li, M., Jee, W. S. S., Ke, H. Z., Tang, L. Y., Ma, Y. F., Liang, X. G., Setterberg, R. B. (1995) Prostaglandin E(2) Administration prevents bone loss induced by orchiectomy in Rats. J. Bone Miner. Res. 10: 66-73.

Li, Y. M., Schacher, D. H., Liu, Q., Arkins, S., Rebeiz, N., McCusker, R. H., Jr., Dantzer, R., Kelley, K. W. (1997) Regulation of myeloid growth and differentiation by the insulin-like growth factor I receptor. Endocrinology 138: 362-368.

Li, Y. P., Chen, W., Liang, Y. Q., Li, E., Stashenko, P. (1999) Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. Nature Genetics 23: 447-451.

Liberman, U., Karpf, D. B., Capizzi, T. (1996) Alendronate in postmenopausal osteoporosis - Reply. N. Engl. J. Med. 334: 734-735.

Liedholm, H. and Linne, A. B. (1996) Alendronate in postmenopausal osteoporosis. N. Engl. J. Med. 334: 733-734.

Lin, S. K., Kok, S. H., Kuo, M. Y., Lee, M. S., Wang, C. C., Lan, W. H., Hsiao, M., Goldring, S. R., Hong, C. Y. (2003) Nitric oxide promotes infectious bone resorption by enhancing cytokine-stimulated interstitial collagenase synthesis in osteoblasts. J. Bone Miner. Res. 18: 39-46.

Lindberg, M. K., Erlandsson, M., Alatalo, S. L., Windahl, S., Andersson, G., Halleen, J. M., Carlsten, H., Gustafsson, J. A., Ohlsson, C. (2001) Estrogen receptor alpha, but not estrogen receptor beta, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-kappa B ligand) ratio and serum interleukin-6 in male mice. J. Endocrinol. 171: 425-433.

Lips, P., Graafmans, W. C., Ooms, M. E., Bezemer, P. D., Bouter, L. M. (1996) Vitamin D supplementation and fracture incidence in elderly persons - A randomized, placebo-controlled clinical trial. Ann. Intern. Med. 124: 400-406.

Liu, X. H., Kirschenbaum, A., Yao, S., Levine, A. C. (2005) Cross-talk between the interleukin-6 and prostaglandin E-2 signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-kappa B (RANK) ligand/RANK system. Endocrinology 146: 1991-1998.

Ljunghall, S., Johansson, A. G., Burman, P., Kampe, O., Lindh, E., Karlsson, F. A. (1992) Low plasma levels of insulin-like growth factor 1 (IGF-1) in male patients with idiopathic osteoporosis. J. Intern. Med. 232: 59-64.

Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z. D., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., Mak, T. W. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Devel. 13: 1015-1024.

Lorch, I. J. (1949) Alkaline phosphatase and the mechanism of ossification. J. Bone Joint Surg. -British Volume 31: 94-99.

Lorenzo, J. A. (1991) The role of cytokines in the regulation of local bone-resorption. Crit. Rev. Immun. 11: 195-213.

Lorenzo, J. A., Kalinowski, J., Voznesensky, O., Pilbeam, C. (1999) Osteoblasts from interleukin 1 receptor type 1 knockout mice have a markedly reduced capacity to support osteoclast formation. J. Bone Miner. Res. 14: S164-S164.

Lorenzo, J. A., Sousa, S. L., Alander, C., Raisz, L. G., Dinarello, C. A. (1987) Comparison of the bone-resorbing activity in the supernatants from phytohemagglutinin-stimulated human peripheral blood mononuclear cells with that of cytokines through the use of an antiserum to interleukin 1. Endocrinology 121: 1164-1170.

Lu, X. H., Gilbert, L., He, X. F., Rubin, J., Nanes, M. S. (2006) Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways. J. Biol. Chem. 281: 6297-6306.

Lubberts, E., van den Bersselaar, L., Oppers-Walgreen, B., Schwarzenberger, P., Coenen-de Roo, C. J. J., Kolls, J. K., Joosten, L. A. B., van den Berg, W. B. (2003) IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-kappa B ligand/osteoprotegerin balance. J. Immunol.170: 2655-2662.

Luukinen, H., Kakonen, S. M., Pettersson, K., Koski, K., Laippala, P., Lovgren, T., Kivela, S. L., Vaananen, H. K. (2000) Strong prediction of fractures among older adults by the ratio of carboxylated to total serum osteocalcin. J. Bone Miner. Res. 15: 2473-2478.

Macdonald, H. M., New, S. A., Fraser, W. D., Campbell, M. K., Reid, D. M. (2005) Low dietary potassium intakes and high dietary estimates of net endogenous acid production are associated with low bone mineral density in premenopausal women and increased markers of bone resorption in postmenopausal women. Am. J. Clin. Nutr. 81: 923-933.

Macdonald, H. M., New, S. A., Golden, M. H., Campbell, M. K., Reid, D. M. (2004) Nutritional associations with bone loss during the menopausal transition: evidence of a beneficial effect of calcium, alcohol, and fruit and vegetable nutrients and of a detrimental effect of fatty acids. Am. J. Clin. Nutr. 79: 155-165.

Maggi-Capeyron, M. F., Ceballos, P., Cristol, J. P., Delbosc, S., Le Doucen, C., Pons, M., Leger, C. L., Descomps, B. (2001) Wine phenolic antioxidants inhibit AP-1 transcriptional activity. J. Agric. Food Chem. 49: 5646-5652.

Maggio, D., Barabani, M., Pierandrei, M., Polidori, M. C., Catani, M., Mecocci, P., Senin, U., Pacifici, R., Cherubini, A. (2003) Marked decrease in plasma antioxidants in aged osteoporotic women: Results of a cross-sectional study. J. Clin. Endocrin. Metab. 88: 1523-1527.

Mann, S. T., Stracke, H., Lange, U., Klor, H. U., Teichmann, J. (2003) Alterations of bone mineral density and bone metabolism in patients with various grades of chronic pancreatitis. Metabolism 52: 579-585.

Manolagas, S. C. (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. End. Rev. 21: 115-137.

Marangella, M., Di Stefano, M., Casalis, S., Berutti, S., D'Amelio, P., Isaia, G. C. (2004) Effects of potassium citrate supplementation on bone metabolism. Calcif. Tissue Int. 74: 330-335.

Martinez, J. and Moreno, J. J. (2000) Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem. Pharmacol. 59: 865-870.

Mattsson, J. P., Schlesinger, P. H., Keeling, D. J., Teitelbaum, S. L., Stone, D. K., Xie, X. S. (1994) Isolation and Reconstitution of A Vacuolar-Type Proton Pump of Osteoclast Membranes. J. Biol. Chem. 269: 24979-24982.

Mccord, J. M. and Edeas, M. A. (2005) SOD, oxidative stress and human pathologies: a brief history and a future vision. Biomed. Pharmacother. 59: 139-142.

McDermott, A. Y., Shevitz, A., Knox, T., Roubenoff, R., Kehayias, J., Gorbach, S. (2001) Effect of highly active antiretroviral therapy on fat, lean, and bone mass in HIV-seropositive men and women. Am. J. Clin.Nutr. 74: 679-686.

McGartland, C. P., Robson, P. J., Murray, L. J., Cran, G. W., Savage, M. J., Watkins, D. C., Rooney, M. M., Boreham, C. A. (2004) Fruit and vegetable consumption and bone mineral density: the northern Ireland young hearts project. Am. J. Clin. Nutr. 80: 1019-1023.

McGrath, H. (1996) Alendronate in postmenopausal osteoporosis. N. Engl. J. Med. 334: 734-734.

McLaughlin, F., Mackintosh, J., Hayes, B. P., McLaren, A., Uings, I. J., Salmon, P., Humphreys, J., Meldrum, E., Farrow, S. N. (2002) Glucocorticoid-induced osteopenia in the mouse as assessed by histomorphometry, microcomputed tomography, and biochemical markers. Bone 30: 924-930.

Mehta, J. L., Saldeen, T. G. P., Rand, K. (1998) Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease. J. Am. Coll. Cardiol. 31: 1217-1225.

Miller, E. A., Pankow, J. S., Millikan, R. C., Bray, M. S., Ballantyne, C. M., Bell, D. A., Heiss, G., Li, R. L. (2003) Glutathione-S-transferase genotypes, smoking, and their association with markers of inflammation, hemostasis, and endothelial function: the atherosclerosis risk in communities (ARIC) study. Atherosclerosis 171: 265-272.

Miyaura, C., Inada, M., Matsumoto, C., Ohshiba, T., Uozumi, N., Shimizu, T., Ito, A. (2003) An essential role of cytosolic phospholipase A2alpha in prostaglandin E2-mediated bone resorption associated with inflammation. J. Exp. Med. 197: 1303-1310.

