EFFECTS OF DIETS RICH IN PHENOLIC COMPOUNDS ON BONE IN OVARIECTOMIZED RATS

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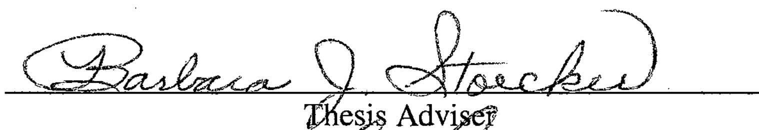

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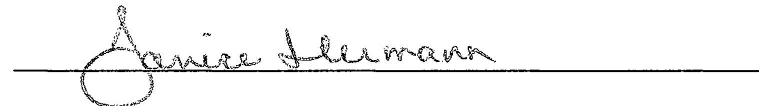

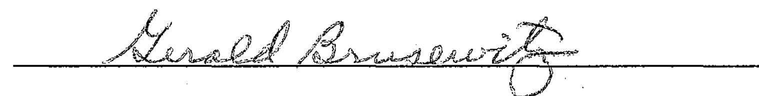

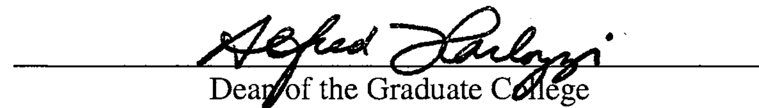

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DEDICATION

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CHAPTER I

INTRODUCTION

Osteoporosis is defined and characterized by micro architectural deterioration of the bone tissue and increased bone fragility (Consensus Development Conference, 1991). It is a disease characterized by low bone mass per unit volume of bone. Osteoporotic bone yields to fracture with relatively low trauma, trauma which would not have been expected to cause fracture in an adult in their prime. As life expectancy is improved and the population continues to age, serious complications of osteoporosis including hip fracture will increase exponentially. For a Caucasian woman, the lifetime risk of an osteoporosis-related fracture is about 30% (Grady et al. 1992). This progressive disease currently affects 26 to 38 million people, causes 300,000 hip fractures, and costs Americans over 14 billion dollars annually (Tolstoi and Levin, 1992; Melton, 1993; Wactawski-Wende et al. 1996; Looker et al. 1997).

Bone mass increases during the first two to three decades, peaks at 25-35 years of age, maintains itself for 10-15 years, and is followed by a gradual decline (Ott, 1990). Such an observation suggests that the higher the peak bone mass, the longer it takes for the bone mass to decline to the stage of pathophysiological imbalance characterizing osteoporosis.

Postmenopausal osteoporosis (type I) is the most common type of osteoporosis. It typically involves females within 20 years after menopause and is characterized by an accelerated decrease in bone mass exacerbated by ovarian hormone deficiency.

Trabecular (cancellous) bone which is found toward the metaphysis and the epiphysis is usually affected in type I osteoporosis (Dodd, 1996). Type II osteoporosis occurs in both sexes at around 70 years of age. In type II osteoporosis, the individual loses both cancellous and cortical bone mass (Dodd, 1996). The focus of this study is to investigate the role of naturally occurring estrogen-like compounds (phytoestrogens) in bone using a rat model which represents osteoporosis type I-related bone loss.

Many studies have established estrogen as a bone anti-resorptive agent, and estrogen replacement therapy (ERT) shortly after menopause retards the loss of bone (Lindsay et al. 1976; Barzel, 1988). However, ERT is associated with side effects and contraindications such as uterine bleeding, endometrial cancer, and breast cancer (Judd et al. 1983; Henderson et al. 1993). Although other therapies such as calcitonin and bisphosphonates may prevent additional bone loss in established osteoporosis (Gruber et al. 1984; Mazzuoli et al. 1986; Turner, 1991), they can not restore bone mass after loss has already occurred. Additionally, the potential bone formation stimulating agents such as parathyroid hormone, vitamin D, and sodium fluoride are either associated with side effects or are ineffective in reducing the risk of fracture (Turner, 1991; Tolstoi and Levin, 1992; Lindsay, 1993; Raisz, 1993). Therefore, investigating the role of alternative approaches, including the use of phytoestrogens, in reducing bone loss or increasing bone mass in ovarian hormone deficiency has merit.

Recent reports indicate that naturally occurring compounds found in soy, such as isoflavones, have estrogen-like activities (Riddle and Estes, 1992; Brandi, 1993; Miksicek, 1993). Isoflavones are a group of phenolic compounds which have been shown

to have beneficial effects on bone, under ovarian hormone deficiency conditions, in both humans (Gambacci et al. 1985) and animals (Arjmandi et al. 1996, Arjmandi et al. 2000).

Phenolic compounds are found in plants, grains, fruits, and vegetables and as antioxidants they inhibit lipid peroxidation. Prunes are an example of fruit rich in phenolic compounds with antioxidant capacity (Bravo, 1998). The antioxidants present in prunes, in part, may protect bone through modulation of oxygen-derived free radical formation either in the bone microenvironment or in the cells that serve as osteoclastic precursors.

Prunes are considered a good source of nutrients including macro-minerals such as potassium and magnesium and trace elements such as boron and copper. These nutrients have been implicated in improvement and/or maintenance of skeletal health (Nielsen et al. 1987; Cohen and Meyer, 1993; Classen et al. 1994). New and colleagues (1997) reported that nutrients found in fruits and vegetables improve bone mass in the presence of normal estrogen. This study was designed to evaluate whether prunes are also effective in reversing bone loss after such loss has already occurred.

HYPOTHESES

The hypotheses of this study are that the consumption of prune is effective in reversing bone loss that has already occurred due to ovarian hormone deficiency. Additionally, we hypothesize that consumption of isoflavones will prevent bone loss in ovarian hormone deficient rats. Four specific aims have been established to test these hypotheses as follows:

Specific Aim 1: To evaluate the effects of prunes on bone mineral density, bone strength, bone calcium, and bone magnesium.

Specific Aim 2: To investigate the effects of prunes on markers of bone formation and bone resorption including serum alkaline phosphatase and tartrate-resistant acid-phosphatase (TRAP) activities and on urinary hydroxyproline and deoxypyridinoline.

Specific Aim 3: To evaluate the effects of isoflavones on bone mineral density, bone strength, bone calcium, and bone magnesium.

Specific Aim 4: To investigate the effects of isoflavones on markers of bone formation and bone resorption including serum alkaline phosphatase and tartrate-resistant acid-phosphatase (TRAP) activities and on urinary hydroxyproline and deoxypyridinoline.

CHAPTER II

REVIEW OF LITERATURE

A. Structure and Biology of Bone

Bone is a dynamic and complex tissue which is made up of connective tissue and is a site for hematopoiesis (Baron, 1993). Bone has internal (endosteal) surfaces and external (periosteal) surfaces which contain osteogenic cells (Baron, 1993). Bone is regulated by local (e.g., prostaglandins and interleukins) factors as well as systemic (e.g., parathyroid hormone, vitamin D, estradiol, and calcitonin) hormones (Raisz, 1990).

The skeleton is composed of two types of bone, cortical bone and trabecular (cancellous) bone. Cortical bone is 80-90% calcified by volume and is found in the shaft of the long bones including both the femur and the tibia. Trabecular bone is 15-25% calcified and is found in the end of the long bones, vertebrae, the pelvis, and flat bones (Baron, 1993).

Bone is made up of four different cell types: osteoblasts, osteoclasts, osteocytes, and lining cells. The primary functions of osteoblasts and osteoclasts are to carry out the remodeling process; osteocytes and lining cells are involved with metabolic functions and providing nutrients to bones (Christiansen, 1993).

Osteoblasts are derived from mesenchymal stem cells, are primarily mononuclear and have well developed Golgi apparatus and extensive rough endoplasmic reticulum. Osteoblasts are involved with the synthesis of collagen and non-collagenous proteins (Baron, 1993). Collagenous proteins are predominately type I collagen, while non-

collagenous proteins include osteocalcin, osteonectin, osteopontin, bone siloprotein, and alkaline phosphatase (Aubin et al. 1995). Bone formation by osteoblasts involves three distinct stages. In the first stage, collagen is deposited and then the rate of mineralization increases to match the rate of collagen synthesis. Finally, the rate of collagen synthesis decreases, whereas the rate of mineralization continues at a rapid rate.

Osteoblasts are regulated by three mechanisms: endocrine hormones including 1,25 $(OH)_2$ vitamin D and parathyroid hormone; paracrine such as insulin-like growth factors, interleukin-6, fibroblast growth factors, and transforming growth factors; and autocrine such as insulin-like growth factors, fibroblast growth factors, and transforming growth factors (Harrison and Clark, 1986; Hesch et al. 1988).

In contrast to osteoblasts, osteoclast cells originate from phagocytic lineage and are giant multinucleated cells with abundant Golgi complexes containing lysosomal enzymes and collagenase (Baron, 1993). Osteoclast activity is influenced by calcium regulating hormones and a number of cytokines and growth factors including interleukin-1 and transforming growth factors (Wener et al. 1972; Roodman et al. 1985).

Bone resorption by osteoclasts is divided into two phases. Phase I is involved in inorganic mineral metabolism conducted by osteoclasts, macrophages, and monocytes (Elion and Raisz, 1978). In phase II, hydrogen ions that are secreted by osteoclasts provide an optimal environment to degrade bone matrix. In this process collagen is broken down and released into the systemic circulation in the form of hydroxyproline, hydroxylysine, pyridinoline, and deoxypyridinoline (Baron, 1993; Risteli and Risteli, 1993).

The osteocytes were originally the bone forming cells that have been trapped in the matrix they synthesized and have become calcified. The lining cells however, provide cell to cell contact through microfilament processes forming a network within the bone matrix (Raisz, 1993). It has been postulated that via these networks cells communicate and receive nutrients (Raisz, 1993).

B. Bone Modeling, Remodeling, and Turnover

Bone modeling is initiated with bone formation at the periosteal surface and is followed by bone resorption at the endosteal surface (Baron, 1993; Arnaud, 1996). Bone remodeling is a cyclic process consisting of osteoclastic bone resorption followed by osteoblastic bone formation. Bone remodeling occurs at discrete sites where osteoclasts excavate the old bone, leaving pits. The osteoblasts move into the region and produce collagen and bone matrix protein filling the pits that become mineralized. This remodeling follows a cyclic process in order to replace old bone with new bone (Kanis, 1997). These remodeling cycles are normally linked to ensure maintenance of bone mass. Lack of coordination between the resorption and formation can result in bone loss if there is a relative excess of resorption over formation.

Bone remodeling is a complex process that happens all through life and follows four sequential steps in each site: activation, resorption, reversal, and formation (Baron, 1993).

1. Activation

Activation of osteoclasts in conjunction with bone remodeling and bone resorption is not well understood. It appears that local and systemic factors, such as interleukin-1, tumor necrosis factor, vitamin D, and parathyroid hormone, are involved in

the activation of osteoclasts (Mundy, 1993). Others have suggested that the activation of osteoclasts may be mediated via osteoblasts such that the osteoblasts would initiate the activation process (Raisz, 1993).

2. Resorption

Bone resorption follows osteoclast activation (Raisz, 1993; Arnaud, 1996). Osteoclasts attach to the bone surface and stimulate the release of enzymes responsible for acidification and digestion of bone cells (Raisz, 1993; Arnaud, 1996). Others believe that collagenase released by osteoblasts prepares the bone surface for resorptive activity by osteoclasts (Mundy, 1993). Once the resorption task is accomplished, the osteoclasts undergo apoptosis which concludes the remodeling phase (Hughes et al. 1995).

3. Reversal

During the reversal period, it is believed that macrophage-like cells appear on the bone surface, creating a cement line between new and old bone. This process limits the resorption area and prevents further resorption (Raisz, 1993; Baron, 1993).

4. Formation

In the bone formation stage, osteoblasts are attracted to the resorptive site and begin proliferation and differentiation and secrete matrix protein substances to be mineralized (Raisz, 1993). In vitro studies have been conducted to evaluate factors involved in differentiation and proliferation of stem cells to synthesis of osteoblasts. At least one factor has been identified in bone formation processes and that is transforming growth factor (TGF). Transforming growth factor stimulates stem cell differentiation, bone morphogenetic protein (BMP) expression, and osteoblast proliferation (Mundy, 1993; Aubin et al. 1995).

C. Osteoporosis and Bone Mass

Osteoporosis is a disorder involving a reduced quantity of bone tissue, change in microstructure, decreased bone strength, and increased fracture risk (Center and Eisman, 1997). The most serious complications of osteoporosis are hip fractures which occur in over a quarter of a million women annually (Melton, 1993). Bone mass is a major determinant of fracture risk and a number of studies have demonstrated that decrease in bone density is associated with an increase in fracture risk (Wasnich et al. 1985; Hui et al. 1988). Wasnich et al. (1985) observed that postmenopausal women with low bone mass had ten times the risk of non-spine fracture. Similarly, Hui et al. (1989) reported a more than two-fold increase in fracture rate for each gram/centimeter decrease in the bone mass of the radius. Decreased bone mass with age followed by superimposed estrogen withdrawal in women after the menopause increased the risk for hip fracture (Cummings et al. 1990; Kanis and Adami, 1994). In several longitudinal studies, authors observed that reduced estrogen production following menopause was the major factor contributing to the significant bone loss (Slemenda et al. 1987; Nilas and Christansen, 1988; Pouilles et al. 1993).

Similar to human studies, ovariectomized rats, a model for studying osteoporosis, have significant decreases in bone density (Arjmandi et al. 1996; Mattila et al. 1998). Peng and co-investigators (1994) observed that the femoral neck was sensitive to ovariectomy. Arjmandi et al. (1996) attributed decrease in bone density to estrogen deficiency, because estrogen treatment following ovariectomy significantly increased bone density.

Bone strength is reported to be a function of bone mass and for this reason low bone mass in the elderly results in greater frequency of fracture (Bartley et al. 1966). To examine bone strength following ovariectomy in rats, Toolan et al. (1992) reported that the maximal load to break the femoral neck does not differ between the sham and ovariectomized groups several months post-surgery (Toolan et al. 1992). In contrast, Mattila et al. (1998) reported that ovariectomies decreased femoral neck strength in rats. Peng et al. (1997) reported that in rats, despite ovariectomy-induced bone loss, an increase in body weight along with other metabolic changes may be responsible for maintaining mechanical strength of the femoral neck. Also, Kiebzak et al. (1988) reported that increase in length and width of bones may reflect architectural adjustment resulting in strength compensation.

D. Bone Resorption Inhibitors

1. Estrogen

Estrogen replacement therapy is used to decrease hot flashes, cardiovascular disease, and bone loss due to ovarian hormone deficiency (Kalu et al. 1991; Mundy, 1993). Estrogen exerts its effects on bone mainly by suppressing bone turnover which is accelerated due to ovarian hormone deficiency (Mundy, 1993). Consequently, estrogen exerts its effects by slowing bone resorption and bone formation (Turner, 1991).

Estrogen deficiency accelerates the rate of bone turnover with resorption exceeding formation resulting in net bone loss (Heaney et al. 1978; Compston, 1994). Administration of estrogen to postmenopausal women (Jensen et al. 1982) and ovariectomized rats (Wronski et al. 1988) prevents this ovarian hormone deficiency-

associated bone loss. However, estrogen administration can not reverse the bone loss that has already occurred (Vedi et al. 1996).

Although estrogen is considered an anti-resorptive agent, there is evidence that estrogen administration increases femoral cortical bone mass in osteopenic adult female rats (Turner, 1991). Tobias and colleagues (1991) also reported increased trabecular bone volume of the tibia in 3-month old Wistar female rats treated with high doses (14.68 mol/kg body weight) of estrogen for two weeks. However, the bone forming ability of estrogen, at best, is questionable and requires further investigation.

