CAN MANGO REVERSE BONE LOSS IN OVARIECTOMIZED MICE?

By

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CAN MANGO SUPPLEMENTATION REVERSE BONE LOSS IN OVARIECTOMIZED MICE?

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CHAPTER I

INTRODUCTION

Osteoporosis and osteopenia are major health threats to more than 50% of the female American population over age 50 [1]. An estimated 44 million Americans are affected by osteoporosis and osteopenia. Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue, resulting in poor bone quality, reduced bone strength, and an increased risk for bone fracture, particularly in the hip, spine and wrist [1, 2].

Fractures of the hip often require hospitalization and surgery and can impair an individual's ability to walk, create a permanent disability, and may even result in death [1, 3]. Vertebral fractures can cause severe back pain, deformity and loss of height [1]. In the USA, approximately 1.5 million fractures are attributable to osteoporosis every year [3] and $19 billion dollars were spent on osteoporosis-related fractures in 2005 [1]. It is predicted that the incidence of osteoporotic fractures will reach catastrophic proportions with a four-fold increase of osteoporosis worldwide over the next 50 years [3]. The costs associated with bone fractures will threaten the viability of health care systems in many countries [3] indicating an emergent need for preventative action.

Postmenopausal osteoporosis accounts for 80% of osteoporosis cases that develop [4]. The decline in sex steroids that occurs with the loss in ovarian function at menopause results in

changes in calcium homeostasis resulting in a negative calcium balance [5]. Estrogen deficiency also increases the production of inflammatory cytokines resulting in a further increase in bone resorption [4, 6, 7]. Considering the high prevalence of postmenopausal osteoporosis and the associated decline in sex hormone production, an option to prevent or reverse postmenopausal osteoporosis is needed. For this study, the ovariectomized mouse is used as a model to emulate the hormonal effects in a postmenopausal state.

Lifestyle modifications through diet may be a significant approach in preventing the development of osteoporosis [1]. Although much research has been conducted on the beneficial effects of dairy products and vitamin D in preventing bone loss, there are some important factors to consider with widespread dairy promotion as a dietary approach to bone health. The first issue is that over two thirds of the world's adult populations (approximately 30 to 50 million Americans; 75 % of African Americans and Native Americans and 90 % of Asian Americans) are lactose intolerant [8]. Also, with the recommended daily allowance (RDA) of calcium set at 1200 mg per day for older adults, many individuals find it difficult to meet calcium requirements through dietary intake. With milk being an excellent source of calcium, it can be challenging for individuals to drink four glasses of milk a day. Another interesting point to consider is that the USA is one of world's greatest consumers of dairy products, yet globally, has one of the highest rates of osteoporosis [3]. When considering these facts, the need for research into other nutrients that may promote bone growth and/or protect an individual from age- or menopause-induced bone loss becomes evident.

Another factor promoting further research into dietary approaches for osteoporosis prevention is the fact that the commonly used osteoporotic medications, although effective, are associated with undesirable side effects. For example, bisphosphonates have been associated

with bone, joint and/or muscle pain and most commonly reported, stomach upset and heartburn [9] . Although uncommon with typical health care administration of the drug, a rapid injection of bisphosphonates can cause renal failure[9]. Oral administration of bisphosphonates, particularly with amines, can cause esophageal and gastrointestinal distress such as nausea, dyspepsia, vomiting, gastric pain, diarrhea, and ulceration[9]. Estrogen therapy, although not encouraged due to its serious side effects, has been associated with endometrial and breast cancer, vaginal bleeding, breast tenderness, gallbladder disease, strokes, heart attacks, venous blood clots and cognitive decline [1]. Selective estrogen receptor modulators (SERM), such as Raloxifene, are more commonly used and do not have the majority of side effects common to estrogen therapy but may induce hot flashes, blood clots in the legs or lungs, and strokes in those with coronary artery disease[10]. Teriparatide, a recombinant parathyroid treatment, has been associated with leg cramps, headache and myalgia[11]. In addition to these side effects, long term oral drug compliance can be challenging for many. Research has shown that women taking oral bisphosphonates stop treatment or take less than what was prescribed, reducing the effectiveness of the medication. Hence, there is a need for alternative prevention strategies with fewer side effects. Dietary interventions that are effective, inexpensive, and can easily be incorporated into an individual's daily diet deserves further research.

Consumption of fruits and vegetables or the phytonutrients derived from them has been associated with reduced risk of osteoporosis in human observational studies or an increase in bone mineral density (BMD) in animals [12], [13] [14, 15] [16], [17],[18]. Fruits and vegetables contain a wide variety of vitamins and minerals as well as bioactive phytochemicals which may contribute to bone health. Phytochemicals are non-nutritive food chemicals that the plant uses to protect itself from the external environment. These same chemicals also may help to prevent disease in

humans by functioning as an antioxidant or hormone, stimulate enzymes, interfere with DNA replication or have an antibacterial affect [19]. Fruits and vegetables have antioxidant and anti-inflammatory properties and reduce the renal acid load, actions which may also function as bone-sparing agents [20-24], [25]. Aging and sex hormone related changes cause inflammation and pro-oxidant conditions in the bone environment which are involved in the development of osteoporosis [26]. Women with osteoporosis have also been shown to have lower levels of plasma dietary and endogenous antioxidant vitamins (i.e., vitamins C, E, and A) and the lower enzymatic activities of superoxide dismutase and glutathione peroxidase when compared to healthy controls[27]. The alkalizing, antioxidant and anti-inflammatory properties of fruits and vegetables are just a few mechanisms by which bioactive food components may play a protective role in preventing bone loss.

Among the fruits, mangos (*Mangifera indica L*) are an excellent source of vitamin C, and carotenoids, and are good sources of dietary fiber, potassium, copper, and vitamin K [28]. Mangos also contain minerals such as magnesium, zinc, copper, manganese, and selenium [29]. Mangos contain carotenoids, triterpenes, and phenolic compounds such as tannins, mangiferin and flavonoids which may contribute to its antioxidant and anti-inflammatory properties. Considering the bioactive compounds found in mango, it is hypothesized that mango may offer some dietary support in preventing and reversing hormone-related bone loss.

The **objective** of this study is to determine if the addition of 5% or 25% freeze-dried mango powder to the daily diet would reverse bone loss in ovariectomized mice. Our **null hypotheses** are that:

1) Mango supplementation will not reverse the loss of bone mineral content (BMC) and bone mineral density (BMD) due to ovariectomy;

2) Trabecular and cortical bone micro-architectural parameters, assessed using micro-computed tomography analyses (μCT), will not be affected by mango supplementation;

3) Bone strength, assessed using finite element analysis, will not be increased by mango supplementation; and

4) Biomarkers of bone formation (i.e., plasma N-terminal propeptide of procollagen type 1 (PINP)) and bone resorption (i.e., plasma pyridinoline (PYD)) will not be affected by mango supplementation.

Identifying dietary factors that can reverse bone loss in experimental animals would establish a strong scientific framework for subsequent clinical trials in humans. The findings of this proposed study will enable us to investigate the efficacy of mango as a novel nutritional strategy to reverse osteoporosis. Furthermore, this study will provide fundamental knowledge related to the health benefits of mango on body composition. Findings of this study will provide a basis for consuming fruits to help combat postmenopausal bone loss. If mango treatment shows effectiveness in reversing bone loss, these findings could potentially help the nation save millions of dollars spent on the treatment of osteoporotic fractures and bone-protecting pharmaceuticals.

CHAPTER II

REVIEW OF LITERATURE

Prevalence of Osteoporosis

By 2020, it is forecasted that there will be over 47 million cases of low bone density in the US and 14 million cases of osteoporosis [1]. In the US, $17 billion was spent on over 2 million fractures in 2005. The majority of these fractures occurred in individuals over 65 years of age. Seventy three percent of these fractures were non-vertebral fractures and accounted for the majority of the cost expenditure. By 2025, annual fractures are projected to increase by 50% with an estimated cost of $25 billion [30]. The increase in the number of hip fractures per year over the past 30 years is partly attributed to the increase in lifespan [31].

Age is not the only important osteoporotic risk factor to consider. Gender and ethnicity also influence bone density. Osteoporotic fractures affect 1 in 3 women and 1 in 5 men over the age of 50 [3, 32]. In the US, over half of all postmenopausal Caucasian women are considered osteopenic with an additional 30% having osteoporosis [3]. The tendency for fracture is higher in the US Caucasian population and lower for other ethnic groups [33]. By the age of 80, up to 70% of white women have osteoporosis and 27% are considered osteopenic [3]. Cauley and colleagues[34] reported that Caucasian women are twice as likely to have hip BMD loss when compared to African-American women with an increase in hip BMD loss with age in both groups.

Bone Remodeling

Bone remodeling occurs as a continuous bone renewal process throughout the life span. In the bone remodeling process, old weakened bone is replaced by mechanically strong bone. A healthy balance is maintained between bone formation and bone resorption. Bone resorbing cells, known as osteoclasts (OC), and bone forming cells, known as osteoblasts (OB), work interdependently in the bone remodeling process [35]. With hormonal and cytokine signaling, OCs degrade bone to liberate mineral ions such as calcium and phosphorous needed by the body to replace old or damaged bone cells. The OC creates an acidic environment with proton pumps and uses enzymes such as tartrate-resistant acid phosphatase and cathepsin K to catabolize the bone matrix minerals and organic components forming resorption pits known as Howship's lacunae[36]. When the resorption phase is complete, the OC under goes apoptosis. Initiating the bone renewal phase are coupling factors such as bone morphogenetic proteins, transforming growth factor-β (TFG-β), insulin like growth factor (IGF)-I and II, platelet derived growth factors and fibroblast growth factor that signal OBs and OB precursor cells to the Howship's lacunae[37]. The OBs then synthesizes the organic bone matrix by secreting osteoid and regulate its mineralization by secreting matrix vesicles which concentrate calcium and phosphorous ions and enzymatically degrade inhibitors of mineralization such as pyrophosphate and proteoglycans[38]. As bone renewal process terminates, OBs can undergo apoptosis, become entombed in the bone matrix and become osteocytes, or become bone-lining cells. When the balance between the bone remodeling of OCs and OBs is uncoupled in favor of bone resorption and osteoclastogenesis, osteopenia or osteoporosis may develop, weakening the bone, and hence, predisposing it to an increased risk for fracture [35].

Osteoblasts and Osteocytes

Bone formation can be categorized in 3 stages; OB proliferation, extracellular matrix development and maturation, and mineralization [39]. Identification of the bone formation stages can be determined by serum biomarkers. For example, alkaline phosphatase and parathyroid hormone 1 receptor (PTH1R) are considered early markers of OB proliferation stage, while serum procollagen I N-terminal propeptide (PINP) and osteocalcin are considered marker of extracellular matrix development and bone mineralization, respectively[40].

For bone formation to occur, OBs need to develop and mature before they can participate in the bone building process. Mesenchymal stem cells in the bone marrow develop in to progenitor cells which may become adipocytes, myocytes, chondrocytes, fibroblasts, stromal cells or OBs. The canonical Wnt/β-catenin pathway indirectly mediates the initial cascade of gene expression for skeletal development, bone formation and OB differentiation. Wnt10b shifts the mesecnchymal stem cells fate to the OB lineage by suppressing adipogenic transcription factors and inducing osteogenic transcription factors such as the runt-related transcription factor 2 (Runx2), D1x5 and Osterix [39, 41]. These pathways are induced in response to extra cellular stimuli such as bone morphogenetic proteins, growth factors, hormones, cytokines, matrix proteins, transcription factors, regulatory co-factors and environmental stress [39].

An active osteoblast is distinguishable by its large nucleus and golgi apparatus, well developed endoplasmic reticulum, and high concentration of alkaline phosphatase enzyme activity in the proliferation stage[40]. OBs are anabolic to bone and function by developing the extracellular bone matrix and directing its mineralization. The OBs secrete a fibrous, non-mineralized protein substance known as osteoid, which contains chondroitin sulfate, osteocalcin and type I collagen and forms the bone matrix. Osteocalcin, osteopontin and bone sialoprotein,

also secreted by the OB, serve to mineralize the extracellular matrix, by binding calcium and phosphates, and regulates both the amount and size of hydroxyapatite crystals in bone [39].

OBs, at the terminal differentiation stage, may develop into osteocytes. Osteocytes are bone cells found throughout the bone matrix which support the structure and metabolic functions of bone [39]. The cellular connection within the bone plays an important role in building strong bones. Cell-to-cell and cell-to-matrix interactions are maintained by adhesion and trans-membrane proteins. Surface lining bone cells, which are composed of flattened inactive OBs, separate the bone from the marrow[39]. Communication exists between the lining cells and the OBs via adherens junctions. In these junctions are cadherins, calcium dependent trans-membrane proteins, which assist in anchoring the surface lining and OB cells through their cytoskeleton [39]. N-cadherin, mediated by bone morphogenetic protein (BMP)2, functions in cell-cell adhesion [39]. These type of cell-cell interactions encourage osteoprogenitor differentiation and OB survival[42]. Integrin, an adhesion protein, joins the bone's extracellular matrix to the structural proteins in the cytoskeleton, and induces OB differentiation, through the mitogen-activated protein kinase (MAPK) pathway[39]. Connexins, integral membrane proteins at gap junctions, allow the osteocytes to communicate with each other in response to mechano-stimulation. Mechano-sensory information stimulates signaling pathways for OB gene transcription.