Mizuno, A., Kanno, T., Hoshi, M., Shibata, O., Yano, K., Fujise, N., Kinosaki, M., Yamaguchi, K., Tsuda, E., Murakami, A., Yasuda, H., Higashio, K. (2002) Transgenic mice overexpressing soluble osteoclast differentiation factor (sODF) exhibit severe osteoporosis. J. Bone Miner. Metab. 20: 337-344.

Mizutani, K., Ikeda, K., Kawai, Y., Yamori, Y. (1998) Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem. Biophys. Res. Commun. 253: 859-863.

Mohan, S. and Baylink, D. J. (1991) Bone growth factors. Clin. Orthop. Relat. Res. 263: 30-48.

Mondy, K. and Tebas, P. (2003) Emerging bone problems in patients infected with human immunodeficiency virus. Clin. Infect. Dis. 36: S101-S105.

Montpied, P., de Bock, F., Rondouin, G., Niel, G., Briant, L., Courseau, A. S., Lerner-Natoli, M., Bockaert, J. (2003) Caffeic acid phenethyl ester (CAPE) prevents inflammatory stress in organotypic hippocampal slice cultures. Mol. Brain Res. 115: 111-120.

Moore, A. L., Vashisht, A., Sabin, C. A., Mocroft, A., Madge, S., Phillips, A. N., Studd, J. W., Johnson, M. A. (2001) Reduced bone mineral density in HIV-positive individuals. AIDS 15: 1731-1733.

Morabito, N., Crisafulli, A., Vergara, C., Gaudio, A., Lasco, A., Frisina, N., D'Anna, R., Corrado, F., Pizzoleo, M. A., Cincotta, M., Altavilla, D., Ientile, R., Squadrito, F. (2002) Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study. J. Bone Miner. Res. 17: 1904-1912.

Moriyama I, Iwamoto J, Takeda T, Toyama Y (2005) Comparative effects of intermittent administration of human parathyroid hormone (1-34) on cancellous and cortical bone loss in tail-suspended and sciatic neurectomized young rats. J. Orthop. Sci. 7: 379-385.

Morton, D. J., Barrett-Connor, E. L., Schneider, D. L. (2001) Vitamin C supplement use and bone mineral density in postmenopausal women. J. Bone Miner. Res.16: 135-140.

Muhlbauer, R. C., Lozano, A., Reinli, A., Wetli, H. (2003) Various selected vegetables, fruits, mushrooms and red wine residue inhibit bone resorption in rats. J. Nutr. 133: 3592-3597.

Mundy GR (1999) Bone remodelling. fourth edition: Primer on the metabolic bone disease and disorders of mineral metabolism pp.30-38.

Muthusami, S., Ramachandran, H., Muthusamy, B., Vasudevan, G., Prabhu, V., Subramaniam, V., Jagadeesan, A., Narasimhan, S. (2005) Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. Clinica Chimica Acta 360: 81-86.

Naghii, M. R. and Samman, S. (1997) The effect of boron supplementation on its urinary excretion and selected cardiovascular risk factors in healthy male subjects. Biol. Trace Ele. Res. 56: 273-286.

Nakagawa, H., Wachi, M., Woo, J. T., Kato, M., Kasai, S., Takahashi, F., Lee, I. S., Nagai, K. (2002) Fenton reaction is primarily involved in a mechanism of (-)-epigallocatechin-3-gallate to induce osteoclastic cell death. Biochem. Biophys. Res. Comm 292: 94-101.

Nakajima, A., Shimoji, N., Shiomi, K., Shimizu, S., Moriya, H., Einhorn, T. A., Yamazaki, M. (2002) Mechanisms for the enhancement of fracture healing in rats treated with intermittent low-dose human parathyroid hormone (1-34). J. Bone Miner. Res.17: 2038-2047.

Nakatani, N., Kayano, S., Kikuzaki, H., Sumino, K., Katagiri, K., Mitani, T. (2000) Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (Prunus domestica L.). J. Agri. Food Chem. 48: 5512-5516.

Nakchbandi, I. A., Mitnick, M. A., Masiukiewicz, U. S., Sun, B. H., Insogna, K. L. (2001) IL-6 negatively regulates IL-11 production in vitro and in vivo. Endocrinology 142: 3850-3856.

Nathens, A. B., Bitar, R., Marshall, J. C., Watson, R. W. G., Dackiw, A. P. B., Fan, J., Hiscott, J., Rotstein, O. D. (2001) Antioxidants increase lipopolysaccharide-stimulated TNF alpha release in murine macrophages: Role for altered TNF alpha mRNA stability. Shock 16: 361-367.

National Osteoporosis Foundation (2007) Osteoporosis, Disease Statistics. NOF fact sheeet (Internet access)

Nelson, H. D., Rizzo, J., Harris, E., Cauley, J., Ensrud, K., Bauer, D. C., Orwoll, E. (2002) Osteoporosis and fractures in postmenopausal women using estrogen. Arch. Int. Med. 162: 2278-2284.

Nesbitt, S. A. and Horton, M. A. (1997) Trafficking of matrix collagens through bone-resorbing osteoclasts. Science 276: 266-269.

New, S. A., Robins, S. P., Campbell, M. K., Martin, J. C., Garton, M. J., Bolton-Smith, C., Grubb, D. A., Lee, S. J., Reid, D. M. (2000) Dietary influences on bone mass and bone metabolism: further evidence of a positive link between fruit and vegetable consumption and bone health? Am. J. Clin. Nutr. 71: 142-151.

Newitt, D. C., Majumdar, S., Van, R. B., von, I. G., Harris, S. T., Genant, H. K., Chesnut, C., Garnero, P., MacDonald, B. (2002) In vivo assessment of architecture and micro-finite element analysis derived indices of mechanical properties of trabecular bone in the radius. Osteoporos. Int. 13: 6-17.

Nielsen, F. H. (2004) The alteration of magnesium, calcium and phosphorus metabolism by dietary magnesium deprivation in postmenopausal women is not affected by dietary boron deprivation. Magnesium Res. 17: 197-210.

Nieves, J. W. (2003) Calcium, vitamin D, and nutrition in elderly adults. Clin. Geriatric Med.19: 321.

NIH (2000) Osteoporosis Prevention, Diagnosis, and Therapy. NIH Consensus Statement 17: 1-36.

Notoya, M., Tsukamoto, Y., Nishimura, H., Woo, J. T., Nagai, K., Lee, I. S., Hagiwara, H. (2004) Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts in vitro. Eur. J. Pharm. 485: 89-96.

O'Gradaigh, D., Ireland, D., Bord, S., Compston, J. E. (2004) Joint erosion in rheumatoid arthritis: interactions between tumour necrosis factor alpha, interleukin 1, and receptor activator of nuclear factor kappa B ligand (RANKL) regulate osteoclasts. Ann. Rheum. Dis. 63: 354-359.

Ohmori, N., Nomura, K., Ohmori, K., Kato, Y., Itoh, T., Takano, K. (2003) Osteoporosis is more prevalent in adrenal than in pituitary Cushing's syndrome. Endocr. J. 50: 1-7.

Ohsaki, Y., Shirakawa, H., Hiwatashi, K., Furukawa, Y., Mizutani, T., Komai, M. (2006) Vitamin K suppresses lipopolysaccharide-induced inflammation in the rat. Biosci. Biotech. Biochem. 70: 926-932.

Okada, Y., Lorenzo, J. A., Freeman, A. M., Tomita, M., Morham, S. G., Raisz, L. G., Pilbeam, C. C. (2000) Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture. J. Clin. Invest. 105: 823-832.

Olthof, M. R., Hollman, P. C. H., Katan, M. B. (2001) Chlorogenic acid and caffeic acid are absorbed in humans. J. Nutr. 131: 66-71.

Orimo, H., Shiraki, M., Tomita, A., Morii, H., Fujita, T., Ohata, M. (1998) Effects of menatetrenone on the bone and calcium metabolism in osteoporosis: A double-blind placebo-controlled study. J. Bone Miner. Metab. 16: 106-112.

Orwoll, E., Ettinger, M., Weiss, S., Miller, P., Kendler, D., Graham, J., Adami, S., Weber, K., Lorenc, R., Pietschmann, P., Vandormael, K., Lombardi, A. (2000) Alendronate for the treatment of osteoporosis in men. N. Engl. J. Med. 343: 604-610.

Orwoll, E. S., Stribrska, L., Ramsey, E. E., Keenan, E. J. (1991) Androgen Receptors in Osteoblast-Like Cell-Lines. Calcif. Tissue Int. 49: 183-187.

Ota, N., Nakajima, T., Nakazawa, I., Suzuki, T., Hosoi, T., Orimo, H., Inoue, S., Shirai, Y., Emi, M. (2001) A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density. J. Human Genetics 46: 267-272.