2. Calcitonin

Calcitonin is a peptide containing 32 amino acids made in the C cells of the thyroid gland. It functions as a regulatory hormone in response to increased serum calcium (Raisz, 1993). Calcitonin decreases serum calcium by increasing urine calcium and inhibiting calcium release from the bone (Raisz, 1993). Calcitonin at pharmacological doses suppresses osteoclastic activity (Raisz, 1993; Arnaud, 1996). Calcitonin apparently inhibits osteoclast activity by binding to specific receptors on osteoclasts and decreases the activity of those cells (Nicholson et al. 1986). Also, calcitonin inhibits osteoclast proliferation of progenitors and differentiation of precursors (Price et al. 1980). In human studies, however, calcitonin supplementation increased bone mineral density of lumbar vertebrae (Nieves et al. 1998). Other studies also support the beneficial effects of calcitonin administration on increased bone mineral content of spine and forearm in postmenopausal women (Gruber et al. 1984; Mazzuoli et al. 1986).

3. Bisphosphonates

Bisphosphonates are synthetic analogues of natural pyrophosphates, which combine with hydroxyapatite crystals in bone and inhibit osteoclastic activity (Tolstoi and Levin, 1992; Mundy, 1993). The therapeutic benefit of bisphosphonates is to reduce vertebral fracture by 50% over a two year period (Turner, 1991). In a separate experiment with postmenopausal women, etidronate was reported to increase bone mass and reduce fracture rate (Meunier et al. 1997). In large doses, however, bisphosphonates may inhibit bone mineralization (Turner, 1991). Therefore, long term benefit of bisphosphonates is limited. However, daily doses of 400 mg for two weeks every three months followed by a daily supplement of 500 mg of calcium and 400 IU of vitamin D supported bone mineralization (Kujdych and Pitman, 1996). Side effects such as nausea, abdominal pain, constipation, diarrhea, and dyspepsia have been reported to occur in a number of individuals (Chestnut et al. 1977; Liberman et al. 1995) limiting their values for long term usage.

E. Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERM) are a class of molecules that provide an alternative to typical estrogen replacement therapy for decreasing postmenopausal bone loss. Selective estrogen receptor modulators are substances that exert an estrogen-like effect at certain sites, like the skeleton, but act as estrogen antagonists at other target tissues such as breast and uterine tissues (Kuiper et al. 1999). Raloxifene is a selective estrogen receptor modulator that has been used widely in both animal and clinical investigations (Evans et al. 1994; Hol et al. 1997). Raloxifene suppressed bone resorption and prevented bone loss in ovariectomized rats (Evans et al.

1994; Li et al. 1998). In a clinical study, raloxifene at doses of 60 and 120 mg daily reduced the risk of vertebral fracture in postmenopausal individuals (Delmas et al. 1993). Similar reduction in vertebral fracture incidence was found in other clinical studies (Hol et al. 1997; Lufkin et al. 1998).

F. Bone Formation Stimulators

1. Parathyroid hormone

Parathyroid hormone (PTH) is one of the major calcium-regulating hormones in humans (Raisz, 1993). Specifically, PTH maintains serum calcium by controlling bone resorption and indirectly regulating renal calcium excretion. It also indirectly affects intestinal calcium absorption by increasing the renal synthesis of $1,25(OH)_2$ vitamin D_3 (Raisz, 1993). Administration of PTH to humans and rodents has been shown to increase the production of growth factors such as IGF-I and transforming growth factor (TGF-β). These growth factors promote osteoblastic activity and hence enhance bone formation (Jackson and Kleerekoper, 1990; Mundy, 1993; Raisz, 1993; Wardlaw, 1993). The efficiency of the bone-forming effect of PTH is enhanced when it is accompanied with $1,25(OH)_2$ vitamin D_3 (Mundy, 1993).

2. Growth hormone and insulin like growth factor

Growth hormone (GH) increases bone formation by enhancing the synthesis of new cortical bone (Francimont et al. 1989; Turner, 1991). An increase in bone mass following GH therapy has been demonstrated in numerous animal models of osteopenia including mice, rats, and dogs (Harris and Heaney, 1969; Arjmandi et al. 1994). Increased bone mass is likely the result of increased cortical bone mass (Francimont et al. 1989). This, in part, may be a direct effect of the GH on osteoblastic cells (Chihara and Sugimoto, 1997)

or mediated indirectly through increased bone or liver production of IGF-I (Ernst and Froesch,1988; Francimont et al. 1989).

In vitro studies also confirm that both GH and IGF-I stimulate osteoblastic activity (Slootweg et al. 1988). Other investigators have shown that an antiserum to IGF-I inhibits GH mediated osteoblast proliferation, suggesting that GH's effect is mediated through local IGF-I production (Ernst and Froesch, 1988). However, GH is associated with a number of side effects that have discouraged scientists and clinicians from using GH therapy as a way of building bone.

3. Vitamin D

Vitamin D plays an important role in the maintenance of normal calcium homeostasis and has an important role in pathogenesis of osteoporosis (Kumar, 1984; Christiansen, 1993). The major organs affected by vitamin D are the small intestine, kidney, and bone. In the small intestine, vitamin D increases the absorption of calcium. In the absence of vitamin D, calcium absorption from the gastrointestinal tract and re-absorption from kidneys declines. In bone, vitamin D facilitates mobilization of calcium from bone and enhances osteoclastic activity (Holtrop et al. 1981). However, it has been reported that mature osteoclasts do not possess vitamin D receptors, whereas osteoblasts contain intracellular receptors (Narbaitz et al. 1983; Suda et al. 1992). Therefore, it is possible to speculate that mobilization of calcium from the bone is due to an increase in osteoclast activity which may in part be mediated through osteoblasts.

In contrast to bone resorptive ability of vitamin D, *in vitro* studies have shown that vitamin D can modulate osteoblast production of alkaline phosphatase and

osteocalcin (Reichel et al. 1989). Vitamin D stimulates synthesis of osteocalcin by osteoblasts, mineralization of bone, and promotion of cell differentiation (Deluca, 1988).

G. Selected Biochemical Markers of Bone Turnover

Bone markers are chemical substances released into the systemic circulation from either osteoblasts or osteoclasts (Piedra et al. 1989; Stein et al. 1990; Risteli and Risteli, 1993). Bone markers are measured in blood or urine as indicators of bone status and along with bone mass measurement are used as non-invasive prognostic assessment tools to evaluate the risk for developing various metabolic diseases, especially osteoporosis. These markers reflect the rate of bone formation and bone resorption. Examples of markers for bone resorption include hydroxyproline, pyridinoline, deoxypyridinoline, and tartrate-resistant acid phosphatase (TRAP), while markers for bone formation include bone specific alkaline phosphatase activity, osteocalcin, and procollagen. Although generally classified as markers of bone formation, these substances reflect different stages during the process of bone formation. For instance, alkaline phosphatase activity increases after cessation of osteoblast proliferation, reaching a maximum level during matrix formation and declining as matrix mineralization commences (Stein et al. 1990). Following bone matrix formation, osteocalcin concentration will increase commencing the mineralization stage of bone development (Risteli and Risteli, 1993).

1. Alkaline phosphatase

Alkaline phosphatase is a membrane-bound glycoprotein enzyme that is synthesized by the liver, kidneys, small intestine, placenta, and bone. The main sources of alkaline phosphatase are the liver and bone. In bone, alkaline phosphatase is involved in

the breakdown of pyrophosphate, which is a potent inhibitor of calcium phosphate deposition (Risteli and Risteli, 1993).

Serum total alkaline phosphatase activity is used as a marker for bone formation by osteoblasts. Increased serum total alkaline phosphatase activity has been observed in ovarian hormone deficient rats (Arjmandi et al. 1996). However, serum total alkaline phosphatase lacks specificity. To improve specificity, a monoclonal antibody immunoassay has been developed that measures bone specific alkaline phosphatase isoenzymes in serum. This appears to be a better choice for detecting osteoblastic activity (Hill and Wolfert, 1989). Increased osteoblastic activity is seen in postmenopausal women, in high bone turnover diseases such as Paget's disease, advanced metabolic bone disease, and fractures (Nilsson and Westlin, 1972; Doty and Schofield, 1976; Delmas et al. 1983).

2. Osteocalcin

Osteocalcin is a non-collagenous protein containing 49 amino acids. Osteocalcin is synthesized by the bones and teeth, is incorporated into the extracellular matrix of bone by osteoblasts and is involved in the mineralization process (Boskey, 1989). It has been reported that circulating osteocalcin levels correlate with the rate of bone formation and bone mineralization (Epstein et al. 1984; Brown et al. 1984). However, the low percentage of released circulatory osteocalcin, release from the teeth, circadian rhythm variation and seasonal differences, along with rapid clearance by the kidney and reduced kidney function in the elderly population makes interpretation of the serum concentration questionable (Price et al. 1981; Nielsen et al. 1990). A better technique for evaluating bone osteocalcin is by quantifying genetic expression of this protein (Turner et al. 1990).

3. Tartrate-resistant acid phosphatase activity (TRAP)

Acid phosphatase is a non-specific lysosomal enzyme present primarily in the prostate, platelets, red blood cells, spleen, and osteoclasts (Minkin, 1982). In an *in vitro* study conducted by Kraenzlin and colleagues (1990), authors reported that osteoclasts release this enzyme during bone resorption. In addition, the activity of this enzyme increases with osteoporosis (Piedra et al. 1989). Similarly, Arjmandi and coworkers (1996) reported an increase in serum TRAP activity with ovariectomized rats. However, lack of specificity of TRAP does limit its validity in osteoporosis management because TRAP isoenzymes originating from various tissues are found resulting in cross reactivity. However, genetic expression of TRAP from bones would be valuable in the assessment of the ability of this marker to predict osteoclastic activity.

4. Hydroxyproline

Hydroxyproline is derived from hydroxylation of proline during collagen synthesis (Prockop et al. 1979). Risteli and Risteli (1993) reported that only 10% of hydroxyproline is excreted by the kidneys. Additionally, it has been reported that only 40 percent of total hydroxyproline is derived from bone collagen, thereby suggesting that the remaining 60% originates from other sources (Delmas, 1993). Such reports make the validity of hydroxyproline questionable as a sensitive marker for collagen breakdown.

5. Pyridinium crosslinks

Pyridinoline and deoxypyridinoline are two nonreducible pyridinium cross-links present in the mature form of collagen (Fujimoto et al. 1978). These cross-links are generated from lysine and hydroxylysine residues, are specific to bones and are derived from collagen (Seibel et al. 1992). Consequently, pyridinium cross-links become useful

in studying osteoporosis because of a good correlation between bone turnover and the urinary excretion of the cross-links (Seibel et al. 1992). It has been reported that urinary concentration of these cross-links increases two fold at the time of menopause (Uebelhart et al. 1991). Delmas and co-workers (1991) have reported that urinary pyridinoline and deoxypyridinoline correlate with bone resorption in patients with osteoporosis.

H. Antioxidants and Free Radical Formation

Free radicals are molecules with one or more unpaired electrons. Free radicals are short lived and are produced in normal cell metabolism (Freeman and Crapo, 1982). Electron acceptors such as molecular oxygen react with free radicals to become free radicals themselves. The first electron reduction of molecular oxygen produces superoxide radicals (Freeman and Crapo, 1982). Dismutation of oxygen derived free radical by superoxide dismutase yields hydrogen peroxide. Hydrogen peroxide can also react with metal ions such as iron and copper to form hydroxyl radical (·OH). Formation of ·OH can directly cause DNA damage (Halliwell and Aruoms, 1991).

Zinc, copper, and manganese are trace minerals involved as cofactors of the scavenger enzyme superoxide dismutase which converts superoxide to peroxides. Superoxide dismutase inhibits the oxidation of Fe^{3+} to Fe^{2+} which otherwise results in the formation of ·OH (Halliwell, 1987; Wilson, 1987). In addition to superoxide dismutase, other enzymes including catalases and peroxidases aid in catalyzing H_2O_2 to form water which otherwise interacts with a reducing agent in generating hydroxyl radical. Collectively, scavenger enzymes require a number of trace minerals including copper, iron, manganese, selenium, and zinc as co-factors to aid in eliminating free radical formation (Kanter, 1994). Deficiencies of these trace minerals may reduce the activity of

scavenger enzymes in catalyzing superoxide formation or may even result in accumulation of ·OH leading to oxidative stress (Cerutti and Trump, 1991). In addition to trace minerals, beta-carotene and vitamins E and C have antioxidant properties. Kelley et al. (1995) reported that vitamin E supplementation reduced oxidative stress upon exposure to an oxidant substance. Vitamin E is reported to protect against oxidative stress by contributing a hydrogen atom from its phenolic hydroxyl group to lipid derived peroxyl radicals. This will prevent the peroxyl radical from attacking the unsaturated fatty acids thereby inhibiting lipid peroxidation (McCay, 1985). Vitamin C is reported to regenerate vitamin E once non-reactive vitamin E is formed. Adding vitamin C to leukocytes and platelets caused α-tocopherol levels to increase after α-tocopherol levels had decreased from oxidative stress. In addition, vitamin C is reported to interact directly with free radicals and stop their progression in cytosol or in plasma (Dustan and Harris, 1994).

Beta-carotene protects against oxidative stress of cells (Meydani et al. 1995). However, the exact mechanism is not clear. It is believed that beta-carotene directly quenches the free radicals (Dustan and Harris, 1994). Zamora et al. (1991) reported that in an *in vitro* assay, beta-carotene prevented lipid peroxidation and oxidative damage to red blood cells.

Similar to other living cells in the body, osteoclasts are capable of generating oxygen-derived free radicals. In a preliminary *in vitro* study, Key et al. (1990) reported that in rat osteoclasts reduction of nitroblue tetrazolium (NBT) to formazan is inhibited by superoxide dismutase in cytosol suggesting that osteoclasts are capable of producing superoxide.

I. Effects of Selected Minerals on Osteoporosis

Age related bone loss is a phenomenon that results in the development of osteoporosis among certain individuals. Several studies have supported the view that poor diet, lack of exercise, genetics, smoking, and excessive alcohol consumption influence the onset of osteoporosis (Krall and Dawson-Hughes, 1991; Holbrook and Barrett-Connor, 1993; Bassey and Ramsdale, 1994). To date research on the influence of nutrients on bone health has been limited primarily to calcium and less attention has been given to other micronutrients (Matkovic, 1991; Reid et al. 1993; Nordin, 1997). New and colleagues (1997) reported that consumption of magnesium, potassium, vitamin C, and fiber improves bone mass among women. It is conceivable that other nutrients found in foodstuffs also have the potential to contribute to development of bone mass.

1. Calcium

Calcium is the most abundant inorganic cation present in the human body. Ninety-nine percent of calcium is stored in the skeletal system. Consequently, calcium deficiency results in osteoporosis and a decrease in bone density (Nordin, 1997). Some studies have found a positive correlation between calcium intake and bone density (Matkovic et al. 1979; Garn et al. 1981). Others have not been able to show a relationship between bone density and calcium (Smith and Frame, 1965; Hurxthal and Vose, 1969; Johnell and Nilsson, 1984). Matkovic et al. (1979) in a longitudinal population study, observed that despite a similarity in rate of bone loss between individuals with high versus low calcium intake, bone mass was higher among those with high calcium (800-1100 mg/day) intake compared to those with low calcium (300-500 mg/day) intake. Such a report suggests that rate of bone loss is independent of bone mass. Low intake of

calcium (less than 500 mg/day) was associated with the risk of femur fracture while moderate increase in calcium intake reduced the risk of femur fracture (Cooper et al. 1988).