Osteoclasts

The bone resorbing OCs are also formed in the bone marrow, but from a monocyte-macrophage myeloid lineage of hemopoietic stem cells [43]. OCs not only function in bone

degradation, but also as immunomodulators in pathological conditions and may also regulate OB function [44].

Cytokines, receptor activator of nuclear factor-kB ligand (RANKL), macrophage colony stimulating factor (M-CSF), and c-Fos, a RANKL activated transcription factor, are necessary for OC differentiation [45]. Both RANKL and M-CSF are produced by bone marrow stromal cells, OBs, T lymphocytes and synovial fibroblasts[46]. RANKL can also be found in other tissues such as in the lymph nodes, thymus gland, lungs, spleen, mammary epithelial cells and in some cancer cells [47]. RANKL is a member of the tumor necrosis super family of proteins and is essential for OC formation. M-CSF contributes to OC precursor differentiation, proliferation, and survival. Osteoclastogenesis can be detected by tartrate-resistant acid phosphatase (TRAP) and proteinase cathepsin K enzyme activity, wich appear 3-5 days after differentiation [48, 49]. Mature OCs can be identified by its large size, approximately 40 micrometer in diameter, multiple nuclei, resulting from cytoplasmic fusion of the precursor monocytes, a "foamy" cytoplasm appearance and extensive golgi complex [50].

OCs attach at resorption sites via actin-rich podosomes securing a tight seal on bone [51]. Integrins act as receptors allowing the OC to bind to the bonding sites in the bone via osteopontin and virtonectin glycoproteins [52]. Approximately 10 – 14 days after OCs attachment to bone, OCs create a pocket which is acidified by a proton pump and a chlorine channel [39]. Hydrochloric acid and proteases degrade the bone matrix while the lysosomal enzyme, cathepsin K, breaks down the mobilized type 1 collagen [39, 48, 50].

The RANKL signaling pathway plays an important role in bone resorption. RANKL, a transmembrane protein on the osteoblast, binds to receptor activator of nuclear factor-kB, RANK, on the OC precursor and OC, increasing OC formation, activity, and bone resoption.

OPG functions to protect bone from breakdown by intercepting RANKL, reducing the rate of OC formation, activity and bone resorption [53] (**Figure 1**). OPG expression is regulated by WNT/β-catenin signaling in OBs [54] and it also expressed in other tissues aside from OBs. Pro-inflammatory cytokines up-regulate RANKL while suppressing the expression of OPG[46].

RANKL stimulates osteoclastogenesis by activating multiple signaling pathways encoding for TRAP, cathepsin K, the calcitonin receptor and β_3-integrin[46]. One influential signaling pathway induces long lasting oscillations of intracellular Ca^{2+} concentration while generating reactive oxygen species (ROS) (**Figure 2**). RANKL mediates stimulation of Rac1, which generates ROS that trigger phospholipase Cγ1 to initiate the Ca^{2+} oscillations. Btk kinase activates phospholipase Cγ1 (PLCγ1), tumor necrosis receptor activator factor 6 (TRAF6), and c-Fos (transcription factor). The Ca^{2+} oscillations then activates calcineurin and nuclear factor of activated T-cells c1 (NFATc1) to stimulate osteoclastogenesis [55] (**Figures 1 & 2**). The role of TRAF6, c-Fos and NFATc1 in the OC precursor nucleus are put in the perspective of the bone remodeling cycle in **Figures 1 and 2**.

Figure 1: Ostoclastogenesis regulation via the receptor activator of nuclear factor-kB ligand (RANKL) signaling pathway [50]

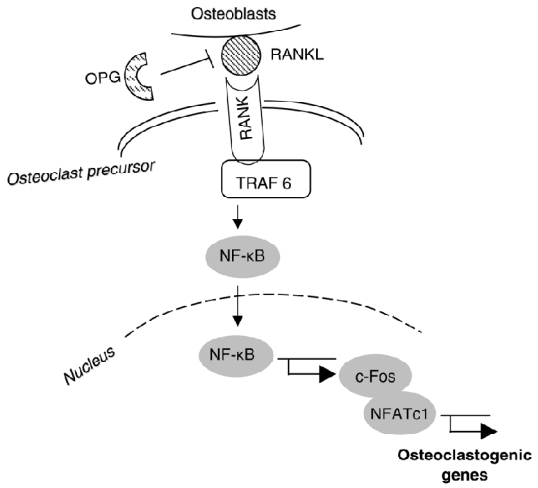
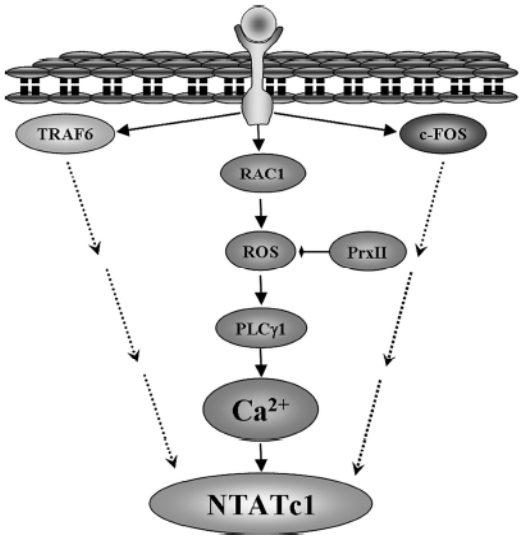


Figure 2: NFATc1 signaling in osteoclastogenesis via ROS and Ca^{2+} oscillations [50]



Factors Affecting Bone Remodeling

The bone remodeling process is regulated by multiple factors. Genetics influence bone remodeling as well as hormones, growth factors and locally produced cytokines [56]. Lifestyle factors such as amount of weight bearing exercise, nutritional status, smoking, alcohol consumption and caffeine intake also affect the bone remodeling process. A disturbance or change in any of the bone regulatory factors can contribute to an uncoupling of the bone remodeling process which can lead to osteoporosis.

Genetics

Genetics play an important role in regulating peak bone mass. Up to 60 -80% of variance in peak bone mass between individuals can be attributed to genetics [56]. Although both elderly men and women lose bone with increasing age, postmenopausal women lose more bone than men [57]. The risk for fracture is doubled in women when compared to men of similar age [57]. Ethnicity also influences BMD and fracture risk. The incidence of reported hip fractures is

lower in Asian than Caucasian populations [58]. Women also vary in terms of the rate and extent of postmenopausal bone loss, some considered fast or slow losers. This variance is in part due to inherited factors. In observational studies, associations between family history of fracture incidence and type of fracture have suggested an inherited correlation in BMD and fracture risk [59-61]. In human and animal studies, high levels of heritability have been shown in bone phenotype as assessed by bone densitometry and ultrasound [62, 63]. Allelic variation in the vitamin D receptor, has shown to influence genetic determination of bone phenotype. The impact of the vitamin D receptor can be mediated by body size and development and by hormonal regulation. Intronic polymorphisms of the collagen Iα1 gene have been related to BMD and fracture risk[57]. Allelic variations in the estrogen receptor, TGFß receptor, TGFß1, insulin-like growth factor-I pathway, IL-4, IL-6, calcitonin, PTHRs and for apolipoprotein E have all been associated with BMD phenotypes [57]. Hormonal factors, nutrition and lifestyle interact with genetic factors over time.

Hormones

Sex steroids, PTH, thyroid hormones, growth hormone, glucocorticoids, and $1,25(OH)_2D_3$ influence the bone remodeling process[64]. Estrogen, have an inhibitory effect on osteoclast function by promoting osteoclast apoptosis [65] and by modulating osteoclast differentiation [66]. Estrogen also decreases the OB progenitor cell population but exerts an anabolic influence on OBs.

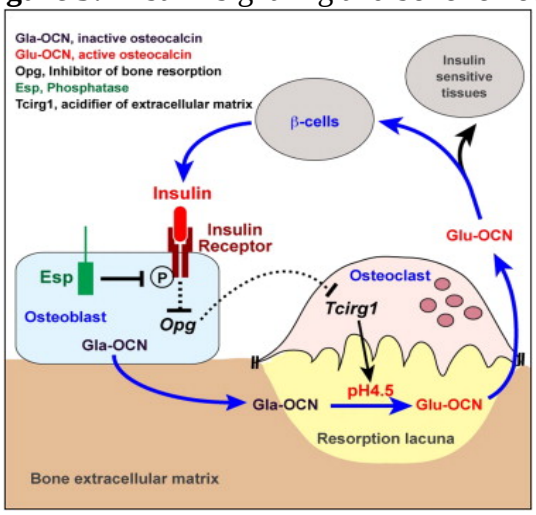
PTH plays a dual role in the bone remodeling process. On one hand, PTH and $1,25(OH)_2D_3$ encourage bone remodeling by increasing the expression of NF- kB, tumor necrosis factor (TNF), RANK, RANKL, and M-CSF [67]. On the other hand, PTH, IGF and other

growth factors stimulate mesenchymal stem cell, osteoprogenitor and osteoblast differentiation. The cell membrane of the parathyroid gland has calcium sensing receptors which regulate PTH secretion. Low serum calcium signals the parathyroid gland to secrete more PTH which enhances renal tubule reabsorption of calcium, increases bone resorption, and increases intestinal calcium absorption indirectly by increasing 1α, $25(OH)_2$ D_3 synthesis in the kidneys [68].

Calcitonin is a peptide hormone produced by the C cells of the thyroid gland which acts to lower blood calcium levels when they are too high by inhibiting bone resorption [69]. The secretion of calcitonin is regulated by serum calcium, gender and age [70, 71]. Calcitonin has been manufactured as a pharmaceutical to inhibit osteoclast bone resorption and hypercalcemia [70].

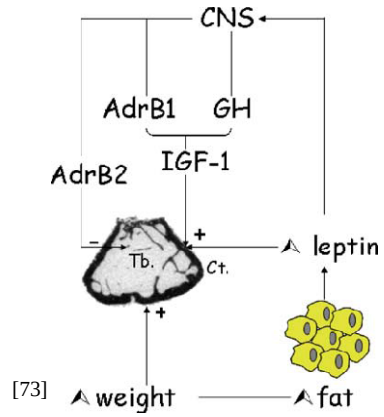
The metabolic hormone, insulin, also regulates bone remodeling. Insulin signaling activates osteoblasts and enhances osteocalcin production[72]. Osteocalcin facilitates bone mineralization and calcium ion homeostasis. Osteocalcin also impacts glucose homeostasis via its interaction with OCs [72]. With the acidic OC environment, osteocalcin, secreted by the osteoblast, is decarboxylated to its active form [72]. Osteocalcin, in turn, mediates insulin secretion and sensitivity (**Figure 3**). Ferron and colleagues (2010)[72] demonstrated that insulin signaling in osteoblasts is a critical link between bone remodeling and energy metabolism.

Figure 3: Insulin signaling and bone remodeling [72]



Leptin, a cytokine-like hormone secreted by adipose tissue, also influences bone remodeling via hypothalamic mediation of the sympathetic nervous system [73]. Leptin has an anabolic effect on osteoblasts and stimulates bone growth indirectly via the central nervous system. Leptin stimulates the growth hormone/ insulin-like growth factor-1 (GH-IGF-1) axis of the central nervous system and suppresses release of neuropeptide Y, an inhibitor of cortical bone formation, from the hypothalamus. The GH-IGF-1 axis stimulation impacts both β1 and β2-adrenergic receptors (**Figure 4**). Although β2- adrenergic stimulation encourages trabecular bone remodeling, β1- adrenergic stimulation increases cortical bone mass. Cortical bone becomes more important when dealing with the excess weight load from increasing amounts of adipose tissue. A study done on leptin deficient mice showed that, not only are leptin deficient mice obese with symptoms of metabolic syndrome, but they also exhibited skeletal abnormalities[74]. Leptin repletion enhanced normal bone growth by increasing femoral length and total bone volume although femoral and vertebral cancellous bone volume decreased [74].

Figure 4 : Leptin's influence on bone [75]



[73]

Ct = cortical bone,;Tb = trabecular bone; GH = growth hormone; AdrB1= adrenergic receptor β1; AdrB2 = adrenergic receptor β2; IGF-1= Insulin like growth factor 1

Cytokines involved in Ovarian Hormone Deficiency

Local cytokines also influence bone remodeling. Immune and bone marrow cells synthesize and secrete cytokines that may have autocrine and paracrine influences on bone metabolism [76, 77]. With lack of estrogen regulation, caused by natural or surgically induced menopause, blood, bone marrow, and monocytic levels of IL-1, IL-6, TNF-α, and the related factors IL-1ra and IL-6R increase [78]. IL-1 and TNF activate OCs indirectly by influencing OB secretory factors such as RANKL and inhibit OC apoptosis. They also enhance OC formation by stimulating the proliferation of the OC precursors and stromal cells by activating IL-6, M-CSF, and granulocyte M-CSF [78].

Lifestyle factors

Nutrition

Nutrition is an important determinant of bone health, yet the effects of nutrients and minerals other than calcium involved in the bone remodeling process are still being elucidated. Bone formation requires adequate supplies of energy, amino acids, and bone-forming minerals such as calcium, phosphorus, magnesium, and zinc.

Adequate amounts of protein are necessary to prevent the development of osteoporosis [79]. Sufficient protein intakes reduced bone loss and fracture risk in the elderly [80]. Excess protein above the recommended allowances, however, can create renal acid load and lead to risks of developing osteoporosis [81]. Excessive renal acid load may be more influential on bone size and mass rather than volumetric bone density [82].