Pacifici, R. (1996) Estrogen, cytokines, and pathogenesis of postmenopausal osteoporosis. J. Bone Miner. Res. 11: 1043-1051.

Palle, S., Vico, L., Bourrin, S., Alexandre, C. (1992) Bone Tissue-Response to 4-Month Antiorthostatic Bedrest - A Bone Histomorphometric Study. Calcif. Tissue Int. 51: 189-194.

Palmer, R. M. J., Hickery, M. S., Charles, I. G., Moncada, S., Bayliss, M. T. (1993) Induction of Nitric-Oxide Synthase in Human Chondrocytes. Biochem. Biophys. Res. Comm. 193: 398-405.

Palmqvist, P., Persson, E., Conaway, H. H., Lerner, U. H. (2002) IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. J. Immunol. 169: 3353-3362.

Pan, G., Wu, X., McKenna, M. A., Feng, X., Nagy, T. R., McDonald, J. M. (2004) AZT enhances osteoclastogenesis and bone loss. AIDS Res. Hum. Retroviruses 20: 608-620.

Pan, W., Quarles, L. D., Song, L. H., Yu, Y. H., Jiao, C., Tang, H. B., Jiang, C. H., Deng, H. W., Li, Y. J., Zhou, H. H., Xiao, Z. S. (2005) Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. J. Cell. Biochem. 94: 307-316.

Park, E. K., Kim, M. S., Lee, S. H., Kim, K. H., Park, J. Y., Kim, T. H., Lee, I. S., Woo, J. T., Jung, J. C., Shin, H. I., Choi, J. Y., Kim, S. Y. (2004) Furosin, an ellagitannin, suppresses RANKL-induced osteoclast differentiation and function through inhibition of MAP kinase activation and actin ring formation. Biochem. Biophys. Res. Comm. 325: 1472-1480.

Patel, M. B., Arden, N. K., Masterson, L. M., Phillips, D. I., Swaminathan, R., Syddall, H. E., Byrne, C. D., Wood, P. J., Cooper, C., Holt, R. I. (2005) Investigating the role of the growth hormone-insulin-like growth factor (GH-IGF) axis as a determinant of male bone mineral density (BMD). Bone 37: 833-841.

Paton, N. I., Macallan, D. C., Griffin, G. E., Pazianas, M. (1997) Bone mineral density in patients with human immunodeficiency virus infection. Calcif. Tissue Int. 61: 30-32.

Peacock, M., Liu, G., Carey, M., McClintock, R., Ambrosius, W., Hui, S., Johnston, C. C. (2000) Effect of calcium or 25OH vitamin D3 dietary supplementation on bone loss at the hip in men and women over the age of 60. J. Clin. Endocrinol. Metab 85: 3011-3019.

Pendurthi, U. R., Meng, F., Mackman, N., Rao, L. V. M. (2002) Mechanism of resveratrol-mediated suppression of tissue factor gene expression. Thrombo. Haemostas.87: 155-162.

Perrault, H. (2006) Efficiency of movement in health and chronic disease. Clin. Invest. Med. 29: 117-121.

Picherit, C., Bennetau-Pelissero, C., Chanteranne, B., Lebecque, P., Davicco, M. J., Barlet, J. P., Coxam, V. (2001) Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats. J. Nutr. 131: 723-728.

Pischon, N., Darbois, L. M., Palamakumbura, A. H., Kessler, E., Trackman, P. C. (2004) Regulation of collagen deposition and lysyl oxidase by tumor necrosis factor-alpha in osteoblasts. J. Biol. Chem. 279: 30060-30065.

Rafferty, K., Davies, K. M., Heaney, R. P. (2005) Potassium intake and the calcium economy. J. Am. Coll. Nutr. 24: 99-106.

Rahman, I., Marwick, J., Kirkham, P. (2004) Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappa B and pro-inflammatory gene expression. Biochem. Pharmacol. 68: 1255-1267.

Raisz, L. G. (2001) Potential impact of selective cyclooxygenase-2 inhibitors on bone metabolism in health and disease. Am. J. Med 110 Suppl 3A: 43S-45S.

Ralston, S. H., Ho, L. P., Helfrich, M. H., Grabowski, P. S., Johnston, P. W., Benjamin, N. (1995) Nitric-Oxide - A Cytokine-Induced Regulator of Bone-Resorption. J. Bone Miner. Res. 10: 1040-1049.

Ramirez-Yanez, G. O., Seymour, G. J., Walsh, L. J., Forwood, M. R., Symons, A. L. (2004) Prostaglandin E-2 enhances alveolar bone formation in the rat mandible. Bone 35: 1361-1368.

Rassi, C. M., Lieberherr, M., Chaumaz, G., Pointillart, A., Cournot, G. (2005) Modulation of osteoclastogenesis in porcine bone marrow cultures by quercetin and rutin. Cell. Tissue Res. 319: 383-393.

Rattanakul, C., Lenbury, Y., Krishnamara, N., Wolwnd, D. J. (2003) Modeling of bone formation and resorption mediated by parathyroid hormone: response to estrogen/PTH therapy. Biosystems 70: 55-72.

Redlich, K., Gortz, B., Hayer, S., Zwerina, J., Doerr, N., Kostenuik, P., Bergmeister, H., Kollias, G., Steiner, G., Smolen, J. S., Schett, G. (2004) Repair of local bone erosions and reversal of systemic bone loss upon therapy with anti-tumor necrosis factor in combination with osteoprotegerin or parathyroid hormone in tumor necrosis factor-mediated arthritis. Am. J. Pathol. 164: 543-555.

Reeve, J., Lunt, M., Felsenberg, D., Silman, A. J., Scheidt-Nave, C., Poor, G., Gennari, C., Weber, K., Lorenc, R., Masaryk, P., Cannata, J. B., Dequeker, J., Reid, D. M., Pols, H. A. P., Benevolenskaya, L. I., Stepan, J. J., Miazgowski, T., Bhalla, A., Armas, J. B., Eastell, R., Lopes-Vaz, A., Lyritis, G., Jajic, I., Woolf, A. D., Banzer, D., Reisinger, W.,

Todd, C. J., Felsch, B., Havelka, S., Hoszowski, K., Janott, J., Johnell, O., Raspe, H. H., Yershova, O. B., Kanis, J. A., Armbrecht, G., Finn, J. D., Gowin, W., O'Neill, T. W. (2003) Determinants of the size of incident vertebral deformities in European men and women in the sixth to ninth decades of age: The European Prospective Osteoporosis Study (EPOS). J. Bone Miner. Res. 18: 1664-1673.

Reeves, P. G., Nielsen, F. H., Fahey, G. C. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing on the reformation of the AIN-76 rodent diet. J. Nutr. 123: 1939-1951.

Rico, H., Roca-Botran, C., Hernandez, E. R., Seco, C., Paez, E., Valencia, M. J., Villa, L. F. (2000) The effect of supplemental copper on osteopenia induced by ovariectomy in rats. Menopause 7: 413-416.

Rifas, L., Arackal, S., Weitzmann, M. N. (2003) Inflammatory T cells rapidly induce differentiation of human bone marrow stromal cells into mature osteoblasts. J. Cell Biochem. 88: 650-659.

Riggins, R. S., Cartwright, A. G., Ruckers, R. B. (1979) Viscoelastic Properties of Copper Deficient Chick Bone. J. Biomech. 12: 197-203.

Riggs, B. L., Khosla, S., Melton, L. J. (2002) Sex steroids and the construction and conservation of the adult skeleton. Endocr. Rev. 23: 279-302.

Ritchlin, C. T., Haas-Smith, S. A., Li, P., Hicks, D. G., Schwarz, E. M. (2003) Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. J. Clin. Invest. 111: 821-831.

Rivera, C. A., Tcharmtchi, M. H., Mendoza, L., Smith, C. W. (2003) Endotoxemia and hepatic injury in a rodent model of hindlimb unloading. J. Appl. Physiol. 95: 1656-1663.

Rodan, G. A. (1992) Introduction to Bone Biology. Bone 13: S3-S6.

Rodan, G. A. and Martin, T. J. (2000) Therapeutic approaches to bone diseases. Science 289: 1508-1514.

Roden, A. C., Moser, M. T., Tri, S. D., Mercader, M., Kuntz, S. M., Dong, H. D., Hurwitz, A. A., Mckean, D. J., Celis, E., Leibovich, B. C., Allison, J. P., Kwon, E. D. (2004) Augmentation of T cell levels and responses induced by androgen deprivation. J. Immunol. 173: 6098-6108.

Roggia, C., Gao, Y. H., Cenci, S., Weitzmann, M. N., Toraldo, G., Isaia, G., Pacifici, R. (2001) Up-regulation of TNF-producing T cells in the bone marrow: A key mechanism by which estrogen deficiency induces bone loss in vivo. Proc. Nat'l. Acad. Sci. U.S.A. 98: 13960-13965.