In postmenopausal women, calcium supplementation has been shown to reduce bone loss (Reid et al. 1993). Benefits were significantly greater among those who were 5 years beyond menopause compared to those who had recently experienced menopause (Dawson-Hughes et al. 1990; Elders et al. 1991).

2. Magnesium

Magnesium is the fourth most abundant cation in the body and comprises 0.5 to 1% of bone ash. Magnesium deficiency has led to reduced plasma 1,25 $(OH)_2$-vitamin D and body magnesium levels, decreased osteoblast and osteoclast activity, decreased calcium metabolism, and bone fragility (Blumenthal et al. 1977; Cohen and Kitzes, 1981). Additionally, magnesium deficiency has led to reduced bone magnesium concentration, abnormal bone formation, and osteoporosis (Cohen and Kitzes, 1981). The exact mechanism by which magnesium deficiency affects osteoporosis is not clear, yet Stendig-Lindberg et al. (1993) reported that magnesium supplementation increases bone density in postmenopausal magnesium-deficient women. Similarly, others have reported that magnesium supplementation in postmenopausal women significantly increased bone formation, reduced bone loss, and prevented bone fractures (Stendig-Lindberg et al. 1993; Dreosti, 1995; Sojeka and Weaver, 1995).

3. Phosphorous

Second to the abundance of calcium, eighty-five percent of phosphorous is stored in the skeletal system, and the remainder is distributed in tissues and membrane

components of the skeletal system. About 80% of phosphorous is present as calcium phosphate crystals in the bones and teeth. Intestinal absorption of phosphate is increased by vitamin D and is reduced by calcium in the intestinal lumen. Reduced plasma phosphate stimulates renal 1α-hydroxylase activity for vitamin D formation, increases intestinal phosphate absorption and bone mobilization, and decreases parathyroid hormone and phosphate excretion (Audran and Kumar, 1985). In contrast to reduced plasma phosphate, increased plasma phosphate increases parathyroid hormone, elevates phosphate excretion, and reduces bone mobilization, intestinal absorption of phosphate, and renal 1α-hydroxylase activity (Audran and Kumar, 1985).

4. Boron

The role of boron in animal growth has been well established (Nielsen, 1988). Boron is absorbed from the intestine and is excreted through the urine, bile, and sweat (Naghii and Samman, 1993). Low concentrations of boron in tissues increase its retention from the diet (Naghii and Samman, 1993).

Boron deprivation has been observed to increase urinary calcium excretion in postmenopausal women (Nielsen et al. 1987). In postmenopausal women, consuming 3 mg of supplemental boron daily for seven weeks decreased the excretion of calcium and magnesium (Nielsen et al. 1987). In contrast, others have reported that boron supplementation at 3 mg/day in postmenopausal women did not change urinary excretion of calcium and magnesium, or urinary excretion of pyridinium cross-links (Beattie and Peace, 1993). Additionally, Nielsen (1994) observed that boron mimics and enhances the effect of estrogen, which may be responsible for reduced urinary calcium excretion. In

postmenopausal women 3 mg/day of supplemental boron increased serum estradiol by two fold.

Boron prevented osteoporosis and loss of bone mass in mice (Rajendran et al. 1995). In an *in vitro* study, the authors supplemented calvaria bone cells with boron and observed increased synthesis of collagen and incorporation of calcium into these cells (Rajendran et al. 1995). Such an observation does implicate boron as a micronutrient that promotes osteoblastic activity.

5. Fluoride

It has been reported that fluoride causes osteoblasts to enhance trabecular bone formation and increase bone mineral density in hip and spine areas (Mundy, 1993). Based on a histomorphometric study, the 10% increase in bone mass was correlated with an increased number of osteoblasts available for the new bone formation. In a separate double blind study, authors reported that number of vertebral fractures was similar between fluoride treated groups and placebo groups but the number of non-vertebral fractures was greater in the fluoride treated group (Tolstoi and Levin, 1992). Additionally, authors reported that 15-37% of patients did not respond to fluoride treatment. Furthermore, excessive fluoride treatment has been associated with abnormal bone formation, gastric bleeding, and pain in the lower extremities (Pak et al. 1989). These results suggest that fluoride treatments are not without risk.

J. Bioactive Plant-derived Components

1. Isoflavones

Until the turn of this century it was assumed that estrogens were exclusively produced by animals. Now it is known that certain plants and plant products contain phytoestrogens with estrogenic activity (Riddle and Estes, 1992; Brandi, 1993). Flavonoids are a group of compounds reported to have estrogenic activity (Miksicek, 1993). Flavonoids, including isoflavones, are found in a limited number of plants and plant products including soy protein isolate.

The bone protective properties of soy protein have received much attention lately (Arjmandi et al. 1996). From the review of the literature it is not clear whether the bone protective effect of soy protein is due to its amino acid composition, nonprotein constituents such as saponins, phytic acid, isoflavones, or a combination of these factors. However, in recent years, there has been rising interest regarding the soy isoflavones and their influence not only on sex hormone metabolism, but also on other biological activities including their effects on bone (Arjmandi et al. 1996). There are several reports indicating that naturally occurring isoflavones, such as those found in soy, may have beneficial effects on bone. Recent studies show that genistein, an isoflavone found predominantly in soy, prevents bone loss in ovariectomized rats (Anderson et al. 1998). The findings of the study by Anderson and colleagues (1998) suggest that the bone-protective effect of genistein has a biphasic dose-response such that either too low or too high a dose is less effective. In a study by Arjmandi et al. (1996), authors reported that the consumption of soy protein isolate with natural isoflavone content prevented bone loss in rats after ovariectomy. This observed beneficial effect may have been due to the

protein component itself, its isoflavone content, or a combination of both. Further studies are needed to elucidate the bone-conserving role of soy isoflavones in ovarian hormone deficiency.

Genistein and related isoflavones have long been recognized as naturally occurring phytoestrogens (Axelson et al. 1984). Both *in vitro* and *in vivo* studies have shown that genistein exerts a weak estrogenic effect, approximately 1×10^{-3} to 1×10^{-5} that of estradiol (Shutt and Braden, 1963). Despite their low estrogenic potency, isoflavone concentrations high enough to produce an estrogenic effect can be achieved from introducing soy into the diet. Arjmandi et al. (1998) reported the importance of the isoflavone component of soy protein in protecting bone in ovarian hormone deficient rats.

Genistein, a natural isoflavonoid phytoestrogen, has been found to have inhibitory effects on femoral bone resorption in tissue culture of female rats (Yamaguchi and Gao, 1997). The mechanism by which genistein has a positive response is believed to be mediated through osteoblast proliferation (Benvenuti et al. 1991). Moreover, genistein added to a femoral-metaphyseal culture increased bone alkaline phosphatase activity, suggesting enhanced osteoblastic activity (Yamaguchi and Gao, 1997). The synthetic isoflavone, ipriflavone, effectively prevents bone loss due to ovarian hormone deficiency in humans (Gambacci et al. 1985; Gennari, 1991) and animals (Yamazaki, 1986; Yamazaki and Kinoshita, 1986; Arjmandi et al. 2000). Ipriflavone has been observed to reduce bone resorption in ovariectomized rats (Cecchini et al. 1997).

2. Prune

There is widespread agreement that fruits are excellent sources of fiber, water-soluble vitamins, trace elements, minerals, and compounds with antioxidant properties such as phenols and flavonoids. There is also ample evidence to suggest that nutrients found in fruits have prophylactic properties against certain debilitating diseases (Ziegler, 1991). Prunes, for example, have been used to lower hypercholesterolemia (Tinker et al. 1991). Tinker et al. (1991) observed that daily consumption of 100 g of prunes lowers serum total- and LDL-cholesterol concentrations in individuals with hypercholesterolemia. Similar observations were noted in a second experiment (Tinker et al. 1994). Beneficial effects of prunes may lie in their nutritional properties. Prunes contain 7.5 g of fiber; 1.76 g of ash; 2.2 mg of boron; 0.42 mg of copper; 745 mg of potassium; and 0.21 mg of manganese per 100 gram of weight (ESHA Research, 1999). These nutrients have been known to improve various health parameters of individuals in separate studies. As an example, increasing supplemental potassium decreased urinary calcium loss (Lemann et al. 1993). Such an effect may preserve the calcium content of bone. Also, magnesium supplementation increased bone formation and prevented bone fractures (Stending-Lindberg et al. 1993; Dreosti, 1995; Sojeka and Weaver, 1995). The trace element selenium has been found to preserve bone mineral density (Dreher et al. 1998).

Although effects of prunes on skeletal health have not been studied in other laboratories, nutrients found in prunes may be beneficial in improving skeletal ability to withstand stress and fractures (Soliman, 1998). Fresh plums and prune extracts are high in nutrients such as potassium, copper, boron, magnesium, and polyphenols with

antioxidant capabilities (Dreosti, 1996; Bravo, 1998) that can influence bone structure and bone strength.

Among the mechanisms thought to influence bone resorption, free radical formation is of particular interest. The exact mechanism of how free radical formation influences the skeletal system is not understood. Free radicals can damage bone cells by forming toxic products such as hydroxy radicals (Florence, 1983). Nutrients involved in the body's defense system against free radical formation are found in prunes. For example, copper enhanced copper-zinc superoxide dismutase activity which otherwise was significantly reduced in rats fed copper deficient diet (Olin et al. 1994). Manganese may also influence Mn-superoxide dismutase activity against free radicals inside mitochondria (Kanter, 1994). Therefore, there is enough evidence to investigate further whether nutrients found in prunes have prophylactic properties against osteoporosis.

CHAPTER III

THE EFFECTS OF PRUNE ON BONE METABOLISM IN OVARIECTOMIZED RATS

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ABSTRACT

Estrogen treatment slows bone loss but is expensive and may have side effects in some post-menopausal women. The current study was undertaken to determine whether varied doses of prunes added to a casein-based diet restore bone loss that has already occurred in ovariectomized (OVX) Sprague-Dawley rats. Fifty-six rats were divided into six groups: Sham-operated (sham), OVX, OVX+Estrogen (daily injection of 10μg/Kg body weight), OVX+5% supplemental prune, OVX+15% supplemental prune, and OVX+25% supplemental prune. Rats were ovariectomixed 40 days prior to initiation of the 60 days of experimental treatments. Serum alkaline phosphatase, Ca, Mg, and whole blood ionized Ca were not affected ($P<0.05$) by treatments. Ovariectomy increased ($P<0.05$) urinary excretion of hydroxyproline and deoxypyridinoline compared to the sham group. Estrogen injection increased ($P<0.05$) uterus weight and decreased ($P<0.05$) hydroxyproline and deoxypyridinoline excretion compared to the OVX group. Consumption of 15% and 25% prunes increased ($P<0.05$) urinary Ca and Mg excretion and decreased ($P<0.05$) duodenal calcium uptake compared to the OVX group. Consumption of 15% and 25% prunes decreased ($P<0.05$) fecal ash and reduced fecal

magnesium excretion per gram of dry matter compared to the OVX group. Consumption of prunes did not affect concentration of magnesium and calcium in femur, tibia, and 4th lumbar vertebrae compared to the OVX group. Consumption of prunes increased ($P<0.05$) bone mineral density compared to the OVX group. Unit yield force (yield force/cortical thickness) decreased ($P<0.05$) with ovariectomy, while, consumption of prunes or estrogen injection tended to improve this parameter compared to the OVX group. The findings of this animal study suggest that bone strength is a function of bone density and consumption of prune indeed increases bone density in ovariectomized rats.
Key Words: Ovariectomy, Prunes, Estrogen, Bone, Rats

INTRODUCTION

Previous research has shown that fruits contain carotenoids, vitamins, minerals, and polyphenolic compounds with antioxidant properties (Bravo, 1998). An inverse relationship has been reported between high intakes of tea, fruits, and vegetables that contain the antioxidant vitamin C and beta-carotene and degenerative diseases such as ocular cataracts and cancer (Taylor, 1992; Dreosti, 1996). These nutrients may be beneficial in reducing the risk of certain debilitating diseases of aging (Ziegler, 1991). In addition to beneficial effects of consumption of fruits and vegetables on general health status, they may improve the bone status of individuals as well. New et al. (2000) reported that the consumption of fruits and vegetables improved bone mass among women 45-55 years of age. One example of a fruit is the prune. Among fruits, prunes are rich in phenolic compounds with antioxidant properties and are considered to be a rich source of magnesium, potassium, and copper (Umegaki et al. 1997; ESHA Research, 1999). The influence of calcium on bone health has received most attention with

substantially less research related to other micronutrients (Matkovic, 1991; Reid et al. 1993). New and colleagues (1997) reported that increased intakes of magnesium, potassium, vitamin C, and fiber improved bone mass. Compared to other fruits, these and other micronutrients are abundant in prunes. Consequently, increased consumption of prunes may aid in improving bone status and protecting against osteoporosis.

Osteoporosis is a disorder involving a reduced quantity of bone tissue, change in microstructure, decreased bone strength, and increased fracture risk (Center and Eisman, 1997). Bone mass is a major determinant of fracture risk and a number of studies have demonstrated that a decrease in bone density is associated with an increase in fracture risk (Wasnich et al. 1985; Hui et al. 1988).

Similar to human studies, in rat models for studying osteoporosis bone density decreases significantly following ovariectomy (Arjmandi et al. 1996; Mattila et al. 1998). Peng and co-investigators (1997) have observed that in rats the femoral neck is sensitive to an ovariectomy. Arjmandi et al. (1996) attributed the decrease in bone density to estrogen deficiency, because estrogen treatment following ovariectomy significantly increased bone density. The purpose of this study was to determine whether the consumption of prunes can reverse the bone loss that occurred due to 40 days of estrogen deficiency in ovariectomized rats.

MATERIALS AND METHODS

Appropriateness of the ovariectomized rat model

Ovariectomized rats were chosen for this study because the osteopenia associated with estrogen (E_2) deprivation in this experimental model is similar to bone loss occurring in postmenopausal women (Frost and Jee, 1992). In this experiment, 90 day-

old female Sprague-Dawley rats were used because they are considered mature female rats and their bones show skeletal sensitivity to ovariectomy (ovx)-induced bone loss in a relatively short period of time (Frost and Jee, 1992). Therefore, the rat skeleton provides a good example for studying human osteoporosis.

B. Experimental design

This study was designed to reverse established bone loss in ovariectomized rats. Six groups (Table I) of female Sprague-Dawley rats were used in the study. On arrival at our institution, the rats were housed in an environmentally controlled animal laboratory and acclimated with a standard laboratory diet for 2 days. The animals were divided into six weight-matched groups using a randomized complete block design. One group of rats was sham operated (sham) and five groups were ovariectomized. All rats received a common control diet for the first 40 days after surgery and thereafter treatment intervention (Table I) began for 60 days. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee of Oklahoma State University were strictly followed.

Rats were fed a semi-purified, powdered casein-based diet, AIN-93M (Teklad, Madison, WI; Table II). Rats had free access to deionized water throughout the study. The sham group, an ovx-control group, and E_2 treatment groups received the casein-based diet. The prune treatment groups received powdered diets that were iso-caloric and iso-nitrogenous to the control diet. Adjustments were made in the diets to account for the carbohydrate, fiber, protein, fat, calcium, and phosphorus contributed by prunes.

TABLE I

Experimental groups

Treatment group	N[1]	Surgery Day 0	Treatment begins Day 40	Sacrifice Day 100
Sham	8	Control diet	Control diet	→
OVX[2]	8	Control diet	Control diet	→
Estrogen	10	Control diet	Control diet	→
LD[3]	10	Control diet	Prune @ 5%	→
MD[4]	10	Control diet	Prune @ 15%	→
HD[5]	10	Control diet	Prune @ 25%	→

[1]N=number of rats per treatment.
[2]OVX=ovariectomized animal; [3]LD=Low dose, prune provided @ 5% of diet by weight [4]MD=Medium dose, prune provided @ 15% of diet by weight; [5]HD=High dose, prune provided @ 25% of diet by weight.