Consuming an alkaline diet may have bone protective effects. Protons are produced during metabolism and are neutralized by the buffering action of anions generated from food or liberated from bone [83]. Since increases in bone resorption lead to greater urinary calcium excretion, a high renal acid load could potentially be associated with increased bone loss in older adults [84]. Foods with a high acid load are those rich in sulfur amino acids, phosphorous or chloride, such as meat, grains, nuts, and dairy products. Alkaline foods are those rich in potassium and magnesium salts of organic acids, as is the case with fruits and vegetables. Considering this information, fruits such as mango, may play a role in maintaining bone health.

Calcium, vitamins D, K, and C, magnesium, boron, copper, fluoride, manganese, potassium, silicon, zinc, and isoflavones are dietary factors contributing to bone formation [29, 85, 86]. Copper, manganese, carbonate, citrate and vitamins C, D and K are involved in crystal and collagen formation, cartilage and bone metabolism and/or the calcium and phosphorus homeostasis [86]. Zinc, copper, iron and manganese are essential cofactors for enzymes involved in the synthesis of the bone matrix [87]. Studies have shown that intake of these minerals has been positively associated with bone mass, while deficiency has been correlated either with reduced bone mass or slow fracture healing [88]. Evidence over the last 30 years strongly suggests that silicon is beneficial to bone and connective tissue health including the synthesis of collagen and/or its stabilization, and bone matrix mineralization [88]. In a human osteoblast cell culture study, vitamin K_2 enhanced osteocalcin production, induced by $1,25(OH)_2D_3$ [89] and the accumulation of γ-carboxyglutamic acid containing osteocalcin [89]. A type of vitamin K_2, addressed as K_2-7, also demonstrated an anabolic effect on bone tissue in osteoblastic (MC3T3-E1) cells [90]. In addition to vitamin K_2 stimulating OB and osteocalcin production, vitamin K_2 has also been shown to attenuate OC formation and activity in rat bone cells in vitro [91].

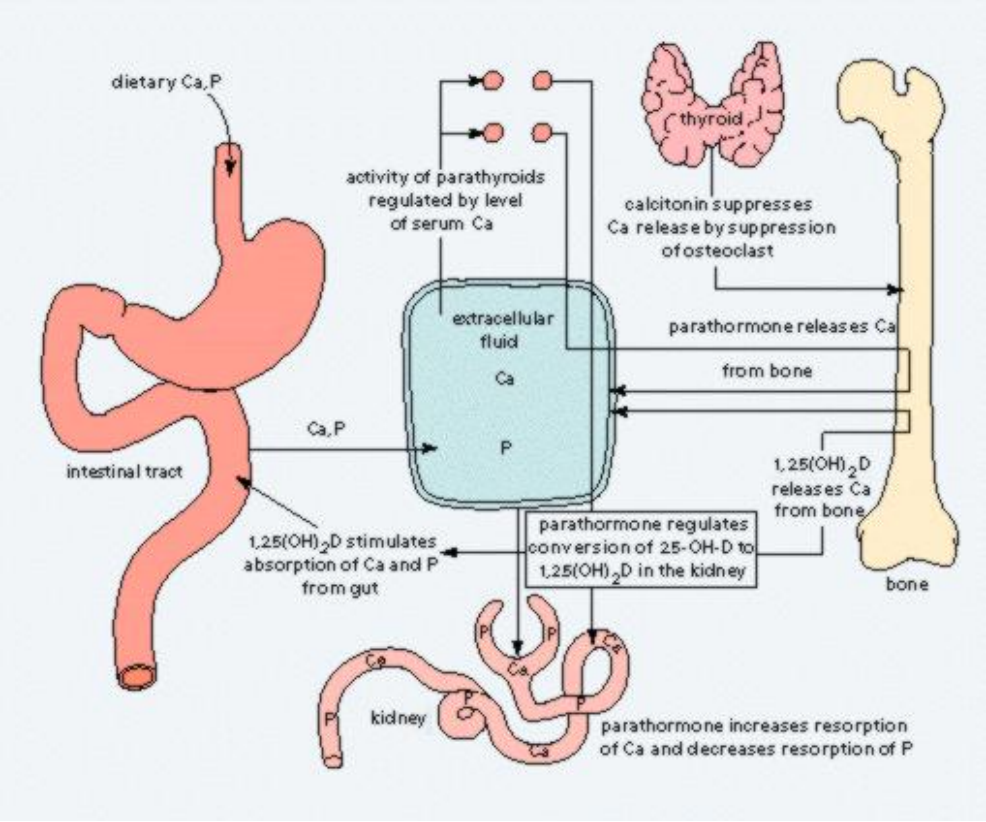
It is important to maintain adequate calcium intakes throughout life. Calcium helps to optimize bone formation during growth and minimizes bone loss in later life. Ninety nine percent of the human body's calcium is stored in the bone as hydroxyapatite. Hydroxyapatite bone crystals are comprised in a ratio of 2:1 calcium to phosphate with trace amounts of other minerals. The hydroxyapatite crystals contribute to the weight bearing and mechanical properties of the bone which can also be mobilized through bone remodeling to meet other biological functions of calcium and phosphorous in the body [68]. According to a meta-analysis [92], every 300 mg increase in calcium intake has been associated with a 4% decrease of fracture risk in postmenopausal women. Another meta-analysis study of randomized controlled trials on post menopausal women showed a 2.02% reduction in bone loss with calcium supplementation of at least 400 mg/day over a two year period [93].

Calcium in the blood is found in the form of free ions or bound to albumin, globulin or phosphate, citrate or other anions. Intracellular calcium concentrations are about 10,000 times less than extracellular concentrations. Calcium increases bone strength by being a principal component of mineralized bone and by reducing serum PTH which lowers bone turnover rate. In addition to building bone, calcium also is involved in blood clotting and intracellular adhesion. Calcium also acts as a signal transducer in muscle contraction, hormone secretion, kinase phosphorylation, neurotransmitter release, vision, glycogen metabolism, cellular differentiation, proliferation and motility [68].

Calcium concentration in the blood is regulated with strict control by parathyroid hormone (PTH) and vitamin D at 2.5 mM/L (**Figure 5**). BMC is sacrificed at the expense of maintaining serum calcium ion concentrations. Low serum calcium and phosphorous levels increase PTH production. PTH encourages bone resorption and encourages renal tubule calcium

re-absorption by converting vitamin D to its active form (**Figure 5**) to meet serum calcium needs. Vitamin D increases calcium absorption in the intestines and reabsortion in the kidneys. $1,25(OH)_2$ vitamin D_3 interacts with vitamin D receptor in the enterocyte and stimulates the synthesis of calcium-binding protein. Calcium receptors are located in the intestine, osteoblast cell lines, calcitionin secreting c-cells of the thyroid gland, parthyroid gland, and the $1,25(OH)_2$ vitamin D_3 producing cells of the renal proximal tubule [94]. Low blood calcium status increases calcium absorption in the intestine. However, chronic low calcium intake will lead to increased bone resorption due to increase in PTH.[71].

Figure 5: Bone Metabolism[95]



Adequate calcium intake for postmenopausal women is 1200 mg/day with most women falling short in meeting this requirement through diet alone[96]. Intestinal calcium absorption declines with age or when calcium intake is high. Calcium absorption is more efficient if calcium is consumed in amount spread throughout the day rather than consuming it all at once. Calcium bioavailability needs to be considered when choosing calcium food sources. Calcium absorption from diet is between 20% and 60 % [71]. Calcium is absorbed more easily when it can disassociate more readily from its ligands. However, low molecular weight calcium salts can be absorbed intact without a vitamin D induced calcium transporter. Enhancers of calcium absorption are soluble salts that prevent precipitation of calcium by phosphates (i.e. calcium citrate malate), inulin and fructooligosaccharides. Some casein and whey peptides also prevent precipitation of calcium by phosphates. Inhibitors to calcium absorption are oxalate and phytic acid[71].

In addition to assuring adequate calcium intake it is also important to consider calcium losses through the urine. Urinary excretion impacts calcium retention by about 50% [97]. However, increases in dietary calcium only affect urinary output of calcium by approximately 6% while dietary sodium exerts a much greater effect on urinary calcium excretion and contributes to low BMD in postmenopausal women [97]. Although high protein intake increases urinary calcium excretion, net calcium retention is not affected because of changes in calcium absorption and endogenous calcium secretion [98]. Urinary calcium tests are not done routinely but conducted to determine the etiology of kidney stones or to check for problems with the parathyroid gland[99]. In addition to low calcium having a negative impact on BMD, low extracellular calcium also increases the risk for hypertension, preeclampsia, premenstrual syndrome, obesity, polycystic ovary syndrome and hyperparathyroidism [68].

Vitamin D is involved in bone formation by regulating calcium and phosphorous amounts in the blood and by promoting bone mineralization. Indirectly, vitamin D is involved in bone remodeling by its role in differentiation and proliferation of hematopoietic cells, keratinocytes, parathyroid secreting cells and beta cells of the pancreas [68]. Vitamin D must be metabolized by both the liver and kidney before becoming biologically active as a steroid hormone. The active metabolite 1α, $25(OH)_2$ vitamin D_3 functions as a steroid hormone by generating biological responses via regulation of gene transcription and activation of signal transduction pathways [68, 100] . The other kidney metabolite of vitamin D, 24R,25 $(OH)_2D_3$ has been shown to improve bone fracture healing in chickens [24]. In the intestine, vitamin D assists with calcium, magnesium and phosphorous absorption [101] (**Figure 5**). Vitamin D receptors are both nuclear and membrane specific and are close to plasma membrane as well as found in bone and bone marrow. Vitamin D participates in bone remodeling through its involvement in OB differentiation, inhibiting OB apoptosis, encouraging calcium mobilization, and inducing osteocalcin production [101]. When plasma calcium and phosphorous levels are low, the kidneys convert 25(OH) vitamin D_3 to 1α, $25(OH)_2$ vitamin D_3, its active steroid hormone form.. The enzyme 1α, 25-hydroxylase is an important regulator of the active metabolite of vitamin D production in the kidneys. It is regulated by 1α, $25(OH)_2$ vitamin D_3 concentrations, serum calcium and phosphate levels and PTH. The enzyme 1α, 25-hydroxylase is also iron dependent. Therefore, variations in iron levels indirectly affect bone by decreasing 1α,$25(OH)_2$ vitamin D_3 production.

Optimal health may require a vitamin D status much greater >25 nmol/L, the threshold for 25-hydroxyvitamin D set to avoid clinical deficiency[102]. The RDA for elderly females is 600 IU/day (10-15 μg/day) [2]. Aside from dietary sources, the skin produces a precursor to the

active vitamin D when the sun's ultraviolet rays transform 7-dehydrocholesterol into cholecalciferol. Cholecalciferol is hydroxylated in the liver to form 25-hydroxycholecalciferol, also known as calcidiol, which is stored in the liver until needed. When needed, calcidiol is hydroxylated to its biologically active form, calcitriol, in the kidney. Minimum levels ranging from 25 nmol/L to >100 nmol/L have been proposed, largely due to the inverse relationship of $25(OH)D_3$ (calcidiol) and PTH. High PTH is a risk factor for osteoporosis in older adults[102]. An estimated 80% of elderly postmenopausal women may have vitamin D deficiency, defined by levels less than 20 ng/mL or 50 nmol/L [103]. Production of vitamin D binding protein, which is responsible for vitamin D (calcitriol) transport in the blood, increases by 50% in high estrogen states [68].

Vitamin C, present in high amounts in mango, plays a critical role in collagen synthesis. Collagen is necessary for bone, skin and tendon formation. Vitamin C is a cofactor which promotes enzyme activity by maintaining metal ions in their reduced form. It is involved in the hydroxylation of proline and lysine residues which form the cross linking necessary to form the triple helical structure of collagen. More specifically, α-ketoglutarate-dependant dioxygenases incorporate one atom of oxygen into succinate and into the oxidized product in the presence of ferrous iron [68]. Ascorbic acid stimulated collagen production in vitro fibroblast cells by 60-100% [104]. A study analyzing the effects of vitamin C supplementation (745 mg/day) on BMD of postmenopausal women, demonstrated that vitamin C users had BMD levels that were 3% higher at the midshaft radius, femoral neck, and total hip than non-users[105]. Women taking both estrogen and vitamin C had significantly higher BMD levels at all sites. Women who took vitamin C plus calcium and estrogen had the highest BMD at the femoral neck, total hip, ultradistal radius, and lumbar spine [105].

The phytonutrients found in fruits and vegetables may play an important role in modulating bone metabolism without the side effects associated with pharmaceutical interventions. Phytonutrients, such as phenolics, may function as antioxidants, donating hydrogen ions, scavenging free radicals, inhibiting radical generation, inhibiting enzyme activity or by specific receptor interactions[19]. Antioxidant functions include enzyme inhibition, metal chelation, hydrogen donation and by oxidizing non-propagating radicals[19]. Phenolics can inhibit xanthine oxidase and other enzymes involved in the production of ROS[19]. They can also function as anti-inflammatory agents by inhibiting lipoxygenase, the enzyme responsible for creating prostaglandins and leucotrienes from arachidonic acid[106]. Some phenolics modulate signal transduction by inhibiting tyrosine kinase[19]. Some flavonoids and isoflavonoids inhibit 17 β – hydroxysteroid oxidoreductase which may influence sex hormone metabolism[107].