Romas, E., Gillespie, M. T., Martin, T. J. (2002) Involvement of receptor activator of NF-kB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. Bone 30: 340-346.

Ronald L.Prior (1999) Can Foods Forestall Aging? Agric. Res. Mag. 47: 14-17.

Roodman, G. D. (1999) Cell biology of the osteoclast. Exp. Hematol. 27: 1229-1241.

Rosen J (2002) Novel, non-steroidal, selective androgen receptor modulators (SARMs) with anabolic activity in bone and muscle and improved safety profile. J. Musculoskelet Neuronal Interact. 2: 222-224.

Rosen, C. J., Chesnut, C. H., Mallinak, N. J. S. (1997) The predictive value of biochemical markers of bone turnover for bone mineral density in early postmenopausal women treated with hormone replacement or calcium supplementation. J. Clin. Endocrinol. Metab. 82: 1904-1910.

Rucker, R. B., Kosonen, T., Clegg, M. S., Mitchell, A. E., Rucker, B. R., Uriu-Hare, J., Keen, C. L. (1998) Copper, lysyl oxidase, and extracellular matrix protein cross-linking. Am. J. Clin. Nutr. 67(suppl): 996S-1002S.

Rucker, R. B., Riggins, R. S., Laughlin, R., Chan, M. M., Chen, M., Tom, K. (1975) Effects of nutritional copper deficiency on the biomechanical properties of bone and arterial elastin metabolism in the chick. J. Nutr. 105: 1062-1070.

Ryan, A. S. and Nicklas, B. J. (2004) Reductions in plasma cytokine levels with weight loss improve insulin sensitivity in overweight and obese postmenopausal women. Diabetes Care 27: 1699-1705.

Safadi, F. F., Dissanayake, I. R., Goodman, G. G., Jago, R. A., Baker, A. E., Bowman, A. R., Sass, D. A., Popoff, S. N., Epstein, S. (2000) Influence of estrogen deficiency and replacement on T-cell populations in rat lymphoid tissues and organs. Endocrine 12: 81-88.

Saidenberg, K. N., Bessis, N., Cohen-Solal, M., de Vernejoul, M. C., Boissier, M. C. (2002) Osteoprotegerin and inflammation. Eur. Cytokine Netw. 13: 144-153.

Saidenberg-Kermanac'h, N., Cohen-Solal, M., Bessis, N., De Vernejoul, M. C., Boissier, M. C. (2004) Role for osteoprotegerin in rheumatoid inflammation. Joint Bone Spine 71: 9-13.

Sakuma, Y., Tanaka, K., Suda, M., Yasoda, A., Natsui, K., Tanaka, I., Ushikubi, F., Narumiya, S., Segi, E., Sugimoto, Y., Ichikawa, A., Nakao, K. (2000) Crucial involvement of the EP4 subtype of prostaglandin E receptor in osteoclast formation by proinflammatory cytokines and lipopolysaccharide. J. Bone Miner. Res. 15: 218-227.

Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., Needleman, P. (1993) Nitric oxide activates cyclooxygenase enzymes. Proc. Natl. Acad. Sci. U.S.A. 90: 7240-7244.

Sampson, H. W. (2002) Alcohol and other factors affecting osteoporosis risk in women. Alcohol Res. Heal. 26: 292-298.

Sato, Y., Honda, Y., Kuno, H., Oizumi, K. (1998) Menatetrenone ameliorates osteopenia in disuse-affected limbs of vitamin D- and K-deficient stroke patients. Bone 23: 291-296.

Scharla, S. H., Strong, D. D., Mohan, S., Chevalley, T., Linkhart, T. A. (1994) Effect of tumor-necrosis-factor-alpha on the expression of insulin-like growth-factor-I and insulin-like growth-factor binding-protein-4 in mouse osteoblasts. Eur. J. Endocr. 131: 293-301.

Schett, G., Redlich, K., Hayer, S., Zwerina, J., Bolon, B., Dunstan, C., Gortz, B., Schulz, A., Bergmeister, H., Kollias, G., Steiner, G., Smolen, J. S. (2003) Osteoprotegerin protects against generalized bone loss in tumor necrosis factor-transgenic mice. Arthritis Rheum. 48: 2042-2051.

Schnitzler, C. M., Schnaid, E., MacPhail, A. P., Mesquita, J. M., Robson, H. J. (2005) Ascorbic acid deficiency, iron overload and alcohol abuse underlie the severe osteoporosis in black African patients with hip fractures - A bone histomorphometric study. Calcif. Tissue Int. 76: 79-89.

Schoppet, M., Preissner, K. T., Hofbauer, L. C. (2002) RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler. Thromb. Vasc. Biol. 22: 549-553.

Sebastian, A., Harris, S. T., Ottaway, J. H., Todd, K. M., Morris, R. C. (1994) Improved mineral balance and skeletal metabolism in postmenopausal women treated with potassium bicarbonate. N. Engl. J. Med 330: 1776-1781.

Sellmeyer, D. E., Schloetter, M., Sebastian, A. (2002) Potassium citrate prevents increased urine calcium excretion and bone resorption induced by a high sodium chloride diet. J. Clin. Endocr. Metab. 87: 2008-2012.

Serrano, S., Marinoso, M. L., Soriano, J. C., Rubies-Prat, J., Aubia, J., Coll, J., Bosch, J., Del, R. L., Vila, J., Goday, A., . (1995) Bone remodelling in human immunodeficiency virus-1-infected patients. A histomorphometric study. Bone 16: 185-191.

Sheng, M. H. C., Taper, L. J., Veit, H., Thomas, E. A., Ritchey, S. J., Lau, K. H. W. (2001) Dietary boron supplementation enhances the effects of estrogen on bone mineral balance in ovariectomized rats. Biol. Trace Ele. Res. 81: 29-45.

Shimon, I., Eshed, V., Doolman, R., Sela, B. A., Karasik, A., Vered, I. (2005) Alendronate for osteoporosis in men with androgen-repleted hypogonadism. Osteoporos. Int. 16: 1591-1596.

Silver, I. A., Murrills, R. J., Etherington, D. J. (1988) Microelectrode Studies on the Acid Microenvironment Beneath Adherent Macrophages and Osteoclasts. Exp. Cell Res. 175: 266-276.

Singleton, V. L., Orthofer, R., Lamuela-Raventos, R. M. (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Oxidants and Antioxidants 299: 152-178.

Smith, B. J., Ethriedge, A., Lucas, E. A., Bellmer, D., Stoecker, B. J., Arjmandi B.H. (2003) Dried plum promotes bone recovery comparable to parathyroid hormone in osteopenic rats following hindlimb unloading. J. Bone Miner. Res. 18: S277.

Smith, B. J., King, J. B., Lucas, E. A., Akhter, M., Arjmandi, B. H., Stoecker, B. J. (2002) Detrimental effects of skeletal unloading and copper depletion on bone in mature rats. J. Nutr. 132: 190-196.

Smith, B. J., Lerner, M. R., Bu, S. Y., Lucas, E. A., Hanas, J. S., Lightfoot, S. A., Postier, R. G., Bronze, M. S., Brackett, D. J. (2006) Systemic bone loss and induction of coronary vessel disease in a rat model of chronic inflammation. Bone 38: 378-386.

Soung DY, Devareddy L, Khalil DA, Hooshmand S, Patade A, Lucas EA, Arjmandi BH (2006) Soy affects trabecular microarchitecture and favorably alters select bone-specific gene expressions in a male rat model of osteoporosis. Calcif. Tissue Int. 78: 385-391.

Speyer, C. L., Rancilio, N. J., McClintock, S. D., Crawford, J. D., Gao, H. W., Sarma, J. V., Ward, P. A. (2005) Regulatory effects of estrogen on acute lung inflammation in mice. Am. J. Physiol. - Cell Physiology 288: C881-C890.

Stacewicz-Sapuntzakis, M., Bowen, P. E., Hussain, E. A., Damayanti-Wood, B. I., Farnsworth, N. R. (2001) Chemical composition and potential health effects of prunes: A functional food? Crit. Rev. Food Sci. Nutr. 41: 251-286.

Staniforth, V., Chiu, L. T., Yang, N. S. (2006) Caffeic acid suppresses UVB radiation-induced expression of interleukin-10 and activation of mitogen-activated protein kinases in mouse. Carcinogenesis 27: 1803-1811.

Stein, B. and Yang, M. X. (1995) Repression of the Interleukin-6 Promoter by Estrogen-Receptor Is Mediated by Nf-Kappa-B and C/Ebp-Beta. Mol. Cell. Biol. 15: 4971-4979.

Stone, K. L., Duong, T., Sellmeyer, D., Cauley, J., Wolfe, R., Cummings, S. R. (1999) Broccoli may be good for bones: Dietary vitamin K-1, rates of bone loss and risk of hip fracture in a prospective study of elderly women. J. Bone Miner. Res. 14: S263-S263.