Beginning at day 40 post-ovariectomy, the estrogen-treated group received daily subcutaneous injections of 10 μg E_2/kg body weight in sesame oil, while the remainder received a similar dosage of solvent vehicle per kg body weight daily. Because E_2 suppresses food intake in ovariectomized rats, all rats were pair-fed to the mean food intake of the E_2 treated rats. Before each feeding, the food remaining was weighed and the amount ingested was calculated. All animals were sacrificed 100 days from the day of surgery.

TABLE II

Composition of diets

Ingredients (g/100g diet)	Control diet	Prune (%) 5	15	25
Carbohydrate				
Total	72.3	72.3	72.3	72.3
Corn starch	46.6	42.3	33.7	25.0
Maltodextrin	15.5	15.5	15.5	15.5
Sucrose	10.2	10.2	10.2	10.2
Prune[1]	-----	4.3	12.9	21.6
Protein				
Total	14.2	14.2	14.2	14.2
Casein	14.0	13.8	13.5	13.2
Prune	-----	0.2	0.5	0.8
Cystine	0.2	0.2	0.2	0.2
Fat				
Total	4.0	4.0	4.0	4.0
Soybean oil	4.0	3.97	3.92	3.86
Prune	-----	0.03	0.08	0.14
Fiber				
Total	4.675	4.68	4.69	4.695
Cellulose	4.675	4.18	3.19	2.195
Prune	-----	0.5	1.5	2.5
Vitamin premix[2]	1.0	1.0	1.0	1.0
Trace mineral[3]	1.34	1.34	1.34	1.34
Choline	0.25	0.25	0.25	0.25
Calcium carbonate	1.235	1.230	1.220	1.215
Prune calcium	-----	0.004	0.0108	0.018
KH_2PO_4	0.5	0.55	0.57	0.58
NaH_2PO_4	0.34	0.34	0.34	0.34
Potassium citrate	0.09	0.09	0.09	0.09

[1]Prune in dry powdered form provided by the California Prune Board (Pleasanton, CA).
[2]Vitamin Mixture Composition (AIN-93; Harlan Teklad, Madison, WI).
[3]Mineral Mixture Composition (g/Kg mix; Harlan Teklad, Madison, WI). magnesium oxide, 24g, Ferric citrate, 6.06g; zinc carbonate, 1.65g; manganous carbonate, 0.63g; cupric carbonate, 0.3g; potassium iodate, 0.01g; sodium selenate, 0.01g; ammonium paramolybdate, 0.007g; chromium potassium sulfate 0.275g; boric acid, 0.0815; sodium fluoride, 0.0635g; nickel carbonate, 0.0318g; ammonium vanadate, 0.0066g.

C. Necropsy

At the termination of the study, animals were weighed and then received an intraperitoneal injection of a combination of xylazine (5 mg/kg body weight) and ketamine (100 mg/kg body weight) anesthesia before they were bled from their abdominal aortas.

D. Body and organ weights

All rats were weighed weekly throughout the experiment. At necropsy weights were recorded for uterine tissue, heart, liver, spleen, and kidneys.

E. Blood processing

An aliquot of heparinized blood was collected and immediately analyzed for ionized calcium using the Nova-8 blood analyzer (Nova Biomedical, Waltham, MASS). Additionally, blood samples were collected without anticoagulant and centrifuged (4°C) at 1500 x g for 15 minutes. Sera were separated immediately and an aliquot of serum was refrigerated for analysis of serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities within 4 hours of sacrifice. The remainder of sera were aliquoted into small volumes and stored at -20°C until required for analysis.

F. Serum alkaline phosphatase and tartrate-resistant acid phosphatase activities

Serum ALP and TRAP activities were determined using the Cobas-Fara II Clinical Analyzer (Roche, Montclair, NJ) following the manufacturer's instructions and using a commercially available calibrator and quality control samples (Sigma Diagnostic Products, St. Louis, MO).

G. Determination of mechanical properties of femur

The left femur was cleaned of soft tissue and stored in a glass vial at –20°C. Femur length was measured by a caliper before strength determination by a three-point bending test utilizing an Instron Universal Testing Machine (Model II; Instron Corp, Canton, MA). Before the mechanical testing, the bones were immersed in normal saline for 48 hours at 4 °C. The bone sample was placed in similar orientation on two rods spaced 20 mm apart and the force was delivered to the mid-shaft by a crosshead moving at a constant speed of 1.0 mm/min. A force versus deformation curve was recorded. Yield force was defined as the force at the yield point (point of deviation from the linear slope on the force versus deformation curve). Force at bone failure was the ultimate force. Yield and ultimate force provided a measure of bending strength of the intact femur. The inner and outer diameters of the anterior-posterior and mediolateral axis were measured at the point of fracture with a micrometer and were used to calculate cortical area, medullary area, and moment of inertia (Kiebzak et al. 1988) assuming elliptical cross section. The property of inertia provided a measure of bone distribution about the neutral axis. Yield stress (measure of bone strength), yield strain (measure of bone bending), and modulus of elasticity (indicator of stiffness) were calculated from the primary data (Kiebzak et al. 1988). Yield and ultimate force adjusted for cortical thickness were calculated using the following formulas:

$$\text{Unit yield force} = \frac{\textit{Yield force}}{\textit{Cortical thickness}}$$

$$\text{Unit ultimate force} = \frac{\textit{Ultimate force}}{\textit{Cortical thickness}}$$

H. Bone processing and bone density

The left tibia, and fourth lumbar vertebra were cleaned of soft tissues and stored in glass vials at –20°C. For determination of bone density, femur, tibia, and vertebra were placed in unstopped glass vials filled with deionized water. The vials were placed into a desiccator connected to a vacuum for 60 minutes so that trapped air diffused out of the bone (Kalu et al. 1988). All bones were weighed before being immersed in deionized water previously equilibrated to room temperature (Kalu et al. 1988). The bone density was calculated by Archimedes' principle as described previously (Kalu et al. 1988):

$$\textit{bone density} = \frac{\textit{bone air weight}}{\textit{bone air weight - bone underwater weight}}$$

Bone mineral density and bone mineral content of left femur and 4th lumbar vertebrae were also measured by dual x-ray absorptiometry (DXA Model 2000; Hologic Inc, Waltham, MA).

I. Bone chemistry

The left femur, left tibia, and 4th lumbar were rinsed with phosphate buffered saline. Bones were then placed in a phosphate buffered saline solution containing 0.01% Triton X-100 (Sigma, St. Louis) and 0.02% sodium azide for 72 hours at 4°C and then centrifuged. Bone ALP activities and bone protein extracts were measured from 100 μL aliquots of bone extract using the Cobas-Fara II Clinical Analyzer (Montclair, NJ).

Bones were placed in 70% alcohol and dried at 100°C for 48 hours, weighed, ashed in covered crucibles at 600°C for 16 hours, cooled, and finally weighed to determine the percent mineral content. Ashed samples were diluted with 0.5% nitric acid and 0.5% lanthanum chloride solution. Bone calcium and magnesium were analyzed using flame atomic absorption spectrophotometry (Model 5100 PC, Perkin-Elmer, Norwalk, CT), and were expressed as milligram of calcium or magnesium per gram of bone.

J. Feces and urine collection

Fourteen days before the termination of the study, rats were transferred to metabolic cages for 12 hours of urine collection from 8 PM to 8 AM. During this 12-hour urine collection period, rats had access to deionized water but no food was provided. Urine was collected in acid-washed tubes, and the total volume was measured and acidified with 0.03 mL of 6 mol/L HCl per 1 mL urine. Samples were frozen at -20°C until analysed. Urine hydroxyproline was determined as described previously (Bergman and Loxley, 1970). Deoxypyridinoline was analyzed using a kit manufactured by Metra Biosystems, Inc. (Mountain View, Calif). Creatinine was measured colorimetrically using a commercially available kit from Roche Diagnostics (Branchburg, NJ). Samples were analyzed using a Cobas-Fara II Clinical Analyzer (Montclair, NJ), and results were expressed based on the 12-hour night urine volume. Urine calcium and magnesium were analyzed by diluting urine samples to appropriate concentration with 0.1% nitric acid and 0.1% lanthanum chloride solution. Samples were analyzed using flame atomic absorption spectrophotometry (Model 5001 PC, Perkin-Elmer, Norwalk, CT) and an air-acetylene flame. Urine samples were expressed as milligrams of calcium or magnesium per 12 hours urine volume.

Rats were fed treatment diets containing an indigestible marker (Aluminum blue, New Brunswick, NJ) a day before fecal collection started and then fed treatment diets for three days followed by one day of feeding diets containing the marker. The collected feces were stored in polyethylene tubes and kept frozen at –20°C. Feces were ground and placed in an oven at 100 °C for 24 hours to determine fecal dry matter. One gram of feces was weighed and ashed in porcelain crucibles at 600°C for 16 hours. The ashed samples were diluted with 0.5% nitric acid and 0.5% lanthanum chloride solution. Fecal calcium and magnesium were analyzed using flame atomic absorption spectrophotometry (Model 5001 PC, Perkin-Elmer, Norwalk, CT) and concentrations were expressed as milligrams of calcium or magnesium per gram dry matter.

K. Duodenal calcium transport

At the termination of the experiment, the duodenum from each rat was excised and trimmed from adhering tissues. A detailed procedure for determining intestinal calcium uptake has been published elsewhere (Arjmandi et al. 1993). Briefly, luminal contents were flushed with an ice cold isolation medium and duodenum was cut lengthwise and washed again with isolation medium which contained 240 mM mannitol, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM NaCl, 0.5 mM β–hydroxybutyrate, 2.5 mM glutamine, 10 mM mannose, 2 mg/ml BSA, and 20 mM HEPES (pH 7.4). After cleaning, the duodenum was cut into 1 to 2 cm lengths and placed in beakers containing an isolation medium containing hyaluronidase (Sigma, St. Louise, MO). Duodenal cells were detached by placing a magnetic stirring bar in the beaker and setting the stirrer at high speed for several seconds. Cells were filtered through cheese cloth and the sediments were washed using isolation medium. Samples were centrifuged at 2500 g for

15 minutes and cells were resuspended in an incubation medium, which contained 140 mM KCl, 10mM HEPES (pH 7.4), 0.5 mM β–hydroxybutyrate, 2.5 mM glutamine, 10 mM mannose, and 2 mg/ml BSA.

Calcium uptake was measured by addition of 100 μl uptake solution consisting of 140 mM KCl, 10 mM HEPES (pH 7.4), 2 mM $CaCl_2$ and 0.5 μCi $^{45}CaCl_2$ (total activity 41.189 mbq; Amersham Pharmacy Biotech, Arlington Heights, IL) to 100 μl cell suspension resulting in a final calcium concentration of 1 mM. Calcium uptake was terminated after 5 minutes using a stopping solution consisting of 140 mM KCl, 10 mM HEPES (pH, 7.4) and 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N,N tetra acetic acid (EGTA). The cells were collected on 5 μm Millipore filters and the radioactivity on the filter was measured using a liquid scintillation counter (Packard scintillation analyzer, Model A1900, Downers Grove, IL).

L. Statistical and data analysis

Data were analyzed by analysis of variance using the General Linear Models procedure of SAS (SAS Institute, 1985) to determine the effects of prune supplementation and estrogen administration on bone density and bone quality as the primary outcome variables. When a significant F statistic ($p \leq 0.05$) from analysis of variance was noted, the least square means procedure was performed for separating means that were significantly ($p \leq 0.05$) different.

RESULTS

Feed intake, body weights, and organ weights

In the present study, initial body weights and food consumption were similar among the six treatment groups (Table III). At the end of the experiment, estrogen administration to ovariectomized rats suppressed ($P<0.05$) body weight gain compared to the ovx-control group. The consumption of prunes did not affect body weight in comparison with the ovx-control group.

The mean weights for heart, liver, kidneys, and uterus per 100 gram body weight were significantly ($P<0.05$) less in the ovx-control group than the sham group. Consumption of prunes decreased ($P<0.05$) the mean weight of the tissues (expressed as percentage of body weight) monitored compared to the sham group (Table III). Estrogen treatment increased ($P<0.05$) mean liver, kidneys, and uterine weights (expressed as percentage of body weight) when compared with those of the ovx-control group. The mean spleen weight was not affected ($P>0.05$) by ovariectomy or consumption of prunes (Table III).

Serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity.

Mean serum ALP activity did not ($P>0.05$) differ between the ovx-control and the sham groups (Table IV). In the present study, estrogen administration did not ($P>0.05$) affect serum ALP activity. The mean TRAP activity, however, was reduced ($P<0.05$) only with estrogen administration compared to the ovx-control and the sham group. Consumption of prunes did not ($P>0.05$) influence serum ALP or TRAP activities in comparison with the sham or ovx-control groups (Table IV).

Serum calcium and magnesium concentration

In this study, no significant differences ($P>0.05$) were detected in serum ionized calcium, serum total calcium, or serum magnesium concentrations in comparison with the sham and ovx-control groups (Table IV).

Duodenal calcium absorption

The mean calcium absorption was not affected ($P>0.1$) in the ovx-control group when compared with the sham group. Likewise, daily estrogen injection did not affect the *in vitro* calcium uptake by the duodenal cells. However, consumption of prunes ($P<0.05$) decreased calcium uptake by the duodenal cells *in vitro* when compared with the sham or the ovx-control groups (Table IV).

Urinary creatinine, hydroxyproline, deoxypyridinoline, calcium, and magnesium

Mean urinary creatinine was not significantly ($P>0.10$) different among treatment groups (Table V). Urinary hydroxyproline and deoxypyridinoline excretion were significantly ($P<0.05$) increased by ovariectomy. Injection of estrogen to ovariectomized rats significantly ($P<0.05$) reduced excretion of hydroxyproline and deoxypyridinoline compared to the ovx-control group. Consumption of prunes significantly ($P<0.05$) reduced the concentration of hydroxyproline in the urine in comparison with the ovx-control group. However, intakes of prunes did not affect the concentration of deoxypyridinoline levels in the urine in comparison with the ovx-control or sham groups.

Mean urinary excretion of calcium and magnesium were not affected with ovariectomy or estrogen administration (Table V). However, increased consumption of prunes dose-dependently increased ($P<0.05$) urinary excretion of calcium. Urinary

magnesium excretion increased ($P<0.05$) only with 15 and 25 percent consumption of prunes in comparison with those of the sham or ovx-control groups (Table V).

Fecal dry matter and fecal mineral content

Mean fecal dry matter or mean total fecal ash did not differ significantly among the treatment groups (Table VI). Similarly, fecal ash as a percentage of dry matter was not affected by ovariectomy or by injection of estrogen in comparison with the sham groups. However, fecal ash as a percentage of dry matter decreased ($P<0.05$) with 15% and 25% consumption of prunes (Table VI).

Mean fecal calcium excretion per gram of fecal dry matter was not affected with ovariectomy, estrogen administration, or consumption of prunes (Table VI). Excretion of fecal magnesium per gram of dry matter, however, was significantly ($P<0.05$) increased in the ovx-control and injection of estrogen decreased ($P<0.05$) magnesium values to those of the sham group. Similar to the estrogen injected group, consumption of prunes at concentrations of 15% and 25% decreased ($P<0.05$) the concentration of magnesium excreted per gram of fecal dry matter when compared to the ovx-control.