Population studies show a positive correlation with fruit and vegetable intake and BMD [25], 109[13, 18, 108]. For example, women who consumed vegetables more than 9 times a week had higher BMD compared to those with lower intakes [109]. Higher BMD was observed at the heel site of women consuming more than 1.5 servings of vegetables a day in a study on a rural population in Iran [108]. The benefits of fruits and vegetables results from an additive and synergistic effect from the combination of phytochemicals[25]. The antioxidant action of the phytonutrients may be responsible for these findings and for this reason it is important to consider the oxidative status associated with osteoporosis.

Oxidative stress is considered as an independent risk factor for osteoporosis[110]. Increased OC activity and decreased OB activity have been associated with an imbalance between oxidant and antioxidant status in postmenopausal osteoporosis[111]. When bone remodeling is imbalanced, as in osteoporosis, ROS production by the OC may overwhelm the

body's endogenous antioxidants defense mechanisms leading to further bone loss. Enhanced OC activity increases superoxide anion generation and inhibits superoxide dismutase and glutathione peroxidase activities[112]. In murine models, superoxide anion was proposed to oxidize calcium binding sites and/or acidify the local environment near OCs, favoring bone loss[113]. Transcription factors, such as nuclear factor-kB (NF-kB) which signals TNF production and inflammation, are sensitive to oxidative changes[19]. Basu and colleagues established a biochemical link between increased oxidative stress and reduced bone density by showing elevated oxidative stress marker 8-iso-prostaglandin $F_{2\alpha}$ and elevated inflammatory marker 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ in osteoporotic subjects when compared to those without osteoporosis[114, 115]. Hence, antioxidant supplementation has been proposed as a novel therapeutic strategy for osteoporosis.

Phytonutrients have been shown to play a protective role in preserving BMD in animals [14, 15, 20, 116, 117] and *in vitro* [16, 118-120]. Orange pulp and citrus fruits in general contain high amounts of vitamin C and flavonoids and have shown to improve bone density in male orchidectomized rats [14, 15]. Citrus hesperidin and olive oleuropein together, were also shown to prevent bone loss and improve peek bone mass in female rats [117]. Green tea polyphenols reduced the extent of bone micro-architecture deterioration in female rats [116]. Pholridzin in apples, rutin, and isoflavones have all been shown to prevent bone loss in ovariectomized rats [20]. Flavonoids, quercetin and kaempferol was shown to inhibit bone resorption *in vitro* [118]. Antioxidants present in fruits and vegetables may reduce oxidative stress in the bone environment by reducing OC differentiation. For example α-lipoic acid was able to inhibit OC differentiation [121], while lycopene was shown to reduce oxidative stress and bone turnover markers in postmenopausal women[122]. Dried plum polyphenols inhibited osteoclastogenesis by

down regulating NFATc1 and inflammatory mediators and attenuated TNF-α on osteoblast function with upregulation of Runx2, Osterix and IGF-1 in MC3T3-E1 cells and in gonadal hormone deficient, inflammation induced animal model [16, 119, 120].

Lifestyle Influences on Bone

Weight-bearing exercise exerts a mechanical stimulation on bone tissue. Resistance and high-impact activities contribute to development of peak bone mass and may reduce risk of falls in older individuals [2]. Many studies have confirmed bone loss resulting from immobilization and micro-gravity in both animals and humans [123-125]. Studies with increased loading activity have resulted in increased bone mass often localized to the bones where the loading took place [126, 127]. Mechano-stimulation to the osetocytes creates a signaling pathway to generate more OBs.

Current research indicates that smoking not only increases the risk for osteoporosis in women but also in men and adolescents [128]. Even second hand smoke has been correlated with an increased risk for osteoporosis and fracture according to a study done on Chinese men and pre-menopausal women [129]. Findings from this study showed that premenopausal women exposed to second- hand smoke had a "threefold higher risk of having osteoporosis and a 2.6 times greater risk for a non-spine fracture" when a family member smoked [129]. Other studies show that second-hand smoke may alter estrogen levels thereby effecting health [128]. A study conducted on young Sweden men demonstrated that 24% of smokers had bone fractures compared to only 14% or those who never smoked [130, 131]. Moreover, smokers had lower bone density in the spine, hip, and whole body when compared to non-smoking peers [130, 131]. Overall, smoking can double osteoporosis risks in men [128].

Alcohol, consumed moderately, may provide benefits to the BMD of postmenopausal women [132, 133] and in men over age 45[133]. In contrast, studies done on alcoholics report lower bone mineral density[134, 135]. In the research conducted by Holrook and colleagues (1993), social drinking of up to 180 g/day of alcohol in men and 120 g/day in women [133] resulted in an increase in BMD at the femoral neck of men and the spine of women in a one week study and in the radial shaft and spine of women in the 24 hour study [133]. Stronger associations on BMD were seen with beer or wine consumption compared to liquor consumption[132]. In men, 2 or more servings per day of liquor resulted in lower BMD [132]. This may suggests that constituents in the wine and beer, other than ethanol, may contribute to bone health. For example, the high silicon content of beer has been associated with increases in BMD [88, 132].

The effects of caffeine on bone are inconclusive. Some studies have shown detrimental effects of caffeine on bone, some of which have disappeared after making statistical adjustments for osteoporotic risk factors [136]. A study conducted by Rapuri and colleagues (2001) [137] found that women who consumed more than 300 mg/day of caffeine had a significantly higher rate of bone loss in the spine. In contrast, Japanese women who drank green tea regularly had significantly higher BMD than those who did not [138]. In addition to the observational findings correlating green tea and BMD, in inflammation induced rats, green tea polyphenols attenuated BMD loss [116] and in bone marrow mesenchymal stem cells green tea catechins encouraged osteogeneis [139]. The interaction of an individual's genotype with caffeine may affect the BMD study outcomes with some caffeine metabolizing genotypes being more susceptible to bone loss than others [140]. This genotypic variation among individuals may help explain some of the controversies and inability of other studies to show clearly the effect of caffeine on bone.

Postmenopausal Osteoporosis

Being postmenopausal or in a state of surgically-induced menopause increases bone resorption [2]. For the most part, this phenomenon is attributed to a decrease in estrogen production. Estrogen production declines several years before the cessation of menstruation which typically occurs between 48 and 50 years of age [141]. Consequently, osteoporosis is most commonly found in the elderly female population. Development of osteoporosis in women has also been associated with the time of menarche, menopause, and the duration of lactation [142]. Li and Zhu (2005) [143] demonstrated that the later menarche, the earlier the menopause, the greater number of child births and the longer the duration of lactation all lead to lower BMD and a higher degree of osteoporosis.

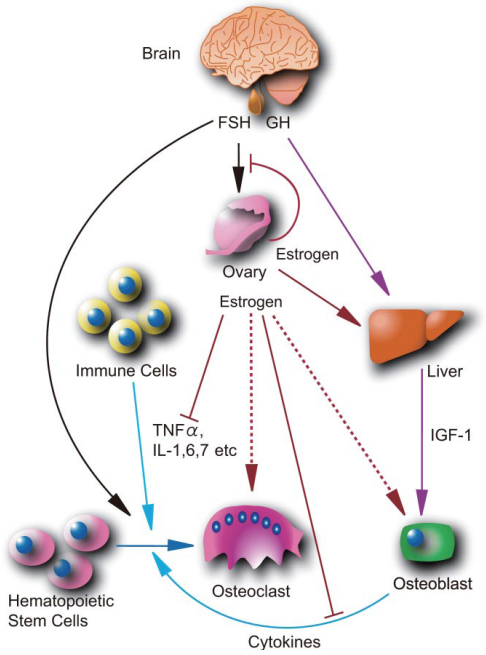
Estrogen has a protective influence on bone by attenuating OC proliferation and activity. Estrogen receptor α (ER α) is present on both the osteoprogenitor cells and the OCs. When 17β-estradiol binds to the ER α, it triggers apoptosis in the OC and inhibits OC differentiation in the progenitor cells[144]. With a dramatic decline in estrogen production during menopause, bone remodeling increases several fold. Postmenopausal bone resorption doubles and biomarkers of bone formation are less than half the levels of that observed premenopause [145].

Estrogen deficiency also increases FSH secretion which encourages the production of inflammatory cytokines, such as TNF-α and several ILs by bone marrow and immune cells. The cytokines, in turn, encourage OC differentiation. The lack of estrogen stimulation during the postmenopausal state complemented with declined levels of GH which occurs with aging leads to weakened signaling on the OB (**Figure 6**).

Estrogen not only exerts an influence on the OCs but also on the OBs. Estrogen attenuates OB progenitor/ mesenchymal stem cell production by as much as 50% [146]. In the low

estrogen environment post menopause, there is an increase in OB progenitor production. OB progenitors are not only involved in OB formation but also the formation of adipocytes, chondroblasts, myoblasts and fibroblasts and play an important role in OC signaling [146]. With aging, OB progenitors preferentially differentiate into adipocytes rather than OB[147] which may also help explain the tendency for increased adiposity post menopause. Although lack of estrogen increases the rate of formation of the OB progenitors, the rate of production is not sufficient enough to counter the increased rate of bone remodeling.

Figure 6: Hormonal stimulation on OCs and OBs [148].



*Lack of estrogen represented by ⊣ and estrogen stimulation represented by →

Although estrogen modulates OB progenitor production, it still exhibits an anabolic effect on OBs. Estrogen encourages OB differentiation, proliferation and increases OB proteins such IGF-1, type I procollagen, transforming growth factor beta (TGF-β) and BMP [141]. Estrogen also inhibits OB and osteocyte apoptosis [149]. Estrogen suppresses bone resorption by increasing OC apoptosis [150] increasing OPG production and decreasing OB/stromal cell production of RANKL[151]. The activation of the Src/Shc/ERK–signaling pathway influences the estrogen receptors in the OB lineage of cells to prevent apoptosis of OB cells [152]. A decrease in the number of cells that support osteoclast development, together with decreased production of osteoclastogenic cytokines by these same cells may be responsible for the potent suppression of osteoclastogenesis by estrogens [146].

Estrogen also increases nitric oxide (NO) and TGF-β levels. An appropriate level of NO is necessary to protect bones and organs from ischemic damage while chronically high levels of NO can cause tissue toxicity, inflammation and carcinomas [153]. NO and TGF-β regulate T cell production of TNF-α and act to inhibit osteoclastogenesis and bone resorption [6]. Estrogen deficiency in postmenopausal women is associated with an increase in pro-inflammatory cytokine production such as IL-1, TNF- α, and IL-6 [4, 6] in the bone environment (**Figure 6**). These pro-inflammatory cytokines are involved in osteoclastogenesis and encourage bone resorption [7]. The inflammatory cytokines also produce oxygen free radicals which contribute to osteoclast formation, differentiation and activation (**Figures 1 & 2**). IL-1 and IL-6 also increase the production of M-CSF which further influences OC differentiation. Estrogen also regulates the release of growth factors and growth hormones such as TGF-β and IGF-I [154].

In addition to the menopausal effects on OB progenitors, OBs, and OCs, estrogen deficiency also affects calcium balance. In an estrogen deficient state, intestinal calcium

absorption is decreased while urinary calcium loss is increased [155]. Intestinal mucosal cells have receptors for 17β-estradiol which increase calcium transport/absorption [156]. The kidneys also reabsorb calcium better when estrogen is present [157]. This is attributed to an increase in circulating 1,25-$(OH)_2D_3$ which is indirectly mediated through stimulation of renal la-hydroxylase by increased serum PTH[158]. In addition to the direct effects of estrogen on calcium absorption, there is also reduced total circulating concentration of 1,25$(OH)_2D_3$ as one ages, which effects both calcium absorption and, indirectly, bone formation.

Estrogen receptors α and β are activated by 17β-estradiol and function as a DNA binding transcription factor. In the absence of estrogen, the inactive α receptor located on the nucleus is associated with several heat shock proteins [159]. In the presence of a receptor stimulus, like 17β-estradiol, the receptor undergoes a conformational change displacing the inhibitory heat shock proteins and forms dimers. The dimers allow the receptor to interact with "specific steroid response elements (SRE) located within the regulatory regions of target promoters [160]. The ligand-activated receptor can interact with the general transcription apparatus (GTA) directly or indirectly through adaptor proteins. Ultimately, these interactions stabilize the transcription preinitiation complex and enhance RNA polymerase activity [160] .

Bone also degrades with age and can be attributed to the degree of mineralization, anisotropy, skeletal geometry and the periosteal response to trabecular bone mass [161]. Growth hormone secretion declines 14% per decade and is one of the primary factors responsible for low serum IGF-I levels which are directly correlated with BMD [103]. Adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA sulfate are also 10-20% lower than young adults further contributing to a lack of bone building stimulus factors[162].