Stoscheck, C. M. (1990) Quantitation of Protein. Methods Enzymol. 182: 50-68.

Strause, L., Saltman, P., Glowacki, J.(1987) The effect of deficiencies of manganese and copper on osteoinduction and on resorption of bone particles in rats. Calcif. Tissue Int. 41: 145-150.

Suda, K., Udagawa, N., Sato, N., Takami, M., Itoh, K., Woo, J. T., Takahashi, N., Nagai, K. (2004) Suppression of osteoprotegerin expression by prostaglandin E-2 is crucially involved in lipopolysaccharide-induced osteoclast formation. J. Immunol. 172: 2504-2510.

Sudo, H., Kodama, H. A., Amagai, Y., Yamamoto, S., Kasai, S. (1983) Invitro Differentiation and Calcification in A New Clonal Osteogenic Cell-Line Derived from Newborn Mouse Calvaria. J. Cell Biol. 96: 191-198.

Sugihara, A., Yamada, N., Tsujimura, T., Iwasaki, T., Yamashita, K., Takagi, Y., Tsuji, M., Terada, N. (2001) Castration induces apoptosis in the male accessory sex organs of Fas-deficient lpr and Fas ligand-deficient gld mutant mice. In Vivo 15: 385-390.

Szulc, P., Chapuy, M. C., Meunier, P. J., Delmas, P. D. (1993) Serum Undercarboxylated Osteocalcin Is A Marker of the Risk of Hip Fracture in Elderly Women. J. Clin. Invest. 91: 1769-1774.

Tamatani, M., Morimoto, S., Nakajima, M., Fukuo, K., Onishi, T., Kitano, S., Niinobu, T., Ogihara, T. (1998) Decreased circulating levels of vitamin K and 25-hydroxyvitamin D in osteopenic elderly men. Metabolism-Clin.Exp. 47: 195-199.

Tan, K. B., Harrop, J., Reddy, M., Young, P., Terrett, J., Emery, J., Moore, G., Truneh, A. (1997) Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. Gene 204: 35-46.

Tang, Q. Y., Kukita, T., Ushijima, Y., Kukita, A., Nagata, K., Sandra, F., Watanabe, T., Toh, K., Okuma, Y., Kawasaki, S., Rasubala, L., Teramachi, J., Miyamoto, I., Wu, Z., Iijima, T. (2006) Regulation of osteoclastogenesis by Simon extracts composed of caffeic acid and related compounds: successful suppression of bone destruction accompanied with adjuvant-induced arthritis in rats. Histochem. Cell. Biol. 125: 215-225.

Tatakis, D. N. (1993) Interleukin-1 and bone metabolism: a review. J. Periodontol. 64: 416-431.

Teague, H. S. and Carpenter, L. E. (1951) The demonstration of a copper deficiency in young growing pigs. J. Nutr. 43: 389-399.

Teitelbaum, S. L. (2000) Bone resorption by osteoclasts. Science 289: 1504-1508.

Terry, K. K., Cappon, G. D., Hurtt, M. E., Tassinari, M. S., Gupta, U. (2004) Reproductive toxicity assessment of lasofoxifene, a selective estrogen receptor modulator (SERM), in female rats. Birth Def. Res. Part B-Devel. Repr. Tox. 71: 150-160.

Theoleyre, S., Wittrant, Y., Tat, S. K., Fortun, Y., Redini, F., Heymann, D. (2004) The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytk. Growth Factor Rev. 15: 457-475.

Trivedi, D. P., Doll, R., Khaw, K. T. (2003) Effect of four monthly oral vitamin D-3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. Brit. Med. J. 326: 469-472.

Tu, Q. S., Valverde, P., Chen, J. (2006) Osterix enhances proliferation and osteogenic potential of bone marrow stromal cells. Biochem. Biophys. Res. Comm. 341: 1257-1265.

Tucker, K. L., Chen, H., Hannan, M. T., Cupples, L. A., Wilson, P. W., Felson, D., Kiel, D. P. (2002) Bone mineral density and dietary patterns in older adults: the Framingham Osteoporosis Study. Am. J. Clin. Nutr. 76: 245-252.

Tucker, K. L., Hannan, M. T., Kiel, D. P. (2001) The acid-base hypothesis: diet and bone in the Framingham Osteoporosis Study. Eur. J. Nutr. 40: 231-237.

Tuntawiroon, M., Sritongkul, N., Brune, M., Rossanderhulten, L., Pleehachinda, R., Suwanik, R., Hallberg, L. (1991) Dose-Dependent Inhibitory Effect of Phenolic-Compounds in Foods on Nonheme-Iron Absorption in Men. Am. J. Clin. Nutr. 53: 554-557.

Turner, R. T. and Sibonga, J. D. (2001) Effects of alcohol use and estrogen on bone. Alcohol Res. Health 25: 276-281.

U.S. Census Bureau (2007) World population information. U.S. Census Bureau, Population Division/International Programs Center.

Uaratanawong, S., Deesomchoke, U., Lertmaharit, S., Uaratanawong, S. (2003) Bone mineral density in premenopausal women with systemic lupus erythematosus. J. Rheumatol. 30: 2365-2368.

Van Dyke, T. E. and Serhan, C. N. (2003) Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. J. Dent. Res. 82: 82-90.

van't Hof, R. J., Armour, K. J., Smith, L. M., Armour, K. E., Wei, X. Q., Liew, F. Y., Ralston, S. H. (2000) Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption. Proc. Nat'l. Acad. Sci. U.S.A. 97: 7993-7998.

Vatanparast, H., Baxter-Jones, A., Faulkner, R. A., Bailey, D. A., Whiting, S. J. (2005) Positive effects of vegetable and fruit consumption and calcium intake on bone mineral accrual in boys during growth from childhood to adolescence: the University of Saskatchewan Pediatric Bone Mineral Accrual Study. Am. J. Clin. Nutr. 82: 700-706.

Venken, K., Boonen, S., Van Herck, E., Vandenput, L., Kumar, N., Sitruk-Ware, R., Sundaram, K., Bouillon, R., Vanderschueren, D. (2005) Bone and muscle protective potential of the prostate-sparing synthetic androgen 7 alpha-methyl-19-nortestosterone: Evidence from the aged orchidectomized male rat model. Bone 36: 663-670.

Vergnaud, P., Garnero, P., Meunier, P. J., Breart, G., Kamihagi, K., Delmas, P. D. (1997) Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: The EPIDOS study. J. Clin. Endocr. Metab. 82: 719-724.

Vermeer C, Wolf J, Craciun AM, Knapen MH (1998) Bone markers during a 6-month space flight: effects of vitamin K supplementation. J. Gravit. Physiol. 5: 65-69.

Vermeer, C., Knapen, M. H. J., Jie, K. S. G., Grobbee, D. E. (1992) Physiological Importance of Extrahepatic Vitamin-K Dependent Carboxylation Reactions. Annal. N.Y. Acad. Sci. 669: 21-33.

Verrier, S., Hogan, A., Mckie, N., Horton, M. (2004) ADAM gene expression and regulation during human osteoclast formation. Bone 35: 34-46.

Vico, L., Collet, P., Guignandon, A., Lafage-Proust, M. H., Thomas, T., Rehaillia, M., Alexandre, C. (2000) Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. Lancet 355: 1607-1611.

von Stechow, D., Zurakowski, D., Pettit, A. R., Muller, R., Gronowicz, G., Chorev, M., Otu, H., Libermann, T., Alexander, J. M. (2004) Differential transcriptional effects of PTH and estrogen during anabolic bone formation. J. Cell. Biochem. 93: 476-490.

WactawskiWende, J., Grossi, S. G., Trevisan, M., Genco, R. J., Tezal, M., Dunford, R. G., Ho, A. W., Hausmann, E., Hreshchyshyn, M. M. (1996) The role of osteopenia in oral bone loss and periodontal disease. J. Periodon. 67: 1076-1084.

Wallach, S., Avioli, L. V., Feinblatt, J. D., Carstens, J. H., Jr. (1993) Cytokines and bone metabolism. Calcif. Tissue Int. 53: 293-296.

Wang, B. L., Dai, C. L., Quan, J. X., Zhu, Z. F., Zheng, F., Zhang, H. X., Guo, S. Y., Guo, G., Zhang, J. Y., Qiu, M. C. (2006) Parathyroid hormone regulates osterix and Runx2 mRNA expression predominantly through protein kinase A signaling in osteoblast-like cells. J. Endocrin. Invest. 29: 101-108.

Wang, L., Orhii, P. B., Banu, J., Kalu, D. N. (2001) Effects of separate and combined therapy with growth hormone and parathyroid hormone on lumbar vertebral bone in aged ovariectomized osteopenic rats. Bone 28: 202-207.