Femoral mineral density and its mineral concentration

Ovariectomy increased ($P<0.05$) the mean femoral length (Table VII) when compared with femurs of the sham group. The mean femoral density for ovx-control however, was significantly ($P<0.05$) reduced in comparison with the sham group. These observations were confirmed using DXA to estimate femoral mineral density. Injection of estrogen or consumption of prunes increased ($P<0.05$) femoral mineral density of ovariectomized rats to be similar to the sham group. This observation was confirmed using DXA to evaluate femoral mineral density of ovariectomized rats.

The dry matter of the left femur did not (P>0.05) differ significantly among treatment groups. Ash as a percentage of dry matter significantly decreased (P<0.05) with an ovariectomy (Table VII). Injection of estrogen tended (P=0.08) to reverse the reduced bone ash content that occurred with ovariectomy. Estrogen injection or consumption of prunes did not (P>0.05) affect femoral ash content compared to the ovx-control group. The mean concentrations of calcium and magnesium in left femurs were not affected with any of the treatments.

Tibia mineral density and its mineral concentration

Similar to femur length, ovariectomy significantly increased (P<0.05) the mean tibial lengths compare to the tibias of the sham group. Tibial density for the ovx-control group however, was significantly reduced (P<0.05) in comparison with those of the sham group. Estrogen administration restored (P<0.05) the decrease in tibial mineral density of ovx-control group to the value of the sham group. Similarly, consumption of prune increased (P<0.05) the tibial mineral density significantly. Tibial mineral density of those rats fed prunes was not different (P>0.10) from the sham group.

The mean tibia dry weight was not (P=0.11) different among treatment groups. Tibia mineral content as estimated by its ash as a percentage of dry matter decreased (P<0.05) in the ovx-control group when compared to the sham group (Table VIII). Injection of estrogen restored the tibia ash content to be not significantly different from that found in tibia of the sham group. Only 25 percent consumption of prunes increased (P<0.05) tibia ash compared to those of ovx-control group. The mean concentration of calcium and magnesium in the tibia were not (P>0.05) different among the treatment groups.

Fourth lumbar mineral density and its mineral concentration

The 4th lumbar mineral density decreased ($P<0.05$) with ovariectomy compared to those of the sham group (Table IX). Despite numerical improvement in 4th lumbar mineral density with estrogen treatment, estrogen at the dosage administered did not restore 4th lumbar bone loss that had already occurred. Increased consumption of prunes increased ($P<0.05$) 4th lumbar mineral density to the level of the sham group. Using DXA to estimate 4th lumbar mineral density confirmed our previous observation that consumption of prunes increases ($P<0.05$) 4th lumbar mineral density (Table IX).

In this study, percentage of 4th lumbar dry weight was not different ($P>0.05$) among treatment groups. However, 4th lumbar ash content of ovx-control decreased ($P<0.05$) compared to those of the sham group. Injection of estrogen at the dosage given, however, was not effective in restoring 4th lumbar ash content to what was found in the sham group. Consumption of prunes at the 25% concentration, significantly increased ($P<0.05$) bone ash content of the 4th lumbar compared to those of ovx-controls. However, consumption of prunes did not restore 4th lumbar ash percentage to the level found in the sham group (Table IX).

In the present study, concentrations of calcium and magnesium in 4th lumbar were not affected by ovariectomy, estrogen injection, or consumption of prunes (Table IX).

Bone alkaline phosphatase activity (ALP)

Femur and tibia ALP activities (U/g protein) were not ($P>0.05$) significantly different among the treatments (Tables VII and VIII). The 4th lumbar vertebrae ALP activity was reduced ($P<0.05$) in the ovx-control group in comparison with the sham

group (Table IX). Estrogen injection or consumption of prunes did not (P>0.05) influence 4th lumbar vertebral ALP activity in comparsion with the ovx-control group (Table IX).

Ovariectomy, estrogen injection, and consumption of prune on biomechanical properties of left femur bone

Irrespective of treatment, mean femoral cortical area increased (P<0.05) after ovariectomy when compared with the sham group. Femur medullary area, however, was not significantly affected by ovariectomy, estrogen injection, or consumption of prunes (Table X).

Distribution of bone about the central axis (second moment inertia; SMI) increased (P<0.05) with ovariectomy when compared with the sham group. Estrogen injection, however, decreased (P<0.05) SMI in ovariectomized rats compared to the ovx controls. Consumption of prunes was ineffective in significantly modulating SMI compared with those of the ovx-control (Table X).

Yield and ultimate modulus of elasticity along with yield and ultimate strain were not affected (P>0.05) with ovariectomy, estrogen injection, or prune consumption (Tables X and XI).

In the present study, more (P<0.05) yield force per unit area was required to bend the femur of rats that were subjected to ovariectomy (Table X). Estrogen treatment similar to sham significantly decreased (P<0.05) the yield force and numerically (P=0.07) reduced the ultimate force (Table XI) required to bend the femur when compared to those of the ovx-control.

Unit yield force, however, was less (P<0.05) for the femur in ovx-control in comparison to those of the sham group. Unit yield forces for estrogen injected rats and those rats which consumed prunes were not significantly (P>0.05) different when

compared with ovx-control (Table X). The mean unit ultimate force did not differ significantly among treatments (Table XI).

DISCUSSION

In the present study, due to pair feeding, no significant differences were observed in feed intake among treatments. Increased body weight of ovariectomized rats compared to those of the sham group is perhaps due to a change in metabolism shifting toward an increase in energy accretion as seen by increase in body weight. Body weight increase of ovariectomized rats may have been due to a decrease in basal metabolism and circulating thyroid hormones (Thomas et al. 1986). Other investigators have reported increased body weight due to ovarian hormone deficiency (Richard 1986; Kalu et al. 1994). The decrease in weight gain with daily estrogen injection also may have been due to alteration in energy metabolism. Other investigators have found that in rats, estrogen administration suppresses the body weight (Kalu et al. 1991) which may have been due to an increase in energy expenditure as suggested previously (Richard, 1986).

In comparison to the sham group, the reason mean weights of the heart, liver, and the kidneys in the ovx-control group and rats consuming prunes were suppressed is attributed to increased body weight gain relative to organ weight gains. Heart and kidney weights of the estrogen administered group were not affected compared to the sham group, perhaps because of suppressed body weight gain. However, increased liver weight with estrogen injection may in part be due to a change in metabolism or increased lipid accumulation in the liver. The increase in uterus weight observed with estrogen administration is due to the sensitivity of this organ to estrogen.

In the present study, in contrast to the consumption of prunes, the decrease in serum TRAP activity with daily estrogen injection suggests that estrogen suppressed bone resorption in ovariectomized rats. These results support the previous suggestion that estrogen slows bone resorption by blocking osteoclastic access to bone for creating new erosion cavities (Kanis, 1996). Furthermore, compared to the ovx-control group, the bone anti-resorptive capacity of estrogen was evident by 62% and 64% decreases in hydroxyproline and deoxypyridinoline, respectively. In contrast to the sham group, ovariectomy significantly elevated both hydroxyproline and deoxypyrindinoline, suggesting increased bone resorption. Consumption of prunes lowered these indices compared to the ovx-control, suggesting that consumption of prunes may have decreased bone resorption.

In the present study, the reason serum ionized calcium, serum total calcium, and serum magnesium were not affected by ovariectomy or consumption of prunes may in part be due to adequate calcium and magnesium consumption. Similarly, Kiebzak and co-workers (1998) observed that the aging did not influence the concentration of calcium in serum.

The reason fecal and urinary calcium excretion were not influenced by ovariectomy may in part be due to these rats being pair-fed and their diets being adjusted for calcium concentration. Based on the current observation, it is not clear what are the mechanisms involved in increased urinary calcium excretion with consumption of prunes. A similar observation has been reported previously (Soliman, 1998). However, increased fecal excretion of magnesium in the ovx-control group may be related to decreased magnesium absorption due to estrogen deficiency. Estrogen injection to ovariectomized rats

normalized magnesium excretion to the level of the sham group. Decreased excretion of magnesium in the feces of rats that were prune-fed compared to the ovx-control group may have been due to increased magnesium absorption.

Increased urinary excretion of magnesium found with 15% and 25% consumption of prunes may be due to increased magnesium consumption and subsequent increase in magnesium excretion. Consequently, net change in urinary magnesium excretion may have resulted in maintaining serum magnesium concentration similar to the sham values. The data presented herein is supported by Soliman (1998).

It is not clear why duodenum intestinal calcium uptake was reduced with consumption of prunes. Fecal excretion of calcium was unaffected by consumption of prunes compared to other treatments which may be indicative of increased calcium absorption along other lower sites in the small and large intestine. However, it is important to note that overall cellular viability was not recorded which may have contributed to overall decrease in intestinal calcium uptake among treatments.

The reason concentration of calcium in femur, tibia, and 4th lumbar vertebrae was not influenced by ovariectomy, estrogen injection, or consumption of prunes may in fact be due to pair feeding method used in this study. Soliman (1998) supports this result in that the concentration of calcium in femur, tibia, and 4th lumbar vertebrae were not influenced by ovariectomies in her study. Similarly, Peng and co-workers (1997) observed that ovariectomy did not influence the concentration of calcium in femur bone.

Additionally, consumption of prunes tended to numerically increase magnesium concentration in the femur and 4th lumbar vertebrae, when compared to the ovx-control group. Such numerical increase in bone concentration of magnesium is partially due to

increased dietary intake of magnesium with increased dietary inclusion of prunes. Consequently, prune being a good source of magnesium has contributed to magnesium deposition in the femur. Soliman (1998) reported that the mean concentration of magnesium in the femur was significantly elevated with 25% consumption of prune. The lack of significant response observed in this study may have been due to the pattern of feeding the rats. In the former study, OVX rats were fed ad libitum, and in the present study, all rats were pair-fed. Previously, it has been reported that magnesium is involved in bone metabolism and bone formation (Blumenthal et al. 1977; Cohen and Kitzes, 1981; Cohen, 1988; Classen et al. 1994).

The decrease in mean femoral, tibia, and 4th lumbar vertebrae densities of the ovx-control group may have been due to reduced bone ash. The trend toward the increase in bone ash of rats injected with estrogen reflected an increase in bone density of ovariectomized rats. Additionally, increased bone ash in prune-fed rats related to bone density, suggesting that bone density is mineral dependent. Sojka and Weaver (1995) reported that magnesium therapy resulted in an increase in bone density in postmenopausal women.

In this study, increase in length of femur and tibia may have reflected to support the increase in weight associated with ovariectomy. Also, change in bone geometry as evident from increased cortical thickness may have been the reason that more force was required to yield and ultimately to cause a fracture in femur bones. Peng and co-workers (1997) reported that the induction of periosteal bone growth in ovariectomized rats increases bone strength. The authors noted that increase in body weight in ovariectomized rats could serve as a protective mechanism through loading and a change

in metabolism. Our data support observations by Peng et al. (1997) in that the geometrical changes associated with ovariectomy influenced bone strength of the osteopenic rat model. Unit yield force was clearly less in the ovx-control group. Perhaps the change in this index is due to decreased bone density in the ovx group. Estrogen injection improved femoral strength by 9.8% over the ovx-control group. Perhaps, such improvement in strength is due to increased bone density. Faulkner (2000) reported that increase in bone density increases bone strength and reduces fracture. Similar to the estrogen injected group, the reason that bone strength in prune-fed rats increased by an average of 6.9% may have been due to increased in bone density. In conclusion, increase in bone strength observed in this study is in part related to bone density.

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TABLE III

Effects of prune and estrogen replacement therapy on growth, feed consumption, and tissue composition of ovariectomized rats.

Variables	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	***P* values**
Body Weights *(g)*							
Initial	205 ± 1	208 ± 1	208 ± 1	207 ± 1	207 ± 1	207 ± 1	0.9896
Final	247 ± 5[b]	299 ± 5[a]	261 ± 4[b]	296 ± 4[a]	295 ± 4[a]	295 ± 4[a]	0.0001
[1]Food Intake *(g/rat/day)*	13.0 ± 0.1	13.1 ± 0.1	13.1 ± 0.1	13.1 ± 0.1	13.1 ± 0.1	13.1 ± 0.1	0.9388
Organ weights *(wt/100g body wt)*							
Heart	0.34 ± 0.01[a]	0.29 ± 0.01[b]	0.33 ± 0.01[a]	0.29 ± 0.01[b]	0.29 ± 0.01[b]	0.29 ± 0.01[b]	0.0046
Liver	2.79 ± 0.08[b]	2.17 ± 0.08[c]	3.02 ± 0.07[a]	2.13 ± 0.07[c]	2.24± 0.07[c]	2.27 ± 0.07[c]	0.0001
Spleen	0.24 ± 0.01	0.23 ± 0.01	0.21 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	0.7900
Uterus	0.24 ± 0.02[a]	0.03 ± 0.02[c]	0.16 ± 0.02[b]	0.03 ± 0.02[c]	0.03 ± 0.02[c]	0.03 ± 0.01[c]	0.0001
Kidneys	0.63 ± 0.02[a]	0.51 ± 0.02[bc]	0.65 ± 0.02[a]	0.50 ± 0.02[c]	0.54 ± 0.02[bc]	0.56 ± 0.02[b]	0.0001

[a-c]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).
[1]Mean food intake from a three day collection.

TABLE IV

Effects of prune and estrogen replacement therapy on serum biochemical values of ovariectomized rats.

Variables	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	***P values***
Serum *(U/L)*							
ALP activity	70.14 ± 10	78. 85 ± 10	90.00 ± 11	79.78 ± 9	88.40 ± 8	93.22 ± 9	0.5269
TRAP activity	2.87 ± 0.35^a	2.90 ± 0.38^a	1.32 ± 0.32^b	2.50 ± 0.30^a	2.77 ± 0.28^a	2.15 ± 0.28^a	0.0104
Serum *(mg/dL)*							
Ionized calcium	1.21 ± 0.07	1.09 ± 0.09	1.12 ± 0.07	1.10 ± 0.07	1.11 ± 0.07	1.08 ± 0.07	0.7254
Total calcium	21.51 ± 0.78	20.00 ± 0.78	21.00 ± 0.68	19.83 ± 0.72	21.05 ± 0.68	20.13 ± 0.67	0.1922
Magnesium	2.65 ± 0.08^{ab}	2.67 ± 0.07^{ab}	2.80 ± 0.06^a	2.51 ± 0.07^b	2.57 ± 0.07^b	2.52 ± 0.07^b	0.0212
Duodenal Ca uptake (nmole/mg of protein/5 min	2.84 ± 0.40^a	3.41 ± 0.40^a	2.36 ± 0.47^{ab}	1.72 ± 0.40^b	1.37 ± 0.35^b	1.47 ± 0.45^b	0.0001

$^{a\text{-}c}$Means ± SEM within a row not sharing a common superscript are significantly different (P<0.05).

TABLE V

Effects of prune and estrogen replacement therapy on selected urinary biochemical excretions of ovariectomized rats.

Variables	Sham	OVX	OVX+E_2	Prune (%)			*P values*
				5	15	25	
Creatinine *(mg/12 hrs urine)*	45.27 ± 6.04	34.49 ± 7.14	34.65 ± 5.05	34.35 ± 5.05	34.26 ± 5.05	47.27 ± 5.32	0.3141
Hydroxyproline *(µg /12 hrs urine)*	9.97 ± 5.16^{b}	36.64 ± 5.96^{a}	13.80 ± 4.86^{b}	17.68 ± 4.61^{b}	16.48 ± 4.61^{b}	20.53 ± 5.16^{b}	0.0342
Deoxypyridinoline *(nM/12 hrs urine)*	1.91 ± 0.29^{bc}	2.47 ± 0.36^{a}	0.90 ± 0.27^{c}	1.74 ± 0.27^{ab}	1.84 ± 0.29^{ab}	1.97 ± 0.32^{ab}	0.0149
Calcium *(mg/12 hrs urine)*	1.03 ± 0.44^{c}	1.72 ± 0.51^{c}	1.60 ± 0.47^{c}	2.00 ± 0.39^{c}	3.69 ± 0.39^{b}	5.17 ± 0.39^{a}	0.0001
Magnesium *(mg/12 hrs urine)*	0.78 ± 0.17^{c}	0.72 ± 0.19^{c}	0.74 ± 0.15^{c}	0.98 ± 0.14^{bc}	1.31 ± 0.14^{ab}	1.91 ± 0.14^{a}	0.0001

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).