Animal Model

Laboratory animals have played a major role in understanding osteoporosis and developing treatment options for the management of osteoporosis[163]. The success of an animal model is based on its ability to successfully predict outcome measures in people. Because the average life-span of the laboratory mouse is about 2 years, one year is roughly equivalent to middle age. A menopausal state, however, can be induced surgically by ovariectomy [163]. Ovariectomized mice are comparable to women who undergo bilateral ovariectomy prior to menopause and prematurely experiences menopausal symptoms soon after surgery [163]. Hence, the ovariectomized mouse experiences an increase in bone turn over, osteopenia and weight gain [163]. It takes the mice 21 days post ovariectomy to reach a state similar to that of early menopause whereas after 35 days corresponds to late menopause in women[164]. Research conducted by Lynch and colleagues demonstrated that the mouse tibia did not experience significant bone loss or architectural adaptation following OVX in growing mice [165]. However, ovariectomy-induced estrogen deficiency resulted in a reduction in vertebral bone mass due to decreased trabecular thickness and increased separation when compared to SHAM control mice at 6 weeks post OVX in 26week old C57Bl/6 mice[166]. The decreases in bone mass did not affect mechanical integrity of lumbar vertebrae, suggesting that a duration longer than 6 weeks is needed before bone mass changes result in deterioration of mechanical properties[166]. Several studies have used the ovariectomized mouse model to study both nutrition and pharmaceuticals in preventing further postmenopausal bone loss[139,[17, 167, 168]. Some nutrition-related examples of research conducted on ovariectomized rodents with successful results in improving or maintaining bone parameters are nano calcium products[168], soy protein [163], and dried plum[17, 167].

Pharmacological Options for Osteoporosis Treatment

Drugs for the prevention and treatment of osteoporosis are classified as anti-resorptive or anabolic depending on their effect on bone remodeling[169]. Anti-resorptive drugs act by inhibiting OC formation, OC activity or by inducing premature osteoclast cell death. Anti-resorptive drugs include SERMs, bisphosphonates (alendronate, risedronate, ibandronate, and zoledronic acid), an estrogen agonist/antagonist (raloxifene), and calcitonin. Some resorption inhibitors, such as SERMs, PTH, some interleukins, and Denosumab, inhibit OC formation by working on OBs since OBs secrete essential factors for OC differentiation such as M-CSF and RANKL [170]. Denosumab is a fully human monoclonal antibody (IgG2 immunoglobulin isotype) antiresorptive drug that has recently been approved for use in the US. It functions by binding to RANKL, preventing it's binding to RANK. This results in a reduction in OC formation, activity and survival, thereby reducing the bone resorption rate [171]. Denosumab also has the advantage of being a subcutaneous medication administered every six months thereby improving long-term adherence to therapy compared with oral treatment options. Calcitonin and aminobisphosphonates, on the other hand, work by inhibiting OC resorption activity, where as the first generation bisphosphonate, clodronate, works by inducing osteoclast apoptosis [170] .

Anabolic agents work by increasing osteoblast activity. The only pharmaceutical anabolic agents to bone are teriparatide, a recombinant PTH, and strontium ranelate [172]. Strontium ranelate has both antiresorptive and anabolic properties [173]. The present study looks at mango as a possible anabolic agent to bone, thereby reversing the effects of ovariectomy induced bone loss.

Nutrient Composition of Mango

Mangos (*Mangifera indica L*) are excellent sources of vitamin C and vitamin A and are good sources of dietary fiber, potassium, copper, and vitamin K [28]. Mangos also contain minerals involved in bone health such as magnesium, zinc, copper, manganese, and selenium [29]. **Table 1** details the nutrient composition and daily value of the mango fruit.

Table 1: Nutrient composition of mango and percent daily values (DV).[ab][28]

Nutrient	DV reference (units/day)	Amount in 100g mango	% DV[c]	Amount in 1 fruit (no peel or pit) (207 g)	% DV[c]
Macronutrient					
Carbohydrate, g	300	17	5.7	35.2	11.7
Protein, g	50	0.51	1.0	1.06	2.1
Total fat, g	65	0.27	0.4	0.56	0.9
Dietary fiber	25	1.8	7.2	3.7	14.8
Minerals					
Calcium, mg	1000	10	1.0	21	2.1
Potassium, mg	3500	156	4.4	323	9.2
Phosphorus, mg	1000	11	1.1	23	2.3
Copper, mg	2	0.110	5.5	.228	11.4
Sodium, mg	2400	2	0.1	4	0.2
Vitamins					
Vitamin A, IU	5000	765	15.3	1584	31.7
Vitamin C, mg	60	27.7	46.2	57.3	95.5
Vitamin K, ug	80	4.2	5.2	8.7	10.9
Thiamin, mg	1.5	0.058	3.9	0.12	8.0
Riboflavin, mg	1.7	0.057	3.4	0.118	6.9
Folate, ug	400	14	3.5	29	7.2
Vitamin E, IU	30	1.12	3.7	2.32	7.7
Niacin, mg	20	0.584	2.9	1.209	6.0

[a]Amount of nutrient a food provides based on DRI (dietary reference intake) guide and reference values for nutrition labeling (2000 calories per day).
[b] Values were obtained from USDA National Nutrient Database for Standard Reference, Release 22 (2009)
[c] Foods that provide %DV of ≥10% are considered a 'good' source; those ≥20% are considered an 'excellent' source.

Mangos also contain carotenoids, triterpenes, and phenolic compounds such as tannins, mangiferin and flavonoids which contribute to its antioxidant and anti-inflammatory functions. Total phenolics in mango vary depending on the cultivar and the processing methods. Total phenolics of dry weight with peel ranges from 350 to 4860 mg/kg, while 60 to 180 mg/kg is common in the fresh fruit [174-177]. Mercadante and colleagues show the following carotenoid profile as detected by HPLC and confirmed by mass spectrometry on a Brazilian mango cultivar, Keitt; β-carotene (all-trans), β-cryptoxanthin (all-trans and cis), zeaxanthin (all-trans), luteoxanthin isomers, violaxanthin (all-trans and cis), and neoxanthin (all-trans and cis) [178]. Carotenoid composition of the mango cultivar Keitt is shown in **Table 2.** β –Carotene is a predominant carotenoid in mango and functions as pro-vitamin A. **Table 3** details the β – Carotene and vitamin A content of various varieties of mango in their fresh and dried forms. Although the ripe mango flesh is not particularly high in flavonoids, higher amounts of flavonoids can be obtained by consuming them unripened and with the peel intact, a common practice done in Indian cuisine know as the spicy mango pickle (**Table 4**).

Table 2: Carotenoid Composition of Mango Cultivar Keitt Bahia, Brazil [178]

carotenoid (µg/g)	range	mean[a]
all-trans-β-carotene	13.4-16.2	15.1 ± 1.5
unidentified compound		0.2 ± 0.0
cis- β -cryptoxanthin	tr-0.1	0.1 ± 0.1
all-trans- β -cryptoxanthin	0.3-0.3	0.3 ± 0.0
all-trans-zeaxanthin	0.6-0.9	0.8 ± 0.2
luteoxanthin isomers	3.1-4.1	3.8 ± 0.6
all-trans-violaxanthin	18.2-23.9	21.1 ± 2.9
9-cis-violaxanthin[b]	9.9-10.3	10.1 ± 0.2
13-cis-violaxanthin[b]	1.3-1.5	1.4 ± 0.1
cis-neoxanthin	tr-0.2	0.1 ± 0.1
all-trans-neoxanthin	1.0-3.6	2.1 ± 1.3
total	49.9-59.8	55.0 ± 5.0
vitamin A value	222-270	251 ± 26

[a] Mean and standard deviation of three sample lots. [b] Tentative identification.

Table 3: β -Carotene Content of Fresh and Dried Mango Flesh of Different Cultivars

Samples …	all-trans- β –carotene µg/100 g DW[c]	9-cis- β –carotene µg/100 g DW	13-cis- β –carotene µg/100 g DW	relative amount of cis-isomers[a] %	Vitamin A value[b] RE/100 g
Kent					
fresh	4580	tr[d]	1120	24.4	142
dried[e]	4270	180	1390	36.8	752
Tommy Atkins					
fresh	3650	nd[f]	940	25.8	114
dried[e]	2510	tr[d]	930	37.1	431
Namdok Mai					
fresh	3650	tr[d]	990	27.1	121
solar-dried[g]	2400	810	730	64.2	425
Kaew					
fresh	11,680	1010	1220	19.1	423
solar-dried[g]	6820	2050	1430	51.0	1011

[a] Calculated as percentage of all-trans-β-carotene.
[b] Retinol equivalent (RE) according to Zechmeister (41).
[c] Dry weight.
[d] In traces.
[e] Standard drying process (σ_a = 75 °C, a_w = 0.6, t_D = 3-3.5 h).
[f] Not detected.
[g] Solar-drying process (a_w = 0.6, t_D = 7-8 h).

Table 4: Total phenolic and total flavonoid content of mango: flesh/ peel, ripe/unripe[179]

Samples	Total phenolic content (*mg* GAE/*g*)	Total flavonoid content (*mg* RE/*g*)
Unripe Mango Flesh	27.8 ± 2.21	8.15 ± 1.54
Unripe Mango Peel	92.6 ± 3.40	22.2 ± 3.32
Ripe Mango Flesh	26.9 ± 3.76	3.30 ± 0.79
Ripe Mango Peel	70.1 ± 4.61	21.2 ± 2.47

All data are presented as mean ± SD of the three replicates. GAE: gallic acid equivalents, RE: rutin equivalents.

Studies on Health Benefits of Mango Pulp

Different parts of the mango fruit and tree has been investigated for its health benefits. In addition to the mango pulp, the kernel, peel, leaves, stem and bark have all been utilized for research. Research on the different parts of mango fruit and tree have explored its antioxidant and anti-inflammatory properties, anti-bacterial, anti-viral, anti-HIV, anti-cancer, anti-diabetic, anti-atherogenic, and immune-modulatory properties as well as functions in bone health [180-183].

The bulk of the research on mango has utilized the stem bark extract of mango that is rich in the phenolic compound mangiferin. **Table 5** lists the pharmacological activity of mangiferin extracted from mango tree bark. Since our study has used mango pulp, this section will focus on research that has been done on mango pulp and some of the research done on mango related to bone.

The pulp of the mango has been investigated as a chemo-protective agent via its antioxidant functions. A study conducted by Percival et al. in 2005[184], showed that mango juice inhibits cancer cell growth. Lupeol, a naturally occurring pentacyclic triterpene present in mango pulp and other fruits, was shown to exhibit strong anti-inflammatory, anti-arthritic, anti-mutagenic and anti-malarial activity [185]. The effects of lupeol/mango pulp extract in testosterone induced oxidative stress in the prostate of male Swiss albino mice were also examined [185]. Lupeol/ mango pulp extract treated mice had lower ROS levels and restored levels of lipid peroxidation and antioxidant enzymes catalase, superoxide dismutase, glutathione reductase, and glutathione-S-transferase. In this study, lupeol/mango pulp extract was effective in combating oxidative stress-induced cellular injury of mouse prostate, indicating a protective role in prostate cancer [185]. The anti-tumor effects of lupeol have also been demonstrated in mouse skin in a study done by Saleem et al. (2004)[186, 187]. The antioxidant properties of lupeol have also demonstrated a hepatoprotective effect using cadmium-induced hepatotoxic rats [188]. Hepatoprotective effects of lupeol and mango pulp extract were also demonstrated in a carcinogen induced (dimethylolbutanoic acid) mouse model [189]. The endogenous antioxidant enzyme status was restored and a decrease in lipid peroxidation was observed.

Mango and Bone Health

We are only aware of a few studies investigating the effect of mango on bone. Er- Xian Decoction, has long been used for the treatment of osteoporosis and menopausal syndrome in China. Bioactivity-guided fractionation of Er Xian decoction has led to the successful isolation of the anti-osteoporotic constituents, identifying one as mangiferin [190].

Another study investigated the effect of the xanthonoid compound found in mangoes, mangiferin, on periodontitis [191]. Periodontitis is a chronic inflammatory disease related to the formation of colonies of microorganisms present in subgingival plaque leading to inflammatory periodontal pockets, destruction of the periodontal ligaments, alveolar bone resorption and tooth loss[192]. Carvalho and colleagues (2008)[191], induced periodontitis in Wistar rats by applying a ligature around the lower right first molar with subsequent oral treatment of 100 mg/kg mangiferin for 1, 4 or 7 days. Oral administration of mangiferin significantly reduced alveolar bone loss and cellularity, inhibited COX-2 expression and the adhesion of leukocytes, while maintaining normal lipoxin A_4 levels. Lipoxin A_4 acts as an endogenous anti-inflammatory mediator by its anti-chemotaxic action in inhibiting the rolling of leukocytes. The mangiferin-treated rats presented an earlier peak of cell proliferation and augmented angiogenesis in the injured region.

Table 5: Pharmacological activity studies on mangiferin from mango bark [193]

Antioxidant	Sanchez *et al.* 2000; Muruganandan *et al.* 2002; Leiro *et al.* 2003; Stoilova *et al.* 2005
Radioprotective	Jagetia and Baliga 2005; Jagetia and Venkatesha 2005
Immunomodulatory	Chattopadhay *et al.* 1987; Guha *et al.* 1996; Moreira *et al.* 2001; Garcia *et al.* 2002, 2003; Leiro *et al.* 2003, 2004a, 2004b; Sarkar *et al.* 2004
Anti-allergic	Rivera *et al.* 2006
Anti-inflammatory and anti-nociceptive	Beltran *et al.* 2004; Garrido *et al.* 2004
Antitumor	Guha *et al.* 1996; Yoshimi *et al.* 2001
Antidiabetic	Ichiki *et al.* 1998; Miura *et al.* 2001a, 2001b, 2001; Muruganandan *et al.* 2005
Inhibitory activities on carbohydrate metabolism enzyme	Yoshikawa *et al.* 2001
Lipolytic activity	Yoshikawa *et al.* 2002
Antibone resorption	Li *et al.* 1998
Antiviral	Zheng *et al.* 1990, 1993; Guha *et al.* 1996
Antibacterial	Srinivasan 1982; Stoilova *et al.* 2005
Antifungal	Stoilova *et al.* 2005
Antiparasitic	Perrucci *et al.* 2006
Monoamine oxidase-inhibiting activity	Bhattacharya *et al.* 1972

CHAPTER III

METHODOLOGY

This study was designed to determine the dose-dependent effects of freeze-dried mango pulp on reversing ovariectomy-induced bone loss. To mimic a post-menopausal state, the ovariectomized mice model was used.