Wattel, A., Kamel, S., Mentaverri, R., Lorget, F., Prouillet, C., Petit, J. P., Fardelonne, P., Brazier, M. (2003) Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. Biochem. Pharmacol. 65: 35-42.

Wattel, A., Kamel, S., Prouillet, C., Petit, J. P., Lorget, F., Offord, E., Brazier, M. (2004) Flavonoid quercetin differentiation decreases osteoclastic induced by RANKL via a mechanism involving NF kappa B and AP-1. J. Cell Biochem. 92: 285-295.

Wei, S., Kitaura, H., Zhou, P., Ross, F. P., Teitelbaum, S. L. (2005) IL-1 mediates TNF-induced osteoclastogenesis. J. Clin. Invest.115: 282-290.

Williams, F. M. K., Cherkas, L. F., Spector, T. D., MacGregor, A. J. (2005) The effect of moderate alcohol consumption on bone mineral density: a study of female twins. Annal. Rheumat. Dis. 64: 309-310.

Wimalawansa, S. J. (2000) Restoration of ovariectomy-induced osteopenia by nitroglycerin. Calcif. Tissue Int. 66: 56-60.

Woo, J. T., Nakagawa, H., Notoya, M., Yonezawa, T., Udagawa, N., Lee, I. S., Ohnishi, M., Hagiwara, H., Nagai, K. (2004) Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. Biol. Pharm. Bull. 27: 504-509.

World Health Organization (1994) World health Organization: Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of the WHO Study Group 843: 1-129.

Wu, C. H., Yang, Y. C., Yao, W. J., Lu, F. H., Wu, J. S., Chang, C. J. (2002) Epidemiological evidence of increased bone mineral density in habitual tea drinkers. Arch. Int. Med. 162: 1001-1006.

Yang, C. S. and Landau, J. M. (2000) Effects of tea consumption on nutrition and health. J. Nutr. 130: 2409-2412.

Yao, W., Jee, W. S. S., Zhou, H., Lu, J., Cui, L., Setterberg, R., Liang, T., Ma, Y. (1999) Anabolic effect of prostaglandin E-2 on cortical bone of aged male rats comes mainly from modeling-dependent bone gain. Bone 25: 697-702.

Zapf, J. and Froesch, E. R. (1986) Insulin-like growth factors/somatomedins: structure, secretion, biological actions and physiological role. Horm. Res 24: 121-130.

Zhao, S., Kato, Y., Zhang, Y., Harris, S., Ahuja, S. S., Bonewald, L. F. (2002) MLO-Y4 osteocyte-like cells support osteoclast formation and activation. J. Bone Miner. Res.17: 2068-2079.

Zheng, H., Yu, X., Collin-Osdoby, P., Osdoby, P. (2006) RANKL stimulates inducible nitric-oxide synthase expression and nitric oxide production in developing osteoclasts - An autocrine negative feedback mechanism triggered by RANKL-induced interferon-beta via NF-kappa B that restrains osteoclastogenesis and bone resorption. J. Biol. Chem. 281: 15809-15820.

Zou, W. and Bar-Shavit, Z. (2002) Dual modulation of osteoclast differentiation by lipopolysaccharide. J. Bone Miner. Res. 17: 1211-1218.

APPENDIX A

SUPPLEMENTAL MATERIALS FOR CHAPTER III

Table 1. Diet composition (g/kg diet) for the control (AIN-93) and dried plum diet

Figure 1. Bone mRNA expression of A) ALP, B) TRAP, C) COL-I, D) IGF-I, E) Runx2, F) RANKL, and G) OPG following 90 days of consumption of control diet (ORX-Control), dried plum (ORX-DP) or parathyroid hormone (ORX-PTH). Values were normalized to the expression of GAPDH. Bars represent the mean ± SE for each treatment group. Bars that share the same superscript letter are not significantly (p <0.05) different from each other (n=3-6/group).

Table 1. Diet composition (g/kg diet) for the control (AIN-93) and dried plum diets

| Ingredients	Control (g/kg diet)	Dried plum (g/kg diet)
Carbohydrate		
Total	720.7	720.7
Cornstarch	465.7	265.7
Maltodextrin	155.0	155.0
Sucrose	100.0	100.0
Dried plum	---	250.0
Protein		
Total	140.0	140.0
Casein	140.0	132.5
Dried plum	---	7.5
Fat		
Total	40.0	40.0
Soybean Oil	40.0	38.75
Dried plum	---	1.25
Fiber		
Total	50.0	50.0
Cellulose	50.0	27.5
Dried plum	---	22.5
Vitamin Mix	10.0	10.0
Mineral Mix		
Total	35.0	35.0
Mineral mix (Ca-P deficient)	13.4	13.4
Ca carbonate	9.88	9.43
Ca from dried plum	---	0.18
K phosphate	5.6	5.0
Na phosphate	3.44	2.84
P from dried plum	---	0.27
K citrate	0.9	0.9
Sucrose	1.78	2.98
L-cystein	1.8	1.8
Caloric density (kJ/g diet)	17.6	17.4

Figure 1

A)
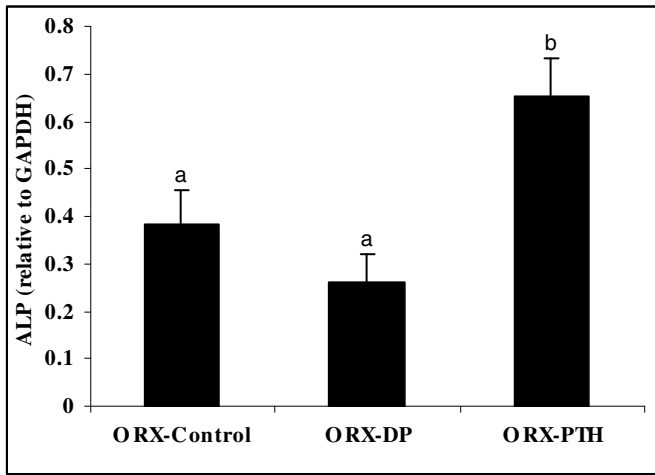
B)
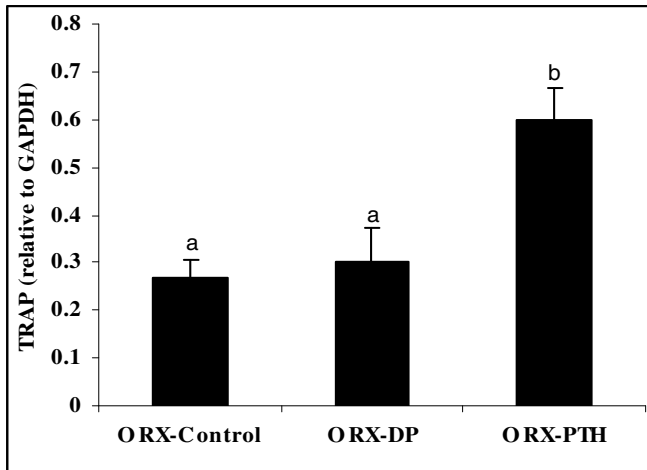
C)
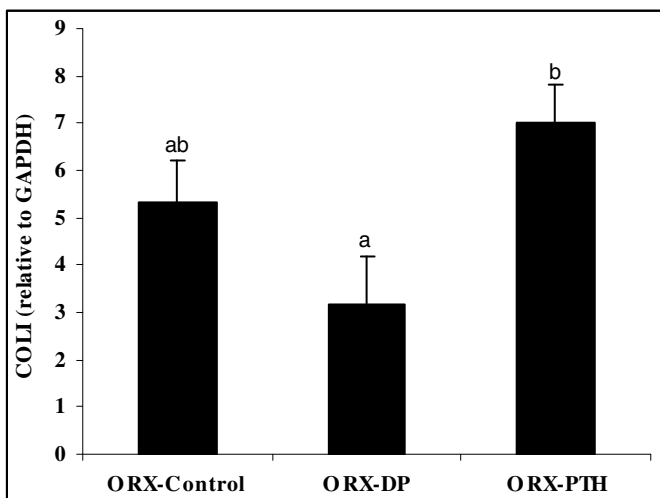
D)
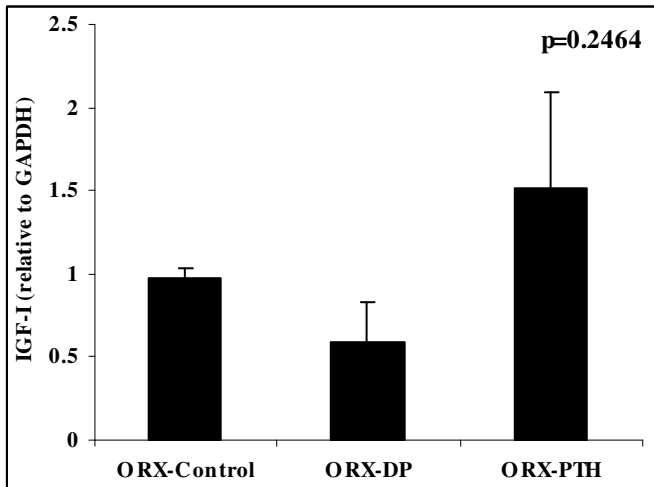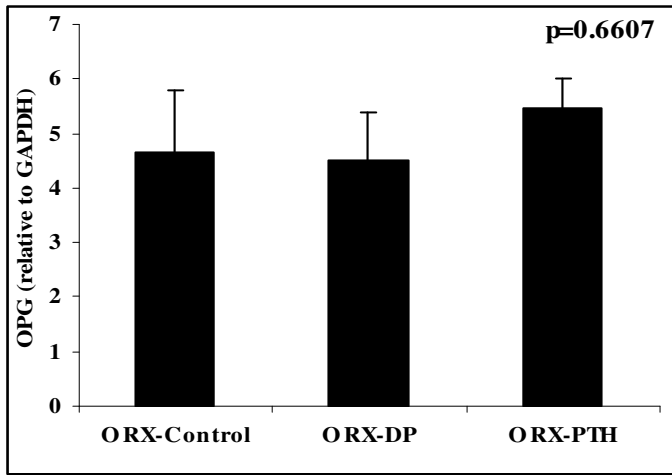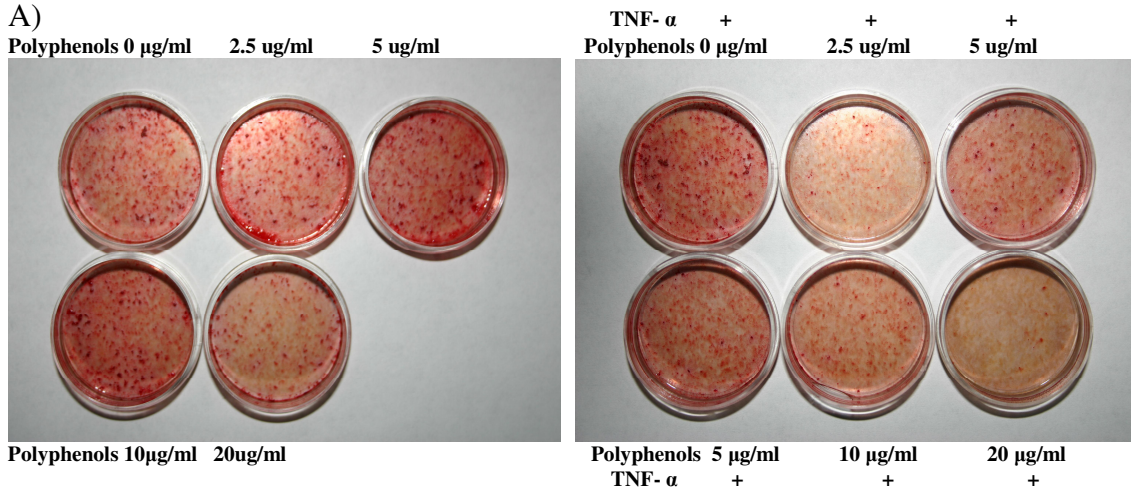