TABLE VI

Effects of prune and estrogen replacement therapy on selected fecal output of ovariectomized rats.

Parameters: Fecal	Sham	OVX	OVX+E_2	Prune (%) 5	15	25	***P values***
Dry matter *(%)*	70.14 ± 10	78.85 ± 10	90.00 ± 11	79.78 ± 9	88.40 ± 8	93.22 ± 9	0.0744
Total ash *(mg excreted)*	448 ± 20	404 ± 20	412 ± 20	431 ± 17	419 ± 17	394 ± 17	0.4170
Calcium *(mg in total ash)*	106 ± 15	78 ± 14	90 ± 13	86 ± 13	95 ± 12	83 ± 12	0.7765
Magnesium *(mg in total ash)*	4.79 ± 0.56[ab]	5.80 ± 0.52[a]	3.70 ± 0.52[bc]	4.81 ± 0.47[bc]	3.75 ± 0.45[bc]	4.38 ± 0.45[bc]	0.0305
Ash *(mg/g dry matter)*	186 ± 5[a]	184 ± 5[a]	175 ± 5[a]	179 ± 4[a]	158 ± 4[b]	144 ± 4[b]	0.0132
Calcium *(mg/g dry matter)*	36.90 ± 6.62	35.70 ± 6.62	38.72 ± 6.19	43.11 ± 5.54	32.91 ± 5.54	31.21 ± 5.54	0.6958
Magnesium *(mg/g dry matter)*	1.96 ± 0.23[bc]	2.66 ± 0.22[a]	1.58 ± 0.22[c]	2.33 ± 0.18[ab]	1.51 ± 0.22[c]	1.70 ± 0.19[c]	0.0015

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).

TABLE VII

Effects of prune and estrogen replacement therapy on femur parameters of ovariectomized rats.

Parameters: Femur	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	***P values***
Femur length *(cm)*	3.31 ± 0.03^{b}	3.53 ± 0.04^{a}	3.43 ± 0.04^{a}	3.44 ± 0.04^{a}	3.45 ± 0.04^{a}	3.51 ± 0.04^{a}	0.0036
[1]BMD (g/cm^3)	1.448 ± 0.020^{a}	1.348 ± 0.022^{b}	1.434 ± 0.019^{a}	1.427 ± 0.018^{a}	1.448 ± 0.018^{a}	1.441 ± 0.018^{a}	0.0125
[2]BMD (g/cm^2)	0.235 ± 0.004^{a}	0.225 ± 0.004^{b}	0.233 ± 0.003^{ab}	0.239 ± 0.003^{a}	0.240 ± 0.003^{a}	0.242 ± 0.003^{a}	0.0162
[3]BMC *(g)*	0.387 ± 0.012^{c}	0.413 ± 0.012^{bc}	0.413 ± 0.011^{bc}	0.430 ± 0.010^{ab}	0.431 ± 0.010^{ab}	0.438 ± 0.01^{a}	0.0105
Dry matter *(%)*	93.2 ± 0.2	93.4 ± 0.2	92.9 ± 0.2	93.4 ± 0.2	93.5 ± 0.2	93.4 ± 0.2	0.0744
Ash/ dry matter *(%)*	68.4 ± 0.6^{a}	66.8 ± 0.6^{b}	67.9 ± 0.5^{ab}	66.6 ± 0.5^{b}	66.4 ± 0.5^{b}	67.5 ± 0.4^{ab}	0.0132
Calcium *(mg/g bone)*	169 ± 33	158 ± 30	196 ± 29	195 ± 29	178 ± 25	212 ± 26	0.7878
Magnesium *(mg/g bone)*	3.35 ± 0.22	2.87 ± 0.24	3.10 ± 0.22	3.00 ± 0.20	3.31 ± 0.20	3.34 ± 0.20	0.3995
[4]ALP activities *(U/g bone protein)*	4.09 ± 0.40	4.34 ± 0.40	3.04 ± 0.32	3.59 ± 0.32	3.24 ± 0.32	3.71 ± 0.31	0.1129

[a-c]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).
[1]BMD: Bone Mineral Density (displacement); [2]BMD: Bone Mineral Density (DXA); [3]BMC: Bone Mineral Content; [4]ALP: Alkaline Phosphatase.

TABLE VIII

Effects of prune and estrogen replacement therapy on tibia parameters of ovariectomized rats.

Parameters: Tibia	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	***P values***
Length *(cm)*	5.89 ± 0.26^{b}	7.06 ± 0.28^{a}	6.72 ± 0.25^{a}	6.94 ± 0.23^{a}	6.66 ± 0.23^{a}	7.34 ± 0.23^{a}	0.0053
[1]BMD (g/cm^3)	1.512 ± 0.014^{a}	1.448 ± 0.018^{b}	1.497 ± 0.013^{a}	1.523 ± 0.013^{a}	1.512 ± 0.013^{a}	1.519 ± 0.013^{a}	0.0275
Dry matter *(%)*	95.8 ± 0.1	95.7 ± 0.1	95.8 ± 0.1	96.1 ± 0.1	95.9 ± 0.1	96.1 ± 0.1	0.1117
Ash/ dry matter *(%)*	66.0 ± 0.4^{a}	64.3 ± 0.6^{c}	65.4 ± 0.5^{ab}	64.5 ± 0.5^{bc}	64.9 ± 0.5^{bc}	66.2 ± 0.4^{ab}	0.0237
Calcium *(mg/g bone)*	259 ± 28	288 ± 34	256 ± 25	235 ± 24	263 ± 24	275 ± 25	0.8263
Magnesium *(mg/g bone)*	3.17 ± 0.29	3.34 ± 0.29	3.07 ± 0.26	3.29 ± 0.26	3.04 ± 0.25	3.32 ± 0.26	0.9387
ALP activities *(U/g protein)*	2.89 ± 0.24	3.18 ± 0.27	2.34 ± 0.23	3.06 ± 0.23	3.02 ± 0.25	2.94 ± 0.23	0.1661

[a-c]Means ± SEM within a row not sharing a common superscript are significantly different (P<0.05).
[1]BMD: Bone Mineral Density (displacement).

TABLE IX

Effects of prune and estrogen replacement therapy on 4th lumbar vertebrae parameters of ovariectomized rats.

Parameters: 4th lumbar	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	***P value***
[1]BMD (g/cm^3)	1.266 ± 0.008[a]	1.231 ± 0.008[b]	1.242 ± 0.007[b]	1.238 ± 0.007[b]	1.247 ± 0.007[ab]	1.260 ± 0.007[a]	0.0248
[2]BMD (g/cm^2)	0.231 ± 0.004[a]	0.201 ± 0.004[c]	0.220 ± 0.004[b]	0.220 ± 0.003[b]	0.227 ± 0.003[ab]	0.232 ± 0.003[a]	0.0001
[3]BMC *(g)*	0.112 ± 0.005[b]	0.098 ± 0.005[d]	0.109 ± 0.004[cd]	0.113 ± 0.004[bc]	0.122 ± 0.004[ab]	124 ± 0.004[a]	0.0037
Dry matter *(%)*	95.2 ± 0.2	95.40 ± 0.2	95.2 ± 0.2	95.4 ± 0.2	95.3 ± 0.2	95.4 ± 0.2	0.8369
Ash/ dry matter *(%)*	58.7 ± 0.6[a]	54.6 ± 0.7[c]	55.5 ± 0.6[bc]	55.5 ± 0.6[bc]	55.4 ± 0.6[bc]	56.7 ± 0.6[b]	0.0015
Calcium *(mg/g bone)*	121 ± 12	109 ± 15	98 ± 15	125 ± 11	110 ± 12	119 ± 14	0.7342
Magnesium *(mg/g bone)*	5.05 ± 0.34	4.07 ± 0.36	5.07 ± 0.34	4.33 ± 0.32	4.67 ± 0.32	4.95 ± 0.34	0.2220
[4]ALP activity *(U/g bone protein)*	2.07 ± 0.14[a]	1.55 ± 0.14[b]	1.62 ± 0.12[b]	1.57 ± 0.12[b]	1.62 ± 0.12[b]	1.41 ± 0.12[b]	0.0271

[a-d]Means ± SEM within a row not sharing a common superscript are significantly different (P<0.05).
[1]BMD: Bone Mineral Density (displacement); [2]BMD: Bone Mineral Density (DXA); [3]BMC: Bone Mineral Content;
[4]ALP: Alkaline Phosphatase.

TABLE X

Effects of prune and estrogen replacement therapy on mechanical properties and yield force strength of ovariectomized rats femur bone.

Variables	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	***P values***
Femur cortical area *(mm^2)*	4.68 ± 0.30[b]	5.64 ± 0.32[a]	5.02 ± 0.28[ab]	5.72 ± 0.27[a]	5.71 ± 0.27[a]	5.70 ± 0.27[a]	0.0494
Femur medullary area *(mm^2)*	2.94 ± 0.17	2.58 ± 0.19	3.04 ± 0.16	2.77 ± 0.16	2.77 ± 0.16	2.69 ± 0.16	0.4400
Moment of inertia *(cm^4)*	2.99 ± 0.26[bc]	4.19 ± 0.30[a]	3.29 ± 0.24[b]	3.99 ± 0.23[a]	3.86 ± 0.24[a]	3.74 ± 0.23[a]	0.0183
Yield modulus of elasticity *(Kgf/cm^2)*	0.32 ± 0.03	0.42 ± 0.03	0.40 ± 0.03	0.35 ± 0.03	0.38 ± 0.03	0.37 ± 0.03	0.4084
Yield force *(N)*	102 ± 4[b]	114 ± 4[a]	104 ± 4[b]	114 ± 4[a]	114 ± 4[a]	114 ± 4[a]	0.0095
Yield strain *(Unitless)*	32.08 ± 2.25	27.04 ± 1.9	29.09 ± 1.79	31.89 ± 1.79	31.21 ± 1.68	31.01 ± 1.79	0.4003
Unit yield force *(N/mm^2)*	22.1 ± 0.9[a]	19.4 ± 0.9[b]	21.3 ± 0.9[ab]	20.8 ± 0.8[ab]	21.3 ± 0.9[ab]	20.1 ± 0.8[ab]	0.0396

[a-c]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).

TABLE XI

Effects of prune and estrogen replacement therapy on ultimate force strength of ovariectomized rats femur bone.

Variables	Sham	OVX	OVX+E_2	Prune (%) 5	Prune (%) 15	Prune (%) 25	*P values*
Ultimate modulus of elasticity *(Kgf/cm²)*	0.33 ± 0.04	0.36 ± 0.04	0.38 ± 0.03	0.32 ± 0.03	0.33 ± 0.03	0.34 ± 0.02	0.7158
Ultimate force *(N)*	109 ± 6[b]	126 ± 6[a]	118 ± 5[ab]	125 ± 4[a]	127 ± 4[a]	127 ± 4[a]	0.0363
Ultimate strain *(Unitless)*	37.6 ± 2.5	37.3 ± 2.7	33.6 ± 2.3	39.9 ± 2.3	38.5 ± 2.2	39.9 ± 2.1	0.4025
Unit ultimate force *(N/mm²)*	23.7 ± 0.81	21.5 ± 0.93	23.3 ± 0.77	22.5 ± 0.73	23.7 ± 0.77	22.2 ± 0.73	0.0653

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).

CHAPTER IV

THE EFFECTS OF ESTROGEN DEPLETION AND ISOFLAVONES ON BONE METABOLISM IN RATS

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ABSTRACT

To examine a potential role for phytoestrogens in postmenopasual bone loss, the ovariectomized (OVX) rat model has been used to investigate whether lower than previously used doses of isoflavones are beneficial in impeding estrogen-dependent bone loss. In this study, thirty-two 95-day-old Sprague-Dawley rats were randomly divided among four treatments for forty days of experimentation. The treatments were sham-operated (sham), OVX, OVX+ low dose isoflavones (0.575 mg/g of protein; LD), and OVX+high dose isoflavones (1.15 mg/g protein; HD). Ovariectomy reduced 4th lumbar vertebrae, tibia, and femur bone mineral density as well as adjusted femoral bone strength when compared with the sham group. Serum Ca and Mg, blood ionized Ca, and urinary Mg excretion were not affected with ovariectomy. Urinary excretion of Ca increased ($P<0.05$) in the ovariectomized group when compared with the sham group. Dietary supplementation of isoflavones at the two doses administered did not significantly influence the bone quality compared to the OVX group.

Key words: Ovariectomy, Isoflavones, Bone, Rats

INTRODUCTION

Decreased bone mass in rats has been shown to be a result of ovarian hormone deficiency (Li et al. 1998). Such decreases in bone mass may be due to increased bone resorption and to reduced bone formation. This imbalance in bone remodeling leads to an increase in bone fragility and bone fracture (Mattila et al. 1998). Although these relationships are poorly understood, there are nutritional factors that prevent the bone loss associated with ovarian hormone deficiency (Arjmandi et al. 1996). Flavonoid administration has been found to prevent bone loss in ovariectomized rats (Arjmandi et al. 1996; Arjmandi et al. 2000).

Flavonoids are benzo-γ-pyrone derivatives of plant origin among which isoflavones have received immense attention as therapeutic agents in recent years (Anderson and Garner, 1997). Isoflavones have been known to have estrogen-like activities in preventing bone loss (Miksicek, 1993; Anderson and Garner, 1997). The most studied derivative of isoflavones, ipriflavone, has been shown to be effective in preventing bone loss in rats (Ettinger et al. 1988; Cheng et al. 1994; Cecchini et al. 1997). In most animal studies, consumption of ipriflavone is in excess of naturally occurring isoflavones found in soy-based diet (Nakamura et al. 1992; Civitelli et al. 1995). For example, in a previous study, the beneficial effects of consumption of ipriflavone were observed when ipriflavone was used at a dosage of 400 mg/kg body weight daily (Civitelli et al. 1995; Cecchini et al. 1997). Others used ipriflavone at a daily dosage of 100 mg/Kg body weight with positive effects on bone (Arjmandi et al. 2000).

The purpose of this study was to determine whether dietary supplementation of isoflavones at the average daily dosages of 3.85 and 7.7 mg/kg of diet would prevent bone loss in ovariectomized rats.

MATERIALS AND METHODS

A. Experimental design

This study was designed to represent preventative intervention on bone loss due to ovarian hormone deficiency. Thirty-two (Table I) female Sprague-Dawley rats, aged 90 days old, were used in the study. On arrival at our institution, the rats were housed in an environmentally controlled animal laboratory and were acclimated with a standard laboratory diet for 2 days. The animals were divided into four weight-matched groups (8 animals/group) using a randomized complete block design. One group of rats was sham operated (sham) and three groups were subjected to bilateral ovariectomy. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee of Oklahoma State University at Stillwater were strictly followed. Treatment intervention (Table I) began two days from the date of surgery and continued for 40 days.

Rats were fed a semi-purified powdered casein-based diet, AIN-93M (Table II). The ingredients used in formulating the basal diet were acquired from Teklad (Madison, WI) (Table II). Rats had free access to deionized drinking water throughout the study. The sham group and one ovx group received the basal diet. The other two ovx groups received either low dose (LD; 0.575 mg/g protein) or high dose (HD; 1.15 mg/g protein) isoflavones.