Animal Grouping

Thirty 12–week old ovariectomized (OVX) mice and 14 sham-operated (SHAM) C57BL/6 mice were purchased from Charles River Laboratory (Kingston, NY) and housed in Oklahoma State University (OSU) animal research laboratory. Mice were fed a standardized AIN-93M powdered rodent diet [194] for two weeks post-ovariectomy. All animal handling and procedures were approved by Institutional Animal Care and Use Committee at OSU.

After a 2 week period, a total of 12 mice (n= 6 SHAM; n= 6 OVX) were necropsied to confirm ovariectomy-induced bone loss. Whole body dual-energy X-ray absorptiometry (DXA) scans were preformed prior to necropsy. The remaining 32 mice were weighed and divided into 4 groups and housed in groups of four mice per cage. Mice were assigned to one of the following dietary treatment groups (n = 8 mice/ group) for 8 weeks: (1) SHAM - control diet; (2) OVX - control diet; (3) OVX - 5% dried mango diet; and (4) OVX - 25% dried mango diet. The OVX groups were match-fed to the SHAM group. All mice were provided deionized water *ad libitum* and were weighed weekly.

Diet Formulation

Ripe Tommy Atkins variety mangoes were purchased from a local grocery store. The pulp was separated from the skin and kernel and freeze-dried. The freeze-dried mango pulp was ground to a powder and sent to Nestle Purina Analytical Laboratories (St. Louis, MO) for analysis of macronutrients and calcium and phosphorous content (**Table 6**). Freeze-dried mango powder was incorporated into the diet at 5% or 25% concentration by weight. The macro-nutrient composition as well as the calcium and phosphorus content of the mango diets were adjusted to be similar to the control diet (**Table 7**). The control diet was an AIN-93M formulation containing 72% carbohydrate, 4% fat and 14% protein by weight [194].

Table 6: Macronutrient, calcium and phosphorus content of freeze dried mango powder (TommyAtkins variety) used in the study. *

Parameter	Amount (*g/100g*)
Carbohydrate	73.70
Protein	4.80
Fat	2.89
Crude Fiber	7.58
Ash	3.00
Calcium	0.0854
Phosphorus	0.115
Calories	340

*Analysis performed by Nestle Purina Analytical Laboratories (St. Louis, MO)

Necropsy, Tissue Processing, and Storage

After eight weeks of dietary treatment, mice were fasted for 12 hours, weighed and injected with ketamine/xylazine cocktail (80 and 8 mg/kg body weight, respectively). Body composition was determined using DXA (GE Lunar Piximus, Fitchburg, WI). Blood was collected via exsanguination from carotid artery and placed into ethylenediamine-tetraacetic acid (EDTA) coated tubes. Plasma was obtained by centrifugation of whole blood for 20 minutes at 1300 X *g* and stored at -80°C for later analyses. Thymus, spleen, liver and uterus were removed

Table 7: Diet composition

	Control (AIN-93M)*	5% Mango	25% Mango
Ingredients (*g/kg*)			
Dried mango[a]		50.0	250
Total Carbohydrate	720.7	720.7	720.7
Cornstarch[b]	465.7	428.9	281.5
Maltodextrin[b]	155.0	155.0	155.0
Sucrose[b]	101.1	101.4	102.4
Dried mango[a]		36.6	184.3
Total Protein	140.0	140.0	140.0
Casein[b]	140.0	137.6	128.0
Dried mango[a]		2.4	12.0
Total Fat	40.0	40.0	40.0
Soybean oil[b]	40.0	38.6	32.8
Dried mango[a]		1.5	7.2
Total Fiber	50.0	50.0	50.0
Cellulose[b]	50.0	46.2	31.1
Dried mango[a]		3.8	18.9
Vitamin Mix[b]	10.0	10.0	10.0
Total Mineral Mix	35.00	35.00	35.00
Mineral Mix[b] (Ca & P deficient)	13.40	13.40	13.40
Calcium carbonate[b]	12.50	12.39	11.97
Calcium from mango[a]		0.04	0.21
Sodium phosphate[c] monobasic (NaH_2PO_4)	5.60	5.48	5.03
Potassium phosphate[d] monobasic (KH_2PO_4)	2.40	2.36	2.16
Phosphorus from mango[a]		0.06	0.29
Choline bitartrate[b]	2.50	2.50	2.50
L-cystein[b]	1.80	1.80	1.80

*Based on AIN-93M formulations containing 72% carbohydrate, 4% fat and 14% protein by calories (Reeves et al., 1997).

[a] Freeze dried Tommy Atkins Mango Pulp

[b] Harlan Teklad Diets (Madison, WI)

[c] Sigma Aldrich Co. (St. Louis, MO)

[d] Fisher Scientific Bio Reagents (Fair Lawn, NJ)

and weighed. A portion of abdominal white adipose tissue was collected and stored in formalin with the remainder snap-frozen in liquid nitrogen and stored at -80°C.

The femurs, tibiae, and spine were removed. Femurs and tibiae were cleaned of surrounding soft tissue. The tibiae were stored in 70% ethanol and the femurs snap frozen in liquid nitrogen and stored at -80°C. Spines were collected and stored at -20°C.

Body Composition and Bone Mineral Density (BMD) Assessment

Whole body, tibial, and vertebral bone mineral density (BMD), bone mineral content (BMC), bone mineral area (BMA) as well as total body composition (i.e. lean and fat mass, and % body fat) were assessed using DXA (Lunar PIXI, GE Medical Systems, Madison, WI) with PIXImus Series Software version 1.4X. Percent fat free mass was calculated using total body composition and lean tissue weight. A quality control phantom mouse was scanned before sample measurements were taken to assure no more than 2% error in measurements. Mice were spread with the frontal/coronal plane down, limbs extended away from the body, and the tail was not included in the scan. A region of interest (ROI) was selected after the scan was taken with the head excluded from analysis as recommended by the manufacturer.

Bone Micro-Architecture Assessment

Micro-architecture trabecular parameters of the spine were assessed using micro-computed tomography (Scanco μCT 40 scanner; SCANCO Medical, Switzerland). Scans were taken using 2048 x 2048 pixels. The area between L4 and L5 lumbar vertebrae was analyzed by acquiring 160 ± 5 slices (600 μm) and evaluating in the volume of interest (VOI). Semi-automated contours were placed starting 10 slices (60 μm) distal to the growth plate to assess secondary spongiosa within the VOI. Trabecular bone volume expressed per unit of total

volume (BV/TV), trabecular number (TbN), trabecular separation (TbSp), and trabecular thickness (TbTh), were determined and connectivity density (ConnDens) and structural model index (SMI) were calculated. The bone volume fraction is a measure of the volume of bone tissue (including internal pores like lacunae and canaliculi) per total volume.

Bone Strength Assessment

Bone strength was assessed using finite element (FE) pressure simulation on the μCT images of the area between L4 and L5vertebra (SCANCO Medical). The FE data is obtained by converting bone voxels, which represent the bone tissue, from the VOI into 8-node brick elements using a meshing technique [194]. FE uses compression on the simulated reconstructed 3-D images of the spine to determine the total force and size-independent stiffness [195].

Biomarkers of Bone Homeostasis

The bone formation marker, N-terminal propeptide of procollagen type 1 (PINP), and bone resorption marker, pyridinoline (PYD), were assessed in the plasma. Both biomarkers were assessed using enzyme-immuno-assays (EIA). PINP was purchased from Immunodiagnostic Systems Ltd. (Fountain Hills, AZ) and PYD from Quidel Corporation (San Diego, CA). The PINP assay used a polyclonal rabbit anti-PINP antibody. Standards, controls, and samples were added to a 96-well plate in duplicate. PINP labelled with biotin was then added and incubated for 1 hour at room temperature. Well contents were then decanted and the wells were washed three times with wash solution. Enzyme conjugate (PBS with avidin linked to horseradish peroxidase) was then added to all wells and incubated for another 30 min. Wells were again washed three times and tetramethylbenzidine substrate was added to all wells and incubated for 30 min. The reaction was stopped with hydrochloric acid and absorbance was measured at 450

nm using a microplate reader. A standard curve was created using the mean of each standard for the ordinate and the PINP concentration (abscissa) and was in read in ng/mL. PINP concentration values were multiplied by the dilution factor used (x10)

In the PYD assay (MicroVue^{TM}), PYD in the samples or standards competes with the PYD on the micro plate for polyclonal rabbit anti-PYD antibody. The bound antibody is then detected by goat anti-rabbit antibody conjugated to alkaline phosphatase. This reaction is detected with pNPP substrate. The plasma sample was filtered through 30k MWCO Spinfilter by centrifugation for 30 min at 3,000xg-10,00xg to obtain the sample filtrate used in the assay. Diluted standard, control or undiluted filtered plasma sample was then added to each well of the coated strip followed by the addition of PYD antibody. The plate was covered with an adhesive plate sealer and incubated overnight in the dark at 2-8ºC. Wells were decanted, washed with wash buffer, and enzyme conjugate was then added to each well followed by incubation for one hour. Wells were washed again with wash buffer followed by addition of working substrate solution and incubation for 40 minutes at room temperature. The reaction was stopped by the addition of sodium hydroxide stop solution and absorbance was read at 405 nm using a microplate reader. The results were analyzed with a 4 parameter calibration curve.

Statistical Analyses

Statistical analyses used a student's *t* - test and were derived using least square means and standard deviation (SD) for each treatment group using SAS version 9.1 (SAS Institute, Cary, NC). Analysis of variance and least square means were computed using the general linear model procedure and the means were compared using Fisher's least significant difference for comparing groups. Differences were considered significant at P values < 0.05.

CHAPTER IV

FINDINGS

Baseline Data

Two weeks post-ovariectomy, a baseline group of SHAM (n = 6) and OVX (n = 6) mice were necropsied to confirm bone loss prior to dietary treatment. A significant decrease in whole body BMD was observed in the OVX mice when compared to that of that of the SHAM mice (**Table 8**). However, no significant differences were observed in the BMD of the tibia or in the lumbar vertebrae (L4 and L5). In addition, there were no significant differences in both BMC and BMA of the whole body, tibia or vertebrae at two weeks post-ovariectomy between OVX and SHAM-operated mice. There were also no significant differences in plasma concentrations of the bone formation marker, N-terminal propeptide for type I procollagen (PINP), or the bone resorption marker, pyridinoline (PYD), in the ovariectomized and SHAM-operated C57/BL6 mice prior to dietary treatment.

Being that the ovaries were removed from the OVX group, the uterine tissue weights of the SHAM mice were higher than that of the OVX mice (**Table 8**) indicating success of the ovariectomy procedure. No significant differences in liver, spleen, and thymus weights were observed between the SHAM and OVX mice. Body composition also did not vary between the SHAM-operated and OVX groups after two weeks of surgery. However, fat mass tended (P=0.0516) to be lower in the OVX mice compared to the SHAM mice.

Effects of dried mango on weekly body weights of ovariectomized mice

Before the dietary treatment initiation, mice had similar body weights. After the first week of feeding, significant changes in body weight were already observed between the SHAM and OVX mice (**Table 9**). All OVX mice experienced similar weight gain during the first four weeks of dietary treatment (**Table 9**). However, during the last four weeks of treatment, the OVX mice receiving the control diet weighed more than the ones receiving the mango diet, despite similar food intake (**Table 9**). Among the OVX group mice, the 25% mango diet group had lower body weights than the OVX control and 5% mango group, although still higher than the SHAM animals. At the time of necropsy, 25% mango group mice had weights that were statistically similar to the SHAM animals. The 5% mango group had body weights that were similar to the 25% mango as well as to the OVX control group.

Effects of dried mango on food intake, tissue weights, whole body bone parameters and body composition of ovariectomized mice

Throughout the duration of the study, OVX mice were matched-fed to the SHAM mice. The average food intake throughout the duration of the study for ovariectomized mice was less than the SHAM mice. The OVX mice were matched-fed the food intake of the SHAM group that is one feeding cycle behind. Despite lower food intake by OVX mice when compared to SHAM mice, the weekly body weights of OVX mice were higher than those of the SHAM mice (**Table 9**).

Spleen, thymus and liver weights were not significantly different among treatment groups. Similar to baseline measurements, uterine weights of SHAM mice were significantly higher than OVX mice **(Table 10).**

The addition of mango to the diet modulated body composition, similar to the effects observed on body weight (**Table 10**). Fat mass and % body fat were highest in the OVX control mice. Both doses of mango reduced fat mass and % body fat but not to the level observed in the SHAM animals. Lean mass was not significantly different among treatment groups, but was different when expressed as a percentage of total mass (% fat-free mass). SHAM mice had the highest percentage of fat-free mass, followed by both mango-fed groups. The OVX control mice had the lowest % fat-free mass.