E)
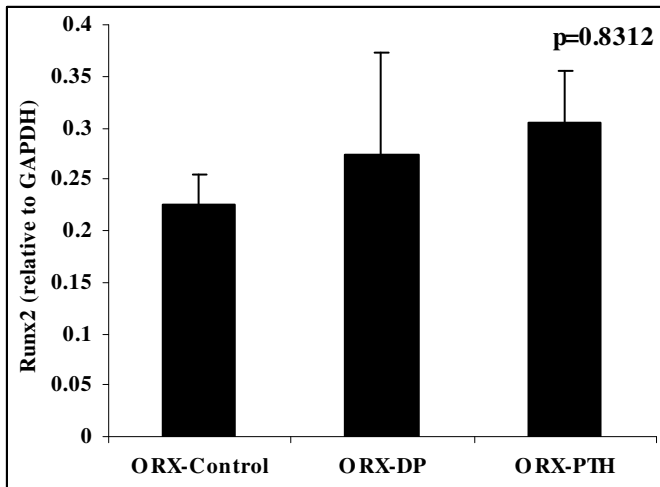
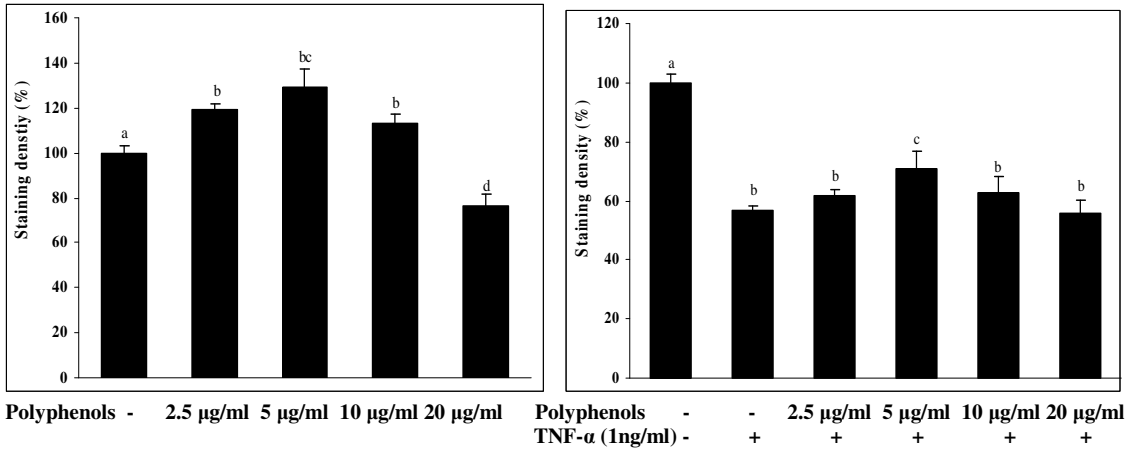
F)
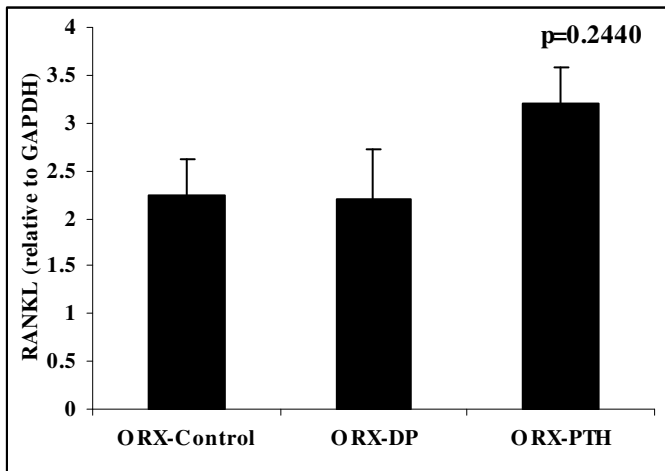
G)

APPENDIX B

SUPPLEMENTAL MATERIALS FOR CHAPTER V

Figure 1. Effect of dried plum polyphenols on mineralized nodule formation at 18 days
Dried plum polyphenols increase mineralized nodule formation as indicated by Alizarin red S staining (red color) density and number of mineralized nodule under inflammatory and normal 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 alone or stimulated with TNF-α (1 ng/ml) for 18 days. Bars that share the same letters are not significantly different from each other.

Figure 2. Microscopic view of mineralized nodules in MC3T3 cells
Dried plum polyphenols increase mineralized nodule formation as indicated by number and size of mineralized nodule under normal (A) and inflammatory (B) 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 alone or stimulated with TNF-α (1 ng/ml) for 28 days. Mineralized nodules were photogaphed under the bright field microscope.