TABLE I

Experimental groups

Surgery Day 0 Treatment group	Treatment begins Day 2		Sacrifice Day 42
Sham	●	Control diet	→
OVX[1]	●	Control diet	→
LD[2]	●	Isoflavones @ 0.575 mg/g protein	→
HD[3]	●	Isoflavones @ 1.15 mg/g protein	→

[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavones provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of dietary protein to OVX animal.

All the rats were pair-fed to the mean food intake of the sham group. Before each feeding, the food remaining was weighed and the amount ingested was calculated. All animals were sacrificed 42 days from the day of surgery.

B. Necropsy

At the termination of the study, animals were weighed and then received an intraperitoneal injection of a combination of xylazine (5 mg/kg body weight) and ketamine (100 mg/kg body weight) anesthesia before they were bled from their abdominal aortas.

TABLE II

Composition of diets

Ingredients(g/kg)	Control diet	Isoflavones	
		LD	**HD**
Corn starch	465.7	464.4	463.9
Maltodextrin	155	155	155
Sucrose	100	100	100
Casein	140	138.6	137.1
Cystine	1.8	1.8	1.8
Corn oil	40	38.6	37.1
Cellulose	49.3	49.3	49.3
Vitamin mix[1]	10	10	10
Choline	2.5	2.5	2.5
Trace mineral mix[2]	13.4	13.4	13.4
Calcium carbonate	12.4	12.4	12.4
KH_2PO_4	5.6	5.6	5.6
NaH_2PO_4	3.4	3.4	3.4
Potassium citrate	0.9	0.9	0.9
Isoflavones	----	3.7	7.5

[1]Vitamin Mixture Composition (AIN-93; Harlan Teklad, Madison, WI).

[2]Mineral Mixture Composition (g/Kg mix; Harlan Teklad, Madison, WI). magnesium oxide, 24g, Ferric citrate, 6.06g; zinc carbonate, 1.65g; manganous carbonate, 0.63g; cupric carbonate, 0.3g; potassium iodate, 0.01g; sodium selenate, 0.01g; ammonium paramolybdate, 0.007g; chromium potassium sulfate 0.275g; boric acid, 0.0815; sodium fluoride, 0.0635g; nickel carbonate, 0.0318g; ammonium vanadate, 0.0066g.

C. Body and organ weights

All rats were weighed initially, weekly, and at the termination of the experiment. At necropsy, uterine tissue, heart, liver, abdominal fat pad, and kidneys were excised and weighed. Left femur, left tibia, and 4th lumbar vertebrae were separated and cleaned from surrounding tissues and an aliquot of whole blood was used immediately to measure ionized calcium using the Nova-8 blood analyzer (Nova Biomedical, Waltham, MA).

D. Blood processing

Blood samples were collected, placed on the laboratory counter top for 4 hours, centrifuged (4 °C) at 1500 x g for 15 minutes, and sera were immediately separated. An aliquot of serum was placed in the refrigerator to be used within four hours of collection for serum alkaline phosphatase (ALP) and tartate-resistant acid phosphatase (TRAP) activities. The remaining aliquots were placed into small tubes and stored at -20°C until required for analysis.

E. Serum alkaline phosphatase and tartrate-resistant acid phosphatase activities

Serum ALP and TRAP activities were evaluated using the Cobas-Fara II Clinical Analyzer (Roche, Montclair, NJ) and commercially available calibrator and quality control samples (Sigma Diagnostic Products, St. Louis, MO).

F. Determination of mechanical properties of femur

The left femur was cleaned of soft tissue and stored in a glass vial at –20°C. Femur length was measured by a caliper before strength determination by a three-point bending test utilizing an Instron Universal Testing Machine (Model II; Instron Corp, Canton, MA). Before the mechanical testing, the bones were immersed in normal saline for 48 hours at 4 °C. The bone sample was placed in similar orientation on two rods spaced 20 mm apart and the force was delivered to the mid-shaft by a crosshead moving at a constant speed of 1.0 mm/min. A force versus deformation curve was recorded. Yield force was defined as the force at the yield point (point of deviation from the linear slope on the force versus deformation curve). Force at bone failure was the ultimate force. Yield and ultimate force provided a measure of bending strength of the intact femur. The inner and outer diameters of the anterior-posterior and mediolateral axis were measured

at the point of fracture with a micrometer and were used to calculate cortical area, medullary area, and moment of inertia (Kiebzak et al. 1988) assuming elliptical cross section. The property of inertia provided a measure of bone distribution about the neutral axis.Yield stress (measure of bone strength), yield strain (measure of bone bending), and modulus of elasticity (indicator of stiffness) were calculated from the primary data (Kiebzak et al. 1988). Yield and ultimate force adjusted for cortical thickness were calculated using the following formulas:

$$\text{Unit yield force} = \frac{\textit{Yield force}}{\textit{Cortical thickness}}$$

$$\text{Unit ultimate force} = \frac{\textit{Ultimate force}}{\textit{Cortical thickness}}$$

G. Bone processing and bone density

The left femur, left tibia, and fourth lumbar vertebrae were cleaned of soft tissues and stored in glass vials at –20°C. For determination of bone density, femur, tibia, and vertebrae were placed in an unstopped glass vial filled with deionized water. The vials were placed into a desiccator connected to a vacuum for 60 minutes so that all trapped air would diffuse out of the bone (Kalu et al. 1988). All bones were weighed before being immersed in deionized water previously equilibrated to room temperature (Kalu et al. 1988).

The bone density was calculated by Archimedes' principle as described previously (Kalu et al. 1988):

$$\textit{bone density} = \frac{\textit{bone air weight}}{\textit{bone air weight - bone underwater weight}}$$

Bone mineral density and bone mineral content of left femur and 4th lumbar vertebrae were also measured by dual x-ray absorptiometry (DXA Model 2000; Waltham, MA).

H. Bone chemistry

The left femur, left tibia, and 4th lumbar were rinsed with phosphate buffered saline (PBS). The bones were then placed in a PBS solution containing 0.01% Triton X-100 (Sigma Diagnostic Products, St. Louis, MO) and 0.02% sodium azide for 72 hours at 4°C and then centrifuged. Bone ALP activities and bone protein extracts were measured from 100 μL aliquots of bone extract using the Cobas-Fara II Clinical Analyzer (Montclair, NJ).

Bones were placed in seventy percent alcohol and dried at 100°C for forty-eight hours, weighed, ashed in covered crucibles at 600°C for 16 hours, cooled, and finally weighed to determine the percent mineral content. Ashed samples were diluted with 0.5% nitric acid and 0.5% lanthanum chloride solution. Bone calcium and magnesium were analyzed using flame atomic absorption spectrophotometry (Model 5100 PC, Perkin-Elmer, Norwalk, CT), and levels were expressed as milligram of calcium or magnesium per gram of bone.

I. Urine collection

Fourteen days before termination of the study, rats were transferred to metabolic cages for twelve hours of urine collection from 8 PM to 8 AM. During this 12-hour urine collection period, rats had access to deionized water but no food was provided. Urine was collected in acid-washed tubes, and the total volume was measured and acidified with 0.03 mL of 6 mol/L HCl per 1 mL urine. Samples were frozen at -20°C until analysis. Creatinine was measured colorimetrically using a commercially available kit from Roche Diagnostics (Branchburg, NJ) and the Cobas-Fara II Clinical Analyzer (Montclair, NJ) and expressed based on the 12-hour night urine volume. Urine calcium and magnesium were analyzed by diluting urine samples with 0.1% nitric acid and 0.1% lanthanum chloride solution. Samples were analyzed using flame atomic absorption spectrophotometry (Model 5001 PC, Perkin-Elmer, Norwalk, CT). Urine samples were expressed as milligrams of calcium or magnesium per 12 hours of urine collection.

J. Statistical and data analysis

Data were analyzed by analysis of variance using the General Linear Model procedure of SAS (version 7; SAS Institute, 1998) to determine the effects of isoflavones supplementation on bone density and bone strength. When a significant F statistic ($p \leq 0.05$) from analysis of variance was noted, the least square means procedure of SAS® was performed for separating means that were significantly ($p \leq 0.05$) different.

RESULTS

Feed intake, body weights, and organ weights

In the present study, initial body weights were similar (P>0.05) among the four treatment groups (Table III). Since all rats were pair-fed to the sham group, feed

consumption was not different among the four treatments. At the end of the experiment, all ovariectomized rats gained more ($P<0.05$) weight than the sham treated control group. Supplementation of isoflavones to the diet did not ($P>0.05$) affect body weight in comparison with ovx-control group.

The mean weights for liver, kidney, and uterus per 100 gram body weight were significantly ($P<0.05$) less in the ovx-control group compared to the sham group (Table III). The mean organ weights of those rats that consumed either dose of isoflavones were not different from the ovx-control group (Table III).

The mean weights for heart and abdominal fat per 100 gram body weight were not different among the treatment groups (Table III).

Serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity.

Mean serum ALP and TRAP activities did not differ ($P>0.05$) between the ovx-control and sham groups (Table IV). Similarly, consumption of isoflavones did not influence serum ALP or TRAP activities ($P>0.05$) in comparison with the sham or ovx-control groups (Table IV).

Serum calcium and magnesium concentration

In this study, no significant differences ($P>0.05$) were detected in serum ionized calcium, serum total calcium, or serum magnesium concentrations of those rats that consumed isoflavones in comparison with those of the sham and ovx-control groups (Table IV).

Urinary creatinine, calcium, and magnesium

Mean urinary creatinine was not significantly ($P>0.05$) different among treatment groups (Table IV). Similarly, the mean urinary excretion of magnesium per 12 hours of

urine was not affected while mean urinary excretion of calcium increased (P<0.05) with ovariectomy compared with the sham group. However, increased consumption of isoflavones did not affect urinary excretion of calcium and magnesium in comparison with the ovx-control group (Table IV).

Femoral length, mineral density, and mineral concentration

Ovariectomies did not affect (P>0.05) the mean femoral lengths when compared with those of the sham group (Table V). The mean femoral density for the ovx-control group however, was reduced (P<0.05) in comparison with the sham group. These observations were confirmed using DXA to estimate femoral mineral density. Bone mineral density was not affected (P>0.1) in rats consuming isoflavones compared to the ovx-control group (Table V).

The mean femoral dry weight percentage did not (P>0.05) differ significantly among treatment groups (Table V). Ash as a percentage of dry matter significantly decreased (P<0.05) with ovariectomy and was not restored with consumption of isoflavones (Table V).

The mean concentrations of calcium and magnesium per gram bone were not (P>0.05) affected by any of the treatments (Table V).

Tibial length, mineral density, and mineral concentration

Ovariectomies increased (P<0.05) the mean tibial lengths compared to the tibias of the sham group. Consumption of isoflavones at the dosage given did not significantly increase tibial length or the tibial mineral density compared to the ovx-control group (Table VI). However, the tibial length was significantly greater for those rats consuming isoflavones than the sham group.

The mean tibial dry weight percentage and ash as a percentage of dry matter were not ($P>0.05$) different among treatment groups. As with the femur, isoflavones did not affect ($P>0.05$) tibial calcium and magnesium concentrations.

Fourth lumbar vertebrae mineral density and mineral concentration

The 4th lumbar mineral density decreased ($P<0.05$) in the ovx-group compared to the sham group. Supplementation of isoflavones to the basal diet did not significantly affect bone mineral density of the 4th lumbar vertebrae compared to the ovx-control group. Using DXA to estimate 4th lumbar mineral density confirmed our previous observation that isoflavones at the dosage provided does not significicatly improve 4th lumbar mineral density over the ovx-control group.

In this study, percentage of 4th lumbar dry matter was not different ($P>0.05$) among treatment groups (Table VII). But, 4th lumbar ash percentages of the ovx-control and those receiving isoflavones decreased ($P<0.05$) compared to the sham group. Consumption of isoflavones did not affect bone ash percentages of 4th lumbar vertebrae compared to those of ovx-controls (Table VII).

In the present study, concentrations of calcium and magnesium were not affected by ovariectomies or by consumption of isoflavones.

Bone alkaline phosphatase activity (ALP)

Tibia and 4th lumbar vertebrae bone ALP activity (U/g protein) were not ($P>0.05$) significantly different among the treatments (Tables VI-VII). However, femur ALP was significantly increased ($P<0.05$) in all ovariectomized rats (Table V). Supplementation with isoflavones did not ($P>0.05$) affect bone ALP activity measured in this study.

Biomechanical properties of left femur bone

The mean femoral cortical area increased ($P<0.05$) after ovariectomy when compared with the sham group. Femur medullary area, however, was not significantly affected by either ovariectomy or isoflavones supplementation (Table VIII).

Second moment inertia (SMI), yield force, and ultimate force in the femurs were not affected ($P>0.05$) by ovariectomy or by isoflavones consumption. Yield and ultimate force required to cause a fracture when adjusted for cortical thickness were significantly ($P<0.05$) less for the ovx-group. Supplementation of isoflavones to the basal diet tended to increase ($P>0.1$) the unit yield and unit ultimate force required for fracture compared to the ovx-control (Table VIII).

DISCUSSION

Increased body weight of ovariectomized rats compared to those of the sham group is perhaps due to a change in metabolism as is evident from an increase in body weight without an increase in food intake. Other investigators have reported increased body weight due to ovarian hormone deficiency (Kalu et al. 1994). Body weight increase of ovariectomized rats may have been due to a decrease in basal metabolism and circulating thyroid hormone (Thomas et al. 1986).

Isoflavones at the concentrations (0.575 and 1.15 mg/g dietary protein) given did not reverse ovx-induced increase in weight gain nor impede uterine atrophy in ovariectomized rats, suggesting that isoflavones used at such dosages did not have estrogenic properties.

In the present study, the decreases in mean weights of the liver and the kidneys in the ovx-control group are due to increased body weights relative to organ weights in

comparison to the sham group. A similar conclusion can be drawn about isoflavones supplemented groups; organ weights of ovariectomized rats consuming isoflavones did not differ from the ovx-controls. The lack of abdominal fat pad response to estrogen deficiency in the ovx-induced group does suggest that, at least in part, the increased weight gain is not due to increased abdominal fat accumulation.

In the present study, it is not clear why serum ALP and TRAP activities were not affected by ovariectomy. However, the lack of specificity of these two markers to ovariectomy has been known for sometime. Alkaline phosphatase is primarily synthesized in the liver and circulates via serum through the body (Risteli and Risteli, 1993). Similarly, tartrate resistant acid phosphatase is a non-specific lysosomal enzyme present primarily in prostate, platelets, red blood cells, spleen, and osteoclasts (Minkin, 1982). Therefore, lack of specificity of both serum ALP and TRAP activity does limit their validity in osteoporosis management.

In the present study, the reason serum and urinary magnesium were not influenced by any of the treatments can be attributed to adequate magnesium intake. Increased urinary excretion of calcium with ovariectomy may in part be due to bone calcium resorption even though serum calcium was not affected.

The decrease in mean femoral, tibia, and 4th lumbar vertebrae densities of the ovx-control group is perhaps due to reduced bone mineral content as is evident from reduced bone ash as a percentage of dry matter.

Because femur, tibial, or 4th lumbar vertebrae mineral densities did not differ from those of the ovx-control group, the dosages of isoflavones used in this study may have been too low. It was recently reported that isoflavones prevented bone loss (Toda et al.

1999). But in that study, oral doses of 50 mg isoflavones per kilogram of body weight were fed daily to ovaiectomized rats for a period of 4 weeks. However, Anderson and colleagues (1998) suggest that the bone-protective effect of genistein, a component of isoflavone, has a biphasic dose-response such that either too low or too high a dose is less effective.