Effects of dried mango on bone parameters: whole body, tibia, and lumbar vertebrae (L4 and L5)

After 8 weeks of dietary treatment, mice in the OVX group lost more bone when compared to SHAM animals. OVX group mice had significantly lower whole body, tibia, and lumbar BMD and BMC when compared to the SHAM control mice (**Table 11**). Whole body, tibial and lumbar BMD of the mice given both mango diets were not different from the OVX mice which received the control diet.

Similarly, whole body and vertebral BMC were not affected by the addition of mango to the diet. Both the mango groups had whole body and vertebral BMC that were not statistically different from the OVX control group mice. In contrast, tibial BMC was increased by both doses of mango, with 5% mango diet increasing it to levels similar to SHAM mice (**Table 11**).

Whole body BMA was similar for all the OVX mice and significantly lower compared to the SHAM animals. Lumbar BMA of OVX control mice and 25% mango group mice were similar to that of the SHAM control mice. Mice receiving the 5% mango dose had the lowest

BMA. The opposite effect was observed with tibial BMA. The mice receiving the mango diets had higher BMA compared to those receiving the control diet (**Table 11**).

As expected, OVX animals had inferior microarchitectural parameters of the vertebra compared to the SHAM mice regardless of dietary treatment (**Table 12**). OVX mice had lower vertebral BV/TV, Conn. D., TbN, TbTH, Mean TV and Mean BV than SHAM animals. Supplementation of mango, at either dose, was not able to restore the deterioration of the micro-architectural parameters resulting from the ovariectomy.

Finite element analyses demonstrate that ovariectomized mice had lower size independent stiffness and total force in comparison to the SHAM mice (**Table 12**). Dietary treatment had no significant effects on vertebral strength of OVX mice

Effects of dried mango on plasma markers of bone formation and resorption

N-terminal propeptide for type I procollagen (PINP), a serum marker of bone formation, was not significantly different between any of the groups but tended ($P = 0.0510$) to be lower in the mango groups (**Table 13**). Plasma pyridinoline (PYD), a marker of bone resorption, was significantly higher in OVX when compared to SHAM mice. PYD values are inversely proportional to the level of bone resorption. Bone resorption rates were similar between all OVX groups. Mango was not able to attenuate changes in bone resorption markers due to ovariectomy.

Table 8: Baseline characteristics of ovariectomized (OVX) and SHAM-operated C57/BL6 mice two weeks post-ovariectomy (prior to dietary treatment).

	Sham	OVX	*P* Value
Bone Parameters			
Whole Body			
BMD *(mg/cm^2)*	49.8 ± 1.1	48.0 ± 1.3	0.0269
BMC (*mg)*	539.5 ± 33.6	539.5 ± 32.7	1.0000
BMA (*cm^2)*	10.83 ± .456	11.24 ± .530	0.1798
Tibia			
BMD (*mg/cm^2*)	44.48 ± 2.26	45.30 ± 2.41	0.5586
BMC (*mg*)	19.00 ± 0.89	18.83 ± 1.17	0.7872
BMA (*cm^2*)	0.43 ± 0.02	0.42 ± 0.01	0.4160
Lumbar Vertebrae (L4 and L5)			
BMD (*mg/cm^2*)	49.85 ± 3.23	50.32 ± 4.2	0.8337
BMC (*mg*)	114.0 ± 21.16	118.83 ± 10.07	0.6243
BMA (*cm^2*)	2.28 ± 0.35	2.36 ± 0.10	0.5921
Tissue Weights			
Uterus (*mg)*	133.3 ± 52.8	33.3 ± 5.2	0.0010
Liver (*mg)*	803.3 ± 43.2	916.7 ± 154.9	0.1150
Spleen (*mg)*	76.7 ± 10.3	81.7 ± 35.4	0.7470
Thymus (*mg)*	51.7 ± 9.8	60.0 ± 16.7	0.3177
Body composition			
Lean mass (*g)*	14.0 ± 0.51	13.71 ± 0.78	0.4739
Fat mass (*g)*	4.07 ± 0.16	3.85 ± 0.18	0.0516
Total mass (*g)*	18.05 ± 0.50	18.22 ± 2.13	0.8559
% Fat	22.45 ± 1.08	21.83 ± 0.68	0.2630
% Fat-free mass	77.54 ± 1.09	76.15 ± 5.01	0.5190
Plasma			
PINP *(ng/ml)*	35.39 ± 6.64	32.94 ± 7.27	0.5564
PYD *(ng/ml)*	1.92 ± 0.65	2.49 ± 0.97	0.2649

Values are mean ± SD (n=6/group), $P < 0.05$ were considered significant. BMD, bone mineral density; BMC, bone mineral content; BMA, bone mineral area.

Table 9: Effect of freeze -dried mango supplementation on weekly body weights of ovariectomized (OVX) mice

	Control SHAM ***(g)***	**Control OVX** ***(g)***	**5% Mango OVX** ***(g)***	**25% Mango OVX** ***(g)***	***P* value**
Baseline	19.7 ± 1.0	20.4 ± 1.0	20.6 ± 0.9	20.5 ± 0.8	0.2477
Week 1	18.7 ± 0.7^{b}	20.1 ± 1.1^{a}	20.2 ± 1.2^{a}	20.6 ±0.9^{a}	0.0047
Week 2	20.6 ± 1.1^{b}	22.4 ± 1.1^{a}	22.3 ± 0.8^{a}	22.6 ± 1.0^{a}	0.0014
Week 3	20.8 ± 1.4^{b}	23.3 ± 1.4^{a}	23.0 ± 1.3^{a}	23.4 ± 1.6^{a}	0.0025
Week 4	21.7 ± 1.1^{b}	25.0 ± 1.2^{a}	24.3 ± 1.8^{a}	23.8 ± 1.6^{a}	0.0006
Week 5	22.4 ± 0.5^{c}	27.1 ± 1.4^{a}	24.9 ± 1.8^{b}	24.5 ± 1.6^{b}	<.0001
Week 6	23.0 ± 1.1^{c}	29.1 ± 1.6^{a}	26.3 ± 2.3^{b}	25.7 ± 2.3^{b}	<.0001
Week 7	23.8 ± 1.8^{c}	30.3 ± 2.0^{a}	28.0 ± 2.7^{ab}	26.4 ± 2.4^{b}	<.0001
Week 8	24.0 ± 2.0^{c}	30.9 ± 1.9^{a}	28.5 ± 3.3^{ab}	27.3 ± 2.5^{b}	<.0001
Final	24.9 ± 1.7^{c}	31.2 ± 2.0^{a}	28.8 ± 4.2^{ab}	28.2 ± 3.0^{bc}	0.0019

Values are mean ± SD, n=8/group; within a row, values that do not share the same letters are significantly different (P<0.05) from each other.

Table 10: Effects of freeze-dried mango supplementation on food intake, tissue weights, and body composition of ovariectomized (OVX) mice

	Control SHAM	**Control OVX**	**5% Mango OVX**	**25% Mango OVX**	***P* Value**
Food intake (g)	4.8 ± 1.1^{a}	3.6 ± 1.0^{b}	3.8 ± 0.8^{b}	3.8 ± 0.9^{b}	<.0001
Tissue Weights (*mg*)					
Uterus	102.5 ± 29.2^{a}	23.1 ± 12.8^{b}	18.6 ± 12.1^{b}	17.5 ± 7.1^{b}	<.0001
Spleen	92.5 ± 15.8	100.0 ± 9.3	106.2 ± 32.0	98.8 ± 14.6	0.5923
Thymus	70.0 ± 24.5	82.5 ± 17.5	85.0 ± 18.5	86.2 ± 14.1	0.3178
Liver	986.7 ± 119.1	1143.3 ± 162.1	991.4 ± 162.8	1091.4 ± 117.5	0.1672
Body Composition					
Lean mass (*g*)	17.29 ± 1.0	18.72 ± 0.6	18.16 ± 1.5	18.25 ± 1.4	0.1288
Fat mass (*g*)	6.19 ± 0.9^{c}	11.68 ± 1.7^{a}	9.58 ± 2.8^{b}	8.66 ± 2.1^{b}	<.0001
Total mass (*g*)	23.50 ± 1.8^{c}	30.41 ± 2.3^{a}	27.74 ± 4.1^{ab}	26.90 ± 3.4^{b}	0.0011
% Fat	26.2 ± 1.8^{c}	38.3 ± 2.7^{a}	33.8 ± 5.3^{b}	31.7 ± 4.4^{b}	<.0001
% Fat free mass	73.7 ± 1.8^{a}	61.7 ± 2.7^{c}	66.1 ± 5.2^{b}	68.3 ± 4.3^{b}	<.0001

Values are mean ± SD, n=8/group; within a row, values that do not share the same letters are significantly ($P < 0.05$) different from each other.

Table 11: Effects of freeze-dried mango supplementation on whole body, tibia and lumbar vertebrae (L4 and L5) bone mineral density (BMD), content (BMC), and area (BMA) of ovariectomized (OVX) mice

	Control SHAM	**Control OVX**	**5% Mango OVX**	**25% Mango OVX**	***P* Value**
Whole Body					
BMD (mg/cm^2)	53.8 ± 1.5^{a}	47.3 ± 1.0^{b}	48.0 ± 1.6^{b}	48.3 ± 1.3^{b}	<.0001
BMC (mg)	564.1 ± 43.0^{a}	414.9 ± 40.0^{b}	444.9 ± 54.8^{b}	450.4 ± 16.7^{b}	<.0001
Area (cm^2)	10.5 ± 0.6^{a}	8.8 ± 0.7^{b}	9.2 ± 0.9^{b}	9.33 ± 0.4^{b}	0.0002
Tibia					
BMD (mg/cm^2)	49.79 ± 1.02^{a}	43.65 ± 1.76^{b}	42.58 ± 1.33^{b}	43.36 ± 0.86^{b}	<.0001
BMC (mg)	18.75 ± 1.16^{a}	16.00 ± 1.51^{c}	17.75 ± 1.58^{ab}	17.38 ± 0.92^{b}	0.0029
BMA (cm^2)	0.38 ± 0.02^{b}	0.37 ± 0.02^{b}	0.42 ± 0.03^{a}	0.40 ± 0.02^{a}	0.0006
Lumbar Vertebrae (L4 and L5)					
BMD (mg/cm^2)	56.66 ± 4.7^{a}	44.13 ± 2.7^{b}	45.51 ± 3.9^{b}	45.61 ± 2.7^{b}	<.0001
BMC (mg)	17.00 ± 1.7^{a}	13.00 ± 1.5^{b}	12.25 ± 1.4^{b}	13.00 ± 1.2^{b}	<.0001
BMA (cm^2)	0.30 ± 0.02^{a}	0.30 ± 0.02^{a}	0.27 ± 0.02^{b}	0.29 ± 0.02^{ab}	0.0049

Values are mean ± SD, n=8/group; within a row, values that do not share the same letters are significantly different ($P < 0.05$) from each other.

Table 12: Effects of freeze-dried mango supplementation on microarchitectural parameters and strength of lumbar vertebra (L4 and L5) of ovariectomized (OVX) mice

	SHAM	**Control OVX**	**5% Mango OVX**	**25% Mango OVX**	***P* Value**
Microarchitectural parameters					
BV/TV *(%)*	19.1 ± 1.0[a]	11.4 ± 1.4[b]	10.3 ± 0.6[b]	11.4 ± 1.4[b]	<.0001
Conn. D. (*1/mm*^{3})	120.0 ± 17.0[a]	85.0 ± 18.2[b]	73.9 ± 9.8[b]	86.3 ± 13.7[b]	0.0002
SMI *(1)*	1.24 ± 0.14[b]	1.82 ± 0.12[a]	1.97 ± 0.11[a]	1.84 ± 0.15[a]	<.0001
TbN (*1/mm*)	3.64 ± 0.13[a]	3.24 ± 0.25[b]	2.94 ± 0.37[b]	3.14 ± 0.23[b]	0.0012
TbTH (*μm*)	56.20 ± 1.13[a]	45.98 ± 2.02[b]	45.55 ± 1.71[b]	46.07 ± 1.68[b]	<.0001
TbSp (*μm*)	275.37 ± 11.11[c]	312.87 ± 26.50[b]	348.07 ± 42.82[a]	322.17 ± 23.65[ab]	0.0025
Mean TV (mg HA/μm)	292.94 ± 12.80[a]	206.98 ± 26.88[b]	194.37 ± 30.13[b]	218.48 ± 15.97[b]	<.0001
Mean BV (mg HA/μm)	1082.99 ± 20.19[a]	1064.85 ± 6.74[ab]	1057.03 ± 15.25[b]	1048.28 ± 31.32[b]	0.0479
Bone strength by finite element analysis					
Von Mises *(MPa x 10^{-3})*	1457.1 ± 400.3	1216.2 ± 162.7	1268.0 ± 263.5	1252.9 ± 238.8	0.4674
Total Force *(N x 10^{-3})*	218.3 ± 82.4[a]	103.6 ± 34.9[b]	102.8 ± 29.9[b]	103.8 ± 32.8[b]	0.0016
Size Independent Stiffness *(N/m)*	20914.2 ± 6222.1[a]	9491.8 ± 2943.4[b]	8894.2 ± 2468.5[b]	9524.6 ± 2995.6[b]	0.0001

Values are mean ± SD, n=6/group; within a row, values that do not share the same letters are significantly different ($P < 0.05$) from each other. BV/TV - bone volume fraction; Conn. D. - connectivity density; SMI - structure model index; TbN - trabecular number; TbTH - trabecular thickness; TbSp - trabecular separation; Mean TV - mean total volume; Mean BV - mean bone volume.