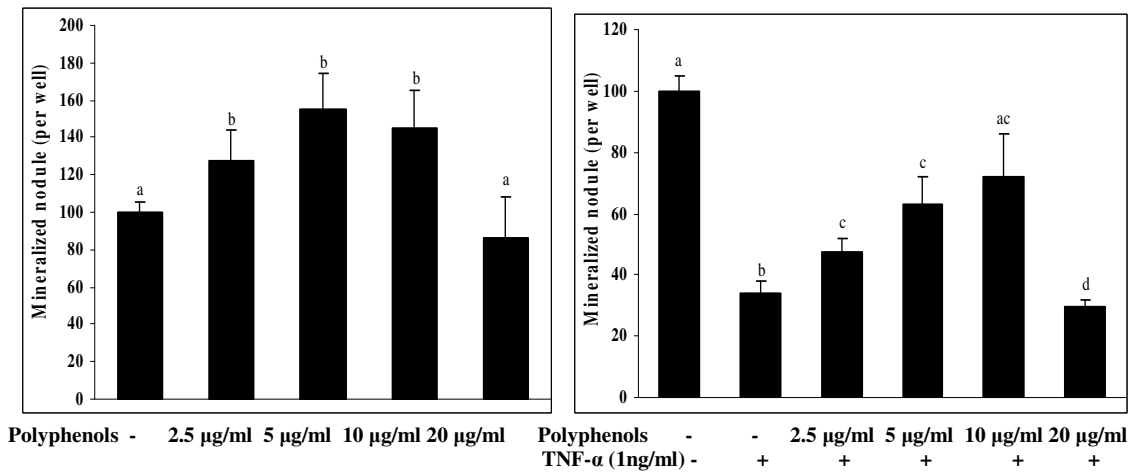## Figure 1. Nodule formation at 18days

A)

Polyphenols 0 µg/ml   2.5 ug/ml   5 ug/ml

Polyphenols 10µg/ml   20ug/ml

TNF- α   +   +   +
Polyphenols 0 µg/ml   2.5 ug/ml   5 ug/ml

Polyphenols 5 µg/ml   10 µg/ml   20 µg/ml
TNF- α   +   +   +
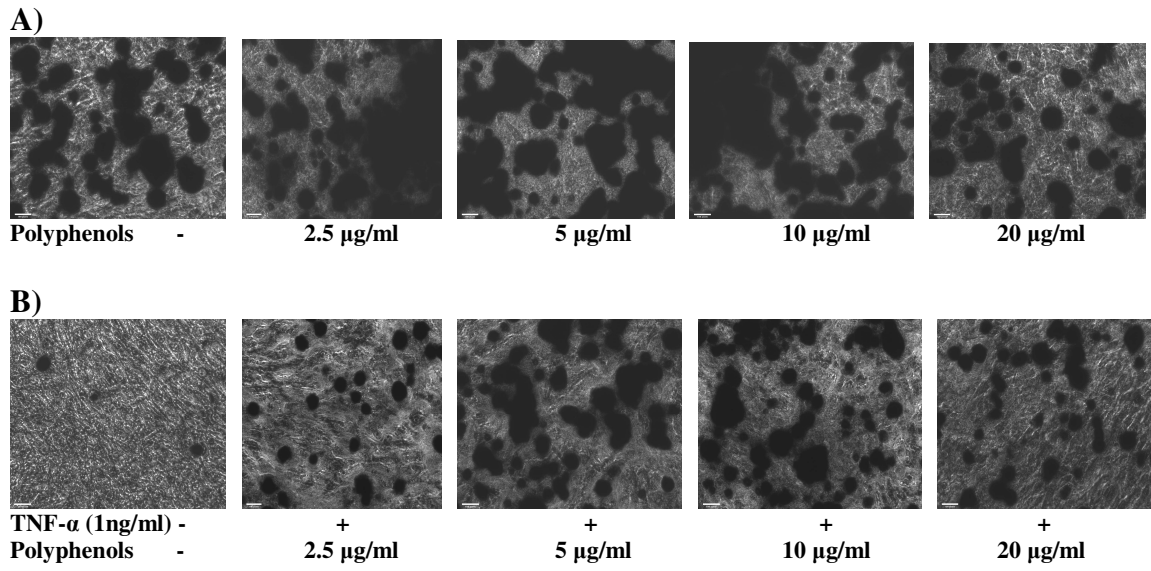
B) AR-S staining density

C) Number of mineralized nodule

Figure 2. Microscopic view of mineralized nodules at 28days

A)

| Polyphenols | - | 2.5 µg/ml | 5 µg/ml | 10 µg/ml | 20 µg/ml |

B)

| TNF-α (1ng/ml) | - | + | + | + | + |
| Polyphenols | - | 2.5 µg/ml | 5 µg/ml | 10 µg/ml | 20 µg/ml |

Oklahoma State University
Institutional Animal Care and Use Committee (IACUC)

Protocol Expires: 2/2/2006

Date : Monday, November 03, 2003   Animal Care and Use Protocol (ACUP) No: HE032

Proposal Title: Can dried plum prevent bone loss in a male model of osteoporosis?

Principal
Investigator:

Brenda J. Smith
Nutritional Sciences
423 HES
Campus

Reviewed and
Processed as: Full Committee   **Modification**

Approval Status Recommended by Reviewer(s) : Approved

_____

Modification approved permitting the transfer and use of 24 rats from HE-02-5.

Signatures :

Dr. Kent Olson, IACUC Chairperson   Monday, November 03, 2003
                                                     Date

cc: Department Head, Nutritional Sciences
       Research Director

VITA

So Young Bu

Candidate for the Degree of

Doctor of Philosophy

Thesis: BONE PROTECTIVE EFFECTS OF DRIED PLUM AND ITS
         POLYPHENOLS UNDER INFLAMMATORY AND OXIDATIVE
         STRESS CONDITIONS

Major Field: Nutritional Sciences

Biographical:

    Personal Data: Born in Jeju, Korea, on November 30, 1975, the daughter of
        Sung Dam Bu and Yun Sook Ko.

    Education: Graduated from Shinseong Girls' High School, Jeju, Korea in
        February 1994; received Bachelor of Science degree in Food and
        Nutrition from Sookmyung Women's University, Seoul, Korea in
        February 1998; received Master of Science degree in Food and Nutrition
        from Sookmyung Women's University, Seoul, Korea in February 2000;
        completed the requirements for the Doctor of Philosophy degree with a
        major in Nutritional Sciences at Oklahoma State University, May, 2007.

    Experience: Graduate research/teaching assistant in Department of Food and
        Nutrition at the Sookmyung Women's University, Seoul, Korea, 1998-
        1999; Graduate research assistant in department of Crop Science at the
        Korea University, Seoul, Korea, 2000-2002; Graduate research assistant
        in Department of Nutritional Sciences at Oklahoma State University,
        Stillwater, Oklahoma, 2002-present.

    Professional/Academic Memberships: Kappa Omicron Nu, American Society of
        Bone and Mineral Research, American Society of Nutritional Sciences,
        The Korean Society for Molecular and Cellular Biology.

Name: So Young Bu   Date of Degree: May, 2007

Institution: Oklahoma State University   Location: Stillwater, Oklahoma

Title of Study: BONE PROTECTIVE EFFECTS OF DRIED PLUM AND ITS
                       POLYPHENOLS UNDER INFLAMMATORY AND OXIDATIVE
                       STRESS CONDITIONS

Pages in Study: 202   Candidate for the Degree of Doctor of Philosophy

Major Field: Nutritional Sciences

Scope and Method of Study: The purpose of this study was to utilize the combination of
    in vivo and *in vitro* studies to investigate the effects of dried plum (DP) on bone and
    bone cell activity under conditions of elevated pro-inflammatory mediators. In Study
    1, 9-month-old male osteopenic orchidectomized rats were randomly assigned to
    control (AIN-93M) or DP diet (25%, w/w) for 90 days. The positive control group
    consumed the control diet and received PTH (80μg/kg). In Study 2, macrophages
    (RAW 264.7) were pretreated with the polyphenols extracted from DP (0, 10, 20, or
    30 μg/ml) cultured with or without RANKL and then stimulated with either LPS or
    hydrogen peroxide (H_2O_2). TRAP-positive osteoclasts and resorption pit formation
    were evaluated and inflammatory mediators such as NO and TNF-α by macrophages
    and during their differentiation into osteoclasts were assessed. In Study 3, MC3T3-E1
    cells, murine pre-osteoblasts, were utilized to determine how DP polyphenols
    influence osteoblast activity, function and signaling under normal and inflammatory
    conditions. Cells were pretreated with polyphenols (0, 2.5, 5, 10 and 20 μg/ml) prior
    to stimulation with TNF-α (0 or 1.0 ng/ml). ALP activity and mineralized nodule
    formation were measured and alterations in gene expression of RANKL, OPG,
    Runx2, Osterix and IGF-I were analyzed.

Findings and Conclusions: Study 1 showed that dietary supplementation with DP
    restored the ORX-induced loss of bone mass and deterioration of bone structure. The
    restoration of bone microarchitecture by dried plum was comparable to PTH. Study 2
    revealed that DP polyphenols inhibit osteoclastogenesis and osteoclast activity which
    may be mediated by the down-regulation of cytokines such as TNF-α and NO. Study
    3 showed that DP polyphenols enhance osteoblast activity and mineralized nodule
    formation under normal and inflammatory conditions. Results *in vitro* studies suggest
    that dried plum which has ability to down-regulate inflammatory mediators, has
    potent effects on osteoclast and osteoblast activity and provide possible mechanisms
    by which dried plum preserves bone mass and microarchitecture as reported in
    current and previous *in vivo* studies.

ADVISER'S APPROVAL: Dr. Brenda J. Smith