In this study, the increase in bone cortical area may have been to support the increase in weight associated with ovariectomy. Peng and co-workers (1997) reported that the induction of periosteal bone growth in ovariectomized rats increases bone strength. The authors noted that an increase in body weight in ovariectomized rats could serve as a protective mechanism through loading and a change in metabolism. Our data support the previous observations of Peng et al. (1997) in that the geometrical changes associated with ovariectomies influence the bone strength in this rat model of osteopenia. When yield and ultimate force were adjusted for femoral bone thickness, it was clear that significantly less force was required to yield a fracture in the ovx-control group compared to the sham controls. The reason that rats that were fed the isoflavones fortified diet showed an average of 9.2% increase in adjusted femur strength may have been due to numerical increases in bone density. Faulkner (2000) reported that decrease in fracture rate is due to increased bone density. In conclusion, additional research at different dosage levels is needed to determine the effect of isoflavones on bone density and bone strength.

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TABLE III

Effects of isoflavones on growth, feed consumption, and tissue composition of ovariectomized rats.

Variables	Sham	OVX[1]	LD[2]	HD[3]	***P values***
Body Weights *(g)*					
Initial	214 ± 4	211 ± 4	211 ± 4	211 ± 4	0.9631
Final	251 ± 6	275 ± 6[a]	278 ± 6[a]	271 ± 6[a]	0.0205
Food Intake[4] *(g/rat/day)*	13.8 ± 0.2	13.9 ± 0.2	13.9 ± 0.2	13.9 ± 0.2	0.9819
Organ weights *(wt/100g body wt)*					
Heart	0.36 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	0.35 ± 0.01	0.5851
Liver	2.83 ± 0.08[a]	2.47 ± 0.08[b]	2.44 ± 0.08[b]	2.46 ± 0.08[b]	0.0097
Uterus	0.25 ± 0.01[a]	0.04 ± 0.01[b]	0.04 ± 0.01[b]	0.04 ± 0.01[b]	0.0001
Kidneys	0.63 ± 0.01[a]	0.56 ± 0.01[bc]	0.55 ± 0.01[c]	0.59 ± 0.01[ab]	0.0117
Abdominal fat	2.22 ± 0.29	2.21 ± 0.27	2.16 ± 0.29	2.74 ± 0.27	0.4079

[a-c]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).
[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavone provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of dietary protein to OVX animal.
[4]Mean food intake from a three day collection.

TABLE IV

Effects of isoflavones on serum and urinary biochemical values of ovariectomized rats.

Variables	Sham	OVX[1]	LD[2]	HD[3]	***P values***
Serum (U/L)					
ALP activity	80.9 ± 6.1	81.6 ± 5.7	75.0 ± 6.1	92.1 ± 5.7	0.2401
TRAP activity	4.98 ± 0.46	5.16 ± 0.43	4.10 ± 0.46	4.79 ± 0.43	0.3834
Serum (mg/dL)					
Ionized calcium	0.97 ± 0.08	1.06 ± 0.08	0.97 ± 0.08	1.12 ± 0.07	0.4099
Total calcium	22.6 ± 1.1	22.2 ± 1.0	20.9 ± 1.2	22.7 ± 1.0	0.6284
Magnesium	2.95 ± 0.14	2.66 ± 0.14	2.77 ± 0.16	2.81 ± 0.14	0.5871
Urinary (mg/12 hrs urine)					
Creatinine	53.3 ± 12	48.4 ± 12	65.8 ± 13	78.2 ± 13	0.3665
Calcium	0.24 ± 0.11[b]	0.67 ± 0.11[a]	0.78 ± 0.12[a]	0.71 ± 0.13[a]	0.0131
Magnesium	0.42 ± 0.10	0.58 ± 0.10	0.67 ± 0.10	0.59 ± 0.10	0.3695

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).
[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavones provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of protein to OVX animal.

TABLE V

Effects of isoflavones on femur parameters of ovariectomized rats.

Parameters: Femur	Sham	OVX[1]	LD[2]	HD[3]	***P values***
Femur length *(cm)*	3.30 ± 0.03	3.39 ± 0.04	3.25 ± 0.05	3.32 ± 0.04	0.1909
[4]BMD (g/cm^3)	1.439 ± 0.014[a]	1.388 ± 0.014[b]	1.412 ± 0.014[ab]	1.407 ± 0.013[ab]	0.0557
[5]BMD (g/cm^2)	0.231 ± 0.002[a]	0.218 ± 0.002[b]	0.220 ± 0.002[b]	0.216 ± 0.002[b]	0.0017
[6]BMC *(g)*	0.381 ± 0.008	0.366 ± 0.008	0.359 ± 0.008	0.355 ± 0.008	0.1804
Dry matter *(%)*	93.1 ± 0.3	93.1 ± 0.3	93.1 ± 0.3	93.3 ± 0.3	0.8920
Ash/ dry matter *(%)*	70.1 ± 0.4[a]	68.4 ± 0.4[b]	68.8 ± 0.4[b]	68.1 ± 0.4[b]	0.0136
Calcium *(mg/g bone)*	131 ± 32	167 ± 35	144 ± 32	151 ± 38	0.9022
Magnesium *(mg/g bone)*	3.65 ± 0.35	3.52 ± 0.33	3.31 ± 0.38	3.62 ± 0.35	0.9161
[7]ALP activities *(U/g bone protein)*	3.94 ± 0.56[b]	5.90 ± 0.60[a]	5.91 ± 0.59[a]	6.14 ± 0.56[a]	0.0305

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P \leq 0.05$).
[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavones provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of dietary protein to OVX animal.
[4]BMD: Bone Mineral Density (displacement); [5]BMD: Bone Mineral Density (DXA);
[6]BMC: Bone Mineral Content; [7]ALP: Alkaline Phosphatase.

TABLE VI

Effects of isoflavones on tibia parameters of ovariectomized rats.

Parameters: Tibia	Sham	OVX[1]	LD[2]	HD[3]	***P values***
Tibia length *(cm)*	3.81 ± 0.03^{b}	3.92 ± 0.03^{a}	3.98 ± 0.03^{a}	3.90 ± 0.03^{a}	0.0394
[4]BMD (g/cm^3)	1.486 ± 0.007^{a}	1.459 ± 0.007^{b}	1.456 ± 0.007^{b}	1.464 ± 0.007^{ab}	0.0523
Dry matter *(%)*	95.7 ± 0.1	95.6 ± 0.1	95.7 ± 0.1	95.6 ± 0.1	0.8887
Ash/ dry matter *(%)*	63.4 ± 0.32	62.5 ± 0.28	62.6 ± 0.32	62.5 ± 0.28	0.1679
Calcium *(mg/g bone)*	306 ± 28	307 ± 29	287 ± 27	308 ± 25	0.9376
Magnesium *(mg/g bone)*	3.91 ± 0.36	3.95 ± 0.34	3.90 ± 0.39	4.17 ± 0.34	0.9393
[5]ALP activities *(U/g bone protein)*	6.41 ± 0.51	7.55 ± 0.38	6.93 ± 0.45	7.68 ± 0.45	0.2307

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P \leq 0.05$).
[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavones provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of dietary protein to OVX animal.
[4]BMD: Bone Mineral Density (displacement); [5]ALP: Alkaline Phosphatase.

TABLE VII

Effects of isoflavones on 4th lumbar vertebrae parameters of ovariectomized rats.

Parameters: 4th lumbar	Sham	OVX[1]	LD[2]	HD[3]	***P values***
[4]BMD (g/cm^3)	1.271 ± 0.016^a	1.204 ± 0.015^b	1.240 ± 0.016^{ab}	1.200 ± 0.015^b	0.0166
[5]BMD (g/cm^2)	0.212 ± 0.004^a	0.198 ± 0.004^b	0.203 ± 0.003^{ab}	0.199 ± 0.003^b	0.0270
[6]BMC *(g)*	0.101 ± 0.03	0.090 ± 0.01	0.093 ± 0.01	0.092 ± 0.01	0.1804
Dry matter *(%)*	94.9 ± 0.4	94.9 ± 0.	94.9 ± 0.4	95.3 ± 0.4	0.8583
Ash/ dry matter *(%)*	64.1 ± 0.7^a	60.3 ± 0.7^b	60.3 ± 0.7^b	60.4 ± 0.7^b	0.0025
Calcium *(mg/g bone)*	173 ± 30	142 ± 30	169 ± 28	128 ± 24	0.5789
Magnesium *(mg/g bone)*	5.71 ± 0.26	5.48 ± 0.28	5.17 ± 0.30	5.33 ± 0.26	0.5675
[7]ALP activities *(U/g protein)*	4.77 ± 1.19	5.03 ± 1.19	4.74 ± 1.34	4.42 ± 1.19	0.9876

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P<0.05$).
[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavones provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of dietary protein to OVX animal.
[4]BMD: Bone Mineral Density (displacement); [5]BMD: Bone Mineral Density (DXA);
[6]BMC: Bone Mineral Content; [7]ALP: Alkaline Phosphatase.

TABLE VIII

Effects of isoflavones on mechanical properties of ovariectomized rats femur bone.

Variables:	Sham	OVX[1]	LD[2]	HD[3]	***P values***
Femur cortical area *(mm^2)*	4.85 ± 0.21^b	5.83 ± 0.20^a	5.52 ± 0.21^a	5.52 ± 0.20^a	0.0164
Femur medullary area *(mm^2)*	3.16 ± 0.18	2.80 ± 0.17	2.70 ± 0.18	2.93 ± 0.17	0.3186
Second moment of inertia *(cm^4)*	3.45 ± 0.23	4.86 ± 0.22	3.78 ± 0.23	3.80 ± 0.22	0.3053
Yield force *(N)*	62.06 ± 3.89	56.39 ± 3.60	64.16 ± 3.89	55.04 ± 3.64	0.2776
Unit yield force *(N/mm^2)*	12.22 ± 0.64^a	10.21 ± 0.50^b	11.28 ± 0.64^{ab}	10.42 ± 0.60^{ab}	0.0539
Ultimate force *(N)*	63.88 ± 2.9	62.03 ± 2.9	66.40 ± 2.8	64.4 ± 2.7	0.7431
Unit ultimate force *(N/mm^2)*	12.66 ± 0.63^a	10.71 ± 0.55^b	11.64 ± 0.63^{ab}	1.74 ± 0.55^{ab}	0.0482

[a,b]Means ± SEM within a row not sharing a common superscript are significantly different ($P \leq 0.05$).
[1]OVX=ovariectomized animal; [2]LD=Low dose, isoflavones provided @ 0.575 mg/g of dietary protein to OVX animal; [3]HD=High dose, isoflavones provided @ 1.15 mg/g of dietary protein to OVX animal.

CHAPTER V

SUMMARY, CONCLUSION, AND RECOMMENDATION

SUMMARY

Experiment 1:

The purpose of this study was to evaluate the effects of consumption of prunes in preventing further bone loss in a rat model of ovarian hormone deficiency. The study examined the effects of the consumption of prune at 5%, 15%, and 25% in the diet. In this study, mode of action of prunes was compared with effects of estrogen injection on bone. Fifty-six Sprague-Dawley 90-day old female rats were used in this study. Rats were divided into six treatment groups as follows: Sham operated (sham), ovx, ovx+estrogen, ovx+5% prune, ovx+15% prune, and ovx+25% prune. Treatments were started 40 days after surgery and continued for 60 days. After the end of the treatment period, rats were sacrificed and bone, blood, liver, kidney, and heart were collected and stored for various analyses. Analysis of variance and least square means tests were used to determine the significant differences between groups.

The findings of this study suggest that ovariectomy deceased bone ash as a percentage of dry matter, bone mineral density of femur, 4th lumbar vertebrae, and tibia, and yield force adjusted for cortical thickness in comparison with the sham group. Estrogen injection improved these indices. Incorporation of prune into the diet restored bone mineral density of 4th lumbar vertebrae, femur, and tibia, bone ash as a percentage of dry matter, and adjusted yield force in comparison with the sham group. From the finding of this study it can be concluded that consumption of prunes increased bone ash

which resulted in an increased bone density that improved adjusted bone strength. However, the protective mechanisms of prunes need further investigation.

Experiment 2:

The purpose of this experiment was to determine if isoflavone prevents bone loss induced by ovarian hormone deficiency. The study was carried out using 32 90-day old female Sprague-Dawley rats. The four treatment groups were as follows: Sham operated (sham), ovx, ovx+0.575 mg of isoflavones per gram protein (LD), ovx+1.15 mg of isoflavones per gram protein (HD) in the diets. Treatment intervention was initiated two days after surgery and continued for 40 days. All animals were given free access to deionized water and were pair fed to the sham group. At the end of the study, rats were weighed and anesthetized with a mixture of ketamine and xyline and were bled from the abdominal aorta. Blood was collected and serum was separated, aliquoted into small vials, and stored at -20 °C. Tissues including liver, kidney, heart, and uterus were collected and weighed. Femur, 4th lumbar vertebrae, and tibia were freed from soft tissues and stored for analysis.

The findings of the present study indicated that ovariectomy decreases bone mineral density, femur and 4th lumbar ash percentage, and adjusted bone strength. Addition of isoflavones to a casein-based diet did not significantly increase bone mineral density and bone strength. Calcium and magnesium concentrations in the bones evaluated were not affected by ovariectomy or isoflavones supplementation. Based on the results of this study it is recommended that additional research be conducted to determine optimum isoflavones concentration in preventing bone loss.

CONCLUSION

Osteoblastic and osteoclastic activities are increased in women during the first few years following menopause (Kanis, 1996). The activities of these cells are reflected by increased serum osteocalcin, alkaline phosphatase, tartrate-resistant acid phosphatase concentrations and urinary indices of bone turnover markers such as urinary hydroxyproline, pyridinoline, and deoxypridinoline. Injection of estrogen attenuates these events and prevents bone loss. Although it has been shown that estrogen injections to ovariectomized rats prevent bone loss, the bone forming properties of these compounds have not been fully explored.

The results of the first study suggested that consumption of prunes increased bone density. Increased bone density with consumption of prunes appeared to be its nutrient contents including its magnesium. Increased magnesium absorption was evident from decreased fecal magnesium excretion in prune-fed rats compared to the ovx-controls. Perhaps other micronutrients found in prune have contributed in increasing bone density of ovariectomized rats. In contrast to consumption of prunes, increased bone density with estrogen injection is due to reduction in bone resorption as is evident from decrease in serum TRAP and urinary hydroxyproline and deoxypridinoline.

The results of the second study suggest that despite ovariectomy induced bone loss, consumption of isoflavones at the dosages provided may not have been sufficient to preserve bone density and prevent bone loss. Perhaps long term consumption of isoflavone may be necessary to elicit a positive response. If long term consumption of isoflavones is needed for restoring bone density, a longer study is needed to establish the role of isoflavones in bone formation and bone preservation.

RECOMMENDATIONS

The finding of the first study suggest that prune consumption can restore bone loss in ovarian hormone deficiency in growing rats. Such benefit can be attributed to minerals, vitamins, fiber, polyphenols, and other chemicals including dihydroxyphenyl isatin found in prune. Chemicals such as dihydroxyphenyl isatin found in prune stimulates gastrointestinal motility which aids in preventing constipation. However, human studies are needed to confirm findings of this animal study. Additionally, other animal studies are needed to investigate the mechanism of action of prunes in protecting from bone loss in an ovarian hormone deficiency-induced rat model of osteopenia.

The findings of the second study suggest that isoflavones at the dosages provided was ineffective in preventing ovariectomy-induced bone loss. Additional research is needed to determine the optimum dosage for a desirable bone-response in dealing with ovarian hormone deficiency in rats.

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