Table 13: Effects of freeze-dried mango supplementation on marker of bone formation, PINP, and bone resorption, PYD, of ovariectomized (OVX) mice

	Control SHAM	**Control OVX**	**5%Mango OVX**	**Mango 25% OVX**	***P* Value**
PINP (*ng/mL*)	27.33 ± 11.26	25.92 ± 6.03	19.56 ± 7.09	18.34 ± 2.72	0.0510
PYD (*ng/mL*)	2.97 ± 0.77[a]	1.70 ± 0.37[b]	1.49 ± 0.41 [b]	1.47 ± 0.22 [b]	<0.0001

Values are mean ± SD, n=8/group; within a row, values that do not share the same letters are significantly different ($P < 0.05$) from each other. PINP, N-terminal propeptide for type I procollagen; PYD, pyridinoline.

CHAPTER V

CONCLUSION

The purpose of this study was to determine whether freeze-dried mango pulp powder supplementation, in 5 and 25% doses, could reverse bone loss in ovariectomized mice. The null hypothesis was accepted in that mango was not able to restore the ovariectomy induced bone loss as evidenced by whole body, vertebral, and tibial BMD measurements, micro-architectural parameters and biomechanical properties of the lumbar vertebrae, and had no effect on serum markers of bone formation (PINP) or bone resorption (PYD). Although mango was not effective in reversing ovariectomy- induced bone loss, it did modulate body weight and % body fat.

Although mango has no effect on BMD, some positive effects were observed on BMC. Whole body and lumbar BMC did not differ from the OVX control diet, but BMC of the tibia was improved with mango supplementation. The 5% dosage even restored tibial BMC to SHAM levels. However, when looking at BMC it is important to consider it in relation to BMA. The higher tibial BMA in the mango groups explains why the BMC is also higher. Although the tibia may not be the best place to detect ovariectomy induced bone loss in mice, according to Lynch and colleagues (2010)[165], in human subjects, the tibia is a valuable assessment measurement for osteoporotic bone loss due to the fact that tibial cortical bone decreases about 5.6–11.0% every 10 years in women while only an average of 5.5% is lost every 10 years at the femoral neck.

Even though tibial BMC and BMA of the mango group are higher than controls, it has been pointed out that BMD accounts for 60-80% of the variations observed in bone strength [3, 196]. It is also important to recognize that the relationship between BMD, BMC and BMA may be different at different bone sites. For example, a study conducted on 1449 women over 30 years of age by Deng and colleagues in 2002[197], demonstrated that, at the spine, 86.2% of BMD variation was attributable to BMC and 12.6% to bone area while at the hip, 98.0% of BMD variation was due to BMC and 1.1% due to bone area.

As expected, micro-architectural parameters in the OVX mice were significantly lower than all SHAM mice as assessed by micro-computed tomography. MicroCT delivers high resolution 3D images which directly measure bone micro-architecture with excellent reproducibility and accuracy [198]. X-ray snapshots at multiple viewing angles are reconstructed to a 3D image which shows the spatial distribution of the bone at the micrometer voxel size. Samples were contoured by hand every ten slices with algorithms used to complete the slices in between with the exclusion of cortical bone. Mango did not exert an influence on the micro-architectural parameters in the secondary spongiosia (trabecular bone) of the lumbar vertebra. Bone volume fraction (BV/TV), the ratio of segmented bone volume to the total volume of the region of interest, was not influenced by mango. Connectivity density, trabecular number, thickness, mean total volume, and mean bone volume were also not affected by mango. The 5% mango group mice did exhibit greater trabecular space which is associated with a weaker micro-architectural arrangement. The structural model index estimates the plate verses rod characteristics of the trabecular bone [198]. Zero values represent plate arrangement while a value of 3 represents rods and 4 represents spheres. All OVX mice exhibited higher SMI values than SHAM mice indicating more rod like arrangements than the SHAM animals. Changes of

trabeculae from plate-like to rod-like are known to occur in aging, bone remodeling, and osteoporosis [199] indicating that the OVX mice were experiencing more bone remodeling than SHAM animals as evidenced by the SMI values.

Bone strength was also not improved by mango supplementation. Von Mises stress is calculated by combining stresses in three dimensions, with the result compared to the tensile strength of the material loaded in one dimension, yielding one numeric measurement[199]. Von Mises stress did not differ between OVX and SHAM animals. This may be due to the fact that animal bones do not tend towards fracture and have a high resiliency to pressure despite their age or hormonal influences [200]. Biomechanical assessment measurements of total force and size independent stiffness, however, were lower in OVX mice indicating that with estrogen withdrawal there is a subsequent loss in bone strength. Mango supplementation did not improve biomechanical properties of the bone when compared to the OVX control diet mice. Using different evaluation tools may help to further evaluate the effect of mango on bone strength. For example, quantitative ultrasound measurements can also reflect bone elasticity, fragility and density closely associated with bone strength and fracture risk [201].

Although many biological compounds may assist in preventing bone loss, few agents are successful at reversing the bone loss associated with osteoporosis. Fruits and vegetables or their bioactive compounds may be effective in alleviating the inflammatory environment associated with the postmenopausal unregulated production of pro-inflammatory cytokines and may also exert a protective antioxidant effect on the bone tissue while supplying important vitamins and minerals necessary for reversing bone loss. Although the OC secretes ROS under normal bone remodeling conditions to assist with the destruction of calcified bone tissue, in a postmenopausal low estrogen state, the increased ROS production with the increase in bone resorption may

overwhelm the bodies endogenous antioxidant system and lead to osteoporosis [202, 203] . Sheweita and Khoshhal suggested that the enhanced OC activity may increase the superoxide anion generation and/or inhibit superoxide dismutase and glutathione peroxidase activities[112]. Mango possesses antioxidants, such as the carotenoids, flavonoids, triterpenes, phenolic compounds and vitamin C, which, in theory, may help attenuate the inflammatory environment associated with osteoporosis.

Some studies have demonstrated the effectiveness of bioactive food compounds in improving bone quality or preventing bone loss but few have shown bone loss reversal. For example, citrus pulp, containing high amounts of flavonoids and vitamin C, improved BMD in male orchidectomized rats [14, 15]. Pholridzin in apples, rutin, and isoflavones have all prevented further bone loss in ovariectomized rats [20]. Green tea polyphenols reduced bone micro-architecture deterioration in female rats [116] and blueberries prevented whole body BMD loss in OVX rats[167]. To our knowledge, only dried plum has been demonstrated to effectively restore bone that has been lost [17]. Dried plum polyphenols not only inhibited osteoclastogenesis by down regulating NFATc1 and inflammatory mediators such as TNF-α, they also upregulated OB transcription factors such as Runx2, Osterix and IGF-1 in MC3T3-E1 cells [16]. In another study involving dried plum in ovariectomized rats, trabecular micro-architectural parameters were improved as well as femoral and tibial BMD by the 5% dose while lumbar BMD was improved at a 25% dose [17].

The pro-vitamin A compound, β-carotene, found in mango may also play a role in OB cell differentiation. Research done by Park and colleagues on mouse osteoblastic cells demonstrated that vitamin A and carotenoids increased OB cell differentiation[204] while another cell culture study showed an inhibited OC like cell formation[205]. In a study involving 334 men

and 540 women over the age of 75, a positive correlation between carotenoid consumption and BMD [18] and a negative correlation with carotenoid consumption and hip fracture rates was observed [206]. A study conducted by Yuyama and colleagues determined that the pro vitamin A carotenoids in mango was absorbed with 82% efficiency in rats[207]. Drammeh and colleagues used dried mango powder along with fat supplementation to successfully improve the vitamin A status in young Gambian children[208]. These findings suggest that pro vitamin A content of the mango in the present study may influence bone. The present study, however, demonstrated that mango, despite being a rich source of antioxidants and β-carotene, was not able to reverse bone loss.

As expected, the OVX mice experienced more bone resorption than the SHAM control mice. Mango did not attenuate bone resorption as evidenced by serum PYD values that were similar between the mango groups and the OVX control mice. Despite PINP being one of the most specific and sensitive indicators of bone formation[209], serum PINP levels were not significantly different between any of the groups. The trend ($P = 0.0510$) was that SHAM mice had the highest PINP levels with 25% mango group exhibiting the lowest values. Considering this trend along with the fact that the mango group mice exhibited a lower body fat body composition, one may want to consider the influence of the metabolically active adipose tissue on bone parameters.

The higher body fat composition in the OVX mice may exhibit a greater hormonal effect contributing to increased rates of bone formation. For example, earlier studies have demonstrated that leptin, secreted by the adipose tissue, stimulates cortical bone formation[74, 75] via its effects on the GH-IGF-1 axis of the central nervous system and by suppressing the release of neuropeptide Y, an inhibitor of cortical bone formation, from the hypothalamus. Leptin's

combined effects of neuropeptide Y suppression and the β1- adrenergic stimulation result in an increase in cortical bone mass [74]. Adiponectin, also secreted by adipose tissue, is known to modulate glucose and fatty acid catabolism [210] with levels inversely associated with percent body fat [211]. In a study conducted on postmenopausal women, increasing adiponectin levels were associated with a decrease in BMD. The percent change in BMD was associated with a doubling in serum adiponectin levels [212]. A study conducted by Richards and colleagues showed that serum adiponectin levels were associated with a 2.7% average decrease in BMD at the hip, femoral neck, forearm and spine[213, 214]. Post-menopausal status increased the magnitude of this inverse relationship. The fact that the decrease in BMD was also evident at non-weight bearing sites indicates an important role of adipose hormones in regulating BMD. Future implications of this research may want to consider the effects of adipose hormones on BMD.

Although mango fed for 8 weeks was not effective in reversing bone loss, mango modulated body weight and body composition. Mango attenuated weight gain and percent body fat. After four weeks of dietary treatment, both mango diet groups weighed less than the OVX control group. Final body weight of the 25% mango diet group was similar to both the 5% mango group and that of the SHAM mice. Similarly, fat mass and % body fat of both mango diet mice groups were less than that of the OVX control mice despite similar food intake patterns between all OVX groups. Following suit, both mango containing diets had more fat free mass than the OVX control mice. Although the fiber content of the mango and control groups were matched, mango may have exerted an effect on digestion or absorption of the diet either by inhibiting fat absorption to a degree or by increasing the metabolism. Stool assessments or direct calorimetry may be ways to assess these possibilities in the future studies. Further investigation

on mango and bone biology and mechanisms in modulating body composition in postmenopausal women are warranted.

The results of our study failed to reject our null hypotheses. Freeze-dried mango powder in 5% and 25% of the daily diet was not effective in reversing ovariectomy induced bone loss in mice. Mango did not improve whole body, tibial or lumbar vertebral BMD , nor did it improve micro-architectural parameters or bone strength in the vertebrae. Plasma markers of bone formation (PINP) and bone resorption (PYD) also were not affected by mango supplementation. Freeze-dried mango powder supplementation in both doses did attenuate weight gain and modulate body composition. Although mango was not able to reverse the bone lose associated with gonadal hormone difficiency, its role in preventing osteoporosis still needs to be explored.

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VITA

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Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in May 2011.

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Experience:
Graduate Research Assistant at; Oklahoma State University, Stillwater, OK Sept. 09 – Dec. 2010

Operated x-ray machinery, analyzed bone micro-architecture (MicroCT), prepared and mixed diet for laboratory animal studies, fed and weighed laboratory mice, participated in necropsies', perform experimental procedures on research tissues such as RNA extraction and ELISA assays, executed cell culture work, assisted in professional journal reviews and report writing

Receptionist, Medical Biller – Davinci Dental Center, Honolulu, HI. Jan 09 – July 09

Prepared medical charts, scheduled appointments, verified insurance coverage, billed insurance companies, processed explanation of benefit statements (EOBs), prepared monthly billing statements.

Nutrition Education – Hawaii Medical Service Association (HMSA), Honolulu, HI. Feb. 08 – Nov. 09

Created and presented nutrition education materials for the HMSA Eat Healthy Campaign. Audiences range from grade school students to senior citizen groups.

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Findings and Conclusions: After menopause or surgically induced menopause, estrogen deficiency results in a pro-oxidant and pro-inflammatory environment which increases bone resorption and hence, fracture risk. Mango, a fruit rich in phytonutrients, vitamins and minerals may help mediate hormone-related bone loss. This study examined the dose- dependent effects of mango on the reversal of bone loss in ovariectomized (OVX) mice. C57BL/6 mice were randomly divided into one of four dietary treatment groups (n=8/group) for eight weeks: SHAM - control diet; OVX - control diet; OVX - 5% or 25% (wt/wt) dried mango diet. Bone mineral density (BMD) of the whole body, lumbar, and tibia of mice in the mango diets were lower than that of SHAM animals ($P < 0.0001$) but were not different from the OVX-control. Similarly, bone micro-architecture and strength parameters of mice receiving the mango diets mice were not statistically different from OVX control and were inferior to the SHAM-control group. Although the mango diets were not able to reverse bone loss in OVX mice, it modulated body weight from week 5 through week 8 and % body fat ($P < 0.0001$) despite similar food intake patterns among OVX mice.